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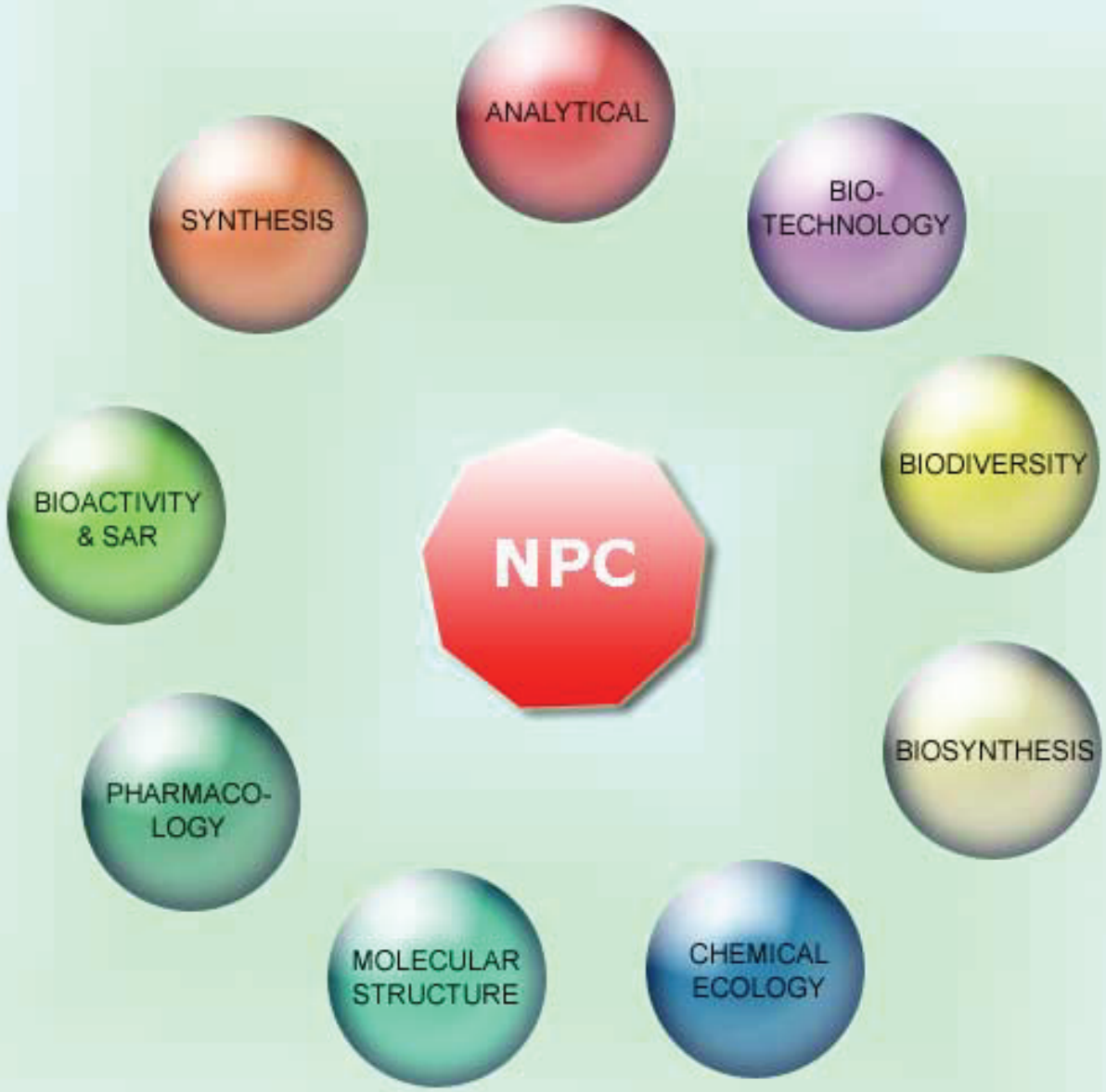

Volume 12. Issue 1. Pages 1-152. 2017
ISSN 1934-578X (printed); ISSN 1555-9475 (online)
www.naturalproduct.us

NPC Natural Product Communications

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Natural Product Communications Vol. 12 (1) 2017
Published online (www.naturalproduct.us)

Editorial Message

The broad scientific scope of *Natural Product Communications* (*NPC*) means that we publish high quality papers across a wide spectrum of natural products research, including organic, medicinal, pharmaceutical, bioorganic, analytical, theoretical, biotechnological and chemotaxonomic aspects. We welcome all types of manuscripts: communications, full papers and reviews. We have a very diverse international authorship, and a readership that is continuously growing, as indicated by the increasing number of submissions and published papers, and also from the web statistics. *NPC* is a SCI-cited journal with an impact factor in 2015 of 0.884. We would like to **thank our authors, readers and referees who are the architects of this success.**

During March 2016, we published a themed issue dedicated to "Traditional Medicines as Sources of Bioactive Compounds (Guest Editors: Profs. Giovanni Vidari and Paola Vita Finzi)". We had four honorarium issues to recognize internationally renowned natural product researchers, Prof. Dr Motto Tori, for his 65th Birthday (February 2016), Prof. Dr. DHC Yoshinori Asakawa, President of the Phytochemical Society of Asia, for his 75th Birthday (July and August 2016) and Prof. Dr Wilhelm Fleischhacker, for his 85th Birthday (October 2016). We are committed to placing this journal at the forefront for the dissemination of new and exciting natural products research. *NPC* will be proud to recognize eminent natural product scientists by dedicating an issue in their honour. Suggestions for themed and/or honorarium issue(s) would be welcomed. We look forward to receiving your submissions and your feedback.

NPC in collaboration with the Phytochemical Society of Asia (PSA) organized an International Symposium on Natural Products for the Future 2016 (ISNPF2016) that was held between September 1-4, 2016 at Tokushima Bunri University, Tokushima, Japan. This attracted scientists, professionals and representatives of companies working in the exciting field of natural product research not only from Asia but also from all over the world. We have also planned a special NPC-ISNPF issue to be published during 2017.

Professor Stefano Serra, National Research Council (CNR), Istituto di Chimica del Riconoscimento Molecolare (ICRM), Milano, Italy, won the 2015 "Gerald Blunden Award" for the best article "The Co-identity of Lipiarmycin A3 and Tiacumicin B" *Natural Product Communications* **9** (2) 237-240 (2014), and co-authored by Angelo Bedeschi, Piera Fonte, Giovanni Fronza, and Claudio Fuganti. The Award wins a free year's subscription to *Natural Product Communications* and is accompanied by US$ 500, sponsored by Natural Product Inc. The award was made for the best article published during the last two years (2014-2015). In 2017, the award will be made for the best review article published during 2015-2016. Each year, the winner will be announced in May at the time of Prof. Blunden's Anniversary, and a similar pattern will be followed in consecutive years.

For the information of our authors and readers, we have to announce some changes to the Editorial Board and Editorial Board of Advisors. Professor De-an Guo, Shanghai Institute of Materia Medica, Shanghai 201203, P. R. China, has been an editor from the time of inception of the journal. He has expressed a wish to step down from his editorial role. His high level of commitment and expertise will be missed, and the editors and publisher thank him for his many valuable contributions. Leaving the Editorial Advisory Board are Professors Viqar Uddin Ahmad, Josep Coll, Imre Mathe and Luca Rastrelli; we thank them for their support and their service. We are, however, pleased to report that as of January 2017, Professor Ping-Jyun Sung, National Museum of Marine Biology and Aquarium Checheng, Pingtung 944, Taiwan, will be joining the Editorial Board, bringing considerable expertise and experience, particularly in marine natural products. Professors Norbert Arnold, Vassya Bankova and Lindros A. Skaltsounis will be joining the Editorial Advisory Board. We welcome them all and look forward to working with them.

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Cytotoxic and Antileishmanial Components from the Bark Extract of *Ruyschia phylladenia* from Monteverde, Costa Rica

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Received: August 3rd, 2016; Accepted: September 7th, 2016

The bark of *Ruyschia phylladenia* was collected from Monteverde, Costa Rica, and extracted with acetone. Bioactivity-directed chromatographic separation of the crude acetone bark extract of *R. phylladenia* led to isolation and identification of lupeol, betulinic acid, and isofraxidin. Lupeol and betulinic acid showed *in-vitro* cytotoxic activity to MCF-7, MDA-MB-231, and 5637 human tumor cell lines. Isofraxidin was not cytotoxic, but did show antileishmanial activity to *Leishmania amazonensis* promastigotes.

Keywords: *Ruyschia phylladenia*, Cytotoxic, *Leishmania amazonensis*, Lupeol, Betulinic acid, Isofraxidin.

Ruyschia phylladenia Sandwith is a Neotropical liana belonging to the Marcgraviaceae that is found in Costa Rica and Panama at an altitude of about 1200 m [1]. Twenty-eight different species of *Ruyschia* are currently recognized [2]. The crude bark extracts of *R. phylladenia* had previously shown antibacterial activity against Gram-positive *Bacillus cereus* and *Staphylococcus aureus* and cytotoxic activity against Hep-G2 (human hepatocellular carcinoma), MDA-MB-231 (human breast adenocarcinoma), and Hs 578T (human breast ductal carcinoma) cell lines [3], as well as antiparasitic activity against *Leishmania amazonensis* promastigotes and amastigotes (IC_{50} <12.5 and = 22.9 μg/mL, respectively) [4]. In this work, we present the bioactivity-directed separation and identification of bioactive components from *R. phylladenia*. To our knowledge, no previous phytochemical studies have been carried out on this plant.

The bark of *R. phylladenia* was collected from the Monteverde region of Costa Rica and extracted with acetone. The crude acetone bark extract was subjected to bioactivity-directed preparative chromatography to give lupeol, betulinic acid, and isofraxidin as the biologically active components (Table 1).

Both lupeol and betulinic acid have shown cytotoxic activity against several tumor cell lines [5], so the cytotoxicity exhibited by these lupane triterpenoids in this study was not surprising. Although these compounds also showed antileishmanial activity against *L. amazonensis* promastigotes, the cytotoxicity against BALB/c mouse macrophages indicates a lack of selectivity; these compounds cannot be considered as potential antileishmanial agents. Lupeol [5b] and betulinic acid [6] have also shown antitrypanosomal and antiplasmodial activity.

The coumarin isofraxidin, on the other hand, showed no cytotoxic activity against the tumor cell lines and minimal toxicity to BALB/c mouse macrophages, while also showing excellent antileishmanial activity. The antiparasitic activity of isofraxidin is consistent with a previous report of antiplasmodial activity of this compound [7].

Table 1: *In-vitro* cytotoxic activities of compounds from *Ruyschia phylladenia* acetone bark extract.

Compounds	Cytotoxicity (IC_{50}, μg/mL)				
	MCF-7	MDA-MB-231	5637	BALB/c mouse macrophage	*L. amazonensis* promastigotes
Lupeol	76.6±2.8	66.1±1.2	32.9±6.8	19.3±3.8	8.5±0.1
Betulinic Acid	15.5±3.9	3.16±1.38	1.29±0.12	10.5±2.9	11.3±4.9
Isofraxidin	inactive	inactive	inactive	51.9±6.2	11.1±1.3
Control	28.6±1.9[a]	25.9±2.4[a]	0.98±0.23[a]	11.7±1.7[b]	0.37±0.01[b]

[a] Doxorubicin. [b] Pentamidine.

This report presents the isolation and identification of lupeol, betulinic acid, and isofraxidin from a new, here-to-fore uninvestigated plant source and reaffirms the biological activities of these compounds.

Experimental

Plant Material: *Ruyschia phylladenia* was collected from the Monteverde region of Costa Rica (10º 17′ 25.24″ N, 84º 49′ 0.82″ W, 1173 m elevation) on 14 May 2008. The plant was identified by W.A. Haber and a voucher specimen (Haber 2921) has been deposited in the herbarium of the Missouri Botanical Garden. The bark was chopped and air dried and the dried bark (462 g) was extracted with refluxing acetone for 5 hours using a Soxhlet extractor. Evaporation of the acetone gave 60.0 g crude bark extract.

Chromatographic Separation: The crude acetone bark extract of *R. phylladenia* (25.0 g) was separated by flash column chromatography (silica gel: 90 cm L × 5 cm D) using a step gradient with *n*-hexane/ethyl acetate, 200-mL fractions: 9:1 *n*-hexane/ethyl acetate, F1-F4; 8:2 *n*-hexane/ethyl acetate, F5-F25; 7:3 *n*-hexane/ethyl acetate, F26-F44; 1:1 *n*-hexane/ethyl acetate, F45-F56; 3:7 *n*-hexane/ethyl acetate, F57-F75; 1:9 *n*-hexane/ethyl acetate, F76-F82; 100% ethyl acetate, F83-F90. Fractions F13-F14 were combined and recrystallized from ethyl acetate/pentane to give 38.8 mg lupeol, which had ^{1}H and ^{13}C NMR and IR spectra in agreement with those previously reported in the literature [8]. Fractions F24-F42 were combined and recrystallized from ethyl acetate/pentane to give 1.70 g betulinic acid, which had ^{1}H and ^{13}C NMR and IR spectra in agreement with those previously reported in

the literature [8ab,9]. Fractions F57-F61 were combined and recrystallized from ethyl acetate/pentane to give 13.8 mg isofraxidin, which had ^{1}H and ^{13}C NMR in agreement with those previously reported in the literature [10].

Cytotoxicity Assay: The isolated compounds from *R. phylladenia* were screened for *in-vitro* cytotoxicity against MCF-7 (human breast adenocarcinoma), MDA-MB-231, and 5637 (human bladder carcinoma) cell lines using the MTT assay as described previously [11].

Antileishmanial Assay: *L. amazonensis* culturing, antileishmanial screening against *L. amazonensis* promastigotes, and cytotoxicity screening against BALB/c mouse macrophages were carried out as previously described [4].

Acknowledgments – We are very grateful to the Monteverde Cloud Forest Preserve and the Tropical Science Center for granting us permission to collect plant materials under a cooperative rights agreement and to the Commission for the Development of Biodiversity of Costa Rica's Ministry of the Environment, Energy, and Telecommunications for Research Permit R-001-2006-OT-CONAGEBIO. This work was performed as part of the activities of the Research Network, Natural Products against Neglected Diseases (ResNetNPND), (http://www.resnetnpnd.org/Start/).

References

[1] Woodson RE, Schery RW, DeRoon AC. (**1970**) Flora of Panama. Part VI. Family 121. Marcgraviaceae. *Annals of the Missouri Botanical Garden*, ***57***, 29-50.

[2] Tropicos.org. (**2016**) Missouri Botanical Garden, 28 May 2016 <http://www.tropicos.org>

[3] Setzer MC, Moriarity DM, Lawton RO, Setzer WN, Gentry GA, Haber WA. (**2003**) Phytomedicinal potential of tropical cloudforest plants from Monteverde, Costa Rica. *Revista de Biología Tropical*, ***51***, 647-674.

[4] Monzote L, Piñón A, Setzer WN. (**2014**) Antileishmanial potential of tropical rainforest plant extracts. *Medicines*, ***1***, 32-55.

[5] (a) Setzer WN, Setzer MC. (**2003**) Plant-derived triterpenoids as potential antineoplastic agents. *Mini Reviews in Medicinal Chemistry*, ***3***, 540-556; (b) Gallo MBC, Sarachine MJ. (**2009**) Biological activities of lupeol. *International Journal of Biomedical and Pharmaceutical Sciences*, ***3***, 46-66; (c) Fulda S. (**2008**) Betulinic acid for cancer treatment and prevention. *International Journal of Molecular Sciences*, ***9***, 1096-1107.

[6] Domínguez-Carmona DB, Escalante-Erosa F, García-Sosa K, Ruiz-Pinell, Gutierrez-Yapu D, Chan-Bacab MJ, Giménez-Turba A, Peña-Rodríguez LM. (**2010**) Antiprotozoal activity of betulinic acid derivatives. *Phytomedicine*, ***17***, 379-382.

[7] Cubukcu B, Bray DH, Warjurst DC, Mericli AH, Ozhatay N, Sariyar G. (**1990**) In vitro antimalarial activity of crude extracts and compounds from *Artemisia abrotanum* L. *Phytotherapy Research*, ***4***, 203-204.

[8] (a) Mahato SB, Kundu AP. (**1994**) ^{13}C NMR spectra of pentacyclic triterpenoids – A compilation and some salient features. *Phytochemistry*, ***37***, 1517-1575; (b) Ahmad VU, Atta-ur-Rahman. (**1994**) *Handbook of Natural Products Data, Volume 2. Pentacyclic Triterpenoids*. Elsevier, Amsterdam; (c) Haque ME, Shekhar HU, Mohamad AU, Rahman H, Islam AKMM, Hossain MS. (**2006**) Triterpenoids from the stem bark of *Avicennia officinalis*. *Dhaka University Journal of Pharmaceutical Sciences*, ***5***, 53-57.

[9] (a) Pînzaru SC, Leopold N, Kiefer W. (**2002**) Vibrational spectroscopy of betulinic acid HIV inhibitor and of its birch bark natural source. *Talanta*, ***57***, 625-631; (b) Tadesse G, Reneela P, Dekebo A. (**2012**) Isolation and characterization of natural products from *Helinus mystachnus* (Rhamnaceae). *Journal of Chemical and Pharmaceutical Research*, ***4***, 1753-1762.

[10] (a) Tsukamoto H, Hisada S, Nishibe S. (**1985**) Coumarins from bark of *Fraxinus japonica* and *F. mandschurica* var. *japonica*. *Chemical & Pharmaceutical Bulletin*, ***33***, 4069-4073; (b) Gao W, Li Q, Chen J, Wang Z, Hua C. (**2013**) Total synthesis of six 3,4-unsubstituted coumarins. *Molecules*, ***18***, 15613-15623.

[11] Chhetri BK, Dosoky NS, Setzer WN. (**2015**) Cytotoxic norhopene triterpenoids from the bark of *Exothea paniculata* from Abaco Island, Bahamas. *Planta Medica Letters*, ***1***(2) e73.

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A New Cardenolide and Other Compounds from *Salsola tetragona*

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Received: August 3rd, 2016; Accepted: November 10th, 2016

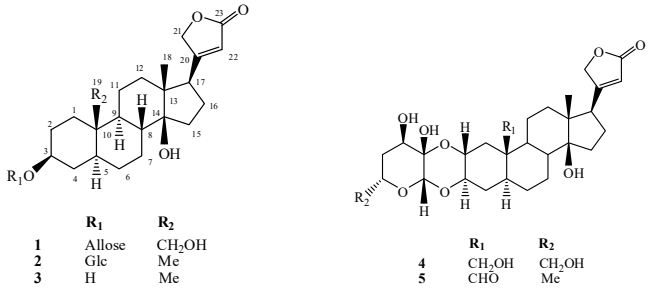
One new cardenolide, 3-*O*-β-D-allopyranosylcoroglaucigenin (salsotetragonin) (**1**), was isolated from the aerial parts of *Salsola tetragona* Delile with four known cardenolides (**2-5**), two known flavonoids (**6-7**), three known phenolic compounds (**8-10**) and two known fatty acids (**11-12**). Their structures were identified by spectroscopic analyses and by comparison of their spectral data with those reported in the literature. Compounds **1-5**, **7**, **10** and **12** were isolated from the genus *Salsola* for the first time. This is the first report on cardenolides identified in the Amaranthaceae family.

Keywords: *Salsola tetragona* Delile, Amaranthaceae, Cardenolides, Phenolic glycosides.

The genus *Salsola* (Amaranthaceae, ex. Chenopodiaceae) consists of over 174 species found in the arid regions of Asia, Europe and Africa [1]. *Salsola* species possess antihypertensive, anti-inflammatory, anticancer, antioxidant, anti-Alzheimer, antidepressant and antimicrobial activities [2-5]. Previous phytochemical investigations of this genus reported the isolation of flavonoids and other phenolic compounds [6], alkaloids [7], fatty acids [8] and triterpene glycosides [9]. In continuation of our works on plants from Algerian Septentrional Sahara [10-11], we report here the isolation and structural characterization of one new cardenolide, 3-*O*-β-D-allopyranosylcoroglaucigenin, named salsotetragonin (**1**), in addition to eleven known compounds from aerial parts of *S. tetragona* (Figure S1).

The hydromethanolic extract of the aerial parts of *S. tetragona* was partitioned successively with light petrolum, CH_2Cl_2, EtOAc and *n*-butanol to give four extracts. Purification of the *n*-butanol extract through repeated silica gel columns, flash chromatography and semi-prep HPLC led to the isolation and identification of a new cardenolide (**1**) and seven known compounds (**3**, **4**, **6-10**). The dichloromethane extract was fractionated by silica gel chromatography to give four known compounds (**2**, **5**, **11-12**). These compounds were identified by extensive spectroscopic methods including 1D-(^{1}H and ^{13}C) and 2D-NMR (COSY, HSQC, HMBC and NOESY) experiments, as well as HR-ESI-MS analysis and by comparison of their spectral data with the literature. The eleven known compounds were identified as four cardenolides: uzarigenin (**2**) [12], desglucouzarin (**3**) [13], 12-dehydroxy-ghalakinoside (**4**), and calactin (**5**) [14] (Figure 1), two flavonoids: kaempferol-3-*O*-β-D-glucopyranoside (**6**) [15] and quercetin-3-*O*-β-D-glucopyranosyl-(1→6)-glucopyranoside (**7**) [16], three phenolic compounds: vanillic acid (**8**) [17], canthoside C (**9**), and canthoside D (**10**) [18] and two fatty acids : oleic acid (**11**) [19], and 2,3-dihydroxypropylpalmitate (**12**) [20] (Figure S1).

Compound **1** was isolated as a white amorphous powder. Its positive HR-ESI-MS showed a pseudomolecular ion peak at *m/z* 575.2829 $[M+Na]^+$, corresponding to the molecular formula $C_{29}H_{44}O_{10}$. The



	R_1	R_2
1	Allose	CH_2OH
2	Glc	Me
3	H	Me

	R_1	R_2
4	CH_2OH	CH_2OH
5	CHO	Me

Figure 1: Structures of compounds **1-5**.

^{1}H NMR spectrum of compound **1** (Table 1) showed characteristic signals of a butenolactone ring at δ_H 4.95 (1H, dd, *J* =18.4, 1.6 Hz, H-21a), 5.07 (1H, dd, *J* = 18.4,1.4 Hz, H-21b) and 5.93 (1H, s, H-22), one oxymethylene at δ_H 3.74 (1H, d, *J* = 11.7 Hz, H-19a) and δ_H 3.88 (1H, d, *J* = 11.7 Hz, H-19b), one oxymethine at δ_H 3.78 (m, H-3), and one methyl signal at δ_H 0.95 (3H, s, H-18). Its ^{13}C NMR spectrum (Table 1) showed twenty-nine carbon signals including six of the sugar part (Figure 1).

The ^{1}H NMR and ^{13}C NMR data of **1** were very similar to those of 3-β-D-glucopyranosylcoroglaucigenin [21]. The COSY and HSQC spectra showed one anomeric proton of a sugar moiety at δ_H 4.78 (d, *J* = 7.9 Hz, H-1′) and δ_C 99.8 with five signals of an hexose unit [H-2′ [δ 3.28 (dd, *J* = 7.9, 3.0 Hz)], H-3′ [δ 4.06 (t, *J* = 3.0 Hz)], H-4′ [δ 3.48 (dd, *J* = 9.5, 3.0 Hz)], H-5′ [δ 3.69 (m)], and H_2-6′, [δ 3.66 (dd, *J* = 11.3, 5.7 Hz) and 3.85 (dd, *J* = 11.3, 1.8 Hz)]; the sugar was identified as an allopyranoside [22]. The large coupling constant (*J* = 7.9 Hz) of the anomeric proton at δ_H 4.78 indicated that the allose had a β-configuration. The linkage of the allose at C-3 of the aglycone was established from the HMBC correlation between allo-H-1′ (δ_H 4.78) and C-3 (δ_C 78.9) (Figure 2). The stereochemistry of **1** was confirmed by the NOE effect (Figure 2) between H-5/H-9 and H-3, which clearly established the α-orientation of H-5.The correlation of H_3-18 to H-21 and H-22 in

the NOESY spectrum indicated the β orientation of the γ lactone at C-17. The chemical shifts of the carbons of the C and D rings of **1** are in agreement with those of coroglaucigenin and madagascarrensilid (A) recorded in deuterated pyidine and in CD_3OD [23,24], indicating a 14-β hydroxy orientation. Thus compound **1** was identified as 3-*O*-β-D-allopyranosyl-coroglaucigenin, which we named salsotetragonin.

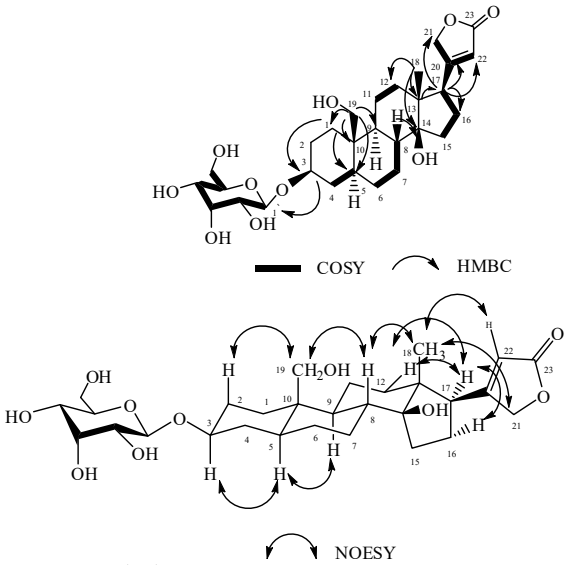


Figure 2: ^1H–^1H COSY, HMBC and NOESY correlations of **1**.

Table 1: ^1H and ^{13}C NMR data for compound **1** (in CD_3OD; ^{13}C: 150 MHz; ^1H: 600 MHz).

Position	**1**	
	δ_H (m, *J* in Hz)	δ_C
1	0.81 (td, 13.6, 3.5), 2.34 (dt, 13.3, 3.5)	32.8
2	1.57 (dm, 14.9), 1.91 (m)	30.9
3	3.78 (m)	78.9
4	1.44 (m), 1.82 (dd, 11.9, 3.4)	35.9
5	1.24 (m)	45.9
6	1.24 (m), 1.34 (m)	29.6
7	2.10 (dd, 12.3, 3.1), 1.15 (m)	28.8
8	1.82 (td, 11.9, 3.4)	43.1
9	1.03 (td, 13.5, 4.5)	51.5
10	-	40.6
11	1.65 (m)	24.1
12	1.41 (td, 13.6, 4.3), 1.53 (dt, 13.5, 3.0)	41.5
13	-	51.2
14	-	86.3
15	1.73 (dd, 11.6, 8.5), 2.14 (m)	33.5
16	2.16 (m), 1.90 (m)	28.1
17	2.85 (dd, 9.5, 5.8)	52.2
18	0.95 (s)	16.7
19	3.74 (d, 11.7), 3.88 (d, 11.7)	59.9
20	-	178.5
21	4.95 (dd, 18.4, 1.6), 5.07 (dd, 18.4, 1.4)	75.3
22	5.93 (s)	117.8
23	-	176.9
Allose (at C-3)		
1′	4.78 (d, 7.9)	99.8
2′	3.28 (dd, 7.9, 3.0)	72.4
3′	4.06 (t, 3.0)	73.1
4′	3.48 (dd, 9.5, 3.0)	69.1
5′	3.69 (m)	75.5
6′	3.66 (dd, 11.3, 5.7), 3.85 (dd, 11.3, 1.8)	63.3

Experimental

General: Optical rotations were measured in DMSO with a Perkin-Elmer 241 polarimeter. ^1H and ^{13}C NMR spectra were recorded on a Bruker Avance DRX III 500 spectrometer (^1H at 500 MHz and ^{13}C at 125 MHz) and on a Bruker Avance III-600 spectrometer equipped with a cryo platform (^1H at 600 MHz and ^{13}C at 150 MHz). 2D-NMR experiments were performed using standard Bruker-microprograms (TopSpin version 3.2 software). HR-ESI-MS and ESI-MS experiments were performed using a Micromass Q-TOF micro-instrument (Manchester, UK). TLC was carried out on pre-coated silica gel 60 F_{254} (Merck). Vacuum liquid chromatography (VLC) was carried out on LiChroprep RP-18 (40-63 μm, Merck). HPFC was performed on a Grace® Reveleris System using Grace® cartridges (Silica gel or RP-C_{18}). The semi-preparative HPLC was performed on a Dionex apparatus equipped with an ASI-100 automated sample injector, a STH 585 column oven, a LPG-3400A pump, a diode array detector UVD-340U and Chromeleon® software.

Plant material: *Salsola tetragona* Delile was collected in Ghardaïa (Algerian Septentrional Sahara) in May 2011 and was identified by Pr. Gérard De Belair (University of Annaba, Algeria). A voucher specimen (LOST.St05/09) was deposited at the herbarium of the Faculty of Sciences, University frères Mentouri- Constantine.

Extraction and isolation: Air-dried and powdered aerial parts (1980 g) of *S. tetragona* were extracted with 80% MeOH (10 L). After evaporating the MeOH under *vacuum*, the residue was dissolved in water (900 mL) and partitioned with PE, CH_2Cl_2, EtOAc, and *n*-BuOH, successively (3 x 300 mL). The *n*-BuOH extract (6 g) was subjected to VLC over RP-18 eluted with H_2O-MeOH (6:4, 5:5, 4:6, 3:7, 2:8 and 0:10). Fractions of 150 mL were collected and pooled according to their similarity in TLC profile to give 6 fractions (F1–F6 respectively). F1 (1.2 g) was submitted to RP-18 flash chromatography eluting with MeCN:H_2O (5:95 to 10:0) to afford 19 sub-fractions. Sub-fraction [20-23] (35 mg), was purified by semi-prep HPLC on RP-18 eluted with MeCN:H_2O (6:94 to 1:9), affording compounds **9** (3 mg) and **10** (1.5 mg). F2 (1.5 g) was chromatographed on a silica gel column eluted with EtOAc:MeOH:H_2O (20:2:1), giving 2 main sub-fractions. Sub-fraction [90] (180 mg) afforded **4** (22 mg) by silica gel CC ($CHCl_3$:MeOH, 9:1). Sub-fraction [115-119] (223 mg) was subjected to silica gel CC eluting with $CHCl_3$:MeOH (8:2) to afford **1** (27 mg) and sub-fraction [35-48] (100 mg), which was purified over silica gel using EtOAc:MeOH:H_2O (20:2:1) to yield compounds **6** (15 mg) and **7** (18 mg). F3 (750 mg) was fractionated by silica gel CC eluted with $CHCl_3$:MeOH (0 to 100% of MeOH). The main sub-fraction [59-63] (212 mg) was further separated by silica gel CC ($CHCl_3$:MeOH, 0 to 100% of MeOH) and **3** (25 mg) was isolated. F4 (302 mg) was subjected to silica gel CC using $CHCl_3$:MeOH (0 to 100% of MeOH) to give 3 sub-fractions. Sub-fraction [72] (111.2 mg) was selected for silica gel CC eluted with $CHCl_3$:MeOH (0 to 100% of MeOH) to yield compound **8** (3 mg).

The CH_2Cl_2 extract (2.5 g) was subjected to silica gel CC, eluted with toluene:$CHCl_3$ (0 to 100% of $CHCl_3$) and $CHCl_3$:EtOAc (0 to 100% of EtOAc). Sub-fraction [27-32] (123 mg) was purified over silica gel using diethyl ether:EtOAc (9:1) to yield compound **11** (1.4 mg). Sub-fraction [160-173] (212 mg) was passed through a silica gel column ($CHCl_3$:EtOAc, 9:1) yielding compounds **2** (15 mg) and **5** (5 mg). Sub-fraction [234-257] (162 mg) was chromatographed on silica gel, eluted with ($CHCl_3$:MeOH, 9:1) that afforded compound **12** (2 mg).

Acid hydrolysis: The pure compounds were treated with 2 M HCl at 100°C for 1 h. The hydrolysates were extracted with EtOAc and the sugars identified in the aqueous residue by comparison with authentic samples by TLC using silica gel impregnated with 0.2 M NaH_2PO_4 and a solvent system of Me_2CO–H_2O (9:1); the compounds were revealed with aniline malonate. The optical rotation of the purified sugar was measured and compared with an authentic sample of D-allose.

3-*O*-β-D-Allopyranosylcoroglaucigenin (salsotetragonin) (1)
White amorphous powder.
$[\alpha]_D^{20}$: -16.9 (*c* 0.18, DMSO).
^{1}H NMR (CD_3OD, 600 MHz): Table 1.
^{13}C NMR (CD_3OD, 150 MHz): Table 1.
HR-ESI-MS: $[M+Na]^+$ *m/z* 575.2829 (calcd for $C_{29}H_{44}O_{10}Na$, 575.2832).

Acknowledgments - The authors would like to thank the DGRSDT-MESRS (Algeria) for financial support and the Groupe Isolement et Structure of the Institut de Chimie Moleculaire de Reims (France) for technical help and registering of NMR data and MS.

Supplementary data: Figure S1 and Supplementary Data are included in the Supporting information.

References

[1] Botschantzev V. (**1974**) A synopsis of *Salsola* (Chenopodiaceae) from South and South-West africa. *Kew Bulletin, **29***, 597-614.
[2] Ahmad Z, Mehmood S, Fatima I, Malik A, Ifzal R, Afza N, Iqbal L, Latif M, Nizami TA. (**2008**) Structural determination of salsolins A and B, new antioxidant polyoxygenated triterpenes from *Salsola baryosma* by 1D and 2D NMR spectroscopy. *Magnetic Resonance in Chemistry*, ***46***, 94-98.
[3] Woo WS, Lee EB, Chi HJ. (**1977**) Biological evaluation of some Saudi Arabian plants. *Journal* of *Korean Pharmaceutical Sciences, **21***, 141-145.
[4] Oueslati MH, Ben JH, Mighri Z, Chriaa J, Abreu PM. (**2006**) Phytochemical constituents from *Salsola tetrandra*. *Journal of Natural Products*, ***69***, 1366-1369.
[5] Tundis R, Loizzo MR, Statti GA, Menichini F. (**2007**) Inhibitory effects on the digestive enzyme alpha-amylase of three *Salsola* species (Chenopodiaceae) *in vitro*. *Pharmazie*, ***62***, 473-475.
[6] Xiang Y, Li YB, Zhang J, Li P, Yao YZ. (**2007**) Studies on chemical constituents of *Salsola collina*. *Zhongguo Zhong Yao Za Zhi*, ***32***, 409-413.
[7] Xiang Y, Li YB, Zhang J, Li P, Yao YZ. (**2007**) A new alkaloid from *Salsola collina. Yao Xue Xue Bao*, ***42***, 618-620.
[8] Jin YS, Du JL, Yang Y, Jin L, Song Y, Zhang W, Chen HS. (**2011**) Chemical and biologically active constituents of *Salsola collina*. *Chemistry of Natural Compounds*, ***47***, 257-260.
[9] Hamed AI, Masullo M, Sheded MG, Mahalel UA, Tawfik MM, Perrone A, Piacente S. (**2011**) Triterpene saponins from *Salsola imbricata*. *Phytochemistry Letters*, ***4***, 353-356.
[10] Boudiar T, Lakhal H, Khalfallah A, Kabouche A, Kabouche Z, Brouard I, Bermejo J, Christian B. (**2010**) A new alkaloid and flavonoids from the aerial parts of *Euphorbia guyoniana. Natural Product Communications*, ***5,*** 35-37.
[11] Lakhal H, Boudiar T, Kabouche A, Kabouche Z, Touzani R, Christian B. (**2010**) New sesquiterpene lactone and other constituents from *Centaurea sulphurea* (Asteraceae). *Natural Product Communications*, ***5***, 849-850.
[12] Shaker KH, Morsy N, Zinecker H, Imhoff JF, Schneider B. (**2010**) Secondary metabolites from *Calotropis procera* (Aiton). *Phytochemistry Letters*, ***3***, 212-216.
[13] Elgamal M, Hani A, Hanna AG, Morsy NAM, Duddeck H, Simon A, Gati T, Toth G. (**1999**) Complete ^{1}H and ^{13}C signal assignments of 5α cardenolides isolated from *Calotropis procera* R. B.R. *Journal of Molecular Structure*, ***477***, 201-208.
[14] Hamed AI, Plaza A, Balestrieri ML, Mahalel UA, Springuel IV, Oleszek W, Pizza C, Piacente S. (**2006**) Cardenolide glycosides from *Pergularia tomentosa* and their proapoptotic activity in Kaposi's sarcoma cells. *Journal of Natural Products*, ***69***, 1319-1322.
[15] Berrehal D, Khalfallah A, Kabouche A, Kabouche Z, Karioti A, Bilia AR. (**2010**) Flavonoid glycosides from *Randonia africana* Coss. (Resedaceae). *Biochemical Systematics and Ecology*, ***38***, 1007-1009.
[16] Shehab NG, Mahdy A, Khan SA, Noureddin SM. (**2011**) Chemical constituents and biological activities of *Fagonia indica* Burm F. *Research Journal* of *Medicinal Plant*, ***5***, 531-546.
[17] Wang XJ, Zhao YX, Jia XH, Ding XB. (**2011**) Studies on the chemical constituents of *Salsola collina*. *Zhong yao cai*, ***34***, 230-231.
[18] Kanchanapoom T, Kasai R, Yamasaki K. (**2002**) Iridoid and phenolic diglycosides from *Canthium berberidifolium*. *Phytochemistry*, ***61***, 461-464.
[19] Turki ZA. (**2000**) Chemotaxonomical studies of the genus *Salsola* (Chenopodiaceae) in Egypt. *Egyptian Journal of Botany*, ***38***, 47-61.
[20] Li Y, An K, Sun J, Wang J, Zhong Y, Sun L. (**2011**) Chemical constituents of vinegar-processed *Kansui radix*. *Food and Drug*, ***13***, 183-186.
[21] Jolad SD, Bastest RB, Cole JR, Hoffmann JJ, Siahaan TJ, Timmerann BN. (**1986**) Cardenolides and a lignan from *Asclepias subulata*. *Phytochemitry*, ***25***, 2581-2590.
[22] Murakami T, Emoto A, Matsuda H, Yoshikawa M. (**2001**) Medicinal foodstuffs. XXI. Structures of new cucurbitane-type triterpene glycosides, goyaglycosides-a, -b, -c, -d, -e, -f, -g, and -h, and new oleanane-type triterpene saponins, goyasaponins I, II, and III, from the fresh fruit of Japanese *Momordica charantia* L. *Chemical and Pharmaceutical Bulletin*, ***49***, 54-63.
[23] Yamauchi T, Abe F, Nish M. (**1978**) ^{13}C NMR of 5α-cardenolides. *Chemical and Pharmaceutical Bulletin*, ***26***, 2894-2896.
[24] Pan E, Harinantenaina L, Brodie PJ, Callmander M, Rakotonandrasana S, Rakotobe E, Resamison VE, TenDyke K, Shen Y, Suh EM, Kingston DGI. (**2011**) Cardenolides of *Leptadenia madagascariensis* from the Madagascar dry forest. *Bioorganic and Medicinal Chemistry*, ***19***, 422-428.

NPC Natural Product Communications

2017
Vol. 12
No. 1
7 - 10

Quantification of Saponins in *Asparagus racemosus* by HPLC-Q-TOF-MS/MS

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Received: October 1st, 2016; Accepted: November 30th, 2016

Asparagus racemosus Willd. or Shatavari (Asparagaceae family) is an important medicinal plant in Ayurvedic medicine as a rejuvenate for women. A method for quantitative analysis of saponin glycosides bioactive constituents in *A. racemosus* is reported. A high performance liquid chromatography quadrupole time of flight mass spectrometry (HPLC-Q-TOF-MS/MS) method was developed and validated for simultaneous determination of five saponin glycosides, asparacoside, shatavarin IX, shatavarin IV, asparanin A and shatavarin V in *A. racemosus* extracted with 70% MeOH. The method was validated through intra- and inter-day precision, with the relative standard deviation (RSD) less than 6%, limits of detection (LOD) and limits of quantification (LOQ) <10 and 50 ng, respectively. Overall recoveries ranged from 95% to 105%, with RSD ranging from 0.7% to 4.5%. The method was applied to saponin glycoside contents in the leaves, stems, and roots of *A. racemosus* sourced from different geographical locations, including four provinces in Thailand, and a sample from India. Saponin glycosides were detected predominantly in the roots, the part used in traditional medicines and these showed wide variations in saponin glycoside profiles from undetectable to 12 mg/g dry weight. The quality control of *A. racemosus* is crucial for reliable and predictable therapies and only methods like the one developed has the necessary flexibility, sensitivity, accuracy, and selectivity for reliable routine quality control.

Keywords: Steroidal saponin, *Asparagus racemosus*, LC-MS/MS, HPLC-Q-TOF-MS/MS, Quantitative analysis.

Asparagus racemosus Willd is among 300 species classified in the family Asparagaceae. The plant is called Shatavari which means "one who possesses a hundred husbands or acceptable to many", implying its ability to increase fertility and vitality [1]. The plant is commonly found in tropical and subtropical forests in India, Thailand and elsewhere. Its medicinal uses are recorded in Indian and British Pharmacopoeias, and in indigenous systems including Ayurveda, and Thai traditional medicine. The powdered dried root of *A. racemosus* is used in Ayurveda to prevent and for the treatment of gastric ulcers [2] and galactogogue [3] and in Thailand as a galactogogue, to induce menstruation, and as a cosmetic tonic. Contemporary studies reveal estrogenic [4], galactagogic [3] effects in animals, anti-fungal [5], anti-diarrheal [6], anti-oxidant [7], and anti-cancer [8] actions *in vitro*.

Steroidal saponins found throughout the plant is characteristic of the *Asparagus* genus and used for chemotaxonomic classification. Many saponin glycosides have been isolated from *A. racemosus* roots and fruits including shavatarins (I, V-X) [9], asparanins [10], asparosides [10], curillins [10], curillosides [10], immunoside [11], racemoside A [12] and shatavaroside C [12]. Among these, Shatavarin I and IV were reported as the major components [9b] in *A. racemosus* collected in India. These saponin glycosides are responsible for *A. racemosus* medicinal affects including, anticancer activity. Immunoside and asparanin A induced apoptosis of cultured tumor cells [8] while shatavarin IV was cytotoxic to several cancer cell lines [13]. Racemoside A, a water-soluble saponin glycoside was a selective and potent inducer of apoptosis in drug resistant donovani promastigotes [14]. We also demonstrated *in vitro* cytotoxicity of asparacoside, shatavarin IX, shatavarin IV, asparanin A and shatavarin V against human hepato- and prostate-carcinoma cell lines [15].

Clearly, these compounds have considerable clinical potential but there lacks a sensitive and reliable way of quantitating these saponin glycosides in crude *A. racemosus* preparations from different sources. The high performance thin layer chromatography (HPTLC) [16] and high performance liquid chromatography (RP-HPLC) [17] with UV detection showed low sensitivity as the saponin glycosides lack chromophores. Recently, we have developed competitive enzyme-linked and immunosorbent assay (ELISA) using monoclonal antibody against shatavarin IV [18] but the method showed cross-reactivity between glycosides sharing the same aglycone. To address these problems, a HPLC-Q-TOF-MS/MS method was developed such that compounds could be identified and quantitated down to 25 ng through a single step extraction process. This provides a quick and easy testing method for quality control as a herbal medicine.

Asparacoside, shatavarin IX, shatavarin IV, asparanin A and shatavarin V were quantitated by HPLC-Q-TOF-MS/MS in the negative ion mode. Their structures, *m/z,* and retention times are shown in Figure 1 and their total ion current (TIC) chromatograms in Figure 2a. The fragmentation pattern of each saponin glycoside shows sequential loss of single monosaccharides leaving one glucose attached to aglycone at *m/z* 577 $[M+Glc-H]^-$ while the

aglycone is undetectable. Peaks corresponding to monosaccharides, glucose (*m/z* 161), rhamnose (*m/z* 145) and arabinose (*m/z* 131) are apparent (Figure 3). The method was validated for linearity, LOQs and LODs, precision, and recovery based on the International Conference on Harmonization (ICH) Guidelines [19] (Tables 1-3). Standard curves of the five saponin glycosides had acceptable linearity in the range of 2.50 - 30.00 µg/mL with correlation coefficients exceeding 0.997. The minimum concentration levels at which analysts could be reliably detected (LODs) and quantified (LOQs) were 5 to 10 ng and 25 to 50 ng, respectively (Table 1). The % RSDs of intra- and inter-day precisions were less than 4% and 6%, respectively (Table 2) and deemed acceptable according to the Horwitz's equation [20] (for analysts at concentration of 6 µg/mL, %RSD should be <12; for 15 µg/mL analyses, RSD should be <11 and for 25 µg/mL analysts, RSD should be <10). For overall accuracy recoveries ranged from 95% to 105%, with RSDs ranging from 0.7 – 4.5 %, again within ICH acceptable values [19] (Table 3). This suggests that sample extraction was efficient. Thus, the method developed is reliable and accurate for quantitation of the five saponin glycosides in *A. racemosus*.

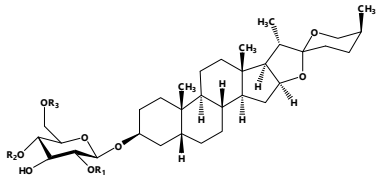
Five saponin glycosides in different parts of *A. racemosus* from five different growing locations were quantified. None of the saponin glycosides were found in the leaves and stems of all *A. racemosus* tested except for trace amounts (less than LOQ) of asparacoside in the Bangkok sample. For root samples, saponin glycoside contents were diverse. Thus asparacoside from Phetchabun had 12 mg/g but

Table 1: Validation parameters of the five saponin glycoside standards using HPLC-Q-TOF-MS/MS

Saponin	[M-H]$^-$	[a]Regression equation (n=3)	Linearity (R^2)	Linear range (µg/mL)	[b]LOD (ng)	[c]LOQ (ng)
Asparacoside	1003.4966	y = 13.20.x-30.43	0.999	5.00 – 30.00	10	50
Shatavarin IX	901.4766	y = 10.13.x-13.75	0.999	2.50 – 30.00	5	25
Shatavarin IV	885.4745	y = 17.79.x-3.16	0.999	2.50 – 30.00	5	25
Asparanin A	739.4162	y = 10.16.x-2.26	0.998	2.50 – 30.00	5	25
Shatavarin V	885.4721	y = 9.47.x-14.95	0.997	2.50 – 30.00	10	25

[a]y is the peak area; x standard solution concentrations, non-weighted. R^2 is the determination coefficient. [b]LOD, limits of detection, S/N = 3.[c]LOQ, limits of quantitation, S/N = 10.

was undetectable in the Indian sample. Other compounds were also erratically distributed (Table 4). Shatavarin IV known to dominate the Indian varieties [9b] was not found in any Thai samples. The Rayong root sample contained most compounds (Table 4, Fig 2b). The reason for this diversity apparently is not genomic because genetic variation in plants harvested at 9 diverse Thai locations [21] and 7 Indian sites [22] showed little variation. This strongly suggests that the growing conditions play a major role in expression of these saponins [23]. It also implies that horticultural conditions can be optimized to produce the most biologically active components.



Compound	Formula	R_1	R_2	R_3	RT, min	[M-H]$^-$
Asparacoside	$C_{49}H_{80}O_{21}$	β-D-glucose	α-L-arabinose	α-L-arabinose	5.38	1003.4966
Shatavarin IX	$C_{45}H_{74}O_{18}$	β-D-glucose	β-D-glucose	H	6.70	901.4766
Shatavarin IV	$C_{45}H_{74}O_{17}$	β-D-glucose	α-L-rhamnose	H	7.04	885.4745
Asparanin A	$C_{39}H_{64}O_{13}$	β-D-glucose	H	H	8.06	739.4162
Shatavarin V	$C_{45}H_{74}O_{17}$	α-L-rhamnose	β-D-glucose	H	8.43	885.4721

Figure 1. Structures of saponin glycosides in *A. racemosus* root with their molecular formula, molecular weights as well as LC/MS retention time and [M-H]$^-$

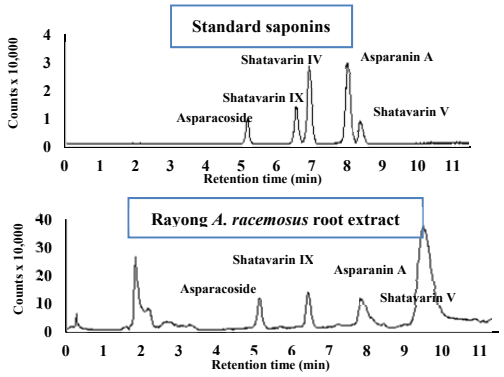


Figure 2. TIC chromatogram of (**a**) five saponin glycosides at concentration 10 µg/mL (**b**) *A. racemosus* root from Rayong, Thailand in 70% MeOH at concentration 20 mg/mL obtained from HPLC-Q-TOF-MS/MS analysis in negative mode.

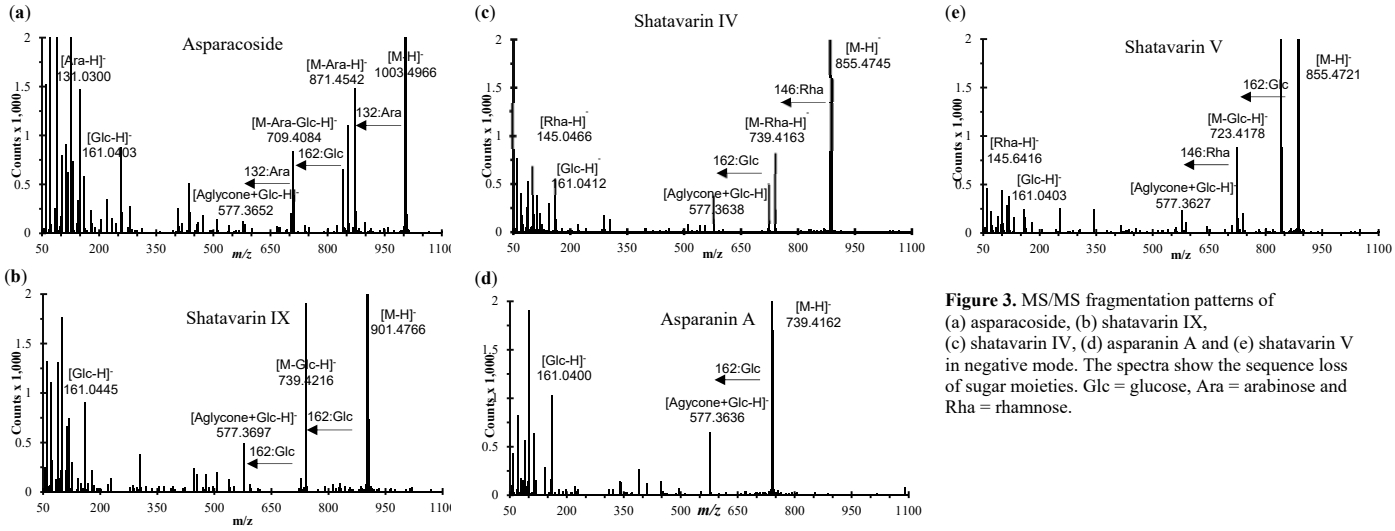


Figure 3. MS/MS fragmentation patterns of (a) asparacoside, (b) shatavarin IX, (c) shatavarin IV, (d) asparanin A and (e) shatavarin V in negative mode. The spectra show the sequence loss of sugar moieties. Glc = glucose, Ara = arabinose and Rha = rhamnose.

Table 2: Intra- and inter-day precisions of the five saponin glycoside standards by HPLC-Q-TOF-MS/MS.

Saponin	Spiked Conc. (µg/mL)	Intraday (n=5)			Interday (n=15)		
		Measured Conc .(µg/mL)	% Accuracy	% RSD	Measured Conc. (µg/mL)	% Accuracy	% RSD
Asparacoside	6	6.3 ± 0.14	104.1 ± 2.38	2.3	6.2 ± 0.14	103.3 ± 2.26	2.2
	15	15.4 ± 0.22	102.6 ± 1.44	1.4	15.6 ± 0.37	104.2 ± 2.48	2.4
	25	25.6 ± 0.12	102.2 ± 0.46	0.5	26.0 ± 0.47	104.1 ± 1.86	1.8
Shatavarin IX	6	6.1 ± 0.19	102.1 ± 3.15	3.1	6.1 ± 0.31	101.3 ± 5.15	5.1
	15	15.4 ± 0.32	102.5 ± 2.10	2.1	15.6 ± 0.72	104.2 ± 4.82	4.6
	25	25.7 ± 1.05	102.8 ± 4.19	4.1	25.7 ± 0.66	102.9 ± 2.63	2.6
Shatavarin IV	6	5.9 ± 0.14	97.8 ± 2.31	2.4	6.2 ± 0.29	103.4 ± 4.89	4.7
	15	15.5 ± 0.35	103.2 ± 2.32	2.3	15.5 ± 0.50	103.0 ± 3.35	3.3
	25	26.2 ± 0.80	104.7 ± 3.18	3.0	26.2 ± 0.51	104.8 ± 2.03	1.9
Asparanin A	6	6.3 ± 0.17	104.3 ± 2.81	2.7	6.2 ± 0.34	102.5 ± 5.61	5.5
	15	15.6 ± 0.38	104.1 ± 2.56	2.5	15.7 ± 0.47	104.8 ± 3.16	3.0
	25	25.9 ± 0.42	103.6 ± 1.70	1.6	25.9 ± 0.76	103.5 ± 3.03	2.9
Shatavarin V	6	6.1 ± 0.24	101.1 ± 4.04	4.0	6.0 ± 0.29	100.7 ± 4.79	4.8
	15	15.6 ± 0.52	103.7 ± 3.45	3.3	15.6 ± 0.39	104.1 ± 2.59	2.5
	25	25.4 ± 0.71	101.6 ± 2.85	2.8	26.0 ± 0.79	103.9 ± 3.15	3.0

Table 3: The accuracy HPLC-Q-TOF-MS/MS method for quantitative analysis of five saponin glycosides when three concentrations of standard saponin glycosides were spiked into the *A. racemosus* root from Bangkok.

Saponin	Measured concentrations of saponins (µg/mL) (n=3)			Recovery (%)	RSD (%)
	Spiked	Original	Detected		
Asparacoside	6	21.5 ± 0.21	26.2 ± 1.03	95.2 ± 4.13	4.3
	15	24.2 ± 0.26*	39.2 ± 0.55*	100. 0 ± 1.21	1.2
	25	24.3 ± 0.23*	52.9 ± 0.71*	105.0 ± 0.75	0.7
Shatavarin IX	6	-	6.2 ± 0.14	103.9 ± 2.30	2.2
	15	-	15.6 ± 0.51	104. 3 ± 3.39	3.3
	25	-	26.2 ± 0.52	104.9 ± 2.07	2.0
Shatavarin IV	6	-	5.9 ± 0.27	98.7 ± 4.46	4.5
	15	-	15.7 ± 0.21	104.4 ± 1.43	1.4
	25	-	25.7 ± 0.32	102.7 ± 1.27	1.2
Asparanin A	6	-	6.2 ± 0.19	104.0 ± 3.22	3.1
	15	-	15.5 ± 0.21	103.1 ± 1.42	1.4
	25	-	26.2 ± 0.38	104.9 ± 1.54	1.5
Shatavarin V	6	-	6.2 ± 0.13	103.4 ± 2.09	2.0
	15	-	15.1 ± 0.45	100.5 ± 3.00	3.0
	25	-	24.8 ± 0.19	99.0 ± 0.74	0.8

* Samples were diluted for 3 times before injected to LC-MS.

The five saponin glycosides have the same aglycone (sarsasapogenin), but differ in monosaccharide moieties. Their bioactive properties require the aglycone but the nature of the monosaccharide residue influences potency as shown by tumor cell susceptibility to cytotoxic actions [24]. Therefore, the saponin profile of *A. racemosus* could dictate its potency and pharmacology and thus have unpredictable therapeutic outcomes. Only the types of methods described here, especially HPLC-Q-TOF-MS/MS, have the flexibility, sensitivity, accuracy, and selectivity for reliable routine quality control.

Table 4: The percentage of five saponin glycosides in roots of *A. racemosus* collected from different locations (n=3).

Sample	Saponin glycosides (mean ± SD, mg/g)				
	Asparacoside	Shatavarin IX	Shatavarin IV	Asparanin A	Shatavarin V
Bangkok	1.1 ± 0.01	nd	nd	nd	nd
Phetchabun	12.3 ± 1.30	< 0.13	nd	nd	nd
Phitsanulok	1.2 ± 0.06	0.5 ± 0.05	nd	nd	nd
Rayong	2.6 ± 0.09	2.5 ± 0.13	nd	0.3 ± 0.02	0.3 ± 0.02
India	nd	0.2 ± 0.01	3.1 ± 0.09	nd	0.4 ± 0.02

*nd: not detected

The quantitative analysis of five saponin glycosides in *A. racemosus* was investigated by HPLC-Q-TOF-MS/MS for the first time. The extremely diverse expression of the compounds from plants taken from different location, highlights the importance of quality control when applied to clinical treatments. Only analyses as described here have the required attributes to provide the all-important quality control of *A. racemosus* formulations for such applications.

Experimental

Chemicals and reagents: MeOH, acetonitrile and water used for LC-MS/MS were LC-MS grades (RCI Labscan, Thailand). Sterile water was purchased from A.N.B. Laboratories Co., Ltd., Thailand. Analytical grade formic acid was purchased from Fisher Scientific (UK), standard grade shatavarin IV from Natural Remedies Pvt. Ltd. (India). Asparacoside, shatavarin IX, asparanin A and shatavarin V were isolated from *A. racemosus* extract as described previously [15] and their structures and purity were confirmed by NMR and MS.

Plant material and sample preparation: *A. racemosus* collected from Bangkok, Phetchabun, Phitsanulok and Rayong Provinces in Thailand and identified by Prof. Wongsatit Chuakul, Faculty of Pharmacy, University of Mahidol, Bangkok, Thailand. The plants were separated into leaves, stems and roots, cut and each dried separately at 45 °C for 3 days before milling into a fine powder. Indian *A. racemosus* dried root powder was purchased from Classic Ayurveda Herbal Products, Aurangabad, India. To extract the plant material, 1 mL of 70% (v/v) MeOH in water was added to 20 mg of each of the fine dried powders of leaf, stem or root samples, and sonicated in a TranssonicTP690 (Elma, Germany) for 15 min at maximum power, filtered through a 0.45 µm syringe filter and injected (10 µL) into the LC-MS system. All samples were prepared in triplicate.

Standard solution preparation: Asparacoside, shatavarin IX, shatavarin IV, asparanin A and shatavarin V were used as saponin glycosides standards in this study. Stock solutions of 30 µg/mL in 70% (v/v) MeOH were appropriately diluted with 70% MeOH to construct standard calibration curves, limits of detection (LOD), and limits of quantification (LOQ) for the five saponins All solutions were stored at 4 ^{o}C until analysis, sample injection volumes 10 µL.

Saponin analysis by HPLC-Q-TOF-MS/MS: Separation used a Phenomenex Luna C8 column (100ºA, 5µm, 150 x 4.6 mm, Phenomenex USA) connected with a SecurityGuard^{TM} C8 guard column (100Å, 5µm, 4 x 3 mm, Phenomenex, USA) and the column temperature was held at 35 ºC. The mobile phase was composed of 0.1% formic acid in water (eluent A) and 0.1% formic acid in acetonitrile (eluent B). The gradient program was: 50:50% eluents A/B at the beginning, then 40:60% A:B for 5 min, then linearly decreased to 10:90% (A/B) over the next 4 min, and held for 3 min. The flow rates were 0.6 mL/min. The liquid chromatograph was coupled to a Q-TOF mass spectrometer with a dual electrospray ion (ESI) source (Agilent Technologies G6540B model, Agilent Technologies, Singapore). The MS parameters were: acquisition-mass range set at 100-1200 *m/z* with a 250 ms/spectrum, the drying gas was N_2 at 350 °C, flowing at 10 L/min, nebulizer pressure 20 psi, capillary voltage 3500 V, the fragmentor voltage 250 V, skimmer 65 V, octapole RFV 750 V. The target MS/MS was set up with collision energy at 10, 20 and 40 V. All the acquisition and analyses of data were controlled by Agilent LC-MS-Q-TOF MassHunter Data Acquisition Software version B.05.01 and Agilent MassHunter Qualitative Analysis Software B 06.0, respectively (Agilent Technologies, USA). Determinations were in both negative and positive ion modes but the negative mode gave higher peaks and was used to quantitate saponin glycosides.

Method validation: The established HPLC-Q-TOF-MS/MS method was evaluated by linearity, LOD, LOQ, intraday and interday precision, and accuracy tests. The calibration curves were constructed with five concentrations of saponin glycoside standards in duplicate. The LOD and LOQ were measured under the LC analytical conditions at a signal-to-noise (S/N) ratio of 3 and 10,

respectively. The repeatability (intraday precision), used three concentrations, (6, 15 and 25 µg/mL) measured 5-times within one day and interday precision by repeating measurements 5x per day for three consecutive days. Matrix effects and accuracy were determined using the *A. racemosus* root from Bangkok by spiking 20 mg of powders with 1 mL of 70% MeOH containing appropriate concentrations of standards (Table 3), and repeated in triplicate. After 15 min of sonication, filtrates were extracted and quantified as above.

Acknowledgments - This work was support by the Royal Golden Jubilee Ph.D. Program (PHD/0298/2552), Naresuan University (R2557B018), and the Center of Excellence for Innovation in Chemistry (PERCH-CIC), Office of Higher Education, Ministry of Education, Thailand. We would like to thank Dr. C. Norman Scholfield, Faculty of Pharmaceutical Science, Naresuan University for his assistance in the manuscript preparation.

References

[1] Bopana N, Saxena S. (**2007**) *Asparagus racemosus*-Ethnopharmacological evaluation and conservation needs. *Journal of Ethnopharmacology*, ***110***, 1-15.

[2] (a) Goel R, Sairam K. (**2002**) Anti-ulcer drugs from indigenous sources with emphasis on *Musa sapientum*, *Tamrahbasma*, *Asparagus racemosus* and *Zingiber officinale*. *Indian Journal of Pharmacology*, ***34***, 100-110; (b) Sairam K, Priyambada S, Aryya N, Goel R. (**2003**) Gastroduodenal ulcer protective activity of *Asparagus racemosus*: an experimental, biochemical and histological study. *Journal of Ethnopharmacology*, ***86***, 1-10.

[3] Sharma S; Ramji S; Kumari S, Bapna J. (**1996**) Randomized controlled trial of *Asparagus racetnosus* (shatavari) as a lactogogue in lactational inadequacy. *Indian Pediatrics*, ***33***, 675-677.

[4] Pandey SK, Sahay A, Pandey RS, Tripathi YB. (**2005**) Effect of *Asparagus racemosus* rhizome (Shatavari) on mammary gland and genital organs of pregnant rat. *Phytotherapy Research*, ***19***, 721-724.

[5] Onlom C, Khanthawong S, Waranuch N, Ingkaninan K. (**2014**) *In vitro* anti-malassezia activity and potential use in anti-dandruff formulation of *Asparagus racemosus*. *International Journal of Cosmetic Science*, ***36***, 74-78.

[6] Venkatesan N, Thiyagarajan V, Narayanan S, Arul A, Raja S, Vijaya Kumar SG, Rajarajan T, Perianayagam JB. (**2005**) Anti-diarrhoeal potential of *Asparagus racemosus* wild root extracts in laboratory animals. *Journal of Pharmacy and Pharmaceutical Sciences*, ***8***, 39-46.

[7] (a) Bhatnagar M, Sisodia SS, Bhatnagar R. (**2005**) Antiulcer and antioxidant activity of *Asparagus racemosus* Willd. and *Withania somnifera* Dunal. in rats. *Annals of the New York Academy of Sciences*, ***1056***, 261-278. (b) Kongkaneramit L, Witoonsaridsilp W, Peungvicha P, Ingkaninan K, Waranuch N, Sarisuta N. (**2011**) Antioxidant activity and antiapoptotic effect of *Asparagus racemosus* root extracts in human lung epithelial H460 cells. *Experimental and Therapeutic Medicine*, ***2***, 143-148.

[8] (a) Bhutani K, Paul A, Fayad W, Linder S. (**2010**) Apoptosis inducing activity of steroidal constituents from *Solanum xanthocarpum* and *Asparagus racemosus*. *Phytomedicine*, ***17***, 789-793. (b) Liu W, Huang XF, Qi Q, Dai QS, Yang L, Nie FF, Lu N, Gong DD, Kong LY, Guo QL. (**2009**) Asparanin A induces G 2/M cell cycle arrest and apoptosis in human hepatocellular carcinoma HepG2 cells. *Biochemical and Biophysical Research Communications*, ***381***, 700-705.

[9] (a) Hayes PY, Jahidin AH, Lehmann R, Penman K, Kitching W, De Voss JJ. (**2008)** Steroidal saponins from the roots of *Asparagus racemosus*. *Phytochemistry*, ***69***, 796-804. (b) Hayes PY, Jahidin AH, Lehmann R, Penman K, Kitching W, De Voss JJ. (**2006)** Structural revision of shatavarins I and IV, the major components from the roots of *Asparagus racemosus*. *Tetrahedron letters*, ***47***, 6965-6969.

[10] Hayes PY, Jahidin AH, Lehmann R, Penman K, Kitching W, De Voss JJ. (**2006)** Asparinins, asparosides, curillins, curillosides and shavatarins: structural clarification with the isolation of shatavarin V, a new steroidal saponin from the root of *Asparagus racemosus*. *Tetrahedron Letters*, ***47***, 8683-8687.

[11] Handa SS, Suri OP, Gupta VN, Suri KA, Satti NK, Bhardwaj V, Bedi KL, Khajuria A, Kaul A, Parikh GG. (**2003**) Process for the isolation of novel oligospirostanoside.US Patents; US6670459, 2003.

[12] Sharma U, Kumar N, Singh B. (**2012**) Furostanol saponin and diphenylpentendiol from the roots of *Asparagus racemosus*. *Natural Product Communications*, ***7***, 995-998.

[13] Mitra SK, Prakash NS, Sundaram R. (**2012**) Shatavarins (containing Shatavarin IV) with anticancer activity from the roots of *Asparagus racemosus*. *Indian Journal of Pharmacology*, ***44***, 732-736.

[14] Dutta A, Ghoshal A, Mandal D, Mondal NB, Banerjee S, Sahu NP, Mandal C (**2007**) Racemoside A, an anti-leishmanial, water-soluble, natural steroidal saponin, induces programmed cell death in *Leishmania donovani*. *Journal of Medical Microbiology*, ***56***, 1196-1204.

[15] Onlom C, Yang Y, Aisa HA, Waranuch N, Phrompittayarat W, Putalune W, Ingkaninan K. (**2017**) Preparative and rapid purification of saponins from *Asparagus racemosus* root by high performance centrifugal partition chromatography, *Natural Product Communications*, ***12***, in press.

[16] (a) Satti N, Suri K, Dutt P, Suri O, Amina M, Qazi G, Rauf A. (**2006**) Evaluation of *Asparagus racemosus* on the basis of immunomodulating sarsasapogenin glycosides by HPTLC. *Journal of Liquid Chromatography and Related Technologies*, ***29***, 219-227. (b) Asmari S, Zafar R, Ahmad S. (**2010**) Production of sarsasapogenin from tissue culture of *Asparagus racemosus* and its quantification by HPTLC. *Iranian Journal of Pharmaceutical Research*, ***3***, 66-67.

[17] Negi JS, Singh P, nee Pant GJ, Rawat M. (**2011)** High performance liquid chromatographic analysis of derivatized sapogenin of Asparagus (RP-HPLC analysis of derivatized sapogenin of Asparagus). *Journal of Medicinal Plants Research*, ***5***, 1900-1904.

[18] Reamyindee N. (**2011**) *The Immunological Methods for Detection of Saponins in Asparagus racemosus* Roots. MS thesis, Naresuan University.

[19] Huber L. (**1998**) Validation of analytical methods. *Validation and Qualification in the Analytical Laboratories, Interpharm Press, Buffalo Grove, IL, **107***.

[20] Rivera C, Rodríguez R. (**2014**) Horwitz equation as quality benchmark in ISO/IEC 17025 testing laboratory. *Private communication*.

[21] Boonsom T, Waranuch N, Ingkaninan K, Denduangboripant J, Sukrong S. (**2012**) Molecular analysis of the genus Asparagus based on matK sequences and its application to identify *A. racemosus*, a medicinally phytoestrogenic species. *Fitoterapia*, ***83***, 947-953.

[22] Vijay N, Sairkar P, Silawat N, Garg R, Mehrotra N. (**2009**) Genetic variability in *Asparagus racemosus* (Willd.) from Madhya Pradesh, India by random amplified polymorphic DNA. *African Journal of Biotechnology*, ***8***, 3135-3140.

[23] Lee MK, Park H; Lee CH. (**1987**) Effect of growth conditions on saponin content and ginsenoside pattern of *Panax ginseng*. In *Korea-Japan Panax Ginseng Symposium*, 89-107.

[24] (a) Mimaki Y, Yokosuka A, Kuroda M, Sashida Y. (**2001**) Cytotoxic activities and structure-cytotoxic relationships of steroidal saponins. *Biological and Pharmaceutical Bulletin*, ***24***, 1286-1289. (b) Man S, Gao W, Zhang Y, Huang L, Liu C. (**2010**) Chemical study and medical application of saponins as anti-cancer agents. *Fitoterapia*, ***81***, 703-714.

NPC Natural Product Communications

2017
Vol. 12
No. 1
11 - 14

New Alkaloids and Anti-inflammatory Constituents from the Leaves of *Antidesma ghaesembilla*

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Received: October 31st, 2016; Accepted: November 22nd, 2016

Using various chromatographic methods, two new alkaloids, antidesoic acids A (**1**) and B (**2**) along with fourteen known compounds (**3-16**) were isolated from the leaves of *Antidesma ghaesembilla* Gaertn. Their chemical structures were elucidated by physical and chemical methods. All the isolated compounds were evaluated for their inhibitory activity on LPS-stimulated nitric oxide (NO) production in BV2 cells and RAW 264.7 macrophages. Bisflavone **8** significantly inhibited LPS-stimulated NO production in BV2 cells and RAW 264.7 macrophages with IC_{50} values of 5.4 and 8.0 μM, respectively. Compounds **1-3**, **7**, **10**, **12**, **14**, and **16** showed moderate inhibitory activities with IC_{50} values ranging from 11.7 to 77.4 μM.

Keywords: *Antidesma ghaesembilla*, Euphorbiaceae, Alkaloid, Anti-inflammation, Antidesoic acid A, Antidesoic acid B.

Nitric oxide (NO) produced in large amounts by inducible nitric oxide synthase (iNOS) is known to be responsible for the vasodilation and hypotension observed during septic shock and inflammation [1]. NO mediates a variety of biological actions ranging from vasodilatation, neurotransmission, inhibition of platelet adherence and aggregation, as well as macrophage and neutrophil-mediated killing of pathogens [2].

Microglia are the resident macrophage-like cells of the central nervous system with a broad role in the brain's innate immunity and in inflammatory neutropathologies. Macrophages are also considered to play essential roles in inflammation. Although inflammation is a general immune system to protect the body from infection or tissue injury, overexpression of inflammatory mediators such as NO can cause cell damage and inflammatory diseases including rheumatoid arthritis [3], diabetes [4], and chronic hepatitis [5].

Antidesma, a genus of tropical plants belonging to the Euphorbiaceae family, comprises about 100 species, 29 of which are found in Vietnam. The leaves of *A. ghaesembilla* Gaertn. have been used in traditional medicine for the treatment of skin diseases and headache [6]. In addition, its fruits have been used to treat sore throat and lung diseases [6]. However, there are few reports about chemical constituents and biological activities of this plant [7].

In a drug discovery program for Vietnamese traditional medicinal plants for finding anti-inflammatory compounds, the methanol extract of *A. ghaesembilla* leaves was found to inhibit NO productions in LPS-stimulated BV2 cells and RAW264.7 macrophages with IC_{50} values of 133.0 and 152.4 μg/mL, respectively. We report herein the isolation, structural elucidation, and evaluation of the inhibitory activity on NO production of two new and fourteen known compounds from the leaves of *A. ghaesembilla*.

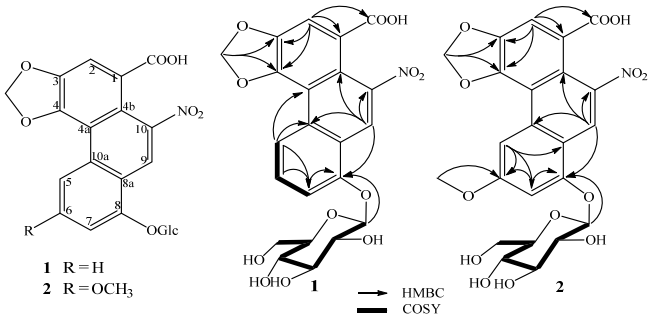

Figure 1: The chemical structures and key HMBC correlations of compounds **1** and **2**

Compound **1** was obtained as a yellow amorphous powder and its molecular formula was determined as $C_{22}H_{19}O_{12}N$ from the HR-ESI-MS pseudo-molecular ion peak at *m/z* 488.0829 $[M - H]^-$ (calcd. for $C_{22}H_{18}O_{12}N$, 488.0834). The ^1H NMR spectrum of **1** showed the presence of five aromatic protons at δ_H 7.45 (d, J = 8.5 Hz, 1H), 7.67 (t, J = 8.5 Hz, 1H), 7.69 (s, 1H), 8.75 (s, 1H), and 8.80 (d, J = 8.5 Hz, 1H); two methylenedioxy protons at δ_H 6.34 (s, 2H); and one anomeric proton at δ_H 5.19 (d, J = 7.5 Hz, 1H). The ^{13}C NMR and DEPT spectra of **1** revealed signals of 22 carbons, including one carboxylic (δ_C 175.0), nine non-protonated carbons (δ_C 118.1, 119.0, 122.0, 131.9, 134.5, 145.4, 147.5, 149.1, and 155.7), ten methines (δ_C 71.3, 74.5, 78.1, 78.3, 102.5, 112.6, 113.6, 119.6, 122.1, and 131.0), and two methylene carbons (δ_C 62.5 and 103.4), suggesting the presence of a phenanthrene glycoside. The ^1H- and ^{13}C-NMR data of **1** (Table 1) were very similar to those of aristolochic acid IIIa-6-*O*-β-D-glucopyranoside [8], except for the position of the sugar moiety at C-8. The HMBC correlations (Figure 1) between H-2 (δ_H 7.69) and C-1 (δ_C 118.1)/C-3 (δ_C 145.4) /C-4 (δ_C 147.5)/COOH (δ_C 175.0); between OCH_2O (δ_H 6.34) and C-3 (δ_C 145.4)/C-4 (δ_C 147.5) confirmed the presence of a carboxylic group at C-1 and a methylenedioxy group at C-3 and C-4. Acid hydrolysis of **1** afforded D-glucose as the sugar component

Table 1: ^{1}H- and ^{13}C-NMR data for compounds **1** and **2**.

C	1		2	
	δ_C[a,b]	δ_H[a,c] (mult., *J* in Hz)	δ_C[a,b]	δ_H[a,c] (mult., *J* in Hz)
Aglycone				
1	118.1	-	118.8	-
2	112.6	7.69 (s)	112.8	7.67 (s)
3	145.4	-	145.5	-
4	147.5	-	147.1	-
4a	122.0	-	12.0	-
4b	131.9	-	133.2	-
5	122.1	8.80 (d, 8.5)	104.5	8.37 (d, 2.0)
6	131.0	7.67 (t, 8.5)	162.9	-
7	113.6	7.45 (d, 8.5)	103.7	7.13 (d, 2.0)
8	155.7	-	157.0	-
8a	134.5	-	116.4	-
9	119.6	8.75 (s)	112.0	8.68 (s)
10	149.1	-	147.1	-
10a	119.0	-	118.7	-
COOH	175.0	-	175.0	-
3,4-OCH_2O	103.4	6.34 (s)	103.4	6.35 (s)
6-OMe			56.2	3.99 (s)
8-*O*Glc				
1′	102.5	5.19 (d, 7.5)	102.5	5.18 (d, 7.5)
2′	74.5	3.71 (dd, 7.5, 8.5)	74.9	3.71 (dd, 7.5, 8.0)
3′	78.1	3.57 (dd, 8.5, 9.0)	78.1	3.57 (dd, 8.0, 9.0)
4′	71.3	3.49 (t, 9.0)	71.4	3.48 (t, 9.0)
5′	78.3	3.57 (m)	78.4	3.57 (m)
6′	62.5	3.76 (dd, 5.5, 12.0)	62.5	3.76 (dd, 5.0, 12.0)
		3.94 (dd, 2.0, 12.0)		3.95 (dd, 2.0, 12.0)

[a]Measured in methanol-d_4, [b]125 MHz, [c]500 MHz, Assignments were made from HSQC, HMBC, and COSY experiments.

Table 2: IC_{50} values of compounds **1**-**16** on LPS-stimulated NO production in BV2 cells and RAW 264.7 macrophages.

Compounds	IC_{50} (μM)	
	BV2	RAW264.7
1	37.3 ± 8.7	77.4 ± 9.1
2	23.8 ± 3.1	20.3 ± 2.4
3	36.3 ± 3.8	53.2 ± 12.5
4	9.5 ± 1.3	>80
5	32.4 ± 9.9	>80
6	62.4 ± 11.2	>80
7	56.6 ± 5.7	54.5 ± 7.8
8	5.4 ± 0.6	8.0 ± 1.0
9	48.3 ± 7.3	>80
10	50.2 ± 5.4	79.1 ± 12.8
11	72.7 ± 20.9	>80
12	21.4 ± 4.4	11.7 ± 1.8
13	44.3 ± 8.9	>80
14	67.9 ± 26.0	76.7 ± 8.4
15	>80	>80
16	48.2 ± 6.8	64.4 ± 10.7

(identified as TMS derivative by GC) [9]. In addition, the HMBC correlation from glc H-1′ (δ_H 5.19) to C-8 (δ_C 155.7); COSY correlations between H-5 (δ_H 8.80)/ H-6 (δ_H 7.67)/ H-7 (δ_H 7.45) were observed. These results indicated that the glucopyranosyl moiety was linked at C-8 of the aglycone. Finally, the position of the nitrogen dioxide group at C-10 was confirmed by HMBC correlation between H-9 (δ_H 8.75) and C-10 (δ_C 149.1), as well as by the HR-ESI-MS of **1**. Based on the above evidence, the structure of **1** was elucidated as aristolochic acid Ia-8-*O*-β-D-glucopyranoside.

Compound **2** was obtained as a yellow amorphous powder and its molecular formula was determined as $C_{23}H_{21}O_{13}N$, by the HR-ESI-MS pseudo-molecular ion peak at *m/z* 542.0910 $[M + Na]^+$ (calcd for $C_{23}H_{21}O_{13}NNa$, 542.0911). The ^{1}H NMR spectrum of **2** showed the presence of four aromatic protons at δ_H 7.13 (d, *J* = 2.0 Hz 1H), 7.67 (s, 1H), 8.37 (d, *J* = 2.0 Hz, 1H), and 8.68 (s, 1H); two methylenedioxy protons at δ_H 6.35 (s, 2H); one anomeric proton at δ_H 5.18 (d, *J* = 7.5 Hz, 1H), and one methoxy group at δ_H 3.99 (s, 3H). Two *meta*-coupled proton signals at δ_H 7.13 (d, *J* = 2.0 Hz) and 8.37 (d. *J* = 2.0 Hz) in the ^{1}H NMR spectrum of **2** in comparison with **1** suggested a 1,2,3,5-tetra-substituted group in the third benzene ring of **2**. The ^{13}C NMR and DEPT spectra of **2** revealed signals of 23 carbons, including one carboxylic, ten non-protonated carbons, nine methines, two methylenes, and one methoxy carbon. The ^{1}H- and ^{13}C-NMR data of **2** (Table 1) suggested that the structure of **2** was similar to that of **1** except for the addition of a methoxy group at C-6. This was further confirmed by the HMBC correlation between the methoxy protons (δ_H 3.99) and C-6 (δ_C 162.9) (Figure 1). Similar to **1**, acid hydrolysis of **2** afforded D-glucose as the sugar component (identified as TMS derivative by GC). Furthermore, the sugar was linked at C-8, confirmed by the observation of an HMBC correlation between glc H-1′ (δ_H 5.18) and C-8 (δ_C 157.0). Thus, the structure of **2** was elucidated as aristolochic acid IVb-8- *O*-β-D-glucopyranoside.

Compounds **1** and **2** were expected to be metabolic products of the related aristolochic acid uptake by the butterfly *Battus polydamas*, using HPLC-MS analysis [10]. However, to the best of our knowledge, this is the first report in which the chemical structure and NMR data of **1** and **2** are clearly provided. Thus, compounds **1** and **2** are considered to be new compounds, named as antidesoic acid A and antidesoic acid B, respectively.

Compounds **3**-**16** were identified as vitexin (**3**) [11], orientin (**4**) [11], isovitexin (**5**) [12], homoorientin (**6**) [11], luteolin-4′-*O*-β-D-glucopyranoside (**7**) [13], amentoflavone (**8**) [14], vanillyl alcohol 4-*O*-β-D-glucopyranoside (**9**) [15], 4-hydroxy-3,5-dimethoxy-benzyl-*O*-β-D-glucopyranoside (**10**), 5-hydroxy-3,4-dimethoxy-phenyl-*O*-β-D-glucopyranoside (**11**) [16], 3,4,5-trimethoxyphenyl-*O*-*β*-D-glucopyranoside (**12**) [17], sinapyl alcohol 4-*O*-β-D-glucopyranoside (**13**) [18], lyoniresinol (**14**) [19], syringaresinol (**15**) [20], and alangioside (**16**) [21] by comparison of their NMR and MS data with the reported values in the literature. This is the first report of compounds **3**-**6**, **9**-**14**, and **16** from the genus *Antidesma.*

All the isolated compounds were tested for their inhibitory activity on LPS-induced NO production in BV2 cells and RAW 264.7 macrophages. First, the compounds were examined at the concentration of 80 μM to screen their cytotoxicity and inhibitory activity on NO production. None of the tested compound showed significant cytotoxic activity (data not shown).

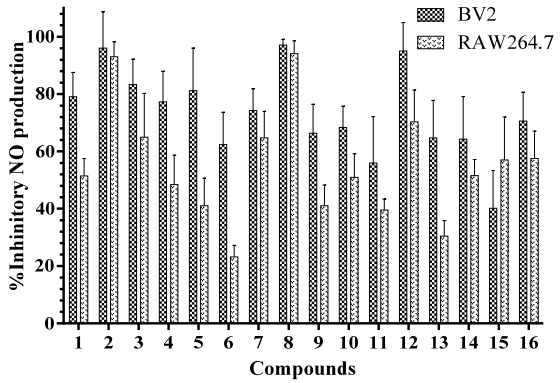

Figure 2: Inhibitory activity of compounds **1** – **16** on LPS-stimulated NO production in BV2 cells and RAW 264.7 macrophages at the concentration of 80 μM.

Nitrite concentrations were measured in the supernatant of BV2 cells and RAW 264.7 macrophages by the Griess reaction [22]. At the concentration of 80 μM, compounds **2** and **8** significantly decreased NO production in both BV2 cells and RAW264.7

macrophages with inhibitory percentages > 80%; compounds **1**, **3**, **5**, **7**, **10**, **12**, **14**, and **16** moderately decreased NO production with inhibitory percentages from 50.8% to 79.0% (Figure 2).

In order to obtain IC_{50} values for the potent inhibitors, dose dependent responses were performed. Bisflavone **8** showed the most potent inhibitory activity on NO production in LPS-stimulated BV2 cells and RAW 264.7 macrophages with IC_{50} values of 5.4 and 8.0 μM, respectively (Table 2). Compounds **1-3**, **7**, **10**, **12**, **14**, and **16** showed moderate inhibitory activities with IC_{50} values ranging from 11.7 to 77.4 μM. Thus, it is possible to demonstrate that bisflavone **8** might be an important anti-inflammatory constituent of this plant.

Experimental

General: Optical rotation, Jasco DIP-370 automatic polarimeter; HR-ESI-MS, Varian 910 FT-ICR-MS 7 tesla; 1D and 2D NMR, Bruker AM500 FT-NMR spectrometer; Column chromatography was performed using either silica gel (Kieselgel 60, 70 – 230 mesh and 230 – 400 mesh, Merck, Whitehouse Station, NJ) or RP-18 resins (150 μm, Fuji Silysia Chemical Ltd.), and thin layer chromatography (TLC) using pre-coated silica-gel 60 F_{254} (0.25 mm, Merck) and RP-18 F_{254S} plates (0.25 mm, Merck).

Plant material: The leaves of *A. ghaesembilla* were collected in Dak Lak province, Vietnam, in March 2013, and authenticated by Dr Nguyen Quoc Binh, Vietnam National Museum of Nature, VAST, Vietnam. A voucher specimen was deposited at the Institute of Marine Biochemistry, VAST.

Extraction and isolation: The dried leaves of *A. ghaesembilla* (2.5 kg) were sonicated in MeOH 3 times to yield 115.0 g of a dark solid extract, which was then suspended in water and successively partitioned with dichloromethane and ethyl acetate (EtOAc) to obtain dichloromethane (AG1, 30.0 g), ethyl acetate (AG2, 20.0 g), and water layers (AG3, 65.0 g) after removal of solvent *in vacuo*. The AG1 layer was chromatographed on a silica gel column with gradient mixtures of *n*-hexane/acetone (100/1 → 0/1, v/v) to obtain 5 fractions, AG1A-AG1E. Three smaller fractions (AG1E1-AG1E3) were obtained from the AG1E fraction by silica gel CC using dichloromethane/MeOH (18/1, v/v) as eluent. Compounds **14** (5.0 mg) and **15** (12.0 mg) was obtained from AG1E3 and AG1E1, respectively, using a RP-18 column and acetone/water (1/1.5, v/v). The EtOAc layer (AG2, 20.0 g) was chromatographed on a silica gel column eluting with dichloromethane/MeOH/water (5/1/0.1, v/v/v) to give 3 fractions, AG2A-AG2C. The AG2A fraction was chromatographed on a RP-18 column with MeOH/water as eluent (2/1, v/v) to yield compound **8** (20.0 mg). The AG2B fraction was chromatographed on a RP-18 column eluting with MeOH/water (1/1.5, v/v) to yield compound **3** (15.0 mg). The AG2C fraction was chromatographed using a RP-18 column then a silica gel column to yield compound **7** (7.0 mg). Compounds **1** (10.0 mg) and **2** (12.0 mg) were obtained from a RP-18 column using methanol/water (1/4.5, v/v), then a silica gel column using dichloromethane/methanol/water (2/1/0.1, v/v/v). The water layer (AG3, 65.0 g) was chromatographed on a Diaion HP-20 column eluting with water to remove sugar, then with an increased concentration of methanol in water (25, 50, 75, and 100 %) to obtain 4 fractions, AG3A-AG3D. The AG3A fraction was chromatographed on a silica gel column eluting with EtOAc/MeOH/water (5/1/0.5, v/v/v) to give 3 fractions, AG3A1-AG3A3. The AG3A1 fraction was chromatographed on a RP-18 column eluting with MeOH/water (1/2, v/v) to yield compounds **9** (6.0 mg) and **11** (5.0 mg). The AG3A3 fraction was chromatographed on a RP-18 column eluting with MeOH/water (1/2, v/v) to yield compound **10** (5.0 mg). The AG3D fraction was chromatographed on a silica gel column eluting with CH_2Cl_2/MeOH/water (5/1/0.1, v/v/v) to give 4 fractions, AG3D1-AG3D4. Compounds **12** (7.0 mg) and **13** (5.0 mg) were obtained from the AG3D2 fraction using a RP-18 column and MeOH/water (1/2.5, v/v) as eluent. The AG3D3 fraction was applied on a silica gel column then a RP-18 column to yield compounds **4** (7.0 mg), **5** (8.0 mg), **6** (5.0 mg), and **16** (8.0 mg).

Antidesoic acid A (**1**)
Yellow amorphous powder.
$[\alpha]_D^{25}$: −45.0 (c 0.1, MeOH).
HR-ESI-MS *m/z*: 488.0829 $[M - H]^-$ (calcd for $C_{22}H_{18}O_{12}N$, 488.0834).
^{1}H- and ^{13}C-NMR (CD_3OD): Table 2.

Antidesoic acid B (**2**)
Yellow amorphous powder.
$[\alpha]_D^{25}$: −63.0 (*c* 0.1, MeOH).
HR-ESI-MS *m/z*: 542.0910 $[M + Na]^+$ (calcd for $C_{23}H_{21}O_{13}NNa$, 542.0911).
^{1}H- and ^{13}C-NMR (CD_3OD): Table 2.

Acid hydrolysis: Each compound (**1-2**, 2.0 mg) was separately dissolved in 1.0 N HCl (dioxane - H_2O, 1:1, v/v, 1.0 mL) and heated to 80°C in a water bath for 3 h. The solvent in acidic solution was removed under a N_2 stream. After extraction with $CHCl_3$, the aqueous layer was concentrated to dryness using N_2. The residue was dissolved in dry pyridine (0.1 mL), followed by addition of L-cysteine methyl ester hydrochloride in pyridine (0.06 M, 0.1 mL). The reaction mixture was heated at 60°C for 2 h. Trimethylsilylimidazole solution (0.1 mL) was then added, followed by heating at 60°C for 1.5 h. The dried product was partitioned with *n*-hexane and H_2O (0.1 mL each), and the organic layer was analyzed by gas chromatography (GC): column DB-5 (0.32 mm ID × 30 m length), detector FID, column temp 210°C, injector temp 270°C, detector temp 300°C, carrier gas He (2 mL/min). Under these conditions, the standard sugars gave peaks at t_R (min) 14.11 and 14.26 for D- and L-glucose, respectively. Peaks at t_R (min) 14.11 of D-glucose for **1** and **2** were observed.

Cell culture: BV2 microglial cells and RAW 264.7 macrophages were maintained at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air at 5 × 10^5 cells/mL in DMEM medium supplemented with 10% heat-inactivated FBS, penicillin G (100 units/mL), streptomycin (100 mg/mL), and L-glutamine (2 mM).

Nitrite production determination: BV2 microglial cells and RAW 264.7 macrophages were pretreated for 3 h with the compounds, and treated for 24 h with LPS (1 μg/mL). The nitrite concentration in the medium was measured as an indicator of NO production by the Griess reaction. In brief, an aliquot (100 μL) of each supernatant was mixed with an equal volume of Griess reagent (0.1%, w/v, *N*-(1-naphthyl)-ethylenediamine and 1%, w/v, sulfanilamide in 5%, v/v, phosphoric acid) for 10 min at room temperature. The absorbance of the final reactant at 525 nm was measured using an ELISA plate reader, and the nitrite concentration was determined using a standard curve of sodium nitrite prepared in DMEM without phenol red. The inhibitory effect on the NO production was estimated by calculating the concentration ratio of nitrite produced in the cells treated with LPS and with/without compounds.

Acknowledgments - This research was supported by the Vietnam National Foundation for Science and Technology Development

(NAFOSTED) under grant number 104.01-2013.05 and Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (NRF-2011-0025129).

References

[1] Sinha B, Eigler A, Baumann KH, Greten TF, Moeller J, Endres S. (**1998**) Nitric oxide downregulates tumour necrosis factor mRNA in RAW 264.7 cells. *Research in Immunology*, ***149***, 139-150.

[2] Ishibe K, Yamanishi T, Wang Y, Osatomi K, Hara K, Kanai K, Yamaguchi K, Oda T. (**2009**) Comparative analysis of the production of nitric oxide (NO) and tumor necrosis factor-α (TNF-α) from macrophages exposed to high virulent and low virulent strains of *Edwardsiella tarda*. *Fish and Shellfish Immunology*, ***27***, 386-389.

[3] Amin AR, Attur M, Abramson SB. (**1999**) Nitric oxide synthase and cyclooxygenases: distribution, regulation, and intervention in arthritis. *Current Opinion in Rheumatology*, ***11*****,** 202-209.

[4] Pavlovic D, Chen MC, Bouwens L, Eizirik DL, Pipeleers D. (**1999**) Contribution of ductal cells to cytokine responses by human pancreatic islets. *Diabetes*, ***48***, 29-33.

[5] Cuzzocrea S, Zingarelli B, Villari D, Caputi AP, Longo G. (**1998**) Evidence for *in vivo* peroxynitrite production in human chronic hepatitis. *Life Sciences*, ***63***, 25-30.

[6] Chi VV. (**2012**) *Dictionary of Medicinal Plants in Vietnam*. Medical Publishing House, Hanoi, ***1***, 441-442.

[7] (a) Maria S, Islam F, Qais N, Hasan CM. (**2013**) Isolation of vomifoliol: a megastigmane from leaves of *Antidesma ghaesembilla*. *Asian Journal of Chemistry*, ***25***, 3533-3534; (b) Gargantiel MF, Ysrael MC. (**2014**) Antioxidant activity and hypoglycemic potential of *Antidesma ghaesembilla* Gaertn (Phyllantaceae). *International Journal of Scientific and Technology Research*, ***3***, 422-431.

[8] Li H, Youji S, Shingo M, Chen X. (**1994**) Eleven aristolochic acid derivatives from *Aristolochia cinnabarin*a. *Phytochemistry*, ***37***, 237-239.

[9] Nhiem NX, Hien NT, Tai BH, Anh Hle T, Hang DT, Quang TH, Kiem PV, Minh CV, Ko W, Lee S, Oh H, Kim SH, Kim YH. (**2015**) New ent-kauranes from the fruits of *Annona glabra* and their inhibitory nitric oxide production in LPS-stimulated RAW264.7 macrophages. *Bioorganic & Medicinal Chemistry Letters*, ***25***, 254-258.

[10] Priestap HA, Velandia AE, Johnson JV, Barbieri MA. (**2012**) Secondary metabolite uptake by the Aristolochia-feeding papilionoid butterfly *Battus polydamas*. *Biochemical Systematics and Ecology*, ***40***, 126-137.

[11] Burns DC, Ellis DA, March RE. (**2007**) A predictive tool for assessing ^{13}C NMR chemical shifts of flavonoids. *Magnetic Resonance in Chemistry*, ***45***, 835-845.

[12] Ramarathnam N, Osawa T, Namiki M, Kawakishi S. (**1989**) Chemical studies on novel rice hull antioxidants. 2. Identification of isovitexin, a C-glycosyl flavonoid. *Journal of Agricultural and Food Chemistry*, ***37***, 316-319.

[13] Lee M, Son Y, Han Y. (**2002**) Tissue factor inhibitory flavonoids from the fruits of *Chaenomeles sinensis*. *Archives of Pharmacal Research*, ***25***, 842-850.

[14] Tchinda AT, Teshome A, Dagne E, Arnold N, Wessjohann LA. (**2006**) Squalene and amentoflavone from *Antidesma iaciniatum*. *Bulletin of the Chemical Society of Ethiopia*, ***20***, 325-328.

[15] Kanho H, Yaoya S, Kawahara N, Nakane T, Takase Y, Masuda K, Kuroyanagi M. (**2005**) Biotransformation of benzaldehyde-type and acetophenone-type derivatives by *Pharbitis nil* hairy roots. *Chemical and Pharmaceutical Bulletin*, ***53***, 361-365.

[16] Takara K, Matsui D, Wada K, Ichiba T, Nakasone Y. (**2002**) New antioxidative phenolic glycosides isolated from Kokuto non-centrifuged cane sugar. *Bioscience, Biotechnology, and Biochemistry*, ***66***, 29-35.

[17] Jin H-G, Kim A, Ko H, Woo E-R. (**2015**) A new megastigmane glycoside from *Akebia quinata*. *Archives of Pharmacal Research*, ***38***, 591-597.

[18] Greca MD, Ferrara M, Fiorentino A, Monaco P, Previtera L. (**1998**) Antialgal compounds from *Zantedeschia aethiopica*. *Phytochemistry*, ***49***, 1299-1304.

[19] Ohashi K, Watanabe H, Okumura Y, Uji T, Kitagawa I. (**1994**) Indonesian Medicinal Plants. XII. Four isomaric lignan-glucosides from the bark of *Aegle marmelos* (Rutaceae). *Chemical and Pharmaceutical Bulletin*, ***42***, 1924-1926.

[20] Min Y, Choi S, Lee K. (**2006**) Aporphine alkaloids and their reversal activity of multidrug resistance (MDR) from the stems and rhizomes of *Sinomenium acutum*. *Archives of Pharmacal Research*, ***29***, 627-632.

[21] De Marino S, Borbone N, Zollo F, Ianaro A, Di Meglio P, Iorizzi M. (**2004**) Megastigmane and phenolic components from *Laurus nobilis* L. leaves and their inhibitory effects on nitric oxide production. *Journal of Agricultural and Food Chemistry*, ***52***, 7525-7531.

[22] Altmann KH, Gertsch J. (**2007**) Anticancer drugs from nature-natural products as a unique source of new microtubule-stabilizing agents. *Natural Product Reports*, ***24***, 327-357.

NPC Natural Product Communications

2017
Vol. 12
No. 1
15 - 18

Chemical Characterization of *Narcissus poeticus* from Sirente –Velino (Apennines - Italy): Galantamine Accumulation and Distribution of Allergenic Compounds in the Flower

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Received: March 14th, 2016; Accepted: November 29th, 2016

Species of *Narcissus* (family *Amaryllidaceae*) are a potential source for large-scale extraction of alkaloids and fragrances. The bulbs typically accumulate a large number of alkaloids, including galantamine, a benzazepine alkaloid proven to be a cholinesterase inhibitor and which is used in the treatment of Alzheimer's disease. The presence of galantamine in *N. poeticus* L. collected in Abruzzo (Italy) was assessed and several levels of alkaloid were found in all parts of the plant (flower, stem, bulb and root) and not only in the bulb. The amount of galantamine obtained was tested by using two different extraction solvents. Extraction of *N. poeticus* absolute from the flowers was also performed, as this product is an important floral note in perfumery, and the distribution of allergenic compounds in the coronas and in the tepals was assessed. Moreover, the *in vitro* propagation of *N. poeticus* was tested as it may be a valuable resource from which to produce biomolecules, as an alternative to chemical synthetic processes.

Keywords: *Narcissus poeticus*, *Amaryllidaceae*, Absolute, Allergenic compounds, Bulbs, Fragrance, Galantamine, *in vitro* Propagation.

Narcissus poeticus L. is a wild monocotyledonous plant belonging to the *Amaryllidaceae* family. It is a native plant of the Mediterranean basin, widespread in the alpine areas of southern Europe, in Spain and Italy; it grows mainly at medium-altitude and on high mountains, in cool, moist and semi-shaded habitats [1]. It is a wild, bulbous, herbaceous plant, from 20 to 30 cm high; the leaves are radical, green, long and narrow (Figure 1a). Geophyta is the plant life form of the *Narcissus* genus; the storage organ contains reserves of carbohydrates and water and helps the plant to survive adverse environmental conditions such as cold or drought. It blooms in April-May, presenting an extremely fragrant flower per stem, characterized by a corona and a perianth tube which is pure white with six tepals fused at the base onto a green floral tube (5 mm diameter and 3 cm long). The corona (1.3 cm in diameter and 2-4 mm long) is light yellow, with a distinct reddish edge as shown in Figure 1b. Geophytes have economic value due to their use as a drug and as a fragrance for the perfume industry [2,3]. In recent years, the absolute fragrance obtained from flowers has been widely used in sophisticated modern perfumes due to its strong, green, woody and deep floral scent, which blends well with many of the floral absolutes, including clove bud carnation, jasmine, neroli, ylang ylang, and mimosa, as well as Karo-karounde. Ferri and co-workers have characterized the essential oil of *N. poeticus* flowers, which contained a great variety of components [4]. However, many of these are allergenic, as stated by Directive 2003/15 /EC, implemented in Italy by Legislative Decree n° 193, 3 February 2005 [5].

N. poeticus is also a valuable source of galantamine [6,7] a tertiary alkaloid, which is one of the therapeutic agents for the symptomatic treatment of Alzheimer's disease, the fourth leading cause of death in developed countries. Recent studies [8,9] supported the cholinergic hypothesis which postulates that memory impairment in patients with Alzheimer's disease results from a deficit of the cholinergic function in the brain. As a lipophilic molecule able to go through the blood-brain barrier (BBB), the action of galantamine is to restore the level of acetylcholine by inhibiting the action of acetylcholinesterase enzymes [10].

Commercial drugs contain galanthamine bromohydrate as the active molecule, which is obtained by chemical synthesis through an expensive and complex process. The high costs and the growing demand for galantamine have driven scientists to obtain the active ingredient from plant matrices. Moreover, plant extracts often have a more effective and durable pharmacological action than mono-therapies, as they contain different active substances that may have a synergistic action.

Previous work on the *in vivo* and *in vitro* propagation of plants containing compounds of particular interest both from Central Apennines (Italy) [11,12] and other sites [13], allowed us to gain knowledge about widely tested technologies. On these premises, in order to exploit the industrial potentialities of *N. poeticus* from Sirente-Velino (Abruzzo region, Italy), the aims of this investigation were: 1) to verify the presence and the amount of allergenic substances in the tepals and corona of the flowers; 2) to investigate the accumulation of galantamine in the different organs of plants; 3) to determine the galantamine content in the bulbs obtained by *in vitro* culture of *N. poeticus*.

Total yield and percentage of absolute of *n*-hexane extraction confirm our preview data [4]. The total yield (concrete) was found to be 0.45% of the weight of fresh flowers, that is higher than the values reported in the literature (0.20 and 0.26%) [1,4]. The percentage of absolute in the *n*-hexane extract was 30.5% of the weight of the concrete, in agreement with the literature [14]. The distribution of allergenic compounds in the tepals and corona are shown in Table 1. Highly allergenic substances, such as cinnamyl

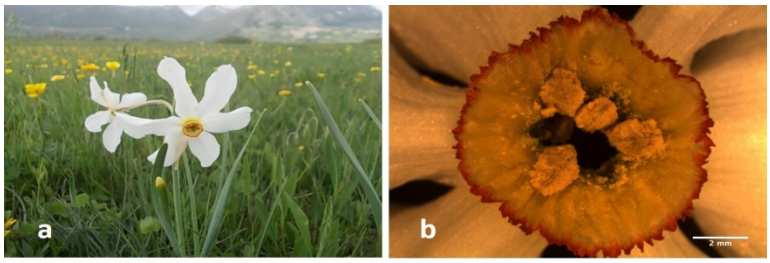

Figure 1: Flowers of *Narcissus poeticus* in Altopiano delle Rocche (a), detail of corona (b).

alcohol, which is the most abundant compound (30.2 μg/g), and cinnamyl aldehyde, are found only in the tepals. Less allergenic substances, such as benzyl benzoate and limonene, are mainly contained in the tepals (70 and 75% respectively), while 70% of isoeugenol, another highly allergenic compound, and 75% of eugenol, a less allergenic substance, are contained in the coronas. The tepals showed a greater amount (total mean value 40 μg/g) of more allergenic compounds compared with the corona (total mean value 15 μg/g). These data are also confirmed for the less allergic components (25 μg/g detected in tepals and 12 μg/g in corona).

Table 1: Percentage distribution of compounds in tepal and corona of *Narcissus poeticus* flowers after *n*-hexane extraction.

Compound	μg/g fresh flower	Tepals %	Coronas %
Cinnamyl alcohol **	30.2	100.0	-
Benzyl benzoate *	28.5	70.0	30.0
Isoeugenol methylether	28.1	40.3	59.7
cis Ocimene	25.2	21.6	77.4
Isoeugenol **	22.9	36.4	63.6
Eugenol methyl ether	20.3	35.5	64.5
α-Terpineol	19.9	1.3	98.7
Eugenol *	16.0	25.0	75.0
Benzyl alcohol	8.8	59.7	40.3
Methyl benzoate	5.8	64.8	35.2
Phenyl propyl alcohol	5.0	30.7	69.3
p-Cresol	3.9	75.0	25.0
Nonaldehyde	3.2	45.8	54.2
Benzyl salicylate	3.0	34.7	65.3
Cinnamyl aldehyde **	1.4	100.0	-
p-Methyl anisole	1.2	53.2	46.8
p-Propenyl anisole	1.2	60.0	40.0
Cinnamyl acetate	0.9	39.9	60.1
Limonene*	0.8	75.0	24.0

* Less allergenic compound; ** More allergenic compound.

As the extraction of absolute from tepals and coronas separately shows that the allergenic substances are mostly found in tepals, thus in the preparation of perfumes a safer absolute can be obtained from the corona only. This is due to the fact that despite preserving its distinctive fragrance it contains a lower quantity of allergenic components. Figure 2 shows the trend of galantamine in roots, bulbs, stems and leaves, and flowers in three phenological stages over four consecutive years. The means show that all parts of the plant contain galantamine, although the greatest amount was found in the underground organs. However, our experimental data showed an accumulation of the alkaloid in stems, leaves and flowers. At blooming stage the galantamine content was highest in both the bulbs and roots (respectively 12 mg /100 g dry weight and 13 mg / 100 g dry weight). The aerial parts, including flowers, stem and leaves (unique value), contain about 9 mg / 100 g dry weight; thus the amount of galantamine in the plant was about 34 mg / 100 g dry weight.

The highest content of galantamine was found in the bulb and root. Table 2 shows the differences observed in the various years at blooming time [15].

The data show slight differences for 2012, 2013 and 2014. In 2015 the lowest contents of alkaloid in both the underground organs

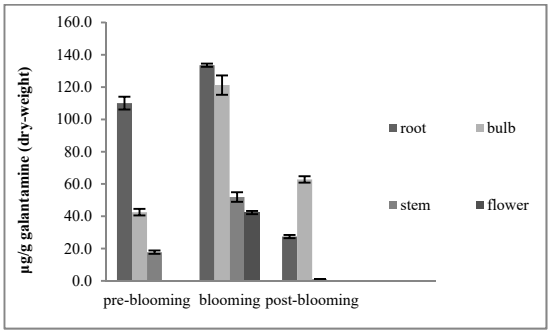

Figure 2: Galantamine distribution at different development stages of *N. poeticus*.

Table 2: Galantamine distribution (μg/g dry weight) in underground organs of *Narcissus poeticus* in different seasons.

Spring	Root	Bulb
2012	122.0 ± 2.2	110.2 ± 10.5
2013	137.5 ± 1.8	134.5 ± 3.2
2014	133.6 ± 1.2	121.3 ± 5.7
2015	77.3 ± 3.1	63.8 ± 2.9

might be due to a sudden rise in temperatures with subsequent drought during the spring (2015 was registered as the warmest year of the last 100 years; http://www.noaa.gov/). A further galantamine extraction both with methanol and ethanol was carried out on one additional sample in order to verify the use of a less-toxic solvent. Bulbs, roots, flowers, stems and leaves, before, during and after blooming were gathered and processed according to the procedures described in the Experimental section: Galantamine extraction.

Figure 3 shows the differences in the efficiency of extraction when ethanol and methanol were used. The distribution of values are plotted separately per group of cases (30) defined by values of categorical (grouping) variables (bulbs, flowers, roots, stems and leaves together). During pre- and post-blooming the content of galantamine extracted from the various organs of the plant was similar using either EtOH or MeOH (in the first case a greater variability among samples was detected). In the blooming stage, when the concentration of alkaloid is greater, the use of MeOH provides the best results in terms of increased efficiency of extraction from the different tissues of the plant, in particular from its roots. From the results of the statistical tests (Supplementary Data: 1-3) we can conclude that the differences between the two samples are statistically significant (p<0.00005). Although the yield obtained by ethanol is good, methanol is a more efficient solvent to extract galantamine from *N. poeticus*.

Primary sterile explants of bulbs of *N. poeticus* were obtained in a medium contained benzylaminopurine (BAP), and naphthalene acetic acid (NAA). Such tests were carried out in order to grow parts of plants *in vitro* (Figure 4) on different media (G) (Supplementary Data: 4).

Bulbs obtained by *in vitro* propagation (Figure 4 b) were extracted with methanol and processed according to the procedure described in the Experimental section. The resulting galantamine content was

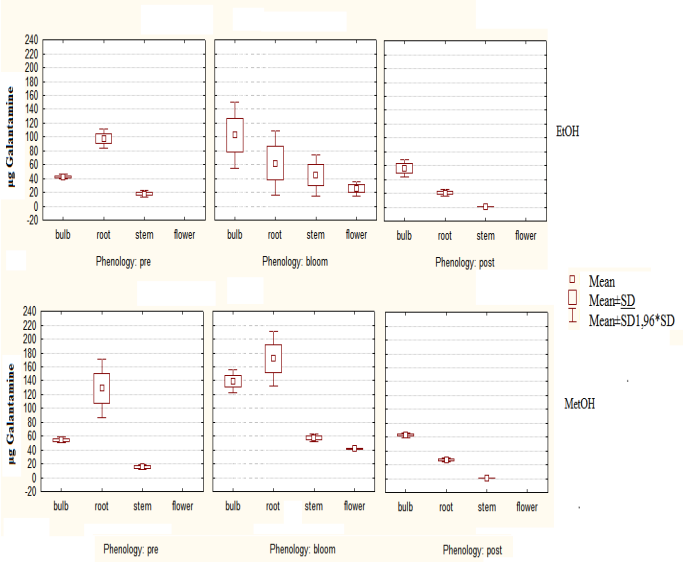

Figure 3: Distribution of galantamine extracted by ethanol (EtOH) and methanol (MeOH) in the three different phenological phases.

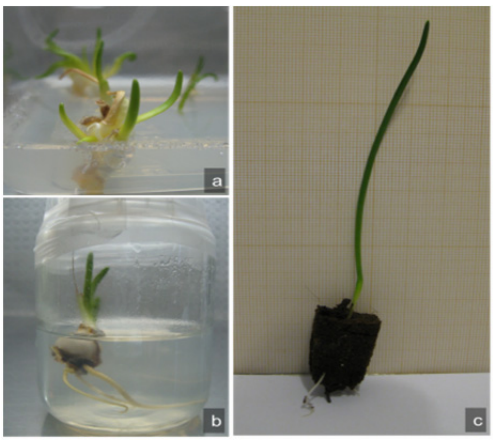

Figure 4: *In vitro* propagation of *Narcissus poeticus*
a) Shoots produced during the multiplication stage.
b) Rooted bulblet exhibiting three leaves.
c) Plants obtained from *in vitro* propagation transplanted to soil

2.5 mg ± 0.9 / 100 g dry weight, five times less than the amount obtained from wild bulbs. This preliminary result encourages us to continue the development of *in vitro* culture of *N. poeticus* bulbs. The organs, including bulbs, microtubers, corms, somatic embryos and shoots are perfect for mass propagation by using bioreactor techniques for large-scale propagation of geophytes, as described for *Gladiolus*, *Narcissus* and *Crocus* [16].

In conclusion, we have demonstrated that all parts of *N. poeticus* contain galantamine, confirming the presence in the species of this important alkaloid. The highest amounts are found in the underground organs (roots and bulbs). The accumulation of alkaloid is also documented in *in vitro* conditions opening new perspectives to biotechnological applications (i.e. bioreactors for mass propagation of adventitious roots), with the advantages to reduce time and costs. Moreover, with the goal to enhance the exploitation of the species for the local economy, the biochemical characterization of the different parts of the flowers pave the way towards a target product for the perfume industry. In summary, *N. poeticus* offers potential opportunities to increase the economy of local mountain communities to the development of marginal areas.

Experimental

Chemicals: Solvent: *N*-hexane HPLC grade, methanol HPLC grade, ethanol HPLC grade, dichloromethane; pure standards, agar, benzylaminopurine, naphthalene acetic acid, starch, sucrose and Murashige and Skoog medium were supplied by Sigma-Aldrich Chemie (Steinheim, Germany).

Plant materials: Wild plants of *Narcissus poeticus* were collected before blooming, during blooming and after blooming in a 300 sq m area of the plateau of Rocca di Mezzo-Sirente Velino Regional Park (L'Aquila, Italy), located at 13°31'35.9''E-42°13'16.3''N-1327 m asl, in 2012, 2013, 2014 and 2015. About 30 plants at each plant stage were collected in April, May and July. Plants were separated into bulbs, roots, flowers and stems. The collected materials were frozen in liquid nitrogen and then powdered using a mortar. The ground materials were freeze-dried for 48 h at -50°C and at about 0 KPa by an EDWARDS 5 Pascal LIO-5PDGT freeze-dryer and then stored at -20°C.

About 2000-2500 fully blooming flowers with 20 cm stems (500 – 650 g) were picked in the early morning during the May campaigns. The samples were preserved at 4°C in closed plastic tanks in a nitrogen atmosphere and extractions were performed the day after collection. Before each extraction the fresh flowers were manually reduced to small pieces of about 0.2 cm size.

Essential oil extraction: With the aim of evaluating the distribution of the allergenic compounds in different parts of the flower, tepals were separated from coronas (Figure 1). About 1 g of fresh flowers, corona and tepals were extracted separately in 10 mL of *n*-hexane for 3 h at room temperature under agitation. Then the solvent was evaporated under reduced pressure using a rotary evaporator and each extract was transferred with *n*-hexane to a volumetric flask, diluted to volume and analysed. To determine the yield of essential oils, 85 g of fresh flowers were extracted with 850 mL *n*-hexane and the concrete obtained (absolute and waxes). The hexane was removed by evaporation and the concrete was treated with ethanol cooled at -18°C to precipitate waxes. In order to improve the separation of waxes from the absolute, the mixture was centrifuged at 1500 x *g* for 30 min using a refrigerated centrifuge cooled at -15°C. The difference in weight between the concrete and waxes determined the absolute.

Galantamine extraction: Fifty mg of bulbs, roots, flowers and stems were transferred to a 1.5 mL Eppendorf tube with 1 mL of methanol adjusted to pH 4 with 25% ammonia solution. Each sample (3 samples were prepared for each vegetable matrix) was macerated with methanol for 24 h by sonication for 30 min every 8 h in an ultrasonic bath at room temperature. After maceration, extracts were centrifuged at 7800 x *g* for 10 min., and supernatants analysed [17]. The same extraction procedure was applied using ethanol instead of methanol, to assess the use of a less toxic solvent. The supernatant was added to 10 mL of $NaHCO_3$ 0.1M, and then galantamine was extracted with 2 mL CH_2Cl_2. The organic phase was dehydrated using Na_2SO_4 and concentrated under a gentle stream of nitrogen to 0.5 mL.

GC-MS analyses: Analyses were performed with a GC-MS-Thermo TraceMS spectrometer using a 60 m long capillary column with an internal diameter of 0.25 mm; the bonded phase was methyl-5% phenylsilane of thickness equal to 0.25 mm (column DB-5 MS, J & W Scientific). The detector was a selection quadrupole mass with electron impact ionization 70 eV, used in scanning (550-50). The operating conditions were: carrier gas helium N55, flow 1 mL min^{-1}; volume injected 1 mL. Injector: splitless (60 sec) at 250°C;

transfer line: 320°C; temperature program: 60°C for 1 min., ramp of 30°C min^{-1} up to 100°C held for 1 min., ramp of 3°C min^{-1} up to 300°C. The instrument was equipped with a NIST software library for the recognition of the mass spectra of the considered analytes. All experiments were carried out in triplicate and on at least 2 separate occasions.

In vitro *culture:* Bulbs of *N. poeticus* were gathered from flowering plants during the spring. The samples without papery scales and roots were washed with soap, rinsed with tap water and then disinfected according to the following scheme: Tween 20 for 15 min then washed several times with sterilized distilled water; 70% ethanol (3 min), followed by 10% commercial bleach (30 min), and finally rinsed several times with sterile distilled water. Bulbs were then vertically cut through two-thirds of their height (leaving one-third of its basal part intact). From each bulb two-thirds of the apical portion was removed, as well as the two external scales; a thin layer of the basal plate was also discarded. The remaining portion of the bulb was longitudinally divided to obtain explants formed by segments of twin-scales and a thick segment (2–3 mm) of the basal plate tissue. Twin-scales were used as primary explants cultured on a modified Murashige and Skoog medium with 2% sucrose supplemented with different growth regulators {benzylaminopurine (BAP) and naphthalene acetic acid (NAA)} at different concentrations [18-20]. The media were solidified with 0.6% agar at pH 5.7 and autoclaved at 121°C (0.1 MPa) (Supplementary data 4). The explants were placed vertically into the culture media with the basal plate tissue inserted into the medium [21-23]. For each culture medium 80 explants were used. About 42 bulbs were used for shoot initiation; from each bulb an average of 8–10 explants were obtained, which were divided into the media. All cultures were incubated in a growth chamber at 22°C with neon Osram L30/41 lamps and a photoperiod of 14 h.

Statistical analysis: The STATISTICA 8 package (Statsoft) was used. Two samples of galantamine were assessed: one sample extracted with methanol and the other with ethanol. Each sample was achieved from plant organs (bulbs, stems plus leaves, roots, flowers) in 3 phenological stages (before blooming, during blooming and after blooming) for a total of 30 cases. Means and standard deviations of data from the 2 samples are shown in box plots (supplementary data 1). In order to evaluate differences between the 2 samples the *t*-test (Supplementary data 2) was used, followed by homoscedasticity and normality of the distributions (Shapiro-Wilk test, Supplementary data 2). Finally, the differences between the means were assessed by the Wilcoxon non-parametric test (Supplementary data 3).

References

[1] Harris R. (**2005**) *Narcissus poeticus*: the heart note. *Perfume & Flavorist*, ***30***, 46-53.
[2] Zaidi N, Khan NH, Zafar F, Zafar SI. (**2000**) Bulbous and cormous monocotyledonous ornamental plants. *In Vitro Science Vision*, ***6***, 58-73.
[3] Heret C, Maupetit P, Petrzilka M. (**1992**) New organoleptically important constituents of *Narcissus* absolute (*Narcissus poeticus* L.). *Journal of Essential Oil Research*, ***4***, 41-47.
[4] Ferri D, Adami M, De Santis A, Ubaldi C. (**2009**) Traditional and supercritical CO_2 extraction of essential oil from *Narcissus poeticus* L. of Rocca di Mezzo (Aq). *Perfumer & Flavorist*, ***34***, 30-33.
[5] Andersen KE, Jensen CD, Paulsen E. (**2005**) Up allergic contact dermatitis from cosmetics. *Giornale Italiano di Dermatologia e Venerologia*, ***140***, 703-712.
[6] Lubbe A, Pomehačova B, Young HC, Verpoorte R. (**2010**) Analysis of metabolic variation and galanthamine content in *Narcissus* bulbs by ^{1}H NMR. *Phytochemical Analysis*, ***21***, 66-72.
[7] Lopez S, Bastida J, Viladomat F, Codina C. (**2003**) Galantamine pattern in *Narcissus confusus* plants**.** *Planta Medica*, ***71***, 1166-1168.
[8] Cahlíková L, Ločárek M, Benešová N, Kučera R, Chlebek J, Novák Z, Opletal L. (**2013**) Isolation and cholinesterase inhibitory activity of *Narcissus* extracts and Amaryllidaceae alkaloid. *Natural Product Communications*, ***8***, 781-785.
[9] Havlasová J, Šafratová M, Siatka T, Štěpánková Š, Novák Z, Ločárek M, Opletal L, Hrabinová M, Jun D, Benešová N, Kuneš J, Cahlíková L. (**2014**) Chemical composition of bioactive alkaloid extracts from some *Narcissus* species and varieties and their biological activity. *Natural Product Communications*, ***9***, 1151-1155.
[10] Kumar A, Singh A, Ekavali (**2015**) A review on Alzheimer's disease pathophysiology and its management: an update. *Pharmacology Reports*, ***67***, 195-203.
[11] Pace L, Grandi S, Marotti M, Piccaglia R, Pacioni G, Spanò L. (**2010**) Terpenoids profiles of *in vitro* regenerated *Artemisia petrosa* subsp. *eriantha* (Apennines' genepì). *Annals of Applied Biology*, ***157***, 309-316.
[12] Reale S, Pace L, D'Archivio AA, De Angelis F, Marcozzi G. (**2014**) Volatiles fingerprint of *Artemisia umbelliformis* subsp. *eriantha* by Head Space –Solid Phase MicroExtraction GC-MS. *Natural Products Research*, ***28***, 61-66.
[13] Reale S, Fasciani P, Pace L, De Angelis F, Marcozzi G. (**2011**) Volatiles fingerprint of artemisinin-rich *Artemisia annua* cultivars by Head Space-Solid Phase Microextraction GC-MS. *Rapid Communications in Mass Spectrometry*, ***25***, 1-6.
[14] Anonis DP. (**1983**) *Narcissus* in perfumery and cosmetics. *Drug Cosmetic Industry*. pp 34-38 and pp 77-78.
[15] Lubbe A, Gude H, Verpoorte R, Choi YH. (**2013**) Seasonal accumulation of major alkaloids in organs of pharmaceutical crop *Narcissus* Carlton. *Phytochemistry*, ***88***, 43-53.
[16] Benschop M, Kamenetsky R, Le Nard M, Okubo H, De Hertogh A. (**2010**) The global flower bulb industry: Production, utilization, research. *Horticultural Reviews*, ***36***, 1-115.
[17] Lopez S, Bastida J, Viladomat F, Codina C. (**2002**) Solid-phase extraction and reversed-phase high performance liquid chromatography of the five major alkaloids in *Narcissus confusus*. *Phytochemical Analysis*, ***13***, 311-315.
[18] Santos A, Fidalgo F, Santos I, Salema R. (**2002**) *In vitro* bulb formation of *Narcissus asturiensis*, a threatened species of the Amaryllidaceae, *Journal of Horticultural Science & Biotechnology*, ***77***, 149-152.
[19] Murashige T, Skoog F. (**1962**) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum*, ***15***, 473-497.
[20] Seabrook EAJ, Cumming BG, Dionne LA. (**1976**) The *in vitro* induction of adventitious shoot and root apices on *Narcissus* (daffodil and narcissus) cultivar tissue. *Canadian Journal of Botany*, ***54***, 814-819.
[21] Tipirdamaz R. (**2003**) Rooting and acclimatization of *in vitro* micropropagated snowdrop (*Galanthus ikariae* Baker) bulblets. *Akdeniz Üniversitesi Ziraat Fakultesi Dergisi*, ***16***, 121-126.
[22] Anbari S, Tohidfar M, Hosseini R, Haddad R. (**2007**) Somatic embryogenesis induction in *Narcissus papyraceus* cv. Shirazi. *Plant Tissue Cultures & Biotechnology*, ***17***, 37-46.
[23] Çiğ A, Başdoğan G. (**2015**) *In vitro* propagation techniques for some geophyte ornamental plants with high economic. *International Journal of Secondary Metabolism*, ***2***, 27-49.

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2017
Vol. 12
No. 1
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A New Imidazole from the Sponge *Dercitus (Halinastra) japonensis*

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Received: September 14th, 2016; Accepted: November 7th, 2016

A new imidazole sulfate (**1**) and three known compounds (**2**-**4**) were isolated from the sponge *Dercitus (Halinastra) japonensis*. The structure of compound **1** was elucidated by spectroscopic means. Compound **2** was confirmed to show anti-HIV activity, whereas compounds **1**, **3** and **4** were inactive.

Keywords: Anti-HIV, Ancorinolate B, 2-Aminoadenosine, 2'-De-*N*-methylaplysinopsin, *Dercitus (Halinastra) japonensis*.

Human immunodeficiency virus (HIV) was discovered in the middle 1980s as a causative agent of acquired immune deficiency syndrome (AIDS). Current therapy, named HAART (Highly Active Anti-Retroviral Therapy), against the disease by administering "cocktails" of drugs prevents the patients from development of the sickness, but they have to keep taking the medication as HAART cannot eradicate the virus [1]. However, there exists a demand for newer drugs for a complete cure.

In our quest for new antiviral drug leads not only for HIV, but for several viruses, we screened a library of 800 extracts of coral reef organisms collected along the islands of Okinawa. Chromatographic study of an anti-HIV extract led to four molecules (**1**-**4**) being obtained. Although the antiviral activity was ascribed to compound **2**, a new compound **1**, separated together with **2**, is the subject of this note.

As the MeOH soluble extract of the sponge *Dercitus japonensis* showed anti-HIV activity, a portion of it was separated by gel filtration and reversed phase HPLC resulting in the isolation of compound **1** with three known compounds: ancorinolate B (**2**) [2], 2-aminoadenosine (**3**) [3], and 2'-de-*N*-methylaplysinopsin (**4**) [4]. Each known compound was identified by comparing its spectroscopic data with those published.

Compound **1** was obtained as a glassy material and its negative HR-ESIMS showed pseudomolecular ions as $C_5H_7N_2O_4{}^{32}S$ ([M-Na]$^-$, Δ -0.27 mmu) and $C_5H_7N_2O_4{}^{34}S$ ([M-Na]$^-$, Δ -1.06 mmu) indicating three degrees of unsaturation without the valence of a sulfur atom. The ^{1}H NMR data contained characteristic low-field signals at δ 7.41 (1H, brs) and 8.87 (1H, d, J = 1.1 Hz), while the ^{13}C NMR spectrum showed three sp^2 signals at δ 116.4 (CH), 131.0 (C), and 133.6 (CH) suggesting the presence of a heteroaromatic moiety. With the presence of two nitrogen atoms in the molecule, it is likely to have a monosubstituted imidazole or pyrazole ring. Another structural feature is two consecutive methylenes at δ 2.91 (t, J = 6.4 Hz) and 3.99 (t, J = 6.4 Hz) showing COSY cross peaks with each other and also ^{13}C NMR signals at δ 24.8 and 63.8. Judging from the chemical shifts, one of the methylenes is oxygenated. The remaining structural unit was assigned as a sulfate (1223, 1062 cm^{-1}) attached to the oxygenated methylene.

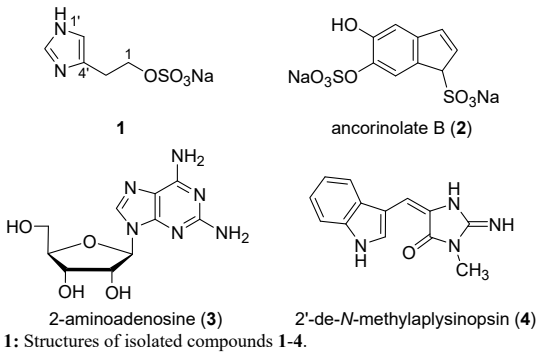

Figure 1: Structures of isolated compounds **1**-**4**.

The sulfate was confirmed as a sodium salt by atomic absorption spectroscopy (AAS). Therefore, the structure of compound **1** was elucidated as an imidazole or a pyrazole with a sulfated ethanol moiety.

HMBC correlations between the aromatic portion and the ethyl group supported the two possible structures, however, it was not conclusive to determine either a pyrazole or imidazole derivative. Since aromatic proton and carbon signals of compound **1** showed close similarity to those (δ 7.39 s, 8.91 d, J = 1.4 Hz; δ 116.1, 131.5, 133.2) of 2-(1*H*-imidazol-4-yl)-ethanol (**5**) and also to those (δ 7.53 s, 9.04 d, J = 1.3 Hz; δ 116.7, 128.9, 133.7) of histamine dihydrochloride (**6**), an imidazole moiety was proved in **1**. By treating compound **5** with sulfuric acid in the presence of dicyclohexylcarbodiimide (DCC) [5], a trace amount of compound **1** was formed, giving a pseudomolecular ion (Δ -1.71 mmu). Thus, we concluded that compound **1** is sodium 2-(1*H*-imidazol-4-yl)-ethyl sulfate.

Figure 2: Structures of reference compounds **5** and **6**.

Table 1: Anti-HIV activity (IC_{50}) and cytotoxicity (CC_{50}) of compounds **1**-**5**.

Compounds	IC_{50}, μM	CC_{50}, μM
1	n.d.	> 93
2	109	$> 2.84\times10^2$
3	$> 2.6\times10^2$	65.2
4	$> 4.2\times10^2$	3.8×10^2
5	n.d.	$> 1.8\times10^2$
AZT (nM)	9.0	> 100.0

n.d.: not determined

The anti-HIV activity of compounds **1**-**5** were evaluated as shown in Table 1. Compound **2** showed moderate activity, but the other compounds did not show any activity.

Experimental

General: HPLC separation was carried out on either a Nacalai Cosmosil C18 (10×250 mm) or HILIC column (4.6×250 mm, 10×250 mm) with an HPLC unit composed of RI and UV detectors. NMR spectra were recorded on a Bruker AVANCE III spectrometer either in dimethylsulfoxide (DMSO)-d_6 or D_2O. ESIMS data were recorded on a Jeol JMR-T-100LP mass spectrometer. FTIR and UV spectra were taken on Jasco FT/IR-6100 and Jasco V-660 spectophometers, while atomic absorption was recorded on a Hitachi Z-2010 instrument. All solvents used were reagent grade (Wako). Compounds **5** (Synthonix) and **6** (TCI) were purchased.

Sponge: A specimen of the sponge *Dercitus* (*Halinastra*) *japonensis* van Soest, Beglinger & de Voogd (2010) was collected by hand with scuba at a reef of Yonaguni Island, Okinawa, September 1992. The specimen was brought back to the laboratory and kept frozen at -20ºC until extraction. The sponge was identified by NJdV and a voucher specimen coded RMNH POR 8684 is deposited at Naturalis Biodiversity Center.

Extraction and isolation: The specimen (0.70 kg, wet weight) was soaked in acetone (1.0 L) 3 times at room temperature. The acetone solution was filtered and concentrated under vacuum, and the resulting material was partitioned between water and ethyl acetate (EtOAc). The water layer was concentrated and the residue washed with methanol (MeOH). The MeOH solution was concentrated to give a crude extract (49.8 g). As this extract showed inhibition against HIV, part of it (505.3 mg) was separated on a Sephadex LH-20 (Pharmacia) column (20 x 300 mm) using aqueous MeOH to give 8 fractions. The sixth fraction was found to contain compound **3** (16.7 mg). While waiting for anti-HIV assay results, we separated the last fraction to obtain compound **4** (11.6 mg) by C18 HPLC (0.01% TFA in CH_3CN). As the fourth fraction showed anti-HIV activity, it was further separated by HILIC HPLC (CH_3CN:H_2O, 9:1) to give pure compound **2** (15.8 mg) and impure compound **1** (2.5 mg). To supply an additional amount of compound **1**, another portion of the extract (303.3 mg) was successively separated by Sephadex LH-20 (aqueous MeOH), C18 HPLC (water), and HILIC HPLC (CH_3CN:H_2O, 9:1) to give pure compound **1** (3.6 mg).

Compound (**1**)
Colorless glass.
IR (film): 3154, 1628, 1223, 1062, 998, 770, 626, 437 cm^{-1}.
UV/Vis λ_{max} (MeOH): 210 nm (log ε) 3.69.
^1H NMR (500 MHz, DMSO-d_6): δ 8.87 (1H, d, J = 1.1 Hz, H-2'), 7.41 (1H, brs, H-5'), 3.99 (2H, t, J = 6.4 Hz, H-1), 2.91 (2H, t, J = 6.4 Hz, H-2).
^{13}C NMR (125 MHz, DMSO-d_6): δ 133.6 (CH, C-2'), 131.0 (C, C-4'), 116.4 (CH, C-5'), 63.8 (CH_2, C-1), 24.8 (CH_2, C-2).
COSY: H-1/H-2.
HMBC: H-1/C-2,4', H-2/C-1,4',5', H-2'/C-4',5', H-5'/C-2',4'.
HRESIMS: *m/z* 191.01238 $[M\text{-}Na]^-$; calcd for $C_5H_7N_2O_4{}^{32}S$ 191.01265; *m/z* 193.00739 $[M\text{-}Na]^-$; calcd for $C_5H_7N_2O_4{}^{34}S$ 193.00845.
AAS: c_{Na} = 0.23 ppm.

Sulfation of 5: Compound **5** (1.0 mg) in dimethyformamide (DMF, 150 μL) was mixed with DCC (63.0 mg) in DMF (300 μL) and sulfuric acid (3.2 μL) in DMF (150 μL). The reaction suspension was stirred overnight at rt. After adding water, the mixture was filtered and the filtrate partitioned against CH_2Cl_2. The water layer was concentrated to give a crude product (56.1 mg), which was separated by HILIC HPLC (CH_3CN:H_2O, 9:1) to give a fraction (4.7 mg) containing compound **1**.
HRESIMS: *m/z* 191.01094 $[M\text{-}Na]^-$; calcd for $C_5H_7N_2O_4{}^{32}S$ 191.01265.

Anti-HIV assay: For evaluation of anti-HIV activities, human T-cell line MT-4 cells were seeded in a 96-well plate at a density of 5 × 10^5 cells/mL in 100 μL/wells of Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum and then cells were infected with HIV-1 strain IIIB at a viral multiplicity of infection of 0.001 in the presence of various concentrations of each compound. Moreover, to confirm the cytotoxic effects, MT-4 cells were inoculated with each compound at various concentrations without virus infection. After incubation at 37ºC in an atmosphere of 5% CO_2 for 5 days, cell viability was determined by WST-1 assay (Dojindo Molecular Technologies, Inc.) that measured absorbance at 450 and 650 nm using the sodium salt of 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate, which produces a highly water-soluble formazan dye. The calculation of the 50% virus inhibitory concentration (IC_{50}) and the 50% cytotoxic concentration (CC_{50}) of each compound were performed by statistical analysis software, GraphPad Prism (GraphPad Software, Inc.).

Acknowledgments - We thank Dr Chiaki Tanaka, Kyushu University, for the database search. This research was supported by a grant from Okinawa Intellectual Cluster Programs of Okinawa Science and Technology Center.

References

[1] Le Douce V, Janossy A, Hallay H, Ali S, Riclet R, Rohr O, Schwartz C. (**2012**) Achieving a cure for HIV infection: do we have reasons to be optimistic? *Journal of Antimicrobial Chemotherapy*, ***67***, 1063-1074.
[2] Meragelman KM, West LM, Northcote PT, Pannell LK, McKee TC Boyd MR. (**2002**) Unusual sulfamate indoles and a novel indolo[3,2-*a*]carbazole from *Ancorina* sp. *Journal of Organic Chemistry*, ***67***, 6671-6677.
[3] Robins MJ, Hansske F, Bernier SE. (**1981**) Nucleic acid related compounds. 36. Synthesis of the 2'-*O*-methyl and 3'-*O*-methyl ethers of guanosine and 2-aminoadenosine and correlation of *O*'-methylnucleoside ^{13}C nmr spectral shifts. *Canadian Journal of Chemistry*, ***59***, 3360-3364.
[4] Djura P, Faulkner DJ. (**1980**) Metabolites of the marine sponge *Dercitus* sp. *Journal of Organic Chemistry*, ***45***, 735-737.
[5] Hoiberg CP, Mumma RO. (**1969**) Preparation of sulfate esters. Reactions of various alcohols, phenols, amines, mercaptans, and oximes, with sulfuric acid and dicyclohexylcarbodiimide. *Journal of the American Chemical Society*, ***91***, 4273-4278.

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Vol. 12
No. 1
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Acetyl-cholinesterase Inhibitory Activity of Methoxyflavones Isolated from *Kaempferia parviflora*

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Received: May 20th, 2016; Accepted: September 28th, 2016

MeOH extracts of *Kaempferia parviflora* Wall. ex. Baker, family Zingiberaceae, were consecutively partitioned with $CHCl_3$, EtOAc, and *n*-BuOH. The $CHCl_3$ fractions were diluted in distilled water with *n*-hexane–CH_2Cl_2 and three methoxyflavones were isolated from the CH_2Cl_2 extract. Based on spectral analysis and comparison of the spectral data with literature values, the compounds were identified as 3,5,7,3′,4′-pentamethoxyflavone (KP1), 5,7-dimethoxyflavone (KP2), and 5,7,4′-trimethoxyflavone (KP3). In relation to their possible effectiveness against Alzheimer's disease, these compounds were tested for their ability to inhibit acetylcholinesterase activity and neurite outgrowth in the PC12 cell line. Of the three compounds, KP1 was the only one to inhibit significantly the acetylcholinesterase activity in a dose-dependent manner.

Keywords: *Kaempferia parviflora*, Zingiberaceae, Acetylcholinesterase inhibitory, Methoxyflavone,

Kaempferia parviflora (KP; family Zingiberaceae) is endemic in Thailand and can be found cultured in the wild in Pitsanulok and Phetchabun. Locally, it is named Krachai Dum [1]. The KP plant grows low to the ground and has short, fleshy, and tuberous dark purple rhizomes and small violet flowers [2]. The black or purple color of KP rhizomes is affected by the conditions of the soil environment. The climatic and soil conditions of South Korea are not ideal for cultivation of this plant.

KP ethanol or methanol extracts are effective for treating human disorders such as visceral fat accumulation, hyperinsulinemia, glucose intolerance, hypertension, diabetes, obesity, peripheral neuropathy, gastric ulcer, and Alzheimer's disease (AD) [3]. Compounds isolated from KP include polymethoxyflavonoids and the phenolic glycoside kaempferiaoside [4]. Moreover, phytochemical screening of KP extracts has produced positive tests for alkaloids, anthrones, and coumarins [5]. 5,7,4′-Trimethoxyflavone, 5,7,3′4′-tetramethoxyflavone, and 3,5,7,4′-methoxyflavones have exhibited significant inhibitory activity against various biological processes [4].

AD is one of the principal causes of disease and death among the elderly [6]. The disease has numerous important confirmed facets, including the hallmark β-amyloid plaques [3]. There have been many different ways and attempts at curing and preventing AD. In recent years, cholinesterases have received increasing attention for the role they play in the formation from β-amyloids during the early stages of AD [7]. Acetylcholinesterase (AChE) serves as the key enzyme in AD, as it hydrolyzes the ester bond (at thiocholine and acetic acid) of the neurotransmitter acetylcholine in the cholinergic synapse [6].

The current study describes the isolation of three methoxyflavones from *K. parviflora*. The molecular structures were determined through ^{1}H NMR spectroscopic, mass spectrometric and literature data. Compounds KP1, KP2, and KP3 were dissolved in DMSO. KP1, at the low concentration of 40 μM, exhibited strong inhibitory activity (80%) against AChE. KP2 and KP3, on the other hand, exhibited weak AChE-inhibitory activity (20%) at a concentration of 100 μM (Figure 1). In fact, the AChE activity increased to 140% in the presence of 40 and 80 μM of KP2 and KP3, confirming that these two compounds did not have high AChE-inhibitory activity.

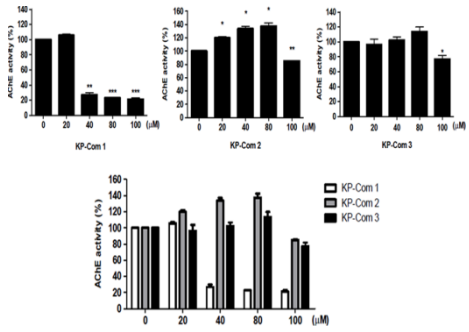

Figure 1: Acetylcholinesterase (AChE) activity in the presence of KP1, KP2 and KP3.

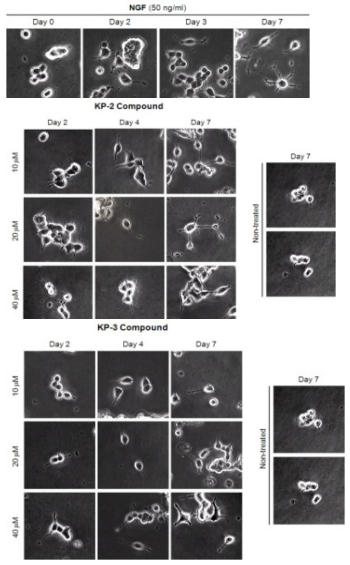

Figure 2: Neurite outgrowth of PC12 cells in the presence of KP2 and KP3. NGF, nerve growth factor.

Although the AChE activity was increased in the presence of 20 μM KP1, the ginger plant compound at 40, 80, and 100 μM concentrations significantly reduced the enzyme activity. It was previously reported that K2 and K3 had high AChE-inhibitory activity, although much lower than that of K1. PC12 cells were treated with the three compounds at the same concentrations to compare their effects on the changes in cell morphology. The cytotoxicity of the compounds in order of strength was KP1 > KP2 > KP3. In the case of KP1, serious cell damage occurred at 40 μM, whereas at the same concentration, KP2 caused only slight cell damage and KP3 did not cause any cell damage at all. PC12 cells were treated with the three compounds dissolved in DMSO to confirm their effects on neurite outgrowth. Almost no neurite outgrowth was observed in the presence of KP1, which was likely an influence of the cell damage caused by the cytotoxicity of this compound. In the cases of KP2 and KP3, neurite outgrowth was similar to or more than that observed in the presence of nerve growth factor (Figure 2), indicating that KP2 and KP3 have the ability to induce neurite outgrowth in PC12 cells. The isolated compounds were identified as 3,5,7,3′,4′-pentamethoxyflavone (KP1), 5,7-dimethoxyflavone (KP2), and 5,7,4′-trimethoxyflavone (KP3). KP2 and KP3 significantly inhibited AChE activity in a dose-dependent manner.

Experimental

General experimental procedures: ^{1}H NMR spectra were recorded with an Agilent MR 400 DD2 400MHz spectrometer. Column chromatography (CC) was conducted using silica gel 60 (40-63 and 63-200 μm particle size) and RP-18 (40-63 μm particle size), both obtained from Merck III.

Plant material: *Kaempferia parviflora* rhizomes were kindly provided by a local farmer from Jang heung, Jeon Nam, South Korea. The rhizomes were identified by Prof. Hyung-In Moon. A voucher specimen (No. 2012-0405) has been deposited in the Herbarium of the Dong-A University (Busan, South Korea).

Extraction and fractionation: The rhizomes of *K. parviflora* (5 kg) were sliced and extracted under reflux with 9 L of MeOH, 3 times at 5°C The MeOH extracts (198 g) were combined and concentrated *in vacuo* at 40°C. The MeOH extract was diluted with distilled water (0.9 L) and then partitioned with $CHCl_3$ (0.6 L×3). The $CHCl_3$-soluble fraction was diluted with distilled water and partitioned with *n*-hexane (0.9 L×3), CH_2Cl_2 (0.7 L×3), EtOAc (0.8 L×3) and *n*-BuOH (0.8 L). The CH_2Cl_2 extract (49 g) was separated by silica gel CC by gradient elution with $CHCl_3$: MeOH (from 30:1 to 0:1 v/v); 12 fractions were obtained (K1-K12). Fractions K1 to K8 were further separated by CC using $CHCl_3$: MeOH (30:1, v/v). All fractions were recrystallized at -20°C from MeOH; KP1 (5.6 g) was obtained from fraction K5, KP2 (3g) from fraction K6 and KP3 (2.7g) from fraction K4.

Acetylcholinesterase inhibitory activity assay: PC-12 cells were obtained from American Type Culture Collection (ATCC; USA). Cells were cultured in a collagen coated culture dish in Roswell Park Memorial Institute 1640 (RPMI1640; GIBCO, Korea) medium supplemented with 10%, v/v, heat-inactivated fetal bovine serum (FBS; WelGENE, Korea), 5%, v/v, heat-inactivated horse serum (HS; WelGENE, Korea), 100 Units/mL penicillin, and 100 mg/mL streptomycin at 37°C in a humidified atmosphere of 5% CO_2. For differentiation, the medium was replaced with RPMI1640 supplemented with 2% FBS and 1% HS. PC-12 cells were treated with 50 ng/mL nerve growth factor (NGF; Sigma) for 7 days. For microscopic observation at 0, 2, 4, and 7 days of neurite outgrowth, PC-12 cells were treated with 10, 20, 40 μM of KP 1~3. Microscopic observations were made after 2, 4, and 7 days of neurite outgrowth.

KP 1~3 were measured for acetylcholinesterase inhibitory activity using the chromogenic method, with slight modification. Acetylcholinesterase from human erythrocytes (10 μg/mL, 860 U/mg, Sigma) was dissolved in 100 mM phosphate buffer (PB, pH 7.0) containing dithiobis (2-nitrobenzoic acid) (DTNB, Sigma), 10 mM (3.96 mg/ mL). Acetylthiocholine iodide, 75 mM (21.67 mg/mL) in the same buffer (pH 7.0), was used as a substrate solution. The enzyme solution (90 μL) and each concentration (0, 20, 40, 80, 100 μM) of the KP compounds (18 μL) were mixed in a well of a microtiter plate and measured (415 nm) at time zero with a microplate reader. After incubation for 15 min at room temperature the substrate solution (90 μL) was added and the reaction left for 10 min at 37°C. The increase in absorbance from zero time was measured.

Statistical analysis: The results were expressed as the mean ± SE. Each value represents the mean of at least 3 independent experiments in each group. The statistical significance of the difference between two cell populations was determined using the two-tailed Student's t-test (Origin software; OriginLab). *P* values equal to or less than 0.05 were considered significant. A difference was considered to be significant at $P<0.05$.

Acknowledgements - This work was supported by the R&D program of MOTIE/KIAT (Establishment of Infra Structure for Anti-aging Industry Support)

References

[1] Supinya T, Sanan S, Sopa K. (**2008**) Anti-allergic activity of compounds from *Kaempferia parviflora*. *Journal of Ethnopharmacology*. ***116***, 191-193.
[2] Denpong P, Junya N, Chie M, Kyoko F, Khaetthareeya S, Bung-orn S, Ryoko Y, Mikihisa T. (**2007**) Modulation of function of multidrug resistance associated-proteins by *Kaempferia parviflora* extracts and their components. *European Journal of Pharmacology*. ***566***, 67-74.
[3] (a) Rujjanawate C, Kanjanapothi D, Amornlerdpison D, Pojanagaroon S. (**2005**) Anti-gastric ulcer effect of *Kaempferia parviflora*. *Journal of Ethnopharmacology*. ***102***, 120-122; (b) Nakata A, Koike Y, Matsui H, Shimada T, Aburada M, Yang J. (**2014**) Potent SIRT1 enzyme-stimulating and anti-glycation activities of polymethoxyflavonoids from *Kaempferia parviflora*. *Natural Product Communications*, ***9***, 1291-1294.
[4] Chavi Y, Khanchara P, Supawadee D, Varima W, Prasat K. (**2003**). Bioactive flavonoids from *Kaempferia parviflora*. *Fitoterapia*, ***75***, 89-92.
[5] Denpong P, Junya N, Ryoko Y, Teruo M, Khaetthareeya S, Bung O.R, Chavi Y, Mikihisa T. (**2007**) Effects of *Kaempferia parviflora* extracts and their flavone constituents on P-glycoprotein function. *Journal of Pharmaceutical Sciences,* ***96***, 223-233.
[6] Kim JY, Lee WS, Kim YS, Curtis-Long M, Lee BW, Ryu YB, Park KI. (**2011**) Isolation of cholinesterase-inhibiting flavonoids from *Morus lhou*. *Journal of Agricultural and Food Chemistry*, ***59***, 4589–4596.
[7] Tanzi RE, Parson AB. (**2013**) Decoding darkness: The search for the genetic causes of Alzheimer's Disease. *The Journal of Undergraduate Neuroscience Education*, ***1***, R12-R13.

NPC Natural Product Communications

2017
Vol. 12
No. 1
23 – 26

Antimutagenic Effects of Polymethoxy Flavonoids of *Citrus unshiu*

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Received: August 4th, 2016; Accepted: October 1st, 2016

Citrus fruits have been used as edible fruit and traditional medicine for various diseases such as cancer. In the courses of our study to find antimutagens, we have found that the ethanolic extract of the peel of *Citrus unshiu* Marc showed antimutagenic effects against several mutagens in the Ames test using *Salmonella typhimurium* TA98 strain. Three polymethoxy flavonoids, nobiletin, 3,5,6,7,8,3',4'-heptamethoxyflavone, and tangeretin, were identified in the extract as major constituents. These three polymethoxy flavonoids showed antimutagenic effects in the Ames test *in vitro* and in the micronucleus test *in vivo*.

Keywords: *Citrus unshiu*, Polymethoxy flavonoids, Antimutagenic effect.

Citrus unshiu Marc., known as cold hardy mandarin, satsuma mandarin, satsuma orange, Christmas orange, and tangerine [1a], is cultivated in the southern regions of Japan and China. In addition, its peel has been traditionally used in East Asia as a drug for the treatment of vomiting and dyspepsia. Reports about the peel show anti-inflammation [1b], hepatoprotective, neuroprotective [2a], and anti-microbial activities [2b].

Polymethoxy flavonoids such as nobiletin (**1**), 3,5,6,7,8,3',4'-heptamethoxyflavone (HMF, **2**), and tangeretin (**3**) are known as the major constituents of *Kaempferia parviflora* and *Citrus* plants. Recent study showed that nobiletin (**1**) has chemopreventive effects against colon carcinogenesis, partly through regulation of leptin levels [3a]. The antimutagenic effects of nobiletin (**1**) were reported against the mutagens 3-amino-1,4-dimethyl-5H-pyrido[4,3-*b*]indole (Trp-P-1) [3b], 3,4-dimethyl-3H-imidazo[4,5-*f*]quinolin-2-amine (MeIQ) [4a] and benzo[*a*]pyrene (BaP) [4b] *in vitro* and against 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) *in vivo*.

In the course of our study to find antimutagens, we found antimutagenic effects of the EtOH extract of *C. unshiu* in the *Salmonella typhimurium* mutagenicity assay against several mutagens. This short-term assay, namely the Ames test, has been extensively used to survey a great variety of environmental substances for mutagenic activity. In this study we evaluated the antimutagenic effects of the ethanolic extract of *C. unshiu,* nobiletin (**1**), HMF (**2**), and tangeretin (**3**) in the Ames test *in vitro* and the micronucleus test *in vivo*.

The EtOH extract of *C. unshiu* was partitioned into an EtOAc-MeOH-H_2O (13:3:16, v/v/v) mixture to give an organic layer and an aqueous layer. The antimutagenic effects of the EtOH extract, the organic layer, and H_2O layer against 1-nitropyrene (1-NP), 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), Trp-P-1, 3-amino-1-methyl-5H-pyrido[4,3-*b*]indole (Trp-P-2), and BaP, were evaluated by the Ames test using *S. typhimurium* TA98 strain. 1-NP and BaP are representative environmental mutagens/carcinogens produced by internal combustion engines, such as diesel engines and emitted into the air. PhIP, Trp-P-1, and Trp-P-2 are mutagenic/carcinogenic heterocyclic amines formed by cooking meat. The EtOH extract showed antimutagenic effects against 1-NP, PhIP, Trp-P-1, and Trp-P-2 (Table 1). The organic layer showed antimutagenic effects at lower concentrations [IC_{50} against1-NP: 0.32 mg/plate, against PhIP: 0.02 mg/plate, against Trp-P-1: 0.02 mg/plate, against Trp-P-2: 0.08 mg/plate] than that of the H_2O layer [IC_{50} against PhIP: 0.21 mg/plate, against Trp-P-1: 0.42 mg/plate, against Trp-P-2: 1.97 mg/plate]. This result suggests that the active constituents were concentrated in the organic layer. On the other hand, the extract did not show an antimutagenic effect against BaP at 0.5 mg/plate.

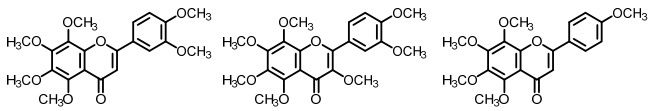

Figure 1: Structures of polymethoxy flavonoids **1**–**3**.

Because polymethoxy flavonoids are reported to be major constituents of Citrus plants, we analyzed the EtOH extract of peels of *C. unshiu* by UV-HPLC to examine whether polymethoxy flavonoids existed in the extract. Peaks of polymethoxy flavonoids **1**–**3** were identified in the extract by comparing retention times and UV spectra. The amounts of **1**–**3** in 1 g of EtOH extract from peels of *C. unshiu* were as follows: nobiletin (**1**), 2.64 mg; HMF (**2**), 7.90 mg; and tangeretin (**3**), 2.42 mg.

To clarify the antimutagenic effects of nobiletin (**1**), HMF (**2**), and tangeretin (**3**), their antimutagenic activities against 1-NP, PhIP, Trp-P-1, and Trp-P-2 were examined by the Ames test using *S. typhimurium* TA98 strain. Compounds **1**–**3** showed antimutagenic effects against PhIP, Trp-P-1, and Trp-P-2 (**1** [IC_{50} against PhIP: 15.1 nmol/plate; against Trp-P-1: 37.0 nmol /plate, against Trp-P-2: 63.2 nmol /plate], **2** [IC_{50} against PhIP: 12.7 nmol/plate; against Trp-P-1: 22.4 nmol /plate, against Trp-P-2: 92.3 nmol /plate], and **3** [IC_{50} against PhIP: 2.9 nmol/plate; against Trp-P-1: 2.6 mol /plate, against Trp-P-2: 28.8 nmol /plate]) (Table 2). Among the tested samples, tangeretin (**3**) showed antimutagenic effects at the lowest concentration against PhIP, Trp-P-1, and Trp- P-2. On the other hand, **1**–**3** did not show significant antimutagenic effects against 1-NP and BaP. The IC_{50} value of **1** against Trp-P-1 in this report was identical to that reported [IC_{50}: between 30 and 60 nmol/plate] [3b].

To examine antimutagenic effects of extract of *C. unshiu*, and polymethoxy flavonoids **1**–**3** *in vivo*, we conducted a micronucleus

Table 1: Antimutagenic effects of EtOH extract, organic layer, and H_2O layer from *C. unshiu*.

Mutagen	With/Without S9 mix	Sample	Dose (mg/plate)	Revertants (number/plates)	Inhibition (%)
1-NP	without	*C. unshiu* ext.	0	1597	0
			0.1	1435	10
			0.2	1224	23
			0.4	791	51
			0.8	552	65
		Organic layer	0	1597	0
			0.05	1387	13
			0.1	1185	26
			0.2	985	38
			0.4	682	57
		H_2O layer	0	1597	0
			0.5	1495	6
			1	1458	9
			2	1466	8
			4	1206	25
PhIP	with	*C. unshiu* ext.	0	1115	0
			0.025	701	37
			0.05	512	54
			0.1	296	74
			0.2	220	80
		Organic layer	0	1031	0
			0.0125	564	45
			0.025	336	67
			0.05	261	75
			0.1	176	83
		H_2O layer	0	1031	0
			0.125	772	25
			0.25	415	60
			0.5	340	67
			1	241	77
Tr-P-1	with	*C. unshiu* extract	0	1198	0
			0.025	820	32
			0.05	586	51
			0.1	378	69
			0.2	234	81
		Organic layer	0	945	0
			0.0125	528	44
			0.025	433	54
			0.05	295	69
			0.1	225	76
		H_2O layer	0	945	0
			0.125	882	7
			0.25	556	41
			0.5	432	54
			1	286	70
Trp-P-2	with	*C. unshiu* ext.	0	1235	0
			0.125	1043	16
			0.25	748	40
			0.5	558	55
			1	344	72
		Organic layer	0	2075	0
			0.05	1300	37
			0.1	915	56
			0.2	663	68
			0.4	607	71
		H_2O layer	0	2075	0
			0.5	1656	20
			1	1389	33
			2	1026	51
			4	695	67
BaP	with	*C. unshiu* ext.	0	495	0
			0.0625	469	5
			0.125	529	ND
			0.25	495	0
			0.5	374	24
		Organic layer	0	495	0
			0.05	473	5
			0.1	508	ND
			0.2	493	0
			0.4	375	24
		H_2O layer	0	495	0
			0.5	446	10
			1	423	15
			2	460	7
			4	514	ND

test using peripheral blood of male ICR mice. The micronucleus test is a test to detect chromosomal damage induced by genotoxic/ carcinogenic compounds, and has been used to evaluate the antimutagenic agents *in vivo* [3b]. In this study, we evaluate the *in vivo* antimutagenic effects of the EtOH extract of *C. unshiu* and

Table 2: Antimutagenic effects of **1**–**3**.

Mutagen	With/Without S9 mix	Sample	Dose (nmol/plate)	Revertants (number/plates)	Inhibition (%)
1-NP	without	Nobiletin	0	1191	0
			5	940	21
			10	956	20
			20	957	20
			40	968	19
		HMF	0	1191	0
			100	1092	8
			200	944	21
			400	976	18
			800	1309	ND
		Tangeretin	0	1191	0
			50	985	17
			100	950	20
			200	926	22
			400	944	21
PhIP	with	Nobiletin	0	818	0
			2.5	730	11
			5	537	35
			10	460	44
			20	360	56
		HMF	0	1031	0
			1.25	914	11
			2.5	984	5
			5	867	16
			10	549	47
			20	427	59
		Tangeretin	0	786	0
			0.47	678	14
			0.9	623	21
			1.86	468	40
			3.72	295	62
Trp-P-1	with	Nobiletin	0	973	0
			10	934	4
			20	596	39
			40	468	52
			80	426	56
		HMF	0	1543	0
			6.25	1317	15
			12.5	998	35
			25	712	54
			50	580	62
		Tangeretin	0	1179	0
			0.625	879	25
			1.25	800	32
			2.5	593	50
			5	441	63
Trp-P-2	with	Nobiletin	0	1086	0
			20	692	36
			40	649	40
			80	467	57
			160	414	62
		HMF	0	2075	0
			12.5	1948	6
			25	1718	17
			50	1593	23
			100	937	55
		Tangeretin	0	1853	0
			6.25	1522	18
			12.5	1214	35
			25	966	48
			50	709	62

polymethoxy flavonoids against PhIP and mitomycin C (MMC). We gave normal feed or sample feed that included the EtOH extract or polymethoxy flavonoids **1**–**3** at low or high dose (5% or 10% for the EtOH extract, and 0.01% or 0.02% for polymethoxy flavonoids). Mouse tail vein blood (5 μL) was taken prior to administration of either PhIP or MMC and after the administration (24, 48 and 72 hours).

In the micronucleus test, the EtOH extract of *C. unshiu* significantly decreased the frequency of micronucleated reticulocytes (MNRTs) treated with PhIP and MMC at 24 and 48 hours after the administration (Figures 2 and 3). The antimutagenic effects of the extract of *C. unshiu* were dose-dependently increased at 5% an 10%. The foods containing 0.02% nobiletin (**1**) produced inhibition significantly at 24 h and 48 hours after administration (Figure 4). The feed containing HMF (**2**) at 0.01 and 0.02% showed significant

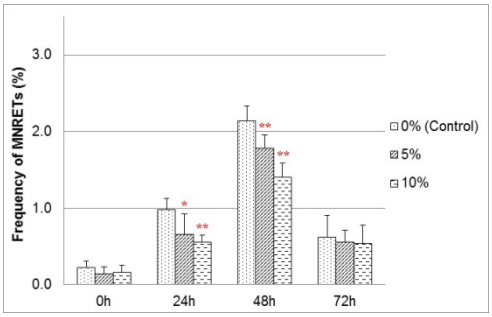

Figure 2: Frequency of micronucleated reticulocytes (MNRETs) from peripheral blood of mice treated with PhIP (50 mg/kg bw) and EtOH extract of *C. unshiu*. Each point represents the mean and standard deviation of five mice.
Significant difference: *0.01<*P*<0.05; **P*<0.01 (Student's t test).

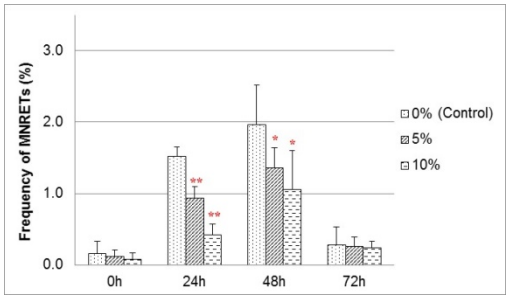

Figure 3: Frequency of MNRETs from peripheral blood of mice treated with MMC (1 mg/kg bw) and EtOH extract of *C. unshiu*. Each point represents the mean and standard deviation of five mice. Significant difference: *0.01<*P*<0.05; **P*<0.01 (Student's t test).

inhibition at 24 and 48 hours after administration (Figure 5), whereas the feed containing tangeretin (**3**) at 0.01 and 0.02% showed significant inhibition at 48 hours after administration (Figure 6). Polymethoxy flavonoids are known inhibitors of cytochrome P450 CYP1 enzymes [5]. Among the cytochrome P450s, CYP1A2 is mainly involved in the bioactivation of heterocyclic amines such as PhIP and Trp-P-1 [6]. Therefore, antimutagenic effects of polymethoxy flavonoids against heterocyclic amines found *in vitro* and *in vivo* tests in this research were mainly induced by inhibitory effects of CYP1A2. On the other hand, MMC has been recognized as a classical DNA damaging agent, on account of its monofunctional and bifunctional DNA alkylating activity [7]. The antimutagenic effects of the EtOH extract of *C. unshiu* against MMC in the micronucleus test may be caused by inhibition of the alkylating activity.

In conclusion, the antimutagenic effects of *C. unshiu* extract were evaluated by the Ames test *in vitro.* The inhibitory effects of polymethoxy flavonoids against chromosomal damage induced by genotoxic/carcinogenic compounds were also evaluated by the micronucleus test *in vivo*. We identified the polymethoxy flavonoids **1**–**3** in the extract of peels of *C. unshiu*. These compounds also showed antimutagenic effects *in vitro* and *in vivo*. A previous report described nobiletin (**2**) as having an inhibitory effect on PhIP-induced colon carcinogenesis in F344 rats [8a]. In this paper, we report for the first time that the polymethoxy flavonoids **1**–**3** show antimutagenic effects against PhIP in an *in vivo* micronucleus test. These results suggest that the peels of *C. unshiu* and polymethoxy flavonoids have a potential to prevent cancer produced by several mutagens.

Experimental

General: For HPLC, a Shimadzu SPD-M10A*vp* was used with UV-VIS detectors (Kyoto, Japan). Nobiletin and HMF were purchased

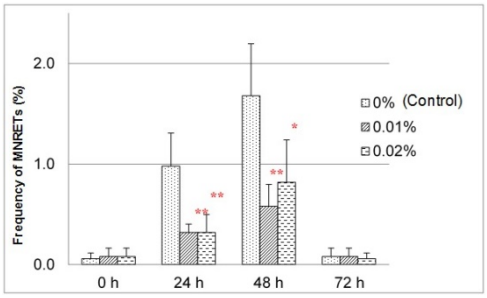

Figure 4: Frequency of MNRETs from peripheral blood of mice treated with PhIP (50 mg/kg bw) and nobiletin (**1**). Each point represents the mean and standard deviation of five mice. Significant difference: **P*<0.01 (Student's t test).

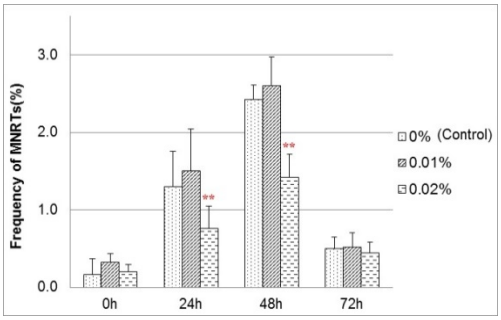

Figure 5: Frequency of MNRETs from peripheral blood of mice treated with PhIP (50 mg/kg bw) and HMF (**2**). Each point represents the mean and standard deviation of five mice. Significant difference: *0.01<*P*<0.05; **P*<0.01 (Student's t test).

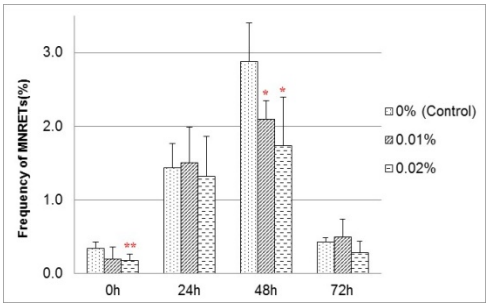

Figure 6: Frequency of MNRETs from peripheral blood of mice treated with PhIP (50 mg/kg bw) and tangeretin (**3**). Each point represents the mean and standard deviation of five mice. Significant difference: *0.01<*P*<0.05; **P*<0.01 (Student's t test).

from Wako Pure Chemical Industries (Osaka, Japan), and tangeretin from INDOFINE Chemical Company. Trp-P-1 (CAS no. 62450-06-0), Trp-P-2, PhIP, and BaP were purchased from Wako Pure Chemical Industries, 1-NP from Sigma–Aldrich (St. Louis, MO, USA), phenobarbital sodium salt from Tokyo Chemical Industry Co (Tokyo, Japan) and β-naphthoflavone from Nacalai Tesqu.

Animals: Male SD rats (7 weeks old, 200 g) and male ICR mice (7 weeks old, 32–34 g) were purchased from Japan SLC (Shizuoka, Japan). The experiments were conducted according to the "Guidelines for Animal Experiments in the Kyoto Pharmaceutical University".

Extraction and partition: The EtOH extract from 1 g of *C. unshiu* peel was partitioned into a EtOAc-MeOH-H_2O (13:3:16, v/v/v) mixture to give organic (167.7 mg) and aqueous layers (766.9 mg).

Identification and quantitative analysis of polymethoxy flavonoids: Quantitative analysis was carried out by the absolute

calibration curve method. The separation was performed on a COSMOSIL 5C18-MS-II (250×4.6 mm) column (Nacalai Tesqu, Kyoto, Japan). The mobile phase consisted of 0.1% formic acid-water/acetonitrile (6:4, v/v) at a flow rate of 0.7 mL/min. The injection volume, column temperature and UV detection wavelength were set at 2 μL, 40°C and 210, 450 nm. Identification of each polymethoxy flavonoid in the test solution was compared with the retention time and UV-visible absorption spectrum of a standard.

In vitro antimutagenic assay: Mutagenicity was examined by the preincubation method [8b] using *Salmonella typhimurium* TA98 with and without S9 mix. The S9 mix (0.5 mL) contained 0.025 mL of S9. The S9 was prepared from the liver of male Sprague-Dawley rats treated with phenobarbital sodium salt and β-naphthoflavone. Dimethyl sulfoxide (DMSO) was used as a negative control. 1-NP (5 μg), PhIP (1 μg), Trp-P-1 (0.04 μg), Trp-P-2 (0.01 μg), and BaP (2.5 μg) were used as positive controls. The antimutagenic effect was examined of several doses of *C. unshiu* extract, and the organic and H_2O layers of the extract. Antimutagenicity was evaluated by the reduction of revertant colonies induced by a mutagen in the presence of samples. The IC_{50} was calculated from the dose responses of the samples. Mutagenicity of 1-NP was examined without S9 mix and that of PhIP, Trp-P-1 and Trp-P-2 with it.

In vivo micronucleus assay: The micronucleus test was carried out according to the method published previously [9]. Micronucleus induction was examined with 5 ICR mice per dose. During the period of taming and experiment, the animals were provided with food (CE-2 pel-let diet; CLEA Japan, Inc., Tokyo, Japan) and tap water *ad libitum*. PhIP (50 mg/kg bw) and MMC (1 mg/kg bw) were administered as the positive control group. The feed containing a test sample was ingested from 2 days before the experiment. *C. unshiu* EtOH extract (5% or 10%, w/w) containing feed, nobiletin (0.01% or 0.02%, w/w) containing feed, tangeretin (0.01% or 0.02%, w/w) containing feed, and HMF (0.01% or 0.02%, w/w) containing feed were used as prepared feeds. As a positive control, CE-2 was provided instead of the feed containing a test sample. Mouse tail vein blood (5 μL) was taken prior to administration of a positive control and after the administration (24, 48 and 72 h). The observation, excitation filter - absorbing filter (Nikon fluorescence block B-2A, Nikon, Ltd.) epi-equipped with a fluorescence microscope [power supply HB-10101AF, OPTIPHOT-2 lens (400 times), Nikon, Ltd.] was used. One thousand reticulocytes were observed per slide, and those including micronuclei were counted. The data were analyzed for significance using Student's t-test. The significance levels of the test were 5 and 1%.

Acknowledgements - The EtOH extract of *C. unshiu* was provided by ORYZA OIL & FAT CHEMICAL CO., LTD.

References

[1] (a) Kim BS, Kim YM, Jae J, Watanabe C, Kim S, Jung SC, Kim SC, Park YK. (**2015**) Pyrolysis and catalytic upgrading of *Citrus unshiu* peel. *Bioresource Technology*, ***194***, 312-319; (b) Oh YC, Cho WK, Jeong YH, Im GY, Yang MC, Hwang YH, Ma JY. (**2012**) Anti-inflammatory effect of *Citrus unshiu* peel in LPS-stimulated RAW 264.7 macrophage cells. *The American Journal of Chinese Medicine*, ***40***, 611-629.

[2] (a) Seo KH, Lee DY, Lee DS, Kang HC, Kim HS, Kim YC, Baek NI. (**2014**) Hepatoprotective and neuroprotective tocopherol analogues isolated from the peels of *Citrus unshiu* Marcovich. *Natural Product Research*, ***29***, 571-573; (b) Min KY. Kim HJ. Lee KA. Kim KT. Paik HD. (**2014**) Antimicrobial activity pf acid-hydrolyzed *Citrus unshiu* peel extract in milk. *Journal of Dairy Science*, ***97***, 1955-1960.

[3] (a) Miyamoto S, Yasui Y, Tanaka T, Ohigashi H, Murakami A. (**2008**) Suppressive effects of nobiletin on hyperleptinemia and colitis-related colon carcinogenesis in male ICR mice. *Carcinogenesis*, ***29***, 1057-1063; (b) Miyazawa M, Okuno Y, Fukuyama M, Nakamura S, Kosaka H. (**1999**) Antimutagenic activity of polymethoxyflavonoids from *Citrus aurantium*. *Journal of Agricultural and Food Chemistry*, ***47***, 5239-5244.

[4] (a) Okuno Y, Miyazawa M. (**2004**) Biotransformation of nobiletin by *Aspergillus niger* and the antimutagenic activity of a metabolite, 4‘-hydroxy-5,6,7,8,3‘-pentamethoxyflavone. *Journal of Natural Products*, ***67***, 1876-1878; (b) Calomme M, Pieters L, Vlietinck A, Berghe DV. (**1996**) Inhibition of bacterial mutagenesis by citrus flavonoids. *Planta Medica*, ***62***, 222-226; (c) Celic A, Eke D, Ekinci SY, Yildirim S. (**2013**) The protective role of curcumin on perfluorooctane sulfonate-induced genotoxicity: Single cell gel electrophoresis and micronucleus test. *Food and Chemical Toxicology*, ***53***, 249-255.

[5] Surichan, Androutsopoulos VP, Sifakis S, Koutala E, Tsatsakis A, Arroo RR, Boarder MR. (**2012**) Bioactivation of the citrus flavonoid nobiletin by CYP1 enzymes in MCF7 breast adenocarcinoma cells. *Food and Chemical Toxicology*, ***50***, 3320-3328.

[6] Oda Y, Aryal P, Terashita T, Gillam EM, Guengerich FP, Shimada T. (**2001**) Metabolic activation of heterocyclic amines and other procarcinogens in *Salmonella typhimurium umu* tester strains expressing human cytochrome P4501A1, 1A2, 1B1, 2C9, 2D6, 2E1, and 3A4 and human NADPH-P450 reductase and bacterial *O*-acetyltransferase. *Mutation Research*, ***492***, 81-90.

[7] Bargonetti J, Champeil E, Tomasz M. (**2010**) Differential toxicity of DNA adducts of mitomycin C. *Journal of Nucleic Acids*, ***2010***, 1-6.

[8] (a) Tang MX, Ogawa K, Asamoto M, Chewonarin T, Suzuki S, Tanaka T, Shirai T. (**2011**) Effects of nobiletin on PhIP-induced prostate and colon carcinogenesis in F344 rats. *Nutrition and Cancer*, ***63***, 227-233; (b) Yahagi T, Nagao M, Seino Y, Matsushima T, Sugimura T, Okada M. (**1977**) Mutagenicities of *N*-nitrosamines on Salmonella. *Mutation Research*, ***48***, 121-130.

[9] Hayashi M, Morita T, Kodama Y, Sofuni T, Ishidate M. (**1990**) The micronucleus assay with mouse peripheral blood reticulocytes using acridine orange-coated slides. *Mutation Research Letters*, ***245***, 245-249.

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Application of Mixture Analysis to Crude Materials from Natural Resources (V) [1]: Discrimination of *Glycyrrhiza uralensis* and *G. glabra* by EI mass spectrometry

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Received: June 23rd, 2016; Accepted: September 6th, 2016

The roots and stolons of some *Glycyrrhiza* species are used worldwide for traditional folk medicines and commercial pharmaceuticals. Phenolic constituents such as flavonoids and coumarins are medicinal and vary according to species. Therefore, species identification is important for quality analysis. In order to identify *Glycyrrhiza* species by chemical fingerprinting, methanol extracts of the root bark of *Glycyrrhiza uralensis* Fischer and *G. glabra* Linné were analyzed using EI-MS. Differences in kinds and quantity of components are reflected in complex EI-MS data and determining characteristic peaks for each species is straightforward. The characteristic peaks were determined statistically by volcano plot, a multivariate analysis method. EI-MS data of *G. uralensis* and *G. glabra* showed differential patterns, and the notable peaks in each pattern were identified. Peaks at *m/z* 153 and 221 are signature peaks of *G. uralensis,* and at *m/z* 173, 309, and 324 are those of *G. glabra*. In conclusion, we found species-specific patterns by EI-MS that distinguish *G. uralensis* and *G. glabra*. This method based on chemical constituent patterns can be applied to identify other *Glycyrrhiza* species and similar natural products.

Keywords: EI-MS, *Glycyrrhiza*, Volcano plot, Metabolomics.

Natural products such as medicinal plants, herbs, and crude drugs vary greatly in quality and composition due to their origin, place of production, and seasonal variation. Therefore, quality control is needed for each natural product.

Multivariate analysis has been applied to natural products. Metabolic profiling of plant extracts provides information about their chemical composition [2]. Many examples of general metabolic profiling have been developed. In general, LC-MS, GC-MS, and NMR are methods used for such analyses.

As part of a series of metabolomics studies, we have reported application of mixture analysis to crude materials from natural products [1a-d]. One of these applications is identification of *Glycyrrhiza* species [1a, d].

The roots and stolons of some *Glycyrrhiza* species are used worldwide in traditional folk medicines and commercial pharmaceuticals. Phenolic constituents such as flavonoids and coumarins are medicinal and vary according to species [3]. These medicinal properties include antimicrobial, anti-inflammatory, and antispasmodic activity [4-7]. Therefore, species identification by constituent pattern has potential for quality analysis.

In a previous study, the bark of the roots and stolons of *G. uralensis* Fischer and *G. glabra* Linné were analyzed using DART (Direct Analysis in Real Time)-MS. The characteristic peaks of each species were determined statistically by volcano plot. DART-MS provides $[M+H]^+$ cation adducts in positive mode, resulting in fewer cleavage reactions. The goal of this study was to demonstrate another methodology for chemical fingerprinting of *Glycyrrhiza* species using electron ionization mass spectrometry (EI-MS). EI-MS is a popular mass spectrometric method that has been applied to discrimination of flavonoids, saponin aglycones, and others. As it provides many fragment peaks in addition to molecular ion peaks, spectral data become complex. The root bark of *G. uralensis* and *G. glabra* contains many compounds. The difference of kinds and quantity of these components are reflected in complex EI-MS data, making it straightforward to determine characteristic peaks of each species with high reproducibility. Once a spectral pattern model is constructed for each species, the chemical fingerprint can be widely shared.

Three kinds of *G. uralensis* (from China, Russia, and Mongolia) and two kinds of *G. glabra* (from Kazakhstan and Uzbekistan) were examined. A methanol extract of the root bark of each *Glycyrrhiza* sample was prepared. Each extract was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 5 mg/mL, and then subjected to EI-MS analysis. Each extract was measured in triplicate.

In the range of *m/z* 50–494, the characteristic peaks of each species were determined statistically by volcano plot. The spectral data when ion contents were at a maximum in total ion chromatography was used for statistical analysis.

Figure 1 shows spectral data for a representative sample of each species. The other samples displayed very similar patterns. Each spectrum is shown in Supplementary data S1-S15. The spectral data for each species were dissimilar, and notable peaks of each pattern were identified. The highest abundance peak was used as standard, and the intensities of the other peaks were calculated relative to the

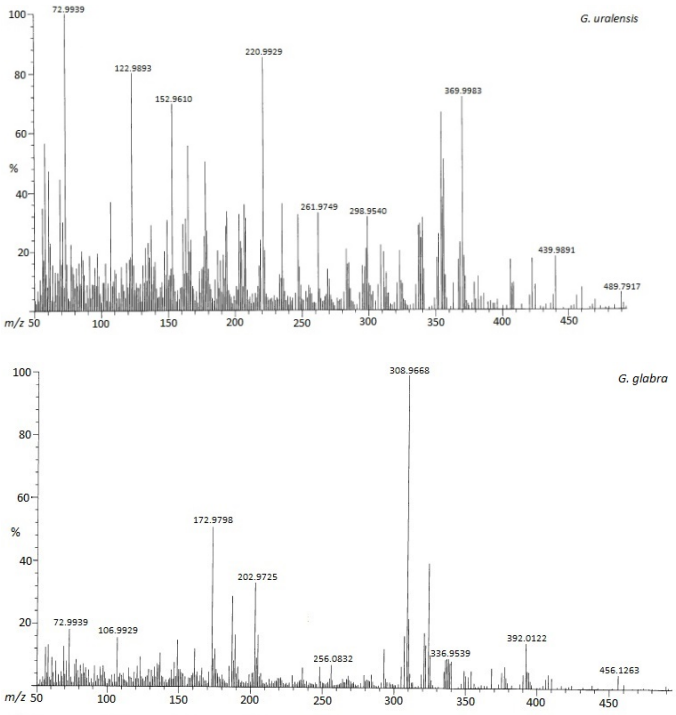

Figure 1: EI-MS data of methanol extracts of root and stolon bark of each *Glycyrrhiza* species.

standard by dividing by the standard deviation (SD) in the sample of the same individual (SD = 1).

The volcano plot summarizes the relationship between the *p*-values of a statistical test and the magnitude of the difference values of the samples in the groups. *p*-Values were calculated by Student's *t*-test. The difference values calculated as [(average of *G. glabra*)−(average of *G. uralensis*)] are plotted on the x-axis. The $-\log_{10} p$ values are plotted on the y-axis.

A volcano plot of the EI-MS data of the *Glycyrrhiza* species is shown in Figure 2. Peaks landing in the upper left region and the upper right region have a small *p*-value with a large absolute difference. In this case, peaks that had *p* values < 0.05 in the *t*-test and absolute differences of ≥ 4 were especially notable.

The volcano plot showed that peaks for *m/z* 153 and 221 are signature peaks of *G. uralensis*, and *m/z* 173, 309 and 324 are those of *G. glabra*. The two *Glycyrrhiza* species can be distinguished using these peaks in the EI-MS data (Figure3).

Moreover, the relative intensities of the above particular peaks of the EI-MS data of the other root barks of *G. uralesis* and *G. glabra*, when applied to the constructed model, verified the process and corresponded with the preliminary identification (Figure 4).

Both species contain specific compounds, for example glycycoumarin for *G. uralensis* and glabridin for *G. glabra* [3].

Kusano *et al*. reported EI-MS data of glabridin; *m/z* 324 (M^+), 309, 187, and 173 [8]. From the view point of this, glabridin has a part role in the signature peaks of *G. glabra*. On the other hand, Demizu *et al*. reported MS data of glycycoumarin: *m/z* 368 (M^+) and 313 [5], which have little relevance to the above signature peaks of *G. uralensis*.

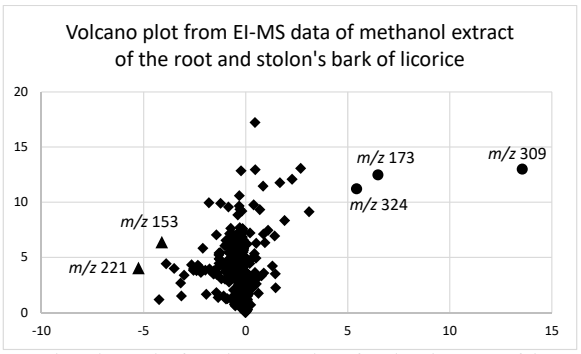

Figure 2: The volcano plot from the EI-MS data of methanol extracts of the root and stolon's bark of licorice. The x-axis is the difference in the average of both groups, and the y-axis is the computed *p*-value in $-\log_{10} p$ scale. Two peaks from the upper left region (characteristic for *G. uralensis*; represented as ▲) and three peaks from the upper right region (characteristic for *G. glabra*; represented as ●) were selected based on the following cutoff thresholds: absolute values of differences ≥ 4 and a *p*-value of 0.05.

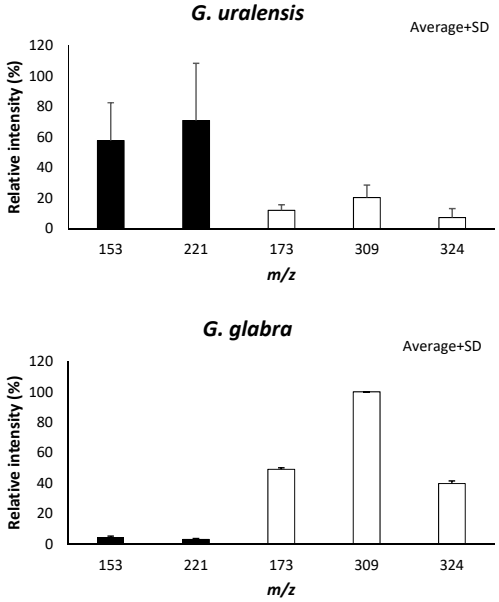

Figure 3: The average selected peak pattern of *G. uralensis* and *G. glabra*. The bars represent the original relative signal intensity. Black bars are signature peaks of *G. uralensis*. White bars are signature peaks of *G. glabra*.

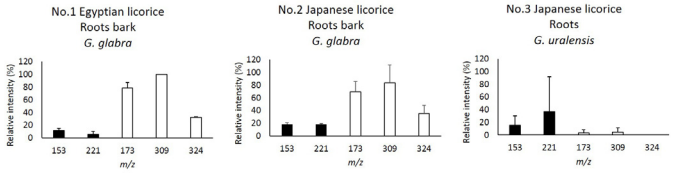

Figure 4: The average selected peak pattern of additional *G. glabra* (from Egyptian and Japan) and *G. uralensis* (from Japan). The bars represent the original relative signal intensity. Black bars are signature peaks of *G. uralensis*. White bars are signature peaks of *G. glabra*.

The issue of identification of compounds generating species specific peaks is not irrelevant, but an important point is that the relative intensity of particular peaks in the EI-MS data led to the discrimination of the two species without identification of any index compounds.

In the same way, the search for potential markers using EI-MS data with multivariate analysis is very useful for quality evaluation of

natural resources, regardless of the existence of an index component.

In conclusion, we found species-specific patterns that distinguish *G. uralensis* and *G. glabra* by EI-MS. This method, based on chemical constituent patterns, can be applied to other *Glycyrrhiza* species and other morphologically similar natural products.

Experimental

Plant material: The roots and stolons of *G. uralensis* Fischer and *G. glabra* Linné were kindly supplied by Mr Fujio Kanai, Kanai Tokichi Shoten Co., Ltd. Voucher specimens were deposited at the Dept. of Natural Medicine and Phytochemistry, Meiji Pharmaceutical University, Tokyo, Japan (No.11G0001, No. 11G0002, No. 11G0003, No.11G0004, and No.11G0005, respectively).

The roots and stolons of additional *G. glabra* were purchased from Tachibana Japan (Tokyo) and identified by Dr Genjiro Kusano, Osaka University of Pharmaceutical Science, and Dr Tohru Okuyama, Meiji Pharmaceutical University. Voucher specimens were deposited at the Dept. of Natural Medicine and Phytochemistry, Meiji Pharmaceutical University, Tokyo, Japan (No. G0001).

The roots and stolons of additional *G. uralensis* (Hokkaido, Japan) and *G. glabra* (Hokkaido, Japan) were kindly supplied and identified by Dr Toshiro Shibata, Research Center for Medicinal Plant Resources in Nayoro, Japan. Voucher specimens were deposited at the Dept. of Natural Medicine and Phytochemistry, Meiji Pharmaceutical University, Tokyo, Japan (No.07G0003, No.07G0002, respectively).

Species identification by HPLC: All samples were identified by HPLC analysis under similar conditions as those described by Shibano *et al.* [9]. A 5C18-AR-II (i.d. 4.6 x 150 mm, Nacalai Tesque Inc.) column was used. HPLC analysis was carried out on D-7000 type software; column oven: L-7300, Diode Array Detector; L-7450H, pump; L-7100 (HITACHI). The presence of species-specific substances was confirmed: glycycoumarin for *G. uralensis* and glabridin for *G. glabra* [3].

Preparation of Glycyrrhiza extracts for EI-MS analysis: Each root bark specimen of 1 g *Glycyrrhiza* was extracted with 30 mL methanol for 1 h under reflux. The methanolic extract was evaporated under vacuum. After evaporation, DMSO was added to a final residue concentration of 5 mg/mL. One µL of each sample was analyzed using EI-MS.

Chemicals: All chemicals were analytical reagent grade. Glabridin was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan), and glycycoumarin was kindly provided by Dr Makio Shibano (Osaka University of Pharmaceutical Science, Japan).

EI mass spectrometry: All experiments were performed using a JMS-GCmate II (JEOL). Accelerating Volts, 2500 V; Mass Scan (Scan Range), *m/z* 50-494; Scan Speed, 0.3 sec/scan; Ion source, 70 eV.

Acknowledgments - The authors would like to express appreciation to Mr Fujio Kanai (Kanai Tokichi Shoten Co., Ltd.) and Dr Toshiro Shibata (Research Center for Medicinal Plant Resources) for providing *G. uralensis* and *G. glabra* and to Dr Makio Shibano (Osaka University of Pharmaceutical Science) for providing glycycoumarin.

References

[1] Part V of the series: Application of mixture analysis to crude materials from natural resources. (a) Part I: Fukuda E, Baba M, Iwasaki N, Uesawa Y, Arifuku K, Kamo O, Tsubono K, Okada Y. (**2010**) Identification of *Glycyrrhiza* species by direct analysis in real time mass spectrometry. *Natural Product Communications*, ***5***, 1755-1758; (b) Part II: Fukuda E, Yoshida M, Baba M, Uesawa Y, Suzuki R, Kamo O, Tsubono K, Arifuku K, Yatsunami K, Okada Y. (**2011**) Application to classification of mulberry leaves using multivariate analysis of proton NMR metabolomic data. *Natural Product Communications*, ***6***, 1621-1625; (c) Part III: Fukuda E, Baba M, Uesawa Y, Kamo O, Arifuku K, Tsubono K, Okada Y. (**2012**) NMR spectral studies to analyze chalcones from *Angelica keiskei. Natural Product Communications*, ***7***, 21-23; (d) Part IV; Fukuda E, Uesawa Y, Baba M, Okada Y. (**2013**) Identification of *Glycyrrhiza* species by direct analysis in real time mass spectrometry (II). *Natural Product Communications*, ***8***, 1721-1724.

[2] Ning Z, Lu C, Zhang Y, Zhao S, Liu B, Xu X, Liu Y. (**2013**) Application of plant metabonomics in quality assessment for large-scale production of traditional Chinese medicine. *Planta Medica*, ***79***, 897–908.

[3] Hayashi H, Hosono N, Kondo M, Hiraoka N, Ikeshiro Y, Shibano M, Kusano G, Yamamoto H, Tanaka T, Inoue K. (**2000**) Phylogenetic relationship of six *Glycyrrhiza* species based on *rbcL* sequences and chemical constituents. *Biological & Pharmaceutical Bulletin*, ***23***, 602-606.

[4] Mitscher LA, Park YH, Clark D, Beal JL. (**1980**) Antimicrobial agents from higher plants. Antimicrobial isoflavanoids and related substances from *Glycyrrhiza glabra* L. var. *typica*. *Journal of Natural Products*, ***43***, 259-269.

[5] Demizu S, Kajiyama K, Takahash K, Hiraga Y, Yamamoto S, Tamura Y, Okada K, Kinoshita T. (**1988**) Antioxidant and antimicrobial constituents of licorice: Isolation and structure elucidation of a new benzofuran derivative. *Chemical and Pharmaceutical Bulletin*, ***36***, 3474-3479.

[6] Chandrasekaran CV, Deepak HB, Thiyagarajan P, Kathiresan S, Sangli GK, Deepak M, Agarwal A. (**2011**) Dual inhibitory effect of *Glycyrrhiza glabra* (GutGard™) on COX and LOX products. *Phytomedicine*, ***18***, 278-284.

[7] Nagai H, Yamamoto Y, Sato Y, Akao T, Tani T. (**2006**) Pharmaceutical evaluation of cultivated *Glycyrrhiza uralensis* roots in comparison of their antispasmodic activity and glycycoumarin contents with those of licorice. *Biological & Pharmaceutical Bulletin*, ***29***, 2442-2445.

[8] Kusano A, Nikaido T, Kuge T, Ohmoto T, Monache GD, Botta B, Botta M, Saitoh T. **(1991)** Inhibition of adenosine 3',5'-cyclic monophosphate phosphodiesterase by flavonoids from licorice roots and 4-arylcoumarins. *Chemical and Pharmaceutical Bulletin*, ***39***, 930-933.

[9] Shibano M, Henmi A, Matsumoto Y, Kusano G, Miyase T, Hatakeyama Y. (**1997**) Studies on the index compounds for HPLC analysis of *Glycyrrhiza uralensis*, *Heterocycles*, ***45***, 2053-2060.

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Comparison of the Qualitative Chemical Composition of Extracts from *Ageratina havanensis* Collected in Two Different Phenological Stages by FIA-ESI-IT-MS^n and UPLC/ESI-MS^n: Antiviral Activity

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Received: March 23rd, 2016; Accepted: September 26th, 2016

The flowers and leaves of *Ageratina havanensis* (Kunth) R. M. King & H. Robinson are traditionally used as a tea to cure several diseases. The production of active secondary metabolites can be affected by several environmental factors such as climate, altitude, rainfall, phenological stage and other conditions that may influence the growth of plants. In this sense, the development of a methodology to compare the chemical composition of plant extracts is needed. The qualitative chemical composition of the ethyl acetate extracts of flowers and leaves, collected in both reproductive and non-reproductive season, was determined by flow injection analysis-electrospray ionization-ion trap tandem mass spectrometry (FIA-ESI-IT-MS^n) and ultra-high-performance liquid chromatography coupled to electrospray negative ionization mass spectrometry (UPLC/ESI-MS^n). The qualitative chemical composition of the ethyl acetate extracts of flowers and leaves was very similar in all cases. Also the antiviral activity of flowers against *human herpes simplex viruses* type 1 and 2 (HSV-1, HSV-2) (*Herpesviridae*) was analyzed. Three glucoside flavonoids were isolated from the ethyl acetate extract of the leaves of *A. havanensis* collected in flowering season using chromatographic methods and their structures were elucidated by physical and spectroscopic data measurements, and by comparing the obtained data with previously published values. The compounds were identified as 3-*O*-β-D-glucosyl-7-methoxyaromadendrin (**5**), 7-*O*-β-D-glucosyl-4'-dihydroxy-5-methoxyflavanone (**6**) and 5-*O*-β-D-glucosylsakuranetin (**7**); this is the first report of the isolation of these compounds in the Asteraceae family**.** Since the qualitative composition of the extracts of *A. havanensis* was similar in all cases, it can be expected that the ethyl acetate extract of the leaves collected in the non-reproductive season has anti-herpetic activity similar to that obtained in the reproductive season.

Keywords: *Ageratina havanensis*, Flavonoids, Tandem mass spectrometry, Anti-herpetic activity.

Ageratina havanensis (Kunth) R. M. King & H. Robinson (common name: Havana snakeroot) is a species of flowering shrub in the family Asteraceae, native of the Caribbean and Texas [1]; and used by humans populations as an antidiarrheal, antirheumatic, aromatic, cholagogue, febrifuge, tonic, and antiparasitic agent [2,3]. This plant is also used to cure diseases of kidneys and bladder [3].

In 1973, Dominguez and de la Fuente isolated sakuranetin and pucherryl acetate from *A. havanensis* grown in Texas [4]. Yu *et al*, in 1987, isolated flavones, flavonols, dihydroflavonols, chalcones and glycosides from dichloromethane and ethyl acetate extracts [5].

In a previous work, our group reported for the first time the antiviral activity of extracts obtained from the leaves and stems of Cuban *A. havanensis* collected in the flowering season. The major metabolites from the most active extract (ethyl acetate extract of the leaves) were sakuranetin (**1**), 7-methoxyaromadendrin (**2**), 4'-*O*-β-D-glucosyl-7-methoxy-eriodictyol (**3**) and 4'-*O*-β-D-glucosyl-sakuranetin (**4**) [6]. However, there are no literature data concerning the chemical composition and pharmacological properties of the flowers and the plant in the non-reproductive season.

A number of environmental factors, such as climate, altitude, rainfall, phenological stage and other conditions may affect the growth of plants, which in turn affects the production of secondary metabolites present in the species, even when produced at one site [7]. The purpose of the present work was to develop a relatively fast, precise and accurate method for an extensive profile of flavonoids in *A. havanensis* suitable for a comparative metabolic profiling of this class of phytochemicals allowing standardization of the plant material, and evaluating its seasonality.

Liquid chromatography-electrospray ionization-tandem mass spectrometry (LC/ESI-MS^n) is considered as a powerful tool for structural elucidation, providing structure specific data for the selective and sensitive identification of either known or unknown molecular structures [8]. Accordingly, an analytical method based on ultrahigh-performance liquid chromatography coupled to electrospray positive ionization mass spectrometry (UPLC/ESI-MS) and flow injection analysis-electrospray ionization-ion trap tandem mass spectrometry (FIA-ESI-IT-MS^n) was implemented for a quick identification of the flavonoids occurring in leaves and flowers of *A. havanensis*.

In order to obtain qualitative information about the chemical composition, ethyl acetate extracts (EtOAc) of the leaves of *A. havanensis* collected in both reproductive (flowering) and non-reproductive seasons, were directly injected into the ESI source of an ion trap spectrometer. Figure 1 shows the ESI-IT full scan mass spectra indicating the [M–H]$^-$ ions of major flavonoids present in the EtOAc extracts of leaves.

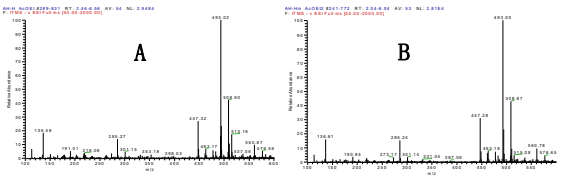

Figure 1: Flow injection analysis ESI-IT-MS fingerprints spectra (enlargement) obtained in negative ion mode of EtOAc extracts of leaves of *Ageratina havanensis* in reproductive season (**A**) and non-reproductive season (**B**).

The direct flow injection ESI-IT-MS fingerprint spectra obtained of the EtOAc extracts were very similar. The ion at *m/z* 285 was assigned to deprotonated sakuranetin (**1**), whose fragmentation (MS^2) led to the base peak at *m/z* 165 ([M–120–H]$^-$). This peak derives from the C-ring cleavage through a retro Diels-Alder (RDA) mechanism [9, 10]. The deprotonated molecule at *m/z* 301 suggested the presence of 7-methoxyaromadendrin (**2**). The product ion spectrum of *m/z* 301 (MS^2) showed the RDA ion at *m/z* 165 ([M–136–H]$^-$). Fragmentation of the deprotonated molecule at *m/z* 447 produced a product ion at *m/z* 285 ([M–162–H]$^-$, loss of a hexose moiety), which was tentatively assigned to the fragmentation of either 4'-*O*-β-D-glucosylsakuranetin (**4**) or another isomer of **4**. Similarly, the deprotonated molecule at *m/z* 463 suggested the presence of either 4'-*O*-β-D-glucosyl-7-methoxy-eriodictyol (**3**) or a glycoside of 7-methoxyaromadendrin. The product ion spectrum of *m/z* 463 showed a [M–162–H]$^-$ ion at *m/z* 301, due to the elimination of a hexose moiety.

A Thermo UPLC C18 analytical column was chosen for the separation of the components present in extracts of the leaves of *A. havanensis* collected in reproductive and non-reproductive seasons. The major compounds were detected in the chromatograms of the EtOAc extract (flowering season) under the UPLC and tandem mass spectrometry conditions (Figure 2).

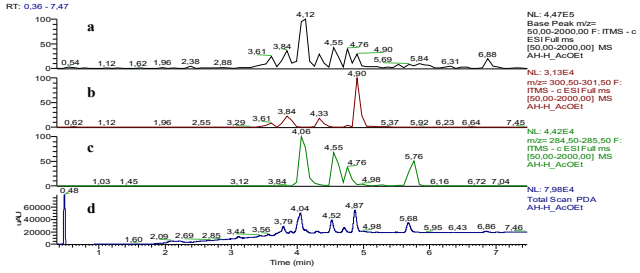

Figure 2: Chromatograms of EtOAc extract of leaves in flowering season. Total ion chromatogram (a), XIC relative to the ions at *m/z* 301 (b), 285 (c) and PDA total scan (d).

The peaks at 5.76 and 4.90 min were assigned to compounds **1** (sakuranetin, *m/z* 285 [M–H]$^-$) and **2** (7-methoxyaromadendrin, *m/z* 301 [M–H]$^-$), respectively. Analysis of the mass spectral data of extracted ion chromatograms (XIC) at *m/z* 285 (Figure 2c) revealed the presence of three components eluting at 4.06, 4.55 and 4.76 min. The MS of these chromatographic peaks showed a predominant fragment ion at *m/z* 447, which was assigned to 4'-*O*-β-D-glucosylsakuranetin (**4**) and other isomers of **4**. The mass spectral data of XIC at *m/z* 301 showed three components eluting at 3.61, 3.84 and 4.33 min. According to the MS, the presence of compound **3** (4'-*O*-β-D-glucosyl-7-methoxyeriodictyol) and another glycoside of either eriodictyol or 7-methoxyaromadendrin are suggested. The behavior of the EtOAc extract from leaves in the non-reproductive season (Figure 3) was very similar to the one of the extract in the flowering season, suggesting that the qualitative chemical composition of both extracts is the same.

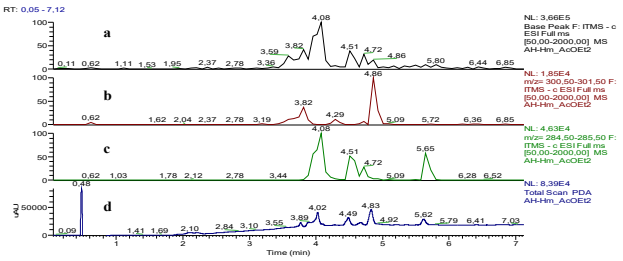

Figure 3: Chromatograms of EtOAc extract of leaves in non-reproductive season. Total ion chromatogram (a), XIC relative to the ions at *m/z* 301 (b), 285 (c) and PDA total scan (d).

To isolate and characterize the glycosides observed in the EtOAc extracts of the leaves of *A. havanensis*, a combination of column chromatographic techniques was used. The ^{1}H- and ^{13}C-NMR spectroscopic data obtained for these compounds (not shown) are consistent with previously reported data. Compound **5** was identified as 3-*O*-β-D-glucosyl-7-methoxyaromadendrin. Compound **6** corresponds to 7-*O*-β-D-glucosyl-4'-dihydroxy-5-methoxyflavanone, isolated for the first time from *Prunus cerasoides* (Rosaceae) by Bahuguna *et al*. in 1987 [11]. Compound **7** data matched those for 5-*O*-β-D-glucosylsakuranetin. This flavonoid occurs commonly in the barks of plants belonging to the *Cerasus* subgenus of the Rosaceae family [12]. Nevertheless, this is the first report of compounds **5**, **6** and **7** in the Asteraceae.

Since compounds **6** and **7** were obtained by HPLC-UV with very close retention times, the peaks observed in the UPLC/ESI-MS^2 analysis at 4.55 and 4.76 min (figure 2) were assigned to compounds **6** and **7**, respectively. Therefore, the component eluting at 4.06 min corresponds to compound **4**.

FIA-ESI-IT-MS^n analysis of flowers was performed under the same conditions as those used for leaves. Compounds **1-7** were identified in the EtOAc extract, so it is concluded that the qualitative chemical composition of leaves and flowers is very similar.

For the determination of kaempherol and quercetin, the ethyl acetate extracts of leaves collected in both reproductive and non-reproductive seasons and flowers were analyzed using an UPLC system with a triple quadrupole tandem mass spectrometer equipped with an ESI source, and Probe Zspray operated in multiple reaction monitoring in negative ion electrospray mode. The chromatograms obtained showed the presence of quercetin (transition 301>151) in the extracts.

Man commonly uses leaf and flower infusions to heal several diseases. As far as is known, there is no report about the antiviral activity of the EtOAc extract of flowers of *A. havanensis.* Taking into account this fact, as well as considering that extracts from leaves and flowers have similar qualitative chemical composition, it was decided to evaluate the antiviral activity of the EtOAc extract of the flowers against *human herpes simplex viruses* types 1 and 2 (HSV-1, HSV-2) (*Herpesviridae*). The antiviral activity of the extract at non-cytotoxic concentrations was assessed by measuring its protective effects on infected Vero cells [13-16]. SI values greater than 2 were considered as an indication of antiviral activity [8]. The EtOAc extract of the flowers showed activity against *human herpes simplex viruses* types 1 and 2 (HSV-1, HSV-2) since in both cases the SI values were greater than 2 (Table 1). The anti-herpetic activity of this extract remains to be investigated, since the maximum concentration tested against HSV-2 did not reach the EC_{50} value (SI< 6.21).

Table 1: Cytotoxicity and antiviral activity of EtOAc extract of *Ageratina havanensis* flowers.

Extract	Cytotoxicity	Antiviral Activity			
		HSV-1		HSV-2	
	CC_{50} (μg/mL)	CE_{50} (μg/mL)	SI	CE_{50} (μg/mL)	SI
AcOEt	1242.8± 37.2	463.4± 12.5	2.7	> 200	< 6.21

Based on the anti-herpetic activity observed in the extract and considering that the qualitative compositions of the extracts from the leaves and flowers are similar, it is suggested that the difference in the anti-herpetic activity is related to the quantitative composition of flavonoids previously identified, and that the presence of these flavonoids has a synergistic effect on the antiviral activity.

Experimental

General: Analytical-grade *n*-hexane, ethyl acetate, *n*-butanol and ethanol were used in this work. For FIA-ESI-IT-MS^n and UPLC/ESI-MS^2, HPLC-grade methanol was purchased from J.T. Baker (Phillipsburg, NJ, USA). HPLC-grade water was prepared using a Milli-Q purification system (Millipore, Bedford, MA, USA). The RP18 cartridge was a Phenomenex Strata C18-E, 55 μm, 70 Å, 500 mg·3 mL^{-1}. The filter membrane (0.22 μm) was of nylon. Sephadex LH-20 and Silica gel 0.06-0.2 mm (70-230 Mesh, Merck) were used for column chromatography and TLC was performed on 0.2 mm-thick Kiesegel 60 F254 layers (Merck). The melting points (m.p.) were determined on a Reichert-Thermovar apparatus. IR spectra were recorded on a Mattson Genesis spectrophotometer, series FT-IR. ^{1}H- and ^{13}C-NMR measurements were obtained on a Varian Inova 500 MHz NMR spectrometer with TMS as internal reference. HPLC-UV separations were performed on an Agilent 1100 series liquid chromatograph, equipped with a G-1312A binary pump, a G-1328 B rheodyne injector with a 0.2 mL sample loop, and a G-1365 B multiple wavelength detector.

Plant material: *Ageratina havanensis* (Kunth) R. M. King & H. Rob., Asteraceae (leaves and flowers), was collected in the eastern region of Havana (Alamar neighborhood) in November 2009 (flowering season) and March 2010 (non-reproductive season). Plant identification and collection were made by Prof. Iralys Ventosa from the Institute of Ecology and Systematics of Plants (Havana, Cuba), where a voucher specimen was deposited (Ref. HAC-42498). The fresh leaves and flowers were separated, oven-dried at 40 ºC, and ground to fine powder.

Extraction: The powdered oven-dried plant sample (leaves from both seasons and flowers) was soaked in *n*-hexane and extracted with ethanol (EtOH). The EtOH extracts were concentrated under reduced pressure to a syrup, further dissolved in water and extracted with ethyl acetate (EtOAc) and *n*-butanol (*n*-BuOH) in a separation funnel. The resulting extracts were concentrated under reduced pressure to obtain the final EtOAc extract.

Sample preparation for mass spectrometric analysis: For mass spectrometric analysis, 2 mL of a MeOH/H_2O 8:2 (v/v) solution of each extract (1 mg mL^{-1}) was subjected to solid-phase extraction using a RP18 cartridge, eluted with MeOH/H_2O 8:2 (v/v). After drying, 1 mg was dissolved in 1 mL of MeOH/H_2O 8:2 (v/v) solution (solution A) and an aliquot (10 μL) was diluted with MeOH/H_2O 8:2 (v/v) up to a final volume of 1 mL, and was filtered through a 0.22 μm membrane of a nylon filter. The solution (150 μL) was diluted with MeOH/H_2O 8:2 (v/v) up to a final concentration of 1 ppm and then introduced by flow injection at 5 μL min^{-1} directly into the ESI source using a syringe pump. For UPLC/ESI-MS analysis, an aliquot (50 μL) of solution A was diluted with MeOH/H_2O 8:2 (v/v) up to a final concentration of 50 ppm.

Mass spectrometric analysis

Flow injection analysis (FIA): Analyses were performed using a Thermo Finnigan LCQ Deca ion trap mass spectrometer (San Jose, CA, USA) equipped with an ESI interface. Mass spectra were obtained both in positive and negative mode. The data were obtained at full scan (range of *m/z* 50–2000) and tandem mass scanning modes. For MS^n analyses, collision energies chosen for each fragmentation was 35%. The optimized instrumental parameters were: capillary temperature 300^{0}C, capillary voltage 13 V, spray voltage 5 kV, sheath gas flow rate 35 (nitrogen, arbitrary units) and auxiliary gas flow rate 10 (arbitrary units).

UPLC-ESI-IT-MS analysis of the extracts: UPLC separations were performed on an Accela UHPLC System, Thermo Fisher Scientific including Accela PDA detector and Thermo Finnigan LCQ Deca ion trap mass spectrometer (San Jose, CA, USA) equipped with Xcalibur 2.2 software. The sample was injected onto a Thermo UPLC column C18 (2.1 x 50 mm x 1.7 μm) with a linear gradient of water (solvent A) and methanol (solvent B), both containing 0.1%, v/v, formic acid, from 0 to 100% B. Elution was performed at flow rate of 400 μL min^{-1} and the volume of the injection was 20 μL. Detection by diode array was performed simultaneously at 3 different wavelengths: 250, 280 and 365 nm. The UV spectra were achieved within a 200–600 nm range. The mass analyses were performed with an ESI interface in negative mode. The data were obtained at full scan (range of *m/z* 50–2000) and tandem mass scanning modes. For tandem mass scanning mode, the percentage of collision energy range was 35%. The optimized instrumental parameters were: capillary temperature 300^{0}C, capillary voltage 13 V, spray voltage 5 kV, sheath gas flow rate 35 (nitrogen, arbitrary units) and auxiliary gas flow rate 10 (arbitrary units).

UPLC-ESI-MS in multiple reaction monitoring mode analysis of the extracts: Analysis was performed on an ACQUITY^{TM} UPLC system (Waters Corp., Milford, MA, USA) with a conditioned auto-sampler. The analytical column used was an ACQUITY^{TM} UPLC Xbridge C18 (2.1 x 50 mm, 2.5 μm column; Waters Corp., Milford, MA, USA). Analysis was carried out with an elution gradient of water containing 0.1% formic acid (A) and methanol (B) at a flow rate of 400 μL/min (10-100% B). The injection volume was 10 μL. Mass spectrometric detection was performed using a Waters ACQUITY^{TM} Xevo TQD triple quadrupole tandem mass spectrometer (Waters Corp., Manchester, UK) equipped with an ESI source, Probe Zspray, operated in multiple reaction monitoring, negative ion electospray mode. The monitored transitions included the following: sakuranetin (285>165), kaempherol (285>151), 7-methoxyaromadendrin (301>165) and quercetin (301>151). The MS conditions were as follows: capillary temperature 200^{0}C, capillary voltage 3.5 kV, cone voltage 39 kV, desolvation temperature 200ºC, source gas flow: desolvation 400 L/h. All data were obtained and processed using Masslynx^{TM} V4.1 software.

Fractionation by gel filtration and adsorption: The EtOAc extract obtained from the leaves of *Ageratina havanensis* collected in flowering season (1.0 g) was dissolved in a *n*-hexane/ chloroform/ methanol (2:1:1) mixture and separated using the same mixture as the mobile phase through a Sephadex LH-20 packed column to give 11 fractions (A-K). Compounds **1**, **2**, **3** and **4** were isolated from fractions I, J, E and G, respectively [6]. Fraction H (328.1mg) was separated on a silica gel packed column with *n*-hexane/ chloroform/ methanol (1:3:1) to give 6 fractions.

HPLC-UV analysis: Fractions 20-24 (silica gel column) from the leaves Sephadex fraction H (89 mg) were analyzed by HPLC-DAD on a C18 reversed-phase column (Phenomenex Luna (2), 250 mm x 4,6 mm i.d, 5 mm), using H_2O+TFA 0.1 % (solvent A) and MeOH+ TFA 0.1 % (solvent B) as mobile phases, at a flow rate of 4.7 mL/min. Elution was performed in 100 min under an isocratic gradient (35% B). The chromatographic separation yielded 3 compounds, which were identified on the basis of IR and NMR experiments, as compounds **5** (4.7 mg, tR = 14.71 min, 206-207ºC), **6** (20.9 mg, tR = 24.01 min, 200-202 ^{0}C), and **7** (29.0 mg, tR = 27.66 min, 200-202ºC). Compounds **5**, **6** and **7** were monitored at 254 nm.

Flower antiviral activity

Cytotoxic assays: Vero cells (ATCC, CCL81) were seeded in 96-well plates at a density of 2×104 cells/ well, and incubated at 37ºC in a 5% CO_2 atmosphere for 48-72 h, until 90% or greater confluence of the monolayers was reached. The cells were then incubated with increasing concentrations of the extracts at 37ºC in a 5% CO_2 atmosphere for 72 h. Afterwards, a MTT solution (0.5 mg/mL) was added and the plates were further incubated for 4 h to allow formazan production. The solid purple precipitate was dissolved in dimethylsulfoxide (Sigma) and the absorbance at 570 nm was measured using a μQUANT Spectrophotometer (Bio-Tek Instruments) with a reference wavelength of 620 nm. The concentration reducing cell viability by 50% (cytotoxic mean concentration, CC_{50}) was calculated by regression analysis [17].

Antiviral assays: Ninety-six well plates containing confluent cell monolayers were pre-incubated for 1 h with increasing non-cytotoxic concentrations of the plant extracts, with a replicate number of 6. Subsequently, the cells were infected with HSV-1 and HSV-2 (10 TCID50). The plates were incubated at 37ºC in a 5% CO_2 atmosphere and daily observed for viral-induced cytopathic effect (CPE) using a light microscope. When CPE was observed in all virus control wells, the percentage of wells with CPE was determined for each treatment concentration, as previously reported [16]. Acyclovir (ACV) at concentrations varying from 0.1-10 μg/mL served as positive control during HSV evaluations.

Statistics: Statistical analyses were performed using Statistic 6.1 software. Differences among antiviral activity of extracts were determined using Student unpaired t-tests. Values of $p<0.05$ were considered indicative of statistical differences. The concentration reducing cell viability by 50% (mean cytotoxic concentration, CC_{50}) and that reducing viral-induced CPE by 50% (antiviral effective mean concentration, EC_{50}) were calculated by regression analyses using the dose-response curves (not shown) generated from the experimental data. A selectivity index (SI) was calculated for each test simple by dividing its CC_{50} by the corresponding EC_{50} value.

Acknowledgements - This research work was supported by the Project CAPES-MES "Sustainable use of Cuban and Brazilian biodiversity: Potentially bioactive: Natural Products from higher plants" and the financial support from CNPq-TWAS Sandwich Postgraduate Fellowships Program (Process No. 190211/2012-1).

References

[1] Diggs GM, Lipscomb BL, O'Kennon RJ, Mahler WF, Shinners LH. (**1999**) *Shinners' and Mahler's illustrated flora of North Central Texas*. Botanical Research Institute of Texas.
[2] Roig JT. (**1974**) *Plantas Medicinales, Aromáticas o Venenosas en Cuba*. Science and Technology, Cuban Book Institute, Havana. 2nd edition.
[3] Torrenegra RD, Pedrozo JA, Robles J, Fuentes O. (**1990**) γ-Palmito lactone from *Ageratina viscosa*. *Phytochemistry*, ***29***, 305-306.
[4] Dominguez XA, Roehll E. (**1973**). Sakuranetin and pulcherryl acetate from *Eupatorium havanense*. *Phytochemistry*, ***12***, 2060.
[5] Yu S, Fang N, Mabry TJ. (**1987**). Flavonoids from *Ageratina havanensis*. *Revista Latinoamericana Química*, ***18***, 20–22.
[6] del Barrio G, Spengler I, García TH, Roque A, Álvarez AL, Calderón JS, Parra F. (**2011**) Antiviral activity of *Ageratina havanensis* and major chemical compounds from the most active fraction. *Brazilian Journal of Pharmacognosy*, ***21***, 915-920.
[7] Gagnier JJ, Boon H, Rochon P, Moher D, Barnes J, Bombardier C. (**2006**) Recommendations for reporting randomized controlled trials of herbal interventions: Explanation and elaboration. *Journal of Clinical Epidemiology*, ***59***, 1134-1149.
[8] Mattoli L, Cangi F, Maidecchi A, Ghiara C, Ragazzi E, Tubaro M, Stella L, Tisato F, Traldi P. (**2006**) Metabolomic fingerprinting of plant extracts. *Journal of Mass Spectrometry*, ***41***, 1534–1545.
[9] Gu L, Kelm MA, Hammerstone JF, Zhang Z, Beecher G, Holden J, Haytowitz D, Prior RL. (**2003**) Liquid chromatographic/electrospray ionization mass spectrometric studies of proanthocyanidins in foods. *Journal of Mass Spectrometry*, ***38***, 1272-1280.
[10] Friedrich W, Eberhardt A, Galensa R. (**2000**) Investigation of proanthocyanidins by HPLC with electrospray ionization mass spectrometry. *European Food Research and Technology*, ***211***, 56-64.
[11] Bahuguna RP, Jangwan JS, Kaiya T, Sakakibara J. (**1987**) Puddumin-A, a new flavanone glucoside from *Prunus cerasoides*. *Journal of Natural Products*, ***50***, 232-234.
[12] Mimaki Y, Sashida Y, Adache T, Yoshinari K. (**1994**) Chemical study on the *Prunus* (Rosaceae): comparative studies on the chemical constituents of the barks of the subgenera *Cerasus*, *Padusand*, *Laurocerasus* plants. *Natural Medicines*, ***48***, 86-89.
[13] Al Jabri AA, Wigg MD, Oxford JS. (**1996**) Initial *in vitro* screening of drug candidates for their potential antiviral activities. In *Virology Methods Manual.* Mahy BWJ, Kangro HO. (Eds), Academic Press, London. Great Britain. p. 303-305.
[14] Cann A. (**1999**) Antiviral testing. In *Virus culture: a practical approach.* Cann A. (Ed). Oxford University Press, New York, 201-219.
[15] del Barrio G, Parra F. (**2000**) Evaluation of the antiviral activity of an aqueous extract from *Phyllanthus orbicularis. Journal of Ethnopharmacolgy*, ***72***, 317-322.
[16] Álvarez AL, Habtemariam S, Juan-Badaturuge M, Jackson C, Parra F. (**2011**) *In vitro* anti HSV-1 and HSV-2 activity of *Tanacetum vulgare* extracts and isolated compounds: an approach to their mechanisms of action. *Phytotherapy Research*, ***25***, 296-301.
[17] Mosmann T. (**1983**) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunology Methods*, ***65***, 55-63.

NPC Natural Product Communications

2017
Vol. 12
No. 1
35 - 42

Characterization of the Polyphenolic Profiles of Peel, Flesh and Leaves of *Malus domestica* Cultivars Using UHPLC-DAD-HESI-MS^n

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Received: August 5th, 2016; Accepted: November 1st, 2016

Qualitative and quantitative analyses of polyphenols extracted from 21 *Malus domestica* cultivars using ultra high performance liquid chromatography with diode array detection coupled to heated electrospray ionization mass spectrometric detection was performed for separation of 27 phenolic compounds on a reversed phase UHPLC column with an optimized gradient consisting of 1% formic acid in water and 1% formic acid in methanol within 20 minutes. According to retention times, UV maxima and mass spectra of the peaks in the chromatograms obtained from extracts of apple peel, flesh and leaves, the polyphenolic compounds were identified and quantified. Based on fragmentation patterns, 6 phenolic acids, 5 flavan-3-ols, 5 dihydrochalcones, 8 flavonols and 3 flavone derivatives were characterized in the studied samples. The method was then employed for analysis of the polyphenolic pattern of 21 apple cultivars, both commercial and autochthonous for the Macedonian region, as well as for monitoring the influence of long term storage on the polyphenolic content and composition of apple fruits and for comparison of polyphenolic profiles of apple cultivars during two years of harvesting. The obtained results revealed minor differences in the quality and major variation in the content of phenolic compounds in the flesh, peel and leaves in the studied apple cultivars that is attributed mainly to cultivar differences and meteorological factors.

Keywords: *Malus domestica*, Polyphenols, HPLC-DAD-HESI-MS^n, Polyphenolic acids, Flavonoids, Procyanidins, Dihydrochalcones.

Interest in the polyphenolic composition and content of different apple varieties and apple products originates from their confirmed contribution to human health [1–3]. It has been demonstrated that qualitative and quantitative differences in the phenolic content of apples can be attributed to many factors such as the variety, environmental conditions, maturity, storage conditions and time [4].

Many studies on polyphenols in apple fruits have been reported [5–11], some of them comparing the polyphenol content of peel and flesh, which is considered important since apples are consumed with and without peel. However, data on the polyphenolic profile of the leaves of apple trees are limited even though it has been shown that their composition might be used as a variety marker as well as an indicator of the health condition of the apple tree [12, 13].

Five major groups of polyphenolic compounds have been characterized in apple fruits: monomeric flavan-3-ols (catechins), polymeric flavan-3-ols (procyanidins), dihydrochalcones (phloretin glycosides), flavonols (quercetin glycosides), hydroxycinnamic acid derivatives and anthocyanins (mainly cyanidin glycosides) present in the red varieties [8, 14]. Apple leaves contain mostly flavonols, few procyanidins, hydroxycinnamic acids, and dihydrochalcones such as phloridzin and phloretin and their derivatives [12, 13]. Unlike from the flesh and the peel, apple leaves also contain flavones [13].

Although the major polyphenolic compounds found in apple varieties have already been identified [6–11], there are not many comprehensive studies on the polyphenolic profiles of large groups of *Malus domestica* varieties where peel, flesh and leaves are discussed. Knowledge on the precise polyphenolic profile of the apple cultivars may contribute to a better understanding of their role in the quality and diversity of apples as agricultural goods and their role in agricultural products derived from them. Additionally, there are not much data on the specific apple varieties grown in southeast Europe, except for recent data for some Croatian [9] and Serbian cultivars [15].

Therefore, in this work, firstly, an efficient UHPLC-DAD-HESI-MS^n method for separation, identification and quantification within 20 minutes was developed and then employed on 21 apple cultivars (11 commercial, 8 autochthonous, and 2 domesticated) growing in the Republic of Macedonia. In that way, a comparative systematic study on the qualitative and quantitative composition of phenolic compounds in lyophilized flesh and peel, and air dried leaves from these regional and international *M. domestica* cultivars from two seasons (2014 and 2015) was carried out providing data on their polyphenolic composition for the first time. Additionally, the method was employed for monitoring the effect of storage on the polyphenolic content and composition of two commercial cultivars (Fuji and Granny Smith).

Twenty-seven phenolic components were determined by the proposed HPLC-DAD method by means of an external standard method. Before quantification, peak identities were confirmed by HPLC-ESI-MS^n (n = up to 3), using mass spectral and absorption data and comparison with standards and literature data. UV chromatograms of water/methanol extracts of peel, flesh and leaves of the cultivars and a mixture of standards were prepared; peaks were assigned based on retention, UV and mass spectral data given in Table 1, which also shows satisfactory chromatographic separation parameters.

Identification of polyphenolic compounds. The polyphenolic compounds detected in all studied samples were classified into five groups: phenolic acids, flavan-3-ols, flavonols, dihydrochalcones

and flavones. Sixteen of them were unambiguously identified by comparing retention times (t_R), UV and MS data with those of the available standards (peaks 1, 3, 5, 6, 8, 10, 11, 12, 16, 17, 22-27). Identification of another 11 polyphenolic compounds, for which standards were not available, was based on the UV absorption and MS data for the deprotonated molecules and their fragmentation pathways [M-H]$^-$ (data in Table 1). Whenever possible, these data and literature were used to support the identification of the peaks.

Phenolic acids: Six compounds were identified as phenolic acid derivatives. According to their UV and MS, four of them (5, 8, 9 and 11) were identified as hydroxycinnamic acid derivatives. Compounds 1, 5, 8 and 11 were confirmed using standards as gallic, chlorogenic, *p*-coumaric and caffeic acids, respectively. Mass spectra of compound 4 indicated fragments related to phenolic acids but in the first step of the fragmentation pattern, the loss of 180 amu implied the presence of one hexose moiety. In the literature it is stated that such phenolic acid derivatives are characteristic for cider apples and have structures with hydroxycinnamic acid moieties [16]. Compound 4 and compound 9 had deprotonated molecular ions [M–H]$^-$ at *m/z* 349 and 343, which fragmented in MS^2 to *m/z* 163, followed by a fragmentation pattern characteristic for gallic (comp. 4) and *p*-coumaric acids. So, they were tentatively identified as gallic and *p*-coumaric acid derivatives.

Flavan-3-ols: Compounds 2 and 7 showed the same deprotonated molecular ions and fragmentation pattern as compound 3, which was confirmed with standard as procyanidin B2. Following their elution order: compound 2 eluted before catechin and epicatechin, whereas compound 7 between catechin and epicatechin, and according to literature data for distinguishing procyanidins by photodiode array detection [17], compounds 2 and 7 were identified as procyanidin B3 and procyanidin B1, respectively.

Dihydrochalcones: Besides phloridzin (12) and phloretin (16), that were confirmed using standards, three other phloretin derivatives were also characterized in all apple cultivars (assigned as peaks 13, 14 and 15). According to their UV spectra, these compounds can be described as phloretin derivatives, whereas the comparison of MS data with those found in literature [18–21] indicated the presence of phloretin glycosides. They were found in different concentrations in all analyzed extracts from peel, flesh and leaves. Compound 13 had a deprotonated molecular ion [M–H]$^-$ at *m/z* 583 and [2M–H]$^-$ at 1166. In the MS^2 spectra, ions at *m/z* 291 [M+H_2O–pentosylhexoside]$^-$ and 273 [phloretin]$^-$ were observed. Because there were no [M+H–glucose]$^-$ fragments it was concluded that the two sugars were not independently attached to the aglycone. The loss of deoxyhexose (–146) indicated that the glucose was directly attached to the aglycone. Thus, peak 13 was tentatively identified as phloretin rhamnosylglucoside (phloretin rutinoside). Compound 14 showed a deprotonated ion [M–H]$^-$ at *m/z* 451 and fragment ions at *m/z* 289 [M-glucose-H]$^-$ and 273 [phloretin]. So, it was tentatively identified as either 3-hydoxyphloretin glucoside or 3-hydroxyphloridzin. Compound 15 had a deprotonated ion [M–H]$^-$ at *m/z* 567, and [2M–H]$^-$ at 1135 and a fragment ion at *m/z* 273 [M–H–pentosylhexose]$^-$. It was tentatively identified as phloretin xyloglucoside. The last two compounds (14 and 15) have been identified in apple pomace as 3-hydroxyphloretin-2′-glucoside (3-hydroxyphloridzin) and phloretin-2'-xyloglucoside [22]. When their UV spectra were examined, it was found that the above three dihydrochalcones all showed UV maxima at 286 nm, but the hydroxyphloretin derivatives exhibited additional shoulders at 262 and 242 nm; also confirmed by Escarpa [5] and Tsao [7].

Flavonols: Eight flavonols were identified according to their UV absorption and MS data (Table 1). The [Y_0]$^-$ ions at *m/z* 317, 301 and 285 indicated three aglycones with the corresponding molecular ions: myricetin (27), quercetin (17–22) and kaempferol (23). Compounds 18, 22, 23 and 27 were determined as quercetin 3-*O*-rutinoside (rutin), quercetin, kaempferol and myricetin, respectively by comparison with standards. Compound 17 had a deprotonated molecular ion at *m/z* 463 and a fragment ion at *m/z* 301 indicating quercetin hexoside. The particular hexose moieties from these flavonol glycoside structures could not be confirmed based on MS information whereas, according to previous reports, glucose was the main hexose linked to flavonols in apples. Compounds 19, 20 and

Table 1: Retention times (t_R), UV maxima, [M–H]$^-$ and fragments, selectivity factor (α), asymmetry and resolution (Rs) of phenolic compounds found in the peel, flesh and leaf extracts of the analyzed 21 *Malus domestica* cultivars.

Peak No	t_R	λ_{max}	[M–H]$^-$	MS^{2a}	MS^{3a}	Compound	Sample	α	Asymmetry	Rs
1[b]	0.43	272	169	**125**		gallic acid	peel, flesh and leaves	1.22	1.13	4.8
4	1.12	316.7	349	181;163; **125**		gallic acid derivative	peel, flesh	1.23	1.22	2.11
5[b]	1.47	235; 325.4	353	**191**		chlorogenic acid	peel	1.09	1.06	5.44
8[b]	2.48	209; 310	163	**119**		*p*-coumaric acid	peel, leaves	2.00	1.16	3.85
9	3.11	209; 310	343	**163;** 119	119	*p*-coumaric acid derivative	peel	1.98	0.96	4.25
11[b]	4.16	220.0; 325.3	179	135		caffeic acid	leaves	1.54	1.23	3.84
2	0.52	277.7	577	**425**; 407; 219	**289;** 149	procyanidin B3	peel, flesh and leaves	1.02	1.15	2.15
3[b]	0.92	277.7	577	**425**; 407; 219	**289;** 149	procyanidin B2	peel, flesh and leaves	1.11	1.32	4.39
6[b]	2.11	279.7	289	**244**; 230; 204	149	catechin	peel, flesh and leaves	1.07	1.15	3.32
7	2.31	278.1	577	**425**; 407; 219	**289;** 149	procyanidin B1	peel, flesh and leaves	1.23	1.15	2.11
10[a]	3.74	279.7	289	**244**; 230; 204	149	epicatechin	peel, flesh and leaves	1.52	1.31	10.88
12[b]	4.76	225; 286	435	297; **273**	167	phloridzin	peel, flesh and leaves	1.23	1.18	6.3
13	4.91	225; 242sh; 262 sh; 286	581	297; **273**	**167**; 151; 125	phloretin rutinoside	peel, flesh and leaves	2.11	1.15	1.55
14	5.11	225; 242sh; 262sh; 286	451	**289**; 271	**167**; 125	3-hydoxyphloridzin	peel, flesh and leaves	1.45	1.43	1.68
15	5.36	225; 286	567	297; **273**; 167	**151**; 125	phloretin pentosylhexoside	peel, flesh and leaves	1.76	1.28	1.98
16[b]	6.51	225; 282.8; 369	273	**167**	**151**; 125	phloretin	peel, flesh and leaves	1.32	0.92	20.77
17[b]	7.92	255; 350	609	**301;** 179; 151	**256**; 151	rutin	leaves	1.11	1.55	1.99
18	7.91	375; 268; 256	463	**301**; 285	**179**; 151	isoquercetin	peel, flesh and leaves	1.09	1.16	13.39
19	8.11	255.3; 357.6	433	**301**; 283; 273; 257	**179**; 151	quercetin pentoside	peel	1.31	1.58	2.01
20	8.54	255.3; 357.6	447	327; **285**	**285**; 255	kaempferol hexoside	peel	1.25	1.12	1.78
21	8.92	255.3; 357.6	417	**285**	**255**; 227	kaempferol pentoside	peel	1.02	1.09	1.25
22[b]	9.64	255.3; 357.6	301	**301**; 179	**179**; 151	quercetin	peel, flesh and leaves	1.54	1.32	12.17
23[b]	10.05	366; 266	285	**255**	151	kaempferol	leaves	1.00	1.17	2.71
27[b]	13.01	353	317	**179**; 151	151	myricetin	leaves	1.27	1.54	12.07
24[b]	10.5	255; 318; 350; 410	285	267; **257**	241	luteolin[b]	leaves	1.23	0.98	3.36
25[b]	12.2	275; 318;401	431	**269**; 224	159; 117	apigenin-7-*O*-glucoside[b]	leaves	1.66	1.23	14
26[b]	12.4	275; 318;401	269	**224**; 151	159; 117	apigenin[b]	leaves	1.98	1.16	1.67

[a]Most abundant ions are marked in bold. [b]Compounds confirmed with standards.

21 showed a deprotonated molecular ion at *m/z* 609 and fragment ions at *m/z* 301. These compounds had the same fragmentation pattern with different abundance of the MS^2 fragments, and considering their retention times, these three compounds eluting after rutin were tentatively attributed to other quercetin rutinoside isomers.

Flavones: Three flavone derivatives were found in the extracts of leaves samples. Their structures were confirmed using standards of luteolin (24), apigenin 7-*O*-glucoside (25) and apigenin (26).

Overall, catechin and epicatechin (flavanols), chlorogenic and caffeic acid (hydroxycinnamic acids), phloridzin (dihydrochalcones) and rutin (flavonols) were identified in both peel and flesh. Some of the varieties analyzed (Srcika (S12), Adam's Pearmain (Parmenka (S13)), Kojce (S14)) showed similar polyphenolic pattern to the ones found for cider apple cultivars reported by Mangas [23]. During sample preparation these cultivars were browning faster, had a bitter taste and showed high content of phenolic acids, especially *p*-coumaric acid.

The same groups of flavonoids present in apple peel and flesh were also found in the leaves, which also had flavones. Polyphenols in apple leaves (caffeic acid, isoquercetin, quercetin, kaempferol, apigenin-7-*O*-glucoside, apigenin, luteolin and myricetin) have been suggested as taxonomic markers for identification of *Malus* L. and *Pyrus* L. genera [12]. Accumulation of polyphenols has been shown as a post infection response to parasites such as scrubs [12, 13].

Quantification of polyphenolic compounds: The validity of the method was examined according to the FDA guidance recommendation [24]. Six standard solutions were prepared for each standard in the concentration range 0.1–200 μg/mL. All polyphenolic compounds showed good linearity (r=0.9986–0.9999) in the investigated concentration range. The limits of detection and quantification were calculated as amounts exhibiting three times the area of the largest noise peak measured with RSD<10% (n=10) and ten times the area of the largest noise peak measured with RSD<2% (n=10), respectively. Limits of detection ranged from 0.11 μg/mL for phloridzin to 0.59 μg/mL for rutin, and limit of quantification from 0.19 μg/mL for phloretin and phloridzin to 2.56 μg/mL for rutin. Standard deviations of 6 replicates of all analytes from the mixed standard solutions in 3 days were used for confirmation of the accuracy and precision of the method. Retention times showed deviation from 0.2 to 1% and peak area deviations were from 0.5%-2%. The accuracy of the method was tested by the standard additions method and recovery values in the range from 92-102% were found satisfactory. Method robustness was tested by varying column dimensions, flow and mobile phase and all tested parameters did not exceed changes in peak area and retention times higher than 25%. So, the method was found suitable for quantification of the phenolic compounds detected in the extracts of apple peel, flesh and leaves.

Overall, 27 compounds listed in Table 1 were detected in the fruits and leaves of 21 *M. domestica* cultivars. Gallic acid, *p*-coumaric acid, procyanidin B3, procyanidin B2, procyanidin B1, catechin, epicatechin, phloridzin, phloretin, rutin, quercetin and their derivatives were detected in both, apple fruit and apple leaves.

All results for the content of every measured compound in all studied cultivars from two seasons of apple harvesting are given in the supplementary tables 1S-6S. The total phenolic content of the 21 apple cultivars analyzed in the two years is given in the graphs in Figure 1, where the polyphenolic patterns are given as distributed in the groups of phenolic acids, flavan-3-ols, dihydrochalcones, flavonols and flavones.

The total polyphenolic content values were in the range from 5.59 to 14.7 mg/g lyophilized peel, from 4.52 to 16.3 mg/g lyophilized flesh, and from 8.07 to 15.1 mg/g dried leaves. As can be seen in Figure 1, flavan-3-ols in the range of 1.95-6.09 mg/g lyophilized sample were most abundant in the apple peel followed by phenolic acids from 0.22 to 4.95 mg/g, and dihydrochalcones from 0.95-3.68 mg/g, whereas apple flesh contained slightly higher flavan-3-ol content than the peel (1.89-9.65 mg/g lyophilized sample) and the same concentration range of phenolic acids and dihydrochalcones as the peel (0.26-4.24 mg/g and 0.71-4.63 mg/g). Leaves also contained flavan-3-ols (2.49-6.52 mg/g dried sample) in highest proportion followed by phenolic acids and flavonols, but also flavones (1.45-2.43 mg/g) that were not detected in apple fruits. The results presented in Figure 1 suggest no significant seasonal variations in the content and distribution of the polyphenolic compounds in the leaves in the two different years (2014 and 2015). However, Figure 1 also suggests that total polyphenol content in the peel was higher, and in the flesh lower in the samples from 2014 compared with the corresponding samples from 2015. Data on the temperature for these years reveal that in 2014 the winter months were with higher temperatures and the temperatures from April to October (the vegetation period) were lower than in 2015, whereas overall 2015 had higher average temperature (Supplementary table 8S with data from the State hydrometeorological service, Skopje). This can be expressed also through the annual temperature sum, which was higher for 2014 (5438.5 vs 5292.5°C in 2015) and the vegetation temperature sum that was lower for 2014 (4215.8 vs 4429.8°C for 2015). As for the air humidity and rainfall, 2014 is characterized by significantly higher rainfall, especially in the vegetation period (543.2 compared with 283.4 mm in 2014). Our results would suggest a positive effect on flesh polyphenol content of higher temperature in the vegetation period and higher humidity, and the reverse effect causes higher polyphenol content in the peel. Still there are no established correlations between polyphenolic composition and climate conditions even though such studies are becoming more relevant due to the increased threat of global warming [25, 26]. Furthermore, Hagen *et al*. [27] have reported that the polyphenolic content, especially of the peel of *M. domestica*, is sensitive to UV irradiation; this was studied by artificially subjecting the fruits to postharvest irradiation. The results from the analysis of the polyphenols in leaf extracts in our study, compared with the ones for the fruit, revealed no seasonal variability.

Tsao *et al*. [7] measured total polyphenols in fresh apple fruits in the range 1.02-2.35 mg/g and Red Delicious contained the highest polyphenolic content in the peel compared with another seven studied apple cultivars popular in Ontario. Also, much lower content of polyphenols was measured in the extracts from the apple flesh (0.53 mg/g for Red Delicious flesh). The Macedonian Red Delicious (S2) cultivar studied in our work contained around 10-12 mg polyphenols/g lyophilized apple peel and around 6 mg polyphenols/g flesh, which is comparable having in mind the water content, but still the flesh polyphenolic content found is higher.

In comparison to our previous work [28], where polyphenols in peel and flesh were measured by spectrophotometric methods, the highest concentrations of polyphenols were found in the flesh of Tetovka (S21) 12.55 mg/g lyophilized sample, which is very similar to the result measured in this study (12.7 mg/g). Moreover, total polyphenols in the peel extracts are in good correlation with the maximal obtained concentrations for the peel of Tetovka, as well as the minimal concentrations measured in this work compared with

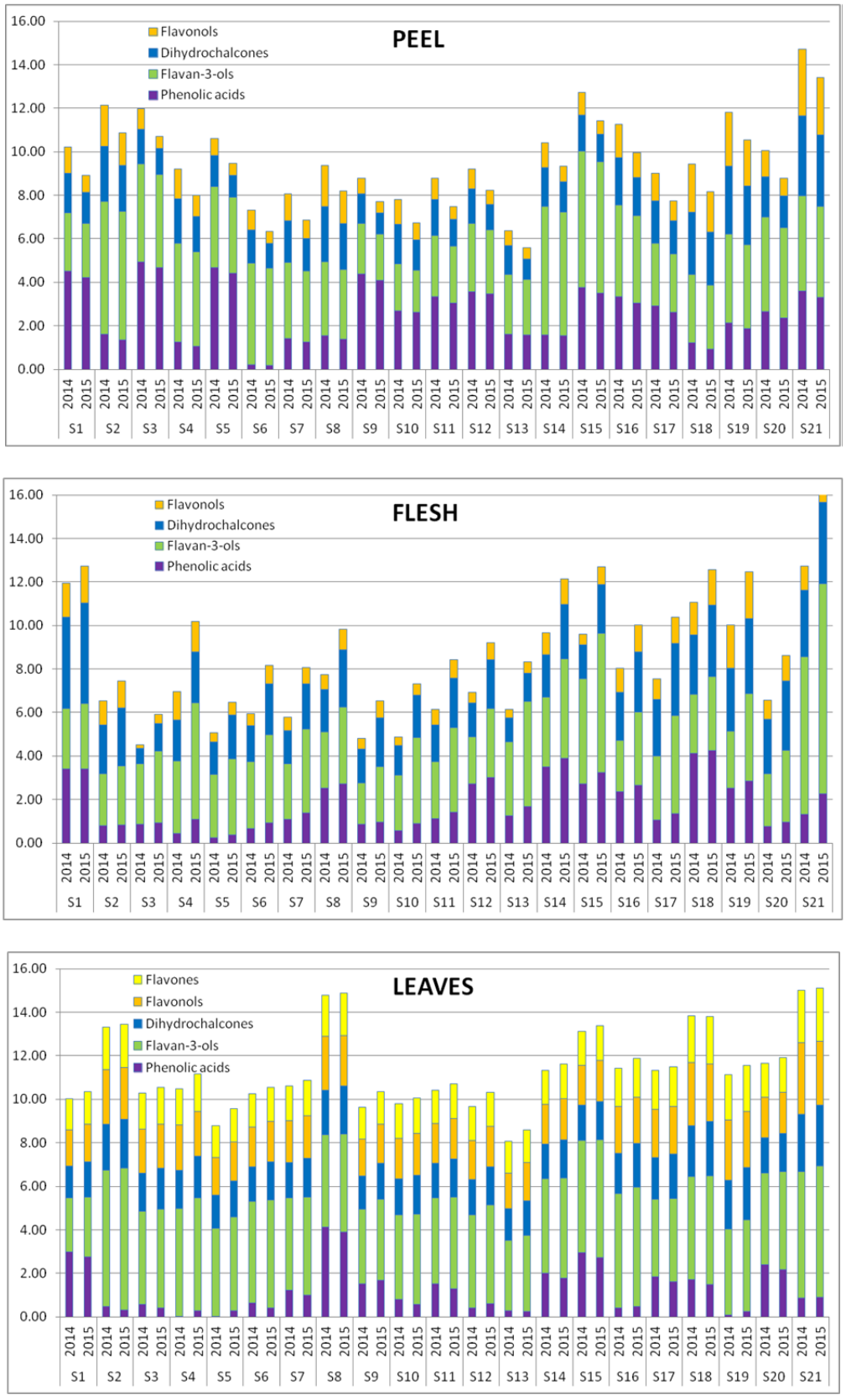

Figure 1: Polyphenolic content (grouped as phenolic acids, flavan-3-ols, dihydrochalcones, flavonols and flavones) in the 21 *Malus domestica* cultivars analyzed in two seasons (2014, 2015) in extracts of leaves, peel and flesh (expressed as mg/g lyophilized peel/flesh and mg/g dried leaves).

the previous work for the peel of Fuji (S10) (about 7 mg/g lyophilized sample). Although the total flavonoid concentration in the flesh was not identical to the results obtained with spectrophotometry, it correlated well to HPLC results with minor differences. Among the five major groups, procyanidins predominated in both peel and flesh in the total polyphenolic profile. Kojce (S14), Livadarka (S15) and Tetovka (S21) had the highest total concentrations of procyanidins in the peel and flesh whereas Golden Delicious (S1) had the lowest amounts, which can also cause differences between the test for total catechins and their separate quantification made here.

Similar results from the quantitative analysis of procyanidins, catechin, epicatechin, caffeic acid and dihydrochalcones for both flesh and peel measured in Golden and Red Delicious and Granny Smith were reported by Escarpa and Gonzales [5]. Analogous polyphenolic contents as the ones obtained in this work were also observed in the study of Basque cider apples where freeze dried samples were investigated [16]. Even though none of the apple cultivar analyzed by Mangas *et al*. was among the cultivars mentioned here, very similar results for the same detected polyphenolic compounds have been observed in this study [23].

Phenolic acids: The highest total amount of phenolic acids in the peel was 4.95 mg/g measured in 2014 and 4.67 mg/g measured in 2015 for the cultivar Chadel (S3), followed by Golden Delicious (S1), which contained about 44% phenolic acids. The highest percentage of gallic acid was found in Chadel (S3) from 2014 and 2015, 2.33 and 2.29 mg/g, respectively. The phenolic acid derivative with 3.25 mg/g in 2014 and 3.19 mg/g in samples from 2015 was found in Granny Smith (S5). *p*-Coumaric acid and its derivative were also found in Chadel (S3) (in 2014, 1.78 mg/g and in 2015 1.75 mg/g) and Golden Delicious (S1) (in 2014, 2.25 mg/g and in 2015, 2.10 mg/g). Minimal concentrations of polyphenolic acids were noticed in the peel of Idaret (S6), where 0.22 mg/g was measured in 2014 and 2015. The lowest concentration of 0.01 mg/g was found for peak 9 (*p*-coumaric acid derivative) found in the apple peel of the Kojce cultivar (S14). The gallic acid derivative (peak 4) was quantified as the highest measured phenolic acid derivative (2.44 mg/g) in the peel of Srcika (S12). Tsao *el al*. [7] found that about 9.3% of the total peel polyphenols were hydroxycinnamic acids. Our results suggest higher contents of total phenolic acids, up to 40%, due to the contribution of the hydroxybenzoic acids detected.

Maximal concentration of phenolic acids was found in the flesh of Pasalma (S18) for both 2014 and 2015 (4.11 and 4.24 per mg/g lyophilized sample). Slightly lower concentration was measured in the flesh of Golden delicious (S1), 3.42 mg/g in 2014 and 2015. Highest amounts of gallic acid and its derivative were found in the flesh of Pasalma cultivar (S18),

Caffeic acid was detected only in apple leaves in the range from 0.01-2.56 mg/g air dried sample with maximum quantity in the Mutsu cultivar (S8) and lowest in Braeburn (S4) and Granny Smith (S5) from 2014.

Flavan-3-ols: The five flavan-3-ols listed in Table 1 were detected as the major group of all analyzed compounds in peel, flesh and leaves. The highest content of flavan-3-ols in the peel was found in the Livadarka cultivar (S15) in 2014 (6.25 mg/g lyophilized sample), which was slightly higher than that measured in 2015 for the same cultivar (6.04 mg/g). Similar contents of flavan-3-ols were noticed in Red Delicious (S2), 6.09 and 5.88 mg/g, for 2014 and 2015 respectively. The peel from apples harvested in 2014 was most abundant in epicatechin (Kojce (S14) with 2.20 mg/g). In our previous work [28], the flesh of Kojce was found to contain the highest amount of total catechins: 3.58 mg/g. The peel of Red Delicious (S2) was most abundant in procyanidin B3 (1.85 mg/g), catechin (1.92 mg/ g in 2014 and 1.81 mg in 2015), and procyanidin B1 (1.22 mg/g). Livadarka (S15) contained the highest amounts of procyanidin B2 (1.85 mg/g), and Kojce the highest amounts of epicatechin in the peel (2.20 mg/g, 2014 and 2.12 mg/g, 2015). These numbers were followed by the contents of flavan-3-ols in Gala (S7), 2.01 mg/g catechin and 0.98 mg/g procyanidin B3. Fuji cultivar (S10) contained the lowest amounts of flavan-3-ols in the peel (2.16 mg/g in 2014 and 1.95 mg/g in 2015). Table 5S shows that Kojce is right after Red Delicious and Livadarka, containing about 60% flavan-3-ols. The lowest content of flavan-3-ols in the peel was measured in Golden Delicious (S1), 2.49 mg/g in 2014 and 2.76 mg/g in 2015, with the lowest measured content of procyanidin B1 (0.01 mg/g in 2014 and 0.06 mg/g in 2015).

As for the flesh, the highest content of these compounds was found in the Tetovka cultivar (S21) with 7.27 mg/g for 2014 and 9.65 mg/g for 2015), followed by half of this content in Kojce (S15), 4.83 mg/g for 2014 and 6.40 mg/g for 2015. The lowest flavan-3-ol content was measured in the flesh of Fuji (S10), 2.16 mg/g in 2014 and 1.95 mg/g in 2015. Procyanidin B3 was found to be the least abundant in Golden Delicious (S1) 0.02 and 0.05 mg/g for 2014 and 2015) and most abundant in the flesh of Tetovka (S21: 0.98 mg/g and 1.01 mg/g in 2014 and 2015). Similar to that, procyanidin B2 was least abundant in Golden Delicious (0.02 and 0.09 mg/g in 2014 and 2015), but most abundant in Mislimka (S17) 0.74 mg/g and 1.11 mg/g in 2014 and 2015). Highest concentrations of catechin were measured in the flesh of Tetovka (S21) from 2014 (3.11 mg/g) and Gala (S7) from 2015 (1.91 mg/g), whereas epicatechin was most abundant in Kojce (S14), 2.20 and 2.12 mg/g in 2014 and 2015). Procyanidin B1 was measured at highest level in the flesh of Tetovka with about 1.2 mg/g for the samples from both years.

Tetovka (S21), as the most famous autochthonous cultivar from Macedonia, contained 3.11 and 4.39 mg/g catechin and 2.35 and 1.08 mg/g epicatechin measured in the samples from 2014 and 2015. Similar as the amounts found in the peel of Tetovka, the flesh of Livadarka, Red Delicious and Kojce contained 40-50% flavan-3-ols from the total polyphenolic compounds in the flesh. Analogous to the flesh, epicatechin in the range from 1.32 to 2.31 mg/g and catechin in the range form 1.09-2.08 mg/g were also the most abundant flavan-3-ols in the apple leaves found in all analyzed cultivars.

Dihydrochalcones: Dihydrochalcones and their derivatives were the second major phenolic class in apple fruit and leaf extracts, also known as taxonomical markers for the *Malus* genus. Similar results for polyphenols extracted from apple peel and flesh have been reported [5, 7]. Total maximal dihydrochalcones measured in the apple flesh in the samples from 2014 and 2015 was 4.21 and 4.63 mg/g, respectively. Lower amounts were measured in the apple peel (about 3.68 in 2014 and 3.28 mg/g in 2015). Maximal concentrations of phloretin, phloretin rutinoside and 3-hydroxy-phloretin were found in the flesh of Golden Delicious (S1) in both years (Figure 1, Table 3S). The third phloretin derivative assigned as peak 15 (Table 3S) was most abundant in the flesh of Tetovka (S21) and Sareno blago (S20). Highest amount of phloridzin was found in the flesh of Belle de Boskoop cultivar (S19), 1.88 mg/g measured in 2014 and 2015. The peel of Tetovka (S21) contained highest amounts of dihydrochalcones, followed by Sareno blago (S20), Belle de Boskoop (S19) and Pasalma (S18).

Lowest concentration of dihydrochalcones when apple peel, flesh and leaves are compared was measured in the air dried leaves (Tetovka from 2014 contained 2.66 mg/g, and from 2015, 2.81 mg/g). Phloretin, phloridzin and phloretin derivatives were also found in apple leaves. Leaves from other cultivars contained amounts in the range from 1.47 to 2.81 mg/g (Table 1S).

Flavonols: Table 1 shows that out of eight identified flavonols, five were found in the peel, three in the leaves and only two were detected in the apple flesh. Quercetin glycosides were also reported by Tsao *et al*. [7]. Although different classes of flavonols are characteristic for the peel and leaves from the same cultivar, the peel (0.51-3.04 mg/g) and leaves (1.65-3.29 mg/g) contained comparable concentrations of flavonols. Detailed analysis of the peel provided identification of 3 flavonol glycosides, assigned as peak 19, 20 and 21 (Table 1) present with highest amounts in Tetovka cultivar (S21) (0.90, 0.60 and 0.55 mg/g, respectively). The flesh contained flavonols in the range from 0.16 to 2.15 mg/g. The flesh of Belle de Boskoop cultivar (S19) was most abundant in isoquercetin (1.77 mg/g), and that of Golden Delicious (S1) from 2015 had the highest content of quercetin (1.28 mg/g). Kaempferol was most abundant in the leaves of Tetovka (S21) from 2014 (1.15 mg/g), followed by quercetin and myricetin.

Flavones: Flavones are group of compounds found only in apple leaves with concentrations from 1.45-2.43 mg/g air dried sample or 14.5-25 % of the total polyphenols measured in the apple leaves. Luteolin was the most abundant flavone in the leaves of Tetovka (S21) with measured concentrations of 1.20 mg/g in 2014 and 1.14 mg/g in 2015 (air dried sample), followed by the leaves of Pasalma ((S18) 1.08 mg/g and 1.02 mg/g in 2014 and 2015) and Karapasa (S16) with about 0.8 mg/g luteolin measured in both years. Lowest amounts of luteolin were found in the leaves of Golden Delicious (S1) (0.73 mg/g and 0.67 mg/g in 2014 and 2015). Apigenin was the second major flavone with highest amounts in the leaves of Tetovka (S21), 0.94 mg/g), Pasalma (S18), 0.88 mg/g) and Red Delicious (S2), 0.83 mg/g); all samples from both years contained apigenin in the range from 0.7-0.94 mg/g (Table 1S). Apigenin-7-glucoside was found in lower concentrations, from 0.02-0.26 mg/g and with concentrations similar to the other two flavones, highest in the leaves of Tetovka (S21) and Karapasa (S16), followed by Pasalma (S18), 0.20 mg/g) and lowest amounts in the leaves of Golden Delicious (S1), 0.02 mg/g).

Influence of storage on polyphenol content: The effect of storage on the polyphenol profile in the apple cultivars Granny Smith and Fuji was tested during 5 months of storage at 0°C and 90% relative humidity. Figure 2 shows that the content of all polyphenols from the peel and the pulp are decreasing during time (data given in Supplementary table 7S). Moreover, the peel of Fuji, containing lower amount of polyphenols, exhibited more intensive decrease in polyphenols in the first 2 months compared with the peel of Granny Smith that was mainly due to the decrease of phenolic acids content. The major decrease in polyphenols in the flesh was observed in the first month and was mainly due to the decrease of flavan-3-ols content, especially epicatechin. The influence of storage has been investigated by Napolitano *et al*. [4] where analysis of the antioxidant activities showed increases during 3 months of storage and then decreases at 4 months of storage. Golding *et al*. showed that although peel phenolics vary among cultivars, they remain relatively stable during storage in air at 0°C and by treatment with diphenylamine indicating a low peel phenolic metabolism during long-term storage [29]. Small but significant differences were observed by Sluis *et al*. in the catechin content of the whole fruit stored in cold chambers for 4 months for Golden Delicious, Cox's Orange and Elstar apples, [30]. Our results demonstrate that there might be cultivar differences that affect the stability of polyphenols during storage that need further studies with suitable analytical methods such as the method developed in this work.

In conclusion, 27 phenolic compounds have been detected and measured in water/methanol (10:90, v/v) extracts of peel, flesh and leaves of 21 apple cultivars from Republic of Macedonia illustrating a complex profile of phenolic acids, procyanidins, dihydrochalcones, flavonols and flavones and their derivatives assayed by UHPLC-DAD-HESI-MS. The methodology developed in this work enabled quantitative determination of the phenolic compounds present not only in the peel and the flesh but also in the leaves from the analysed apple cultivars. The total polyphenolic contents, as well as the major groups and individual compounds, varied significantly among the 21 studied apple cultivars. The autochthonous cultivar Tetovka was the variety with the highest content of polyphenolic compounds in the flesh and the peel, whereas the widespread cultivar Golden Delicious exhibited the lowest polyphenolic content. In all cases apple peel showed higher phenolic content than flesh extracts, except in the case of phloretin, which was found to be more abundant in the apple flesh. The results

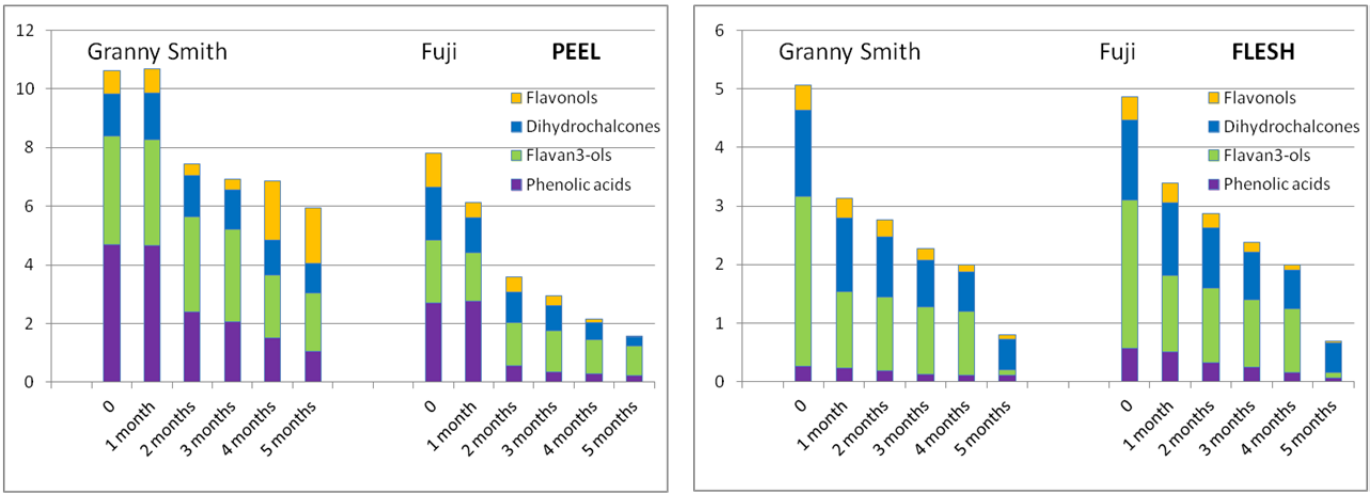

Figure 2: The effect of 5 months of storage on the polyphenolic content and distribution of classes of polyphenolic compounds in the peel and flesh studied on two commercial apple cultivars (Granny Smith and Fuji).

obtained in this study will contribute to the understanding of the polyphenolic composition of apples cultivars, especially for autochthonous cultivars, as well as to their protection.

Experimental

Reagents and standards: Methanol and acetone of HPLC grade were from Merck (Darmstadt, Germany). Water used in all procedures (extraction, separation) was of HPLC quality. Standards of gallic acid, procyanidin B2, chlorogenic acid, catechin, *p*-coumaric acid, epicatechin, caffeic acid, phloridzin, phloretin, rutin, quercetin, kaempferol, luteolin, apigenin-7-*O*-glycoside, apigenin and myricetin were from Sigma Aldrich (Steinheim, Germany).

Table 2: List of the studied apple cultivars.

Code	Cultivar	Type	Region
S1	Golden Delicious	commercial	Resen
S2	Red Delicious	commercial	Resen
S3	Cadel	commercial, local	Resen
S4	Braeburn	commercial	Resen
S5	Granny Smith	commercial	Skopje
S6	Idared	commercial	Resen
S7	Gala	commercial	Resen
S8	Mutsu	commercial	Resen
S9	Jonatan	old variety	Resen
S10	Fuji	commercial	Skopje
S11	Melrose	commercial	Resen
S12	Srcika	autochthonous	Resen
S13	Adam'sPearmain (Parmenka)	domesticated	Resen
S14	Kojce	autochthonous	Resen
S15	Livadarka	autochthonous	Resen
S16	Karapasa	autochthonous	Resen
S17	Mislimka	autochthonous	Resen
S18	Pasalma	autochthonous	Resen
S19	Belle de Boskoop (Kozara)	domesticated	Resen
S20	Sareno blago	autochthonous	Resen
S21	Tetovka	autochthonous	Resen

Materials: The list of the 21 sample of apple cultivars analyzed in this study is presented in Table 2. Besides Granny Smith and Fuji, which were grown in the region of Skopje (R. Macedonia), all other cultivars used in this study were grown in the region of Resen (R. Macedonia). From each apple cultivar (S1 to S21) peel, flesh and leaves harvested during 2014 and 2015 were separately analyzed.

After achieving full maturity, apple fruits were harvested during September 2014 and 2015. Sampling was randomly made by picking fruits from the top, central and bottom parts of the trees. Leaves from the same apple cultivar were also collected from the apple trees in the same manner during July 2014 and 2015.

Apple fruits of the two cultivars Granny Smith and Fuji were stored in an industrial refrigerator with controlled conditions: temperature 0°C and relative humidity 90%. Samples were taken for analysis of polyphenols every month during 5 months.

Sample preparation: As proposed in our previous work [28], 15-20 apple fruits were collected from each tree. Prior to analysis, apple fruits were washed with distilled water and brushed while washing to avoid contamination. Fruits were then manually skinned; the peel was separated from the flesh and blotted on paper. Averaged samples from peel and flesh of all apples from each cultivar were prepared. Peel and flesh were then lyophilized as fast as possible to avoid browning. Peel and flesh were placed in plastic plates (about 20 g of fresh sample) and were lyophilized for 24 h at –45°C and 0.055 mBar. After lyophilisation, residuals from each apple cultivar were ground with a blender at 400 W. After blending, all samples (leaves, flesh and peel) were kept at –20°C till analysis.

About 50 g of fresh leaves were collected from each apple tree cultivar. After collection, leaves were air dried and then ground in a blender.

Extraction of the polyphenols from the peel, flesh and leaves of the 21 apple cultivars was made with methanol/water (90:10, v/v). About 0.5 g flesh/peel/leaves was extracted with 2 portions (2.5 mL) of the solvent using ultrasound at room temperature for 1 h. After centrifugation (3000 rpm/min) for 10 min the supernatants were combined and made up to a final volume of 5 mL. All samples were prepared in triplicate. The extracts were filtered through 0.45 μm membrane filters (PTFE, Agilent) and then analyzed by spectrophotometry [28], and by HPLC. A balance sensitive to 0.1 mg (Sartorius, Germany), an ultrasonic bath Elmasonic S100H, Freeze-dryer Freezone 2.5 (Labconco, USA), a BOSH blender and a centrifuge Harrie 15/80 MSE were used for sample preparation.

UHPLC-DAD-HESI-MS^n apparatus and conditions: The analysis was performed on a Thermo Scientific Dionex Ultimate 3000 UHPLC-UV-DAD system coupled to a Thermo Scientific LTQ XL linear ion trap mass spectrometer with heated electrospray ionisation (HESI) probe and controlled by Chromeleon 5.0 acquisition and Xcalibur 2.0 data analysis software. Zorbax Eclipse plus C18 column (Agilent Technologies), 2.1 x 50 mm i.d., and 1.8 μm particles were used for separation. Elution was performed at a flow rate of 0.7 mL/min, with a mobile phase consisting of 1%, v/v, formic acid in water (phase A) and 1% formic acid in methanol (phase B) with the following gradient: 0-1.5 min 10% B; 1.5-4.5 min linear gradient from 10 to 35% B; 4.5-5.5 min 35% B; from 5.5 to 8.5 min 40% B; from 8.5-9.0 min gradient to 45% B, from 9.0-10.0 min 45% B; from 10.0-10.5 linear gradient to 80% B; from 10.5-14.5% 80% B. The injection volume was 5 μL, and the column temperature was 40°C, UV detection was carried out at wavelengths of 260, 280, 330, 350 and 520 nm. Peak areas for quantification were taken at 330 nm for all detected polyphenolic compounds since all exhibited absorption at this wavelength. Ion source parameters were as follows: source voltage 4 kV, capillary voltage –40 V, tube lens voltage –80 V, heater temperature 285°C, capillary temperature 275°C, sheet and auxiliary gas flow (N_2) 42 and 11 psi. Double heating of the probe (heater temperature) was employed in order to use higher flow rate of the mobile phase. MS were acquired by full range acquisition covering *m/z* 100-1500. Fragmentation studies, data dependent scans and "in source" fragmentations were performed by developing collision induced dissociation (CID). The normalized collision energy of the collision induced dissociation (CID) was set at 35 eV. Fragmentation mechanisms and characteristic fragments were confirmed using Mass Frontier spectral interpretation software 7.1.

Supplementary data: Supplementary tables: 1S-6S contain quantitative results for all individual polyphenolic compounds; 7S contains data with the content of polyphenolic compounds in apple peel and flesh during 5 months of storage of the cultivars Granny Smith and Fuji; 8S contains meteorological data for 2014 and 2015.

Acknowledgement - The authors would like to acknowledge the support of ALKALOID A.D. Skopje for providing some of the instrumentation and reagents used in this study.

References

[1] Scalbert A, Manach C, Morand C, Rémésy C, Jiménez L. (**2005**) Dietary polyphenols and the prevention of diseases. *Critical Reviews in Food Science and Nutrition*, ***45***, 287–306.

[2] Scalbert A, Johnson IT, Saltmarsh M. (**2005**) Polyphenols: antioxidants and beyond., *American Journal of Clinical Nutrition, **81***, 215S–217S.

[3] Vita JA. (**2005**) Polyphenols and cardiovascular disease: effects on endothelial and platelet function. *American Journal of Clinical Nutrition*, ***81***, 292S–297S.

[4] Napolitano A, Cascone A, Graziani G, Ferracane R, Scalfi L, Di Vaio C, Ritieni R, Fogliano V. (**2004**) Influence of variety and storage on the polyphenol composition of apple flesh. *Journal of Agricultural and Food Chemistry*, ***52***, 6526–6531.

[5] Escarpa A, Gonzalez MC. (**1998**) High-performance liquid chromatography with diode-array detection for the determination of phenolic compounds in peel and pulp from different apple varieties. *Journal of Chromatography A*, ***823***, 331–337.

[6] Khanizadeh S, Tsao R, Rekika D, Yang R, Charles MT, Vasantha Rupasinghe HP. (**2008**) Polyphenol composition and total antioxidant capacity of selected apple genotypes for processing. *Journal of Food Composition and Analysis*, ***21***, 396–401.

[7] Tsao R, Yang R, Young JC, Zhu H. (**2003**) Polyphenolic profiles in eight apple cultivars using high-performance liquid chromatography (HPLC). *Journal of Agricultural and Food Chemistry*, ***51***, 6347–6353.

[8] Vrhovsek U, Rigo A, Tonon D, Mattivi F. (**2004**) Quantitation of polyphenols in different apple varieties. *Journal of Agricultural and Food Chemistry*, ***52***, 6532–6538.

[9] Jakobek L, Barron AR. (**2016**) Ancient apple varieties from Croatia as a source of bioactive polyphenolic compounds. *Journal of Food Composition and Analysis*, ***45***, 9–15.

[10] Stracke BA, Rufer CE, Weibel FP, Bub A, Watzl B. (**2009**) Three-year comparison of the polyphenol contents and antioxidant capacities in organically and conventionally produced apples (*Malus domestica* Bork. cultivar 'Golden Delicious'). *Journal of Agricultural and Food Chemistry*, ***57***, 4598–4605.

[11] Lachman J, Šulc M, Sus J, Pavlíková O. (**2006**) Polyphenol content and antiradical activity in different apple varieties. *Horticultural Science (Prague)*, ***33***, 95–102.

[12] Kislichenko VS, Novosel EN. (**2007**) Flavonoids from leaves of *Pyrus communis*, *Malus sylvestris*, and *Malus domestica*. *Chemistry of Natural Compounds*, ***43***, 584–585.

[13] Petkovsek MM, Slatnar A, Stampar F, Veberic R. (**2011**) Phenolic compounds in apple leaves after infection with apple scab. *Biologia Plantarum*, ***55***, 725–730.

[14] Ceymann M, Arrigoni E, Schärer H, Bozzi Nising A, Hurrell RF. (**2012**) Identification of apples rich in health-promoting flavan-3-ols and phenolic acids by measuring the polyphenol profile. *Journal of Food Composition and Analysis*, ***26***, 128–135.

[15] Šavikin K, Živković J, Zdunić G, Gođevac D, Đorđević B, Dojčinović B, Đorđević N. (**2014**) Phenolic and mineral profiles of four Balkan indigenous apple cultivars monitored at two different maturity stages. *Journal of Food Composition and Analysis*, ***35***, 101–111.

[16] Alonso-Salces RM, Korta E, Barranco A, Berrueta LA, Gallo B, Vicente F. (**2001**) Determination of polyphenolic profiles of Basque cider apple varieties using accelerated solvent extraction. *Journal of Agricultural and Food Chemistry*, ***49***, 3761–3767.

[17] Bartolomé B, Hernández T, Bengoechea ML, Quesada C, Gómez-Cordovés C, Estrella I. (**1996**) Determination of some structural features of procyanidins and related compounds by photodiode-array detection. *Journal of Chromatography A*, ***723***, 19–26.

[18] Rak G, Fodor P, Abrankó L. (**2010**) Three-step HPLC–ESI-MS/MS procedure for screening and identifying non-target flavonoid derivatives. *International Journal of Mass Spectrometry*, ***290***, 32–38.

[19] Stobiecki M. (**2000**) Application of mass spectrometry for identification and structural studies of flavonoid glycosides. *Phytochemistry*, ***54***, 237–256.

[20] March RE, Lewars EG, Stadey CJ, Miao X-S, Zhao X, Metcalfe CD. (**2006**) A comparison of flavonoid glycosides by electrospray tandem mass spectrometry. *International Journal of Mass Spectrometry*, ***248***, 61–85.

[21] Zhao Y, Li X, Zeng X, Huang S, Hou S, Lai X. (**2014**) Characterization of phenolic constituents in *Lithocarpus polystachyus*. *Analytical Methods*, ***6***, 1359-1363.

[22] Lu Y, Foo LY. (**1997**) Identification and quantification of major polyphenols in apple pomace. *Food Chemistry*, ***59***, 187–194.

[23] Mangas JJ, Rodríguez R, Suárez B, Picinelli A, Dapena E. (**1999**) Study of the phenolic profile of cider apple cultivars at maturity by multivariate techniques. *Journal of Agricultural and Food Chemistry*, ***47***, 4046–4052.

[24] Analytical Procedures and Methods Validation for Drugs and Biologics Guidance for Industry, U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Biologics Evaluation and Research, *Pharmaceutical Quality*, ***2015***, 1–15.

[25] Darbyshire R, Webb L, Goodwin I, Barlow EWR. (**2014**) Challenges in predicting climate change impacts on pome fruit phenology. *International Journal of Biometeorology*, ***58***, 1119–1133.

[26] Nicholas KA, Matthews MA, Lobell DB, Willits NH, Field CN. (**2011**) Effect of vineyard-scale climate variability on Pinot noir phenolic composition. *Agricultural and Forest Meteorology*, ***151***, 1556–1567.

[27] Hagen SF, Borge GIA, Bengtsson GB, Bilger W, Berge A, Haffner K, Solhaug KA. (**2007**) Phenolic contents and other health and sensory related properties of apple fruit (*Malus domestica* Borkh, cv. Aroma): Effect of postharvest UV-B irradiation. *Postharvest Biology and Technology*, ***45***, 1–10.

[28] Petkovska A, Gjamovski V, Stefova M. (**2016**) Comparison of different extraction solvents for assay of the polyphenol content in the peel and pulp of apple cultivars from Macedonia. *Macedonian Journal of Chemistry and Chemical Engineering*, ***35***, 29-38.

[29] Golding JB, McGlasson WB, Wyllie SG, Leach DN. (**2001**) Fate of apple peel phenolics during cool storage. *Journal of Agricultural and Food Chemistry*, ***49***, 2283–2289.

[30] van der Sluis AA, Dekker M, de Jager A, Jongen WMF. (**2001**) Activity and concentration of polyphenolic antioxidants in apple: effect of cultivar, harvest year, and storage conditions. *Journal of Agricultural and Food Chemistry*, ***49***, 3606–3613.

NPC Natural Product Communications

2017
Vol. 12
No. 1
43 - 45

Preparation and Validated Analysis of Anthocyanin Concentrate from the Calyces of *Hibiscus sabdariffa*

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Received: October 15th, 2016; Accepted: November 1st, 2016

Extracts of *Hibiscus sabdariffa* calyces have been shown to have various medicinal properties, some of which have been reported to be due to anthocyanins present in the calyces. To study whether these claims are valid, it is necessary to produce an extract with a high anthocyanin content and to have available a method to identify and quantify the individual compounds in the product. A method of extraction and purification has been developed based on a polyamide column chromatographic purification step. Using this method, anthocyanin concentrates were produced containing from 57 to 64% of delphinidin-3-sambubioside plus cyanidin-3-sambubioside. A rapid, efficient and validated HPLC analytical method was developed and used for the analysis of the anthocyanin concentrate.

Keywords: *Hibiscus sabdariffa*, Malvaceae, Calyces, Anthocyanins, Polyamide purification, Validated HPLC analysis.

Hibiscus sabdariffa L. (family Malvaceae) is commonly known in English as roselle, rosella and red sorrel, and in Arabic as karkadeh. Aqueous extracts of the red calyces of the flower are drunk both hot and cold as beverages, but they are also used in folk medicine against many illnesses. The phytochemical, pharmacological and toxicological aspects of *H. sabdariffa* were reviewed by Ali *et al*. in 2005 [1]. Since then, other beneficial effects resulting from the use of the calyx extract have been reported [2, and references quoted therein].

The red color of the calyces is due to the presence of anthocyanins and these compounds have been linked to many of the reported medicinal properties [for example, 2, 3a-e]. The anthocyanins found in *H. sabdariffa* have been shown to vary in different collections. The compounds reported include delphinidinpentoside-glucoside [4a], delphinidin [4b,d], delphinidin-3-sambubioside [4c,d,e,h], delphinidin-3-monoglucoside [4d,e], cyanidin [4b], cyanidin-3-monoglucoside [4d,e], and cyanidin-3-sambubioside [4e,h]. In *H. sabdariffa* var. *altissima*, the anthocyanins reported were cyanidin-3,5-diglucoside and cyanidin-3-glucosylrutinoside [4f]. In the calyces of five strains of *H. sabdariffa* var. *sabdariffa*, cyanidin-3-sambubioside was the major pigment, followed by cyanidin-3-monoglucoside. Delphinidin glycosides were not detected in one strain, but were present in the other four. During calyx growth, the anthocyanin content reached 1.7% to 2.5% of the dry weight in all strains [4g].

We have been studying the biological effects of aqueous extracts of *H. sabdariffa* calyces and trying to determine whether these are due, at least in part, to the anthocyanins present in the extract. For this, it has been necessary to purify the extracts to obtain products with a high content of anthocyanins. An analytical procedure to determine the compounds present and their contents was also necessary. Here we report the development of the isolation and preparation of a high anthocyanin containing product from the calyces of *H. sabdariffa* and an analytical procedure to identify and quantify the compounds present.

The plant material used was from the same batch as that used in an earlier study. The major anthocyanins were delphinidin-3-sambubioside and cyanidin-3-sambubioside, although these were accompanied by much smaller quantities of other delphinidin and cyanidin glycosides, as well as rutin and chlorogenic acid [4h].

For extraction of the anthocyanins, water was found to be a less suitable solvent than ethanol, because the calyces of *H. sabdariffa* contain mucilage and pectins [5a,b]; these substances increase the solution viscosity and significantly complicate filtration. The ethanolic extract of the calyces was evaporated to dryness and the residue dissolved in water. This aqueous solution was then purified by passage through a column of polyamide resin. This process was chosen so that the final mixture would contain a minimum of cyanidin, delphinidin and their monoglucosides, as these were eluted from the column easily and relatively quickly with water. Both delphinidin- and cyanidin-3-sambubiosides were subsequently eluted by 95% ethanol. After evaporation of the eluate, the residue was re-dissolved in 95% ethanol and the solution mixed with diethyl ether. The resulting precipitate, after removal by filtration and drying was powdered to provide the final product.

Table 1: Composition of anthocyanin concentrate prepared from the calyces of *H. sabdariffa*.

Compound	Content range
Delphinidin-3-sambubioside	46.1 ± 1.1 to 51.2 ± 0.9
Cyanidin-3-sambubioside	11.1 ± 0.8 to 13.5 ± 1.0
Rutin	2.8 ± 0.1 to 3.9 ± 0.3
Chlorogenic acid	0.9 ± 0.07 to 1.14 ± 0.08

*all data were calculated on dry weight of anthocyanin mixture; every analysis was repeated three times.
**three separate samples from each anthocyanin mixture were weighed and each sample was analyzed twice (total 6 analyses of each anthocyanin mixture).

A rapid and efficient HPLC method for separation and analysis of the individual components of the anthocyanin concentrate was developed. All the major components were well separated. The predominant compounds, as before, were delphinidin-3-sambubioside and cyanidin-3-sambubioside, along with small quantities of rutin and chlorogenic acid [4h] (Table 1).

Polyamide is an optimal adsorbent for the separation of water-soluble phenolic and non-phenolic compounds. It was used earlier to separate the high concentration of aliphatic carboxylic acids in the calyxes of *H. sabdariffa* [5c,d]. However, in our work, total removal of these acids did not seem to be effective and during an attempt to increase further the anthocyanin content of the concentrated extract by repeating the column chromatographic purification step, decomposition products were produced resulting in a grey-blue color indicating the negative impact of higher pH. However, both the delphinidin-3- sambubioside (57.4 ± 2.1 to 63.2 ± 2.4) and cyanidin-3-sambubioside (16.1 ± 0.7 to 17.4 ± 1.5) contents were increased, as well as those of rutin (3.2 ± 0.2 to 4.3 ± 0.3) and chlorogenic acid (1.05 ± 0.10 to 1.63 ± 0.09).

The identities of the non-specified components of the anthocyanin concentrate were not established. Some authors report that protocatechuic acid is an important phenolic acid present in *H. sabdariffa* extract [5e, f]; this was isolated from the dried flowers of the plant [5g,h]. The content in *H. sabdariffa* was reported as 1.19 ± 0.12% [5i]. Chlorogenic acid has also been previously recorded (0.27% in the calyces) [5j], as have rutin (0.21%) and quercetin (0.32%) [5j].

Using the described method, an extract of *H. sabdariffa* calyces was produced which contained greater than 55% of the major two anthocyanins. This product is the one being used in experiments to elucidate the potential pharmacological role of the anthocyanins of *H. sabdariffa.*

Experimental

Plant material: Dried *Hibiscus sabdariffa* calyxes were imported from Sudan. Their identification was verified by comparison with authentic material. A sample has been lodged in the Herbarium of the Faculty of Pharmacy, Hradec Králové, under the following number CUFPH16130/625.

Adsorbent preparation: Polyamide pearls {Silamid (Nylon N6), Považske chemické závody, Slovak Republic; 150 g} were dissolved in 1.5 L of glacial acetic acid under reflux (about 1 h). The resulting slightly yellow, viscous solution was poured into a 5 L beaker and allowed to cool for several hours. The polyamide precipitated as a waxy white suspension. Water (400-500 mL) was added to the suspension, which was well stirred, then filtered on a Büchner funnel, and washed with water. The sediment on the filter was suspended in 2 L of water and alkalized carefully by the addition of 5M NH_4OH (pH ~8). After 30 min the suspension was poured into a chromatographic column and washed with distilled water until the eluate was neutral. The adsorbent was then suspended in 95% ethanol, filtered on a Büchner funnel and the insoluble material dried at room temperature, until the moisture content was 25%. The mass was then passed through a sieve (630 µm) and the material dried again at 60°C to a moisture content of 15%. Final screening was through vibration sieves of 200 µm and 500 µm. For separation of anthocyanins the 200-500 µm fraction was used.

Column preparation: A chromatographic column (average : height 19 : 20 cm) was filled to one third of its volume with water and then, portion-wise, a thick slurry of 600 g of pulverized polyamide (200-500 µm), prepared as above, was slowly added to the column (the adsorbent must be suspended for at least 15 min in the water before pouring).

The column was easily regenerated by washing with boiled water (7 L) at room temperature (air-free). The regenerated column can be used maximally 4 times without change in separation characteristics.

Preparation of anthocyanins concentrate: Milled calyxes (300 g) were added to 2.5 L of boiling 95% ethanol, and the mixture heated, while stirring, for 15 min under reflux. The extract was then decanted and the residue extracted in the same way two more times. The combined extracts were filtered and the filtrate evaporated to dryness. The residue was dissolved in 1 L of water (60°C), with brief sonication, and, after cooling, filtered through a Büchner funnel (filter paper Macherey Nagel MN 640D); the volume of filtrate was made up to 1.5 L. This red solution was filtered through a polyamide column (elution 26-30 mL/min), which was then washed with 6-7 L of water. Then the anthocyanin sambubiosides were eluted by 6-7 L of 95% ethanol.

The red-violet eluate, after evaporation (10-11 g), was dissolved in 100 mL of 95% ethanol and mixed with 750 mL diethyl ether. The precipitate formed was removed by filtration after 15 min, and after a short period of air-drying was dried in a vacuum desiccator (~0.7 kPa, 12 h) over silica gel and then powdered (6-7.6 g).

Table 2: Method validation.

Parameter	Delph-3-samb	Cyan-3-samb	Chlorog acid	Rutin	Criteria
SST					
Repeatability - t_R[a]	0.23	0.19	0.23	0.03	X < 1%
Repeatability – peak areas[a]	0.84	0.57	0.65	0.12	X< 1%
Theoretical plates[b]	2.370	2.768	6.462		
Peak capacity[b]	-	-	-	29	
Resolution[b]	3.13	4.38	5.94	13.07	R_{ij} > 1.5
Symmetry factor[b]	1.01	0.97	1.15	1.12	0.8 -1.5
VALIDATION					
Precision[c] RSD (%)	1.69	4.86	2.32	4.22	RDS (%) < 5%
Linearity					
Correlation coefficient	0.999	0.999	0.999	0.999	R > 0.999
Calibration curve	10480x + 88472	20301x + 78142	1966x + 2576	17053x + 14065	
Calibration	10-120 mg/L	5.0-70 mg/L	2.5-20 mg/L	6.8-72 mg/L	
Accuracy recovery[d] (%)	99.89	100.69	97.56	103.43	X = 100 ± 5%
Accuracy RSD[d] (%)	0.56	1.23	1.50	2.83	X < 5%

Delph-3-samb = delphinidin-3-sambuboside; Cyan-3-samb = cyanidin-3-sambuboside; Chlorog acid = chlorogenic acid.
[a] made in six replicates at 50 mg/L delphinidin-3-sambubioside and cyanidin-3-sambubioside, 15 mg/L chlorogenic acid and 40 mg/L for rutin. [b] made in three replicates. [c] six solutions injected three times each. [d] samples spiked with: 20 mg/L delphinidin-3-sambubioside, 20 mg/L cyanidin-3-sambubioside, 5 mg/L chlorogenic acid and 15 mg/L rutin

Chromatographic analysis: HPLC analysis was performed on a Shimadzu LC-2010 C system (Shimadzu, Kyoto, Japan) equipped

with a built-in UV–VIS detector. A Supelco Discovery HS C18 column (250 x 4.6 mm, 5 μm) was used for the analysis of the extract. Isocratic elution continued for 9 min with a mobile phase composed of 10% acetonitrile (ACN) and 90% formic acid (2%); thereafter isocratic elution was changed to gradient mode and the concentration increased within 8 min to 60% ACN followed by a 7 min equilibration to the initial conditions. The mobile phase flow rate was 1 mL/min. The detection was set at 520 nm for anthocyanins and 371 nm for chlorogenic acid and rutin.

Preparation of standard solution: A standard solution was prepared by dissolving 0.5 mg of delphinidin-3-sambubioside chloride (Extrasynthese, Genay, France), 0.5 mg of cyanidin-3-sambubioside chloride (Extrasynthese, Genay, France), 0.15 mg of chlorogenic acid (Sigma-Aldrich/Merck, Czech division, Prague) and 0.4 mg of rutin trihydrate (Sigma-Aldrich/Merck, Czech division, Prague) in 10 mL of methanol.

Sample preparation: The anthocyanin concentrate (0.5 mg) was dissolved in 1 mL of methanol and filtered through a 0.45 μm PTFE filter into an amber vial prior to analysis by HPLC.

Method validation: Method validation was performed to demonstrate the suitability of the chosen method for its intended use. The validation included a system suitability test (SST) and the determination of method precision, accuracy and linearity. The standard solution was injected 6 times to calculate the SST parameters. Relative standard deviations (% RSD) of retention times and area, tailing factor, number of theoretical plates (peak capacity for rutin analyzed in a gradient mode), and resolution, were calculated.

The standard calibration curves were evaluated using 6 calibration levels. Correlation coefficients were > 0.999 for all analytes. The precision was verified on *H. sabdariffa* anthocyanin concentrate. The sample was prepared 6 times and analyzed using HPLC. Relative standard deviations (% RSD) of areas obtained by the recalculation to the same sample amount were evaluated. The accuracy (% of recovery, % of RSD) was verified using standard additions procedure with samples spiked with a reference standard Method validation results are depicted in Table 2.

References

[1] Ali BH, Al Wabel N, Blunden G. (**2005**) Phytochemical, pharmacological and toxicological aspects of *Hibiscus sabdariffa* L.: A review. *Phytotherapy Research*, ***19***, 369-375.

[2] Inuwa I, Ali BH, Al-Lawati I, Beegam S, Ziada A, Blunden G. (**2012**) Long-term ingestion of *Hibiscus sabdariffa* calyx extract enhances myocardial capillarization in the spontaneously hypertensive rat. *Experimental Biology and Medicine*, ***237***, 563-569.

[3] (a) Wang CJ, Wang JM, Lin WL, Chu CY, Chou FP, Tseng TH. (**2000**) Protective effect of *Hibiscus* anthocyanins against tert-butyl hydroperoxide induced hepatic toxicity in rats. *Food and Chemical Toxicology*, ***38***, 411-416; (b) Ojeda D, Jimenez-Ferrer E, Zamilpa A, Herrera-Arellano A, Tortoriello J, Alvarez L. (**2010**) Inhibition of angiotensin enzyme (ACE) activity by the anthocyanins delphinidin- and cyanidin-3-*O*-sambubiosides from *Hibiscus sabdariffa*. *Journal of Ethnopharmacology*, ***127***, 7-10; (c) Lo CW, Huang HP, Lin HM, Chien CT, Wang CJ. (**2007**) Effect of *Hibiscus* anthocyanins-rich extract induces apoptosis of proliferating smooth muscle cells via activation of P38 MAPK and p53 pathway. *Molecular Nutrition and Food Research*, ***51***, 1452-1460; (d) Jonadet M, Bastide J, Bastide P, Boyer B, Carnat AP, Lamaison JL. (**1990**) *In vitro* enzyme inhibitory and *in vivo* cardioprotective activities of hibiscus (*Hibiscus sabdariffa* L.). *Journal de Pharmacie Belgique*, ***45***, 120-124; (e) Ali BH, Al Lawati I, Beegam S, Ziada A, Al Salam S, Nemmar A, Blunden G. (**2012**) Effect of *Hibiscus sabdariffa* and its anthocyanins on some reproductive aspects in rats. *Natural Product Communications*, **7**, 41-44.

[4] (a) Yamamoto R, Oshima Y. (**1936**) Coloring matter of *Hibiscus sabdariffa* L. (hibiscin). *Scientific Papers of the Institute of Physical and Chemical Research (Tokyo)*, ***30***, 258-262; from *Chemical Abstracts*, (**1936**), ***31***, 3048; (b) Forsyth WGC, Simmonds NW. (**1954**) Survey of the anthocyanins of some tropical plants. *Proceedings of the Royal Society (London) B*, ***142***, 549-564; (c) Seshadri TR, Thakur RS. (**1961**) Constitution of gossytrin, a new glycoside from the flower petals of *Hibiscus sabdariffa*. *Journal of the Indian Chemical Society*, ***38***, 649-651; (d) Shibata M, Furukawa M. (**1969**) Reexamination of the structure of the so-called hiviscin. *Botanical Magazine Tokyo*, ***82***, 341-347; (e) Du CT, Francis FJ. (**1973**) Anthocyanins of rosella (*Hibiscus sabdariffa* L.). *Journal of Food Science*, ***38***, 810-812; (f) Subramanian SS, Nair AGR. (**1972**) Flavanoids of four Malvaceous plants. *Phytochemistry*, ***11***, 1518-1519; (g) Khafaga E-SR, Koch H. (**1980**) Stage of maturity and quality of roselle (*Hibiscus sabdariffa* L. var. *sabdariffa*). 2. Anthocyanins. *Angewandte Botanik*, ***54***, 295-300; (h) Cahlíková L, Ali BH, Havlíková L, Ločárek M, Siatka T, Opletal L, Blunden G. (**2015**) Anthocyanins of *Hibiscus sabdiffera* calyces from Sudan. *Natural Product Communications*, ***10***, 77-79.

[5] (a) Müller VBM, Franz G. (**1990**) Hibiscusblüten – eine Schleimdroge? *Deutsche Apotheker Zeitung*, ***130***, 299–333; (b) Müller BM, Franz G. (**1992**). Chemical structure and biological activity of polysaccharides from *Hibiscus sabdariffa*. *Planta Medica*, ***58***, 60–67; (c) Franz M, Regensburg G, Franz G. (**1988**). *Hibiscus sabdariffa* – Hibiscusblüten. *Zeitschrift fur Phytotherapie*, ***1159***, 1163–1166; (d) Eggensperger H, Wilker M. (**1996**). Hibiscus-Extrakt – Ein hautvertraglicher Wirkstoffkomplex aus AHA'a und polysacchariden. *Parfumerie und Kosmetik*, ***9***, 540–543; (e) McKay D. (**2009**). Can hibiscus tea lower blood pressure? *AfroFood Industry Hi-Tech*, ***20***, 40–42; (f) Williamson EM, Driver SB, Baxter K. (**2013**). *Stockley's herbal medicines interactions: a guide to the interactions of herbal medicines, dietary supplements and nutraceuticals with conventional medicines*. Pharmaceutical Press, London; (g) Osman AM, El-Garby-Younes M, Mokhtar A. (**1975**). Chemical examination of local plants. XIII. Elucidation of the structure of a new glycoside from the leaves of Egyptian *Hibiscus sabdariffa*. *Australian Journal of Chemistry*, ***28***, 217–220; (h) Tseng TH, Wang CJ, Kao ES, Chu HY. (**1996**). Hibiscus protocatechuic acid protects against oxidative damage induced by tert-butylhydroperoxide in rat primary hepatocytes. *Chemico-Biological Interactions*, ***101***, 137–148; (i) Chao C-Y, Yin M-C. (**2009**) Antibacterial effects of roselle calyx extracts and procatechuic acid in ground beef and apple juice. *Foodborne Pathogens and Disease*, ***6***, 201-206; (j) Alarcon-Alonso J, Zamilpa A, Aguilar FA, Herrera-Ruiz M, Tortoriello J, Jimenez-Ferrer E. (**2012**). Pharmacological characterization of the diuretic effect of *Hibiscus sabdariffa* Linn Malvaceae) extract. *Journal of Ethnopharmacology*, ***139***, 751–756.

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The Influence of Extraction Parameters on Antimicrobial Activity of Propolis Extracts

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Received: November 27th, 2015; Accepted: September 12th, 2016

The extraction optimization of the poplar-type propolis was performed in order to improve the isolation of flavonoids as well as the corresponding antimicrobial activity of the products obtained. The efficiency of flavonoids extraction depended upon the type of extraction media used, following the rank 80% ethanol >40 ethanol>> water, regardless of pH value. Ultrasound assisted extraction was as efficient as the maceration procedure, offering additional benefits such as short duration time and low extraction temperature. The antimicrobial efficiency of extracts prepared with 80 and 40% ethanol against the tested microbial stains was comparable, regardless of the extraction technique used, while aqueous extracts mainly showed scarce activity. Observed activity against the yeast *Candida albicans* strongly correlated with flavones and flavonols content in extracts prepared (r^2=0.8217), while regression analysis showed that beside flavonoids, some other components which were successfully extracted from the crude propolis contributed to the observed antimicrobial efficiency against *Bacillus subtilis* and *Staphylococcus aureus*.

Keywords: Poplar-type propolis, Flavonoids, Maceration, Ultrasound extraction, *Bacillus subtilis*, *Staphylococcus aureus*, *Candida albicans*.

Propolis, a resinous substance collected and enzymatically modified by honeybees (*Apis mellifera* L.) from exudates and buds of plants, has been used as a traditional remedy in folk medicine since ancient times, especially in Europe and Asia. To date, more than 300 different constituents have been identified in propolis, resulting in a wide spectrum of biological activities. The chemical composition of propolis is highly dependent on the phytographic characteristics of the flora present around the collection site, leading to formulation of several chemical types of propolis, based on their plant source [1-3]. Its documented efficiency in the treatment of cold and upper respiratory tract infections, wound healing, treatment of burns, acne, herpex simplex infection and neurodermitis [2,3], as well as in prevention and treatment of dental diseases [4] puts propolis in the focus of interest of the pharmaceutical industry, especially taking into account that the number of pathogens that are developing resistance to known antibiotics is rising [5].

The antimicrobial efficiency of propolis varies greatly depending on the extraction procedure of the crude material and the solvents used in this process. In this paper, the efficiency of maceration, as a standard propolis extraction technique, was compared with that of ultrasound assisted extraction, in order to select rationally the best extraction technique. In order to optimize additionally the extraction of the bioactive compounds from the crude propolis, a series of extraction media were used, containing variable amounts of ethanol and water at different pH values. Total flavonoid content in the extracts prepared, determined by two complementary colorimetric methods [6], was taken as an indicator of the extraction efficiency. This selection was made taking into account that hydrophobic flavonoid-aglycones are the main constituents of poplar type propolis, which is characteristic for raw propolis samples collected from inland Croatia [7] and are responsible for the majority of the propolis biological activities [1, 8]. The results obtained for extracts prepared are shown in Table 1. Surprisingly, for some extracts prepared by maceration (namely M-80-50-3, M-80-25-7, M-80-25-3 and M-40-25-7), the method with 2,4-dinitrophenylhydrazine resulted in unrealistically high values (data not shown). Since it was previously shown that flavones, flavonols and isoflavonols with a C2-C3 double bond could not react with 2,4-dinitrophenylhydrazine [6], the observed results were attributed to unknown interferences, but a detailed study of these was not within the scope of this paper.

When analyzing the amount of flavonoids extracted (Table 1), it is obvious that the efficiency of their extraction was manly dependent upon the ethanol concentration in the extraction media, while pH and temperature of extraction were not critical parameters. In the case of flavones and flavonols, the highest extraction efficiency was obtained when 80% ethanol was used as the extraction medium, while 40% ethanol was somewhat less effective. Water, regardless of the pH used, appeared to be the least efficient extraction medium, resulting in extracts containing only around 0.6%, w/v, of flavones and flavonols (Table 1). Such a result can be related to the rather poor aqueous solubility of those compounds and is in agreement with literature data [2]. When comparing the efficiency of the two extraction methods used, ultrasound assisted extraction resulted in comparable amounts of flavones and flavonols extracted, regardless of the type and pH of the extraction media. However, compared with maceration, the use of ultrasound significantly reduced the extraction time and allowed the preparation of propolis extract at rather low temperature (4°C), thus clearly demonstrating its advantages over maceration. When comparing the amounts of flavanones in the extracts prepared, maceration with 80 and 40% ethanol yielded comparable amounts of flavanones, which were in the range previously found to be characteristic of the propolis from the western part of continental Croatia [7]. Ultrasound assisted extraction using 80% ethanol resulted in comparable amounts of flavanones, while somewhat lower amount of flavanones were extracted when 40% ethanol was used as extraction medium. When water was the extraction medium, both methods resulted in comparable amounts of flavanones extracted, ranging from 6.1 to 6.9% (Table 1).

Finally, when comparing the amounts of flavonoids extracted (Table 1) to their quantity in some commercially available propolis

Table 1: Parameters of the extraction process and flavonoids content in liquid extract prepared.

Sample code	extraction method	solvent	temperature (°C)	pH	Flavonoids (%)*	
					Flavones and flavonols[a]	Flavanones[b]
M-80-50-7	maceration	ethanol 80%	50	7	22.31	12.51
M-80-50-3		ethanol 80%	50	3	21.88	*
M-80-25-7		ethanol 80%	25	7	21.25	*
M-80-25-3		ethanol 80%	25	3	22.38	*
M-40-50-7		ethanol 40%	50	7	6.94	15.63
M-40-50-3		ethanol 40%	50	3	5.63	10.91
M-40-25-7		ethanol 40%	25	7	3.85	*
M-40-25-3		ethanol 40%	25	3	4.32	13.94
M-W-50-7		water	50	7	0.59	6.57
M-W-50-3		water	50	3	0.59	6.92
M-W-25-7		water	25	7	0.67	6.34
M-W-25-3		water	25	3	0.40	6.43
UZV-80-4-7	ultrasonication	ethanol 80%	4	7	20.63	12.23
UZV-80-4-3		ethanol 80%	4	3	22.13	11.97
UZV-40-4-7		ethanol 40%	4	7	4.73	7.13
UZV-40-4-3		ethanol 40%	4	3	5.06	7.18
UZV-W-4-7		water	4	7	0.51	6.39
UZV-W-4-3		water	4	3	0.44	6.13

[a] Expressed as quercetin equivalent; [b] Expresed as naringenin equivalent; * Flavonone content determination was hindered by interferences.

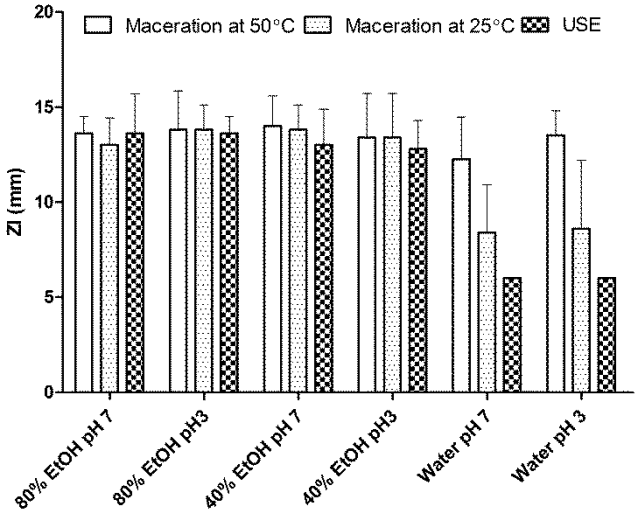

Figure 1: Antibacterial activity of prepared propolis extracts against *Bacillus subtilis* NCTC 8236 expressed as zones of inhibition (ZI) measured in mm (mean±SD, n=6; USE=Ultrasound assisted extraction).

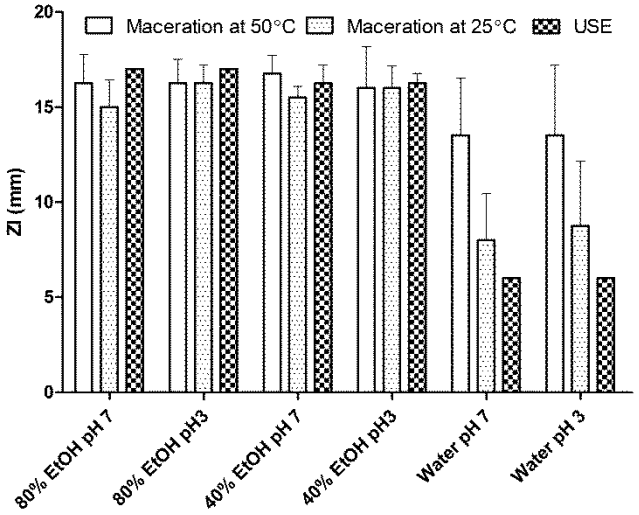

Figure 2: Antibacterial activity of prepared propolis extracts against *Staphylococcus aureus* ATCC 29213 expressed as zones of inhibition (ZI) measured in mm (mean±SD, n=6; USE=Ultrasound assisted extraction).

tinctures [9], maceration and ultrasound assisted extraction with hydroethanolic media resulted in 10 to 60 times higher concentrations of flavones and flavonols, while levels of flavanones were comparable. This clearly showed the need for careful adjustment of the extraction parameters and the methodology used in order to extract successfully the poorly soluble compounds from the crude propolis.

The antimicrobial activity of the propolis extracts prepared against *Bacillus subtilis* NCTC 8236, *Staphylococcus aureus* ATCC 29213, and *Candida albicans* ATCC 10231 strains was evaluated by measuring the zones of inhibition using a cylinder-plate diffusion method. The obtained results are shown in Figures 1, 2 and 3. In the diffusion assay, all the media used for propolis extraction showed no antimicrobial action against the microbial species tested (data not shown). On the contrary, propolis extracts prepared with 80 or 40% ethanol, regardless of the pH and extraction technique used for preparation, showed a pronounced antibacterial activity against *B. subtilis* and *S. aureus* (Figures 1 and 2). In fact, *S. aureus* presented inhibition zones ranging from 15-17 mm, while *B. subtilis* was somewhat less susceptible to ethanolic extracts of propolis, presenting inhibition zones ranging from 13 to 14 mm. In general, such results are in agreement with previously published data which showed good antimicrobial activity of ethanolic propolis extracts against those microbial species [10].

Analysis of the antibacterial action of propolis extracts against *B. subtilis* and *S. aureus* showed that the ethanol concentration (40 or 80%) and the pH value of the extraction media, as well as the technique used for the extract preparation did not have any statistically significant influence on the observed antimicrobial activity of the extract prepared (p>0.05; Figures 1 and 2). This further confirmed the high efficiency of ultrasound assisted extraction in the extraction of antibacterial compounds from the crude propolis, when hydroethanolic extraction media were used. Surprisingly, even the aqueous propolis extracts, prepared by maceration at 50°C (M-W-50-7 and M-W-50-3, respectively), showed comparable antimicrobial efficiency against both microbes

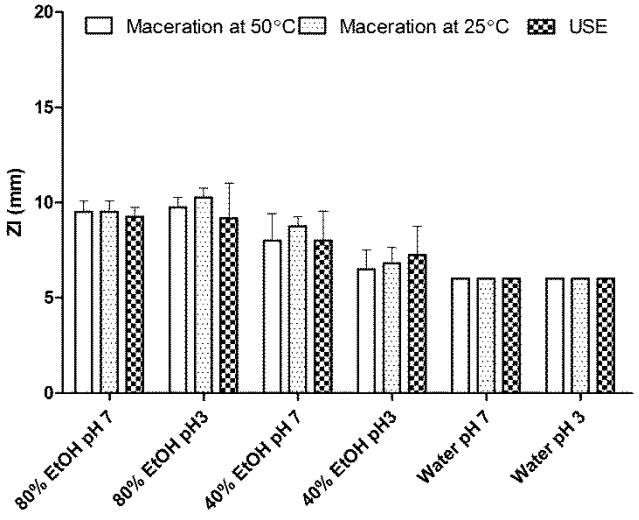

Figure 3: Antifungal activity of prepared propolis extracts against *Candida albicans* ATCC 10231 expressed as zones of inhibition (ZI) measured in mm (mean±SD, n=6; USE=Ultrasound assisted extraction).

as observed for ethanolic extracts (p>0.05). However, when maceration with water was performed at lower temperatures (25°C), the prepared extracts, namely M-W-25-7 and M-W-25-3, showed approximately 30% lower antimicrobial activity (p<0.01), indicating that at this temperature, the extraction of the antibacterial compounds from the crude propolis was less efficient. When aqueous extracts of crude propolis were prepared by ultrasound assisted extraction, the obtained extracts (US-W-4-3 and US-W-4-7) showed no antibacterial effect against *B. subtilis* and *S. aureus* (Figures 2 and 3). Probably, the low temperature at which extraction was performed hampered the successful extraction of the antimicrobial compounds from the raw propolis. Finally, when comparing the antimicrobial efficiency of all extracts prepared, it might be concluded that the pH value of the extraction media is not a critical parameter which would influence the antibacterial activity of the extract prepared.

When the observed antibacterial activity of extracts prepared was correlated with the content of flavones and flavonols, a weak but significant positive correlation was found (r^2=0.3734 and 0.3150, for *B. subtilis* and *S. aureus*, respectively). No statistically significant correlation was found between antimicrobial activity of extracts prepared and flavanones content. This indicated that, beside flavones and flavonols, some other components of the propolis with antibacterial action were successfully extracted from the crude material, and contributed to the antimicrobial action of the extracts prepared. Furthermore, such an assumption would also explain the lack of antimicrobial activity of aqueous propolis extracts prepared by ultrasound assisted extraction (US-W-4-7 and US-W-4-3), which contained comparable amount of flavones and flavonols as M-W-50-3 and M-W-50-7 (Table 1), but lacked any antimicrobial activity. The in depth study of their chemistry, especially in the case of aqueous propolis extracts prepared by maceration at 50°C, is the focus of upcoming studies.

In the case of the activity of propolis extracts against the yeast *C. albicans*, somewhat different results were observed (Figure 3). In general, *C. albicans* is less susceptible to the propolis extracts prepared, showing inhibition zones ranging from 9 to 11 mm. Again, extracts prepared with 80% ethanol, regardless of the pH of the extraction media and extraction technique used were the most active (Figure 3). When 40% ethanol at pH 7 was used as the extraction medium, for both maceration and ultrasound assisted extraction, the obtained extracts (M-40-50-7, M-40-25-7 and US-40-4-7) showed lower action against *C. albicans* (p<0.001), with zones of inhibition around 9 mm, while the extracts prepared with 40% ethanol at pH 3 (M-40-50-3, M-40-25-3 and US-40-4-3) were practically inactive (Figure 3). Aqueous propolis extracts, regardless of the preparation method used, showed no antifungal effect against *C. albicans* (p<0.001).

When the observed antimicrobial efficiency of extracts against *C. albicans* was correlated with the content of flavones and flavonols, a statistically significant positive correlation was found (r^2 = 0.8217), while no statistically significant correlation was found between antimicrobial activity and flavanones content. This indicated that flavones and flavonols have an important role in the activity of the propolis extracts prepared, but they must be present in a sufficient concentration in order to express an anti-*Candida* effect, as was also shown by earlier investigations [9]. Therefore, the extracts of the crude propolis prepared with rather polar extraction media would be less effective. Also, it is possible that some other constituents of propolis could contribute to its anti-*Candida* action. For example, recent studies demonstrated that in the case of ethanolic extracts of Mediterranean propolis, only samples which contained significant amounts of terpenyl esters and hydroxybenzoic acid displayed activity against *C. albicans in vitro*, while others were ineffective [11]. All this demonstrates a need of further research in order to determine the individual contribution of each propolis constituent with regard to its anti-*Candida* action and possible synergism, as well as to understand the variability in propolis samples of different geographical origin. Only better knowledge of their chemistry would allow us to develop novel and more efficient extraction procedures, which would lead to standardized and more efficient propolis preparations for use in medicine and in the food industry.

Experimental

Propolis sample: The raw poplar-type propolis was collected from beehives in the outskirts of Zagreb, Croatia.

Preparation of propolis extracts: Prior to extraction, the raw material was frozen at -20°C and then homogenized and pulverized by grinding in a chilled mortar. The extraction was performed by maceration and an ultrasound method, using 1:5 weight ratios between crude propolis and the extraction medium. The extraction solvents used were water and 40 or 80%, v/v, ethanol/water mixtures, with the pH adjusted to 3 or 7 by addition of 0.1M HCl or NaOH solution. The maceration of raw propolis was performed in hermetically sealed Erlenmeyer flasks in a horizontal shaker thermostated at either 25 or 50°C at a shaking frequency of 120 min^{-1} for 24 h. Ultrasound extraction was performed by introducing an ultrasonic processor probe (Misonix Sonicator® S-4000, Cole-Parner, USA) operating at 60 W for 30 min directly into the sample, immersed in an ice-bath to prevent sample overheating. After the extraction process, the residual solids were separated from the liquid extracts by centrifugation at 3500 rpm for 10 min, followed by filtration through a Whatman cellulose filter paper No.1. The obtained liquid extracts were stored protected from light at 4°C in airtight containers.

Determination of flavones, flavonols and flavanones: Quantification of flavones and flavonols in extracts prepared was performed by a colorimetric method using aluminum chloride, as described by Woisky and Salatino [12]; results are expressed as quercetin equivalents, while flavanones contents were determined

using a 2,4-dinitrophenylhydrazine colorimetric assay proposed by Nagy and Grancai [13]; results are expressed as (±)-naringenin equivalent.

In vitro antimicrobial assays: The antimicrobial activity of the prepared propolis extracts against *Bacillus subtilis* NCTC 8236, *Staphylococcus aureus* ATCC 29213 and *Candida albicans* ATCC 10231 was evaluated by a cylinder-plate diffusion method following the procedure described by Kosalec *et al*. [9] and using 60 μL aliquots of undiluted propolis extracts. Prepared plates were incubated under aerobic conditions first at +4°C for 1 h and then at +37°C for 18 h in the case of bacteria and 48 h in the case of *C. albicans*. The same procedure was utilized for all extraction media used. The diameter of the inhibition zone around each hole was measured and recorded. All values are expressed as mean±SD of 6 separate experiments. Data were compared by one-way ANOVA, followed by Tukey's multiple comparison post-test. The differences were considered statistically significant when $P<0.05$. Calculations were performed using the GraphPad Prism program (GraphPad Software, Inc., San Diego, CA; www.graphpad.com).

References

[1] Farooqui T, Farooqui A. (**2010**) Molecular mechanism underlying the therapeutic activities of propolis: A critical review. *Current Nutrition & Food Science*, ***6***, 186-199.
[2] (a) Sforcin JM, Bankova V (**2011**) Propolis: is there a potential for the development of new drugs? *Journal of Ethnopharmacology*, ***133***, 253-260.
[3] Bankova V, Popova M, Trusheva B. (**2006**) Plant sources of propolis: an update from a chemist's point of view. *Natural Product Communications*, ***1***, 1023-1028
[4] Parolia A, Thomas MS, Kundabala M, Mohan M. (**2010**) Propolis and its potential uses in oral health. *International Journal of Medicine and Medical Sciences*, ***2***, 210-215.
[5] Spellberg B, Bartlett JG, Gilbert DN. (**2013**) The future of antibiotics and resistance. *New England Journal of Medicine*, ***368***, 299-302.
[6] Chang CC, Yang MH, Wen HM, Chern JC. (**2002**) Estimation of total flavonoid content in propolis by two complementary colorimetric methods. *Journal of Food and Drug Analysis*, ***10***, 178-182
[7] Kosalec I, Bakmaz M, Pepeljnjak S, Vladimir-Knezevic S. (**2004**) Quantitative analysis of the flavonoids in raw propolis from northern Croatia. *Acta Pharmaceutica*, ***54***, 65-72.
[8] Viuda-Martos M, Ruiz-Navajas Y, Fernandez-Lopez J, Perez-Alvarez JA. (**2008**) Functional properties of honey, propolis, and royal jelly. *Journal of Food Science*, ***73***, 117-124.
[9] Kosalec I, Pepeljnjak S, Bakmaz M, Vladimir-Knezevic S. (**2005**) Flavonoid analysis and antimicrobial activity of commercially available propolis products. *Acta Pharmaceutica*, ***55***, 423-430.
[10] Vardar-Unlu G, Silici S, Unlu M. (**2008**) Composition and *in vitro* antimicrobial activity of *Populus* buds and poplar-type propolis. *World Journal of Microbiology and Biotechnology*, ***24***, 1011-1017.
[11] Popova M, Trusheva B, Antonova D, Cutajar S, Mifsud D, Farrugia C, Tsvetkova I, Najdenski H, Bankova V (**2011**) The specific chemical profile of Mediterranean propolis from Malta. *Food Chemistry*, ***126***, 1431-143.
[12] Woisky RG, Salatino A (**199**8) Analysis of propolis: some parameters and procedures for chemical quality control. *Journal of Apicultural Research*, ***37***, 99-105.
[13] Nagy M, Grancai D. (**1996**) Colorimetric determination of flavanones in propolis. *Pharmazie*, ***51***, 100-101.

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Comparative Evaluation of Phytochemicals, and Antidiabetic and Antioxidant Activities of *Cuscuta reflexa* Grown on Different Hosts in Northern Thailand

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Received: January 4th, 2016; Accepted: November 27th, 2016

For the first time, total phytochemical profiles of methanol crude extracts of *Cuscuta reflexa* grown on three different hosts, *Coccinia grandis*, *Ficus racemosa* and *Samanea saman*, that were cultivated in northern Thailand were examined, along with their antidiabetic and antioxidant activities. The highest level of total flavonoids (114.4 mg QE/ g extract) and total phenolic content (90.8 mg GAE/ g extract) were observed in the extract of *Cu. reflexa* that was grown on *Co. grandis*. The GC-MS results showed that various types of phenolic compounds, hydrocarbons, saturated fatty acids and methyl ester of fatty acids, unsaturated fatty acids and methyl ester of fatty acids, vitamin E, terpenes and sterol contained in the extracts of *Cu. reflexa* were capable of being grown on three hosts. Moreover, the HPLC results showed the presence of gallic acid, catechin, vanillic acid, rutin and quercetin in all *Cu. reflexa* samples. The extract of *Cu. reflexa* that was grown on *Co. grandis* represented the highest antidiabetic activity with a percent inhibition of 51.2. Moreover, the extract also possessed the greatest antioxidant activity (DPPH; IC_{50} 168.6 μg/mL, FRAP; 40.5 mg GAE/ g extract).

Keywords: *Cuscuta reflexa*, Phytochemical, Total flavonoid content, Total phenolic content, Antidiabetic activity, Antioxidant activity.

The parasitic plant, *Cuscuta*, is commonly used as an ingredient in functional food and in medicinal tonics as a nutrient. It is also often used in alcoholic beverages [1]. In northern Thailand, one of the most widely distributed *Cuscuta* plants is *Cu. reflexa* Roxb. This can grow on a wide variety of plant hosts including a number of perennial plants and annual crop species. Previous research reported that the chemical constituents of *Cuscuta* depend on the type of host [2–3]. Various types of phenolic compounds and their glycosides, terpenes, sterols, saturated aliphatic hydrocarbon, saturated fatty acids and unsaturated fatty acids were found in *Cu. reflexa* [4]. The study of Perveen *et al*. [5] revealed that the phenolic acid and other phenolic components, such as flavonoids found in *Cu. reflexa*, are often extracted in higher amounts in more polar solvents. Furthermore, previous research studies indicated that the stem of *Cu. reflexa* possessed antibacterial, antiviral, anticancer and antioxidant activities [6–7]. The objectives of this study were to identify the total flavonoid content (TFC) and total phenolic content (TPC), and chemical constituents in the methanol crude extracts of *Cu. reflexa* that were grown on three different hosts, *Coccinia grandis* (L.) Voigt., *Ficus racemosa* Linn. and *Samanea saman* (Jacq.) Merr., as well as to evaluate the antidiabetic and antioxidant activities of the above mentioned crude extracts.

Total flavonoid and total phenolic contents of the extracts of *Cu. reflexa* were evaluated, and the results are shown in Table 1. TFC values ranged from 67.5 to 114.4 mg QE/ g extract, while the TPC values ranged from 84.4 to 90.8 mg GAE/ g extract. The highest value of TFC and TPC was exhibited in the extract of *Cu. reflexa* grown on *Co. grandis*. Anjum *et al*. [1] demonstrated the TFC and TPC in methanol extracts of *Cuscuta* sp. that were grown on 9 different hosts. The results revealed that the TFC ranged from 47.0 to 81.0 mg catechin/ g of the dry weight, while the TPC value was in the range of 39.4 to 66.2 mg/g.

Table 1: Total flavonoid and total phenolic contents of the methanol crude extracts of *Cuscuta reflexa* grown on *Coccinia grandis*, *Ficus racemosa* and *Samanea saman*.

Host plant	Total flavonoid contents (mg QE/ g extract)	Total phenolic contents (mg GAE/ g extract)
Coccinia grandis	114.4±0.7a	90.8±2.9a
Ficus racemosa	79.0±1.8b	86.6±0.5b
Samanea saman	67.5±2.6c	84.4±1.4b

*Average ± standard deviation from three replicates.
The different letters in the same column are considered significantly different according to Duncan's multiple comparison test ($P < 0.05$).

The phytochemicals of the all extracts of *Cu. reflexa* were analyzed by gas chromatography and mass-spectrometry (GC-MS). Each compound was identified based on mass spectral matching (≥ 90%) from W8N08 and Wiley7n libraries and is reported in Table 2. The compounds identified in all of the *Cu. reflexa* extracts were (1) phenolic compounds (4-vinylphenol, 4-vinylguaiacol, methyl cinnamate, methyl 4-hydroxycinnamate and methyl-3-(3,5-ditertbutyl-4-hydroxyphenyl) propionate), (2) hydrocarbons (cyclopentadecane, nanocosane and squalene), (3) saturated fatty acids and methyl esters of fatty acids (palmitic acid methyl ester, steric acid methyl ester, palmitic acid amide, arachidic acid methyl ester, stearic acid amide, and lignoceric acid methyl ester), (4) unsaturated fatty acids and methyl esters of fatty acids (linoleic acid methyl ester, linolenic acid methyl ester and oleic acid amide), (5) vitamin E (α- and γ-tocopherol), (6) terpenes (phytol and β-amyrin), and (7) sterols (campesterol, stigmasterol, (24*RS*)-24, 27-dimethylcholesta-5,26-dien-3β-ol and β-sitosterol). However, methyl ferulate, palmitic acid, pentadecanoic acid methyl ester, palmitoleic acid methyl ester, *E*-9-heptadecenal and baccharan-3β-ol were only found in the extract of *Cu. reflexa* grown on *Co. grandis*. Additionally, docosanoic acid methyl ester was only detected in the extract of *Cu. reflexa* grown on *S. saman*. As in the study of Bais and Kakkar [3], the chemical constituents of *Cu. reflexa* vary with the type hosts. In the same way, linolenic acid, linoleic acid, oleic acid, stearic acid, palmitic acid, β-sitosterol,

stigmasterol, campesterol and β-amyrin, were reported in the list of chemical constituents isolated from species of *Cuscuta* [4, 7]. Moreover, 4-vinylphenol, which is widely used as a flavoring agent [8], was found as the major component in the extract of *Cu. reflexa* grown on *S. saman* (18.5%). On the other hand, unsaturated fatty acids and methyl esters of fatty acids were found to be the major components of the extract of *Cu. reflexa* grown on *F. racemosa* (46.9%), *Co. grandis* (33.1%) and *S. saman* (31.6%), respectively. The essential fatty acids (linoleic acid and linolenic acid) that cannot be synthesized in the human body and must be obtained from the diet were found in the extracts of *Cu. reflexa*; therefore, this plant can be considered a good source of essential fatty acids. Moreover, linolenic acid has been associated with health benefits in the treatment of several diseases such as coronary artery disease and rheumatoid arthritis [9–10].

Table 2: Chemical compositions of the methanol crude extracts of *Cuscuta reflexa* grown on *Coccinia grandis*, *Ficus racemosa* and *Samanea saman*.

Peak no.	RT (min)[a]	Components[b]	Total peak (%)		
			CCG	CFR	CSS
1	7.7	4-Vinylphenol	13.3	14.0	18.5
2	10.0	4-Vinylguaiacol	4.7	3.3	4.4
3	11.8	Methyl cinnamate	0.2	0.3	0.2
4	21.0	Methyl 4-hydroxycinnamate	0.8	0.6	0.8
5	22.0	*E*-9-Heptadecenal	3.2	ND	ND
6	22.5	Pentadecanoic acid methyl ester	0.5	ND	ND
7	23.1	Methyl ferulate	0.4	ND	ND
8	24.6	Palmitoleic acid methyl ester	0.4	ND	ND
9	24.9	Palmitic acid methyl ester	12.6	10.1	10.7
10	25.1	Methyl-3-(3,5-ditertbutyl-4-hydroxyphenyl) propionate	1.1	0.5	1.0
11	25.8	Palmitic acid	0.5	ND	ND
12	28.3	Linoleic acid methyl ester	8.6	6.0	7.5
13	28.5	α-Linolenic acid methyl ester	9.9	7.5	7.3
14	28.7	Phytol	0.4	0.5	0.8
15	29.0	Steric acid methyl ester	3.7	2.7	3.0
16	30.1	Palmitic acid amide	1.8	2.8	1.7
17	32.5	Arachidic acid methyl ester	1.0	0.6	0.6
18	33.3	Oleic acid amide	14.1	33.4	16.8
19	33.6	Stearic acid amide	1.2	1.8	1.1
20	35.2	Cyclopentadecane	1.0	0.5	1.2
21	35.7	Docosanoic acid methyl ester (behenic)	ND	ND	0.4
22	38.6	Lignoceric acid methyl ester	0.9	0.6	1.0
23	39.8	Squalene	3.2	2.6	2.1
24	40.9	Nanocosane	2.1	5.1	2.4
25	43.0	γ-Tocopherol	0.9	1.0	0.9
26	44.1	α-Tocopherol	1.1	0.7	1.0
27	45.8	Campesterol	1.8	0.9	3.2
28	46.3	Stigmasterol	1.1	0.5	1.6
29	47.2	(24*RS*)-24,27-dimethylcholesta-5,26-dien-3β-ol	3.3	1.7	4.9
30	47.5	β-Sitosterol	2.1	0.8	4.4
31	48.3	β-Amyrin	1.2	1.3	2.6
32	49.4	Baccharan-3β-ol	2.5	ND	ND
Phenolic compounds			**20.5**	**18.7**	**24.9**
Hydrocarbons			**6.3**	**8.2**	**5.8**
Saturated fatty acids and methyl ester of fatty acids			**22.2**	**18.7**	**18.4**
Unsaturated fatty acids and methyl ester of fatty acids			**33.1**	**46.9**	**31.6**
Fatty aldehydes			**3.2**	**ND**	**ND**
Vitamin E			**2.1**	**1.6**	**1.8**
Terpenes			**1.6**	**1.8**	**3.4**
Sterols			**10.9**	**3.9**	**14.0**
Total			**99.9**	**99.8**	**99.9**

[a]Retention time (minutes). [b]Compounds listed in order of elution from a HP-5 MS column.
"ND" = not detected
Host plants; CCG = *Cu. reflexa* grown on *Co. grandis*, CFR = *Cu. reflexa* grown on *F. racemosa*, CSS = *Cu. reflexa* grown on *S. saman*

Additionally, as shown in Table 3, 20 phenolic compounds were identified and quantified by high performance liquid chromatography (HPLC). Gallic acid, catechin, vanillic acid, rutin and quercetin could be found in the *Cu. reflexa* extracts grown on *Co. grandis* ranging from 0.34 to 6.96 mg/ g extract of which the greatest one was rutin. The extract of *Cu. reflexa* that was grown on *F. racemosa* contained high contents of catechin (5.81 mg/ g extract), quercetin (4.53 mg/ g extract) and rutin (3.95 mg/ g extract). On the other hand, the extract of *Cu. reflexa* that was grown on *S. saman* contained lower amounts of gallic acid, catechin, vanillic acid, rutin and quercetin than when grown on the other hosts, with contents ranging from 0.34 to 1.65 mg/ g extract. Previous studies reported that rutin and quercetin were usually found in extracts of *Cuscuta* species [5, 8, 11].

Table 3: Phenolic compound contents in the methanol crude extracts of *Cuscuta reflexa* grown on *Coccinia grandis*, *Ficus racemosa* and *Samanea saman.*

Compounds	Retention times*	Content (mg/ g extract)		
		Co. grandis	*F. racemosa*	*S. saman*
Gallic acid	11.7	0.90±0.02b	1.25±0.01a	0.35±0.02c
Catechin	23.1	5.97±0.05a	5.81±0.06a	1.14±0.13b
Vanillic acid	24.7	0.34±0.02c	1.27±0.01b	1.64±0.11a
Rutin	32.2	6.96±0.04a	3.95±0.03b	1.44±0.06c
Quercetin	46.6	0.87±0.06c	4.53±0.10a	1.45±0.03b

*HPLC conditions: column Eclipse-C_{18} (150 × 4.6 mm, i.d.5 μM), gradient solution (A) 0.1% formic acid in water and (B) 100% acetonitrile (B. 30%, 0-50 min, 50%, 51-60 min, UV detector 280 nm, flow rate 0.8 mL/min and injection volume 20 μL.
Values are expressed as means ± S.D.
The different letters in the same column are considered significantly different according to Duncan's multiple comparison test ($P < 0.05$).

The results of the antidiabetic assay of the *Cu. reflexa* extracts (1.0 mg/mL) based on the α-glucosidase inhibition model showed that the extract of *Cu. reflexa* that was grown on *Co. grandis* exhibited the highest inhibition percentage of 51.2%, followed by *F. racemosa* (45.7%) and *S. saman* (31.2%). Previously, Rahmatullah *et al*. [12] investigated the hypoglycemic effects of the methanol and chloroform extracts of *Cu. reflexa* based on oral glucose tolerance tests. The results showed that both methanol and chloroform extracts of *Cu. reflexa* demonstrated significant oral hypoglycemic activity in glucose-loaded rats. Previous studies have suggested that the phenolic and flavonoid compounds that are present in plant extracts are responsible for the antidiabetic activity [13–14].

The methanol extracts of *Cu. reflexa* grown on three different hosts were also investigated for their possible antioxidant activities by DPPH free radical (DPPH$^{\bullet}$) scavenging and ferric reducing antioxidant power (FRAP) assays, and the results are displayed in Table 4. In the DPPH$^{\bullet}$ scavenging system, the IC_{50} of *Cu. reflexa* extracts grown on different hosts varied from 279.7 to 168.6 μg/mL. The highest activity was noticed in *Cu. reflexa* grown on *Co. grandis*, and this specimen displayed potential antioxidant activity with a statistical difference ($P < 0.05$) when compared with the other hosts. In the FRAP system, the extract of *Cu. reflexa* grown on *Co. grandis* still possessed the highest FRAP value of 40.5 mg GAE/g dry extract), which was significantly higher than that of the extracts from the other hosts ($P < 0.05$). Amol *et al*. [15] evaluated the antioxidant activities of the methanol extract of *Cu. reflexa* using the DPPH$^{\bullet}$ scavenging and reducing power assays. The extract showed antioxidant activity with an IC_{50} of 359.5 μg/mL, and also exhibited high reducing capacity in the reducing power system. Based on our results, the high phenolic and flavonoid contents of the extract of *Cu. reflexa* grown on *Co. grandis* could be responsible for its greater antioxidant activities. This finding has also been suggested in many earlier reports [16–17]. Previous research studies have suggested that the antioxidant activity of plants extracts can be partly attributed to some constituents other than fatty acids, such as phenolic compounds and vitamin E [18–19]. According to the GC-MS results of the extract of *Cu. reflexa* grown on *Co. grandis*, the presence of methyl ferulate and vitamin E, which are already known to possess high antioxidant activities [20–21], may be related to the greater antioxidant activities. Moreover, previous research reports have revealed that phenolic compounds can exhibit high antioxidant activity [22–23], especially rutin, catechin and quercetin, which were found in the extracts of *Cu. reflexa* grown on *Co. grandis* and *F. racemosa*. However, the unidentified active phenolic compounds that present in the methanol crude extracts of *Cu. reflexa* may also play a role in antioxidant activity.

Table 4: Antioxidant activities of the methanol crude extracts of *Cuscuta reflexa* grown on *Coccinia grandis*, *Ficus racemosa* and *Samanea saman*.

Host plant	Antioxidant activity	
	DPPH scavenging activity (IC_{50} µg/mL)	Ferric reducing antioxidant power (mg GAE/ g extract)
Coccinia grandis	168.7±1.0a	40.5±0.8a
Ficus racemosa	201.6±0.5b	35.0±1.4b
Samanea saman	279.7±6.0c	26.8±0.3c

*Average ± standard deviation from three replicates.
The different letters in the same column are considered significantly different according to Duncan's multiple comparison test ($P < 0.05$).

Experimental

Plant material: *Cu. reflexa* grown on *Co. grandis*, *F. racemosa* and *S. saman* was collected locally in Suthep District, Muang, Chiang Mai, Thailand. The plants were dried at 45°C, ground into powder and stored at room temperature for further extraction.

Preparation of the extracts: Ten g of dried stem material of *Cu. reflexa* was extracted with 100 mL of methanol, and left overnight at room temperature. The extract was sonicated using an ultrasonicator (Crest, USA) for 30 min, filtered through Whatman no. 1 filter paper and evaporated under vacuum at 40°C using a rotary evaporator until dry. The dry extracts were kept at room temperature in the dark until required.

Determination of TFC: TFC was determined using the method of Kaewnarin *et al*. [24], with slight modification. The extract (0.5 mL) was mixed with 2 mL of methanol, followed by the addition 0.15 mL of 50 g/L $NaNO_2$. After 5 min, 0.15 mL of 100 g/L $AlCl_3$ was added and the mixture was incubated at room temperature for 15 min before the absorbance was measured at 415 nm. Quercetin solution was used as a standard for the determination and the results were expressed as mg QE/g dry extract.

GC-MS analysis: Analysis of the extracts of *Cu. reflexa* grown on three different hosts was performed using a 6890 Agilent Technologies/MSD 5973 Hewlett Packard, equipped with a MS detector and an HP-5MS capillary column (bonded and cross-linked 5% phenyl-methylpolysiloxane, 30 m × 0.25 µm, film thickness 0.25 µm). The injector and detector temperatures were set at 270 and 280°C, respectively. The oven temperature was set at 80°C and held for 2 min, and then increased at a rate 10°C/min to 120°C and held for 4 min. The oven temperature was then increased at a rate 10°C/min to 155°C and held for 4 min, and then increased at a rate 5°C/min to 280°C and held for 12.50 min. The total running time was 55 min. Helium was used as carrier gas at a flow rate of 1 mL/min. The sample (1 µL) was injected in the splitless mode. GC-MS detection in an electron ionization system with an ionization energy measurement of 70 eV was used. Injector and MS transfer line temperatures were set at 270 and 290°C, respectively. The components were identified based on a comparison of their relative retention times and the mass spectra with W8N08 and Wiley 7th libraries data of the GC-MS system.

Determination of TPC: TPC was estimated using the protocol of Thitilertdecha *et al*. [25], with slight modification. The procedure involved combining 0.25 mL of sample with 2.5 mL deionized water and 0.5 mL of Folin-Ciocalteu reagent. After 5 min, 0.5 mL of 20%, w/v, Na_2CO_3 was added, and the solution was incubated for 1 h at room temperature. Absorbance was then measured at 760 nm. Gallic acid solution was used as a standard for the determination and the results were expressed as mg GAE/g dry extract.

HPLC analysis: The sample extracts were measured using an HPLC technique (Agilent 1200). An Eclipse-C_{18} column was used (150 x 4.6 mm, i.d.5 µM). The flow rate was set to 0.8 mL/min. The mobile phase was 0.1% formic acid in water (solvent A) and 100% acetonitrile (solvent B). Gradient elution was performed as follows: 0-50 min, 30% B and 51-60 min, 50% B. The injection volume was 20 µL. The fingerprint profiles were recorded at an optimized wavelength of 280 nm. The quantification of gallic acid, catechin, vanillic acid, protocatechuic acid, syringic acid, caffeic acid, chlorogenic acid, sinapic acid, ferulic acid, *m*-coumaric acid, *o*-coumaric acid, ellagic acid, rosmarinic acid, rutin, myrecetin, quercetin, kaempferol, apigenin, luteolin and isorhamnetin was determined based on peak area measurements.

Antidiabetic assay: α-Glucosidase (AGH) solution was prepared from rat intestinal acetone powder by partial modification of the procedure reported by Oki *et al*. [26]. One hundred mg of intestinal acetone powder was added to 3 mL of 0.9% NaCl solution, homogenized by sonication and kept in an ice bath. After centrifugation at 6,000 rpm for 30 min at 4°C, the resulting supernatant was kept cold and directly subjected to inhibitory assay. The method of Adisakwattana *et al*. [27] was used to determine AGH inhibitory assay. The assay was defined as the percent inhibition under the assay conditions, which was calculated according to the formula:

Percent inhibition = $(A_o - A_s / A_o) \times 100$

where A_o is the absorbance of the control, and A_s is the absorbance of the mixture containing the test compound.

Determination of antioxidant activities

DPPH• scavenging assay: The free radical scavenging ability was determined according to the method of Gülçin *et al.* [28], with slight modification. 2,2′-Diphenyl-1-picrylhydrazyl radical solution in ethanol (0.1 mM, 1.5 mL) was mixed with 0.5 mL of different concentrations of each extract, and methanol was used as the control. The mixtures were well shaken and kept at room temperature for 30 min in the dark. The absorbance was measured at 517 nm. The percent of DPPH• discoloration of the samples was calculated according to the formula:

Percent inhibition = $(A_o - A_s / A_o) \times 100$

where A_o is the absorbance of the control (containing all reagents except the test compound), and A_s is the absorbance of the mixture containing the test compound. The test sample concentrations providing 50% inhibition (IC_{50}) were calculated from the plot of inhibition percentage against extract concentration values. The radical scavenging ability was presented as IC_{50} values.

FRAP assay: The FRAP assay was determined according to the protocol of Li *et al*. [29], with some modification. The FRAP reagent containing 10 mM TPTZ solution in 40 mM hydrochloric acid (20 mL), 20 mM ferric (III) chloride (20 mL) and acetate buffer (5 mL, 300 mM, pH 3.6) was prepared freshly prior to being used. Different concentrations of each extract (0.1 mL) were mixed with the FRAP reagent (1.5 mL) and 1.4 mL of acetate buffer (300 mM, pH 3.6) and then incubated at room temperature for 30 min. The absorbance was measured at 593 nm. Gallic acid was used as a standard and FRAP value was calculated as the gallic acid equivalent (mg GAE/ g dry extract).

Statistical analysis: The results of all experiments were expressed as mean ± standard deviation. Analysis of variance was performed by ANOVA procedure and Duncan's multiple comparison test was used to determine any significant differences ($P < 0.05$) identified between treatments.

Acknowledgments - We are grateful to Chiang Mai University and Biology Program, Faculty of Science and Technology, Pibulsongkram Rajabhat University and TRF Research-Team Association Grant (RTA5880006) for the financial support.

References

[1] Anjum F, Bukhari SA, Shahid M, Anwar S, Afzal M, Akhter N. (**2013**) Comparative evaluation of antioxidant potential of parasitic plant collected from different hosts. *Journal of Food Processing and Technology*, ***4***, 1-6.

[2] Lin HB, Lin JQ, Lu N, Lin JQ. (**2007**) Study of quality control on *Cuscuta chinensis* and *Cuscuta australia*. *Journal of Chinese Medicinal Materials*, ***30***, 1446-1449.

[3] Bais N, Kakkar A. (**2013**) Comparative phytochemical analysis of *Cuscuta reflexa* parasite grown on *Cassia fistula* and *Ficus benghlensis* by GC-MS. *International Journal of Pharmacy and Pharmaceutical Sciences*, ***5***, 350-355.

[4] Patel S, Sharma V, Chauhan NS, Dixit VK. (**2012**) An updated review on the parasitic herb of *Cuscuta reflexa* Roxb. *Journal of Chinese Integrative Medicine*, ***10***, 249-255.

[5] Perveen S, Bukhari IH, Ain QU, Kousar S, Rehman J. (**2013**) Antimicrobial, antioxidant and minerals evaluation of *Cuscuta europea* and *Cuscuta reflexa* collected from difference hosts and exploring their role as functional attribute. *International Research Journal of Pharmaceutical and Applied Sciences*, ***3***, 43-49.

[6] Pal DK, Mandal M, Senthilkumar GP, Padhiari A. (**2006**) Antibacterial activity of *Cuscuta reflexa* stem and *Corchorus olitorius* seed. *Fitoterapia*, *77*, 589-591.

[7] Vijikumar S, Ramanathan K, Devi BP. (**2011**) *Cuscuta reflexa* Roxb. – A wonderful miracle plant in ethnomedicine. *Indian Journal of Natural Sciences*, ***11***, 676-683.

[8] Bernini R, Mincione E, Barontini M, Provenzano G, Setti L. (**2007**) Obtaining 4-vinylphenols by decarboxylation of natural 4-hydroxycinnamic acids under microwave irradiation. *Tetrahedron*, ***63***, 9663-9667.

[9] Freeman MP. (**2000**) Omega-3 fatty acids in psychiatry: a review. *Annual Review of Clinical Psychology*, ***12***, 159-165.

[10] Rennie KL, Hughes J, Lang R, Jebb SA. (**2003**) Nutritional management of rheumatoid arthritis: a review of the evidence. *Journal of Human Nutrition Dietetics*, ***16***, 97-109.

[11] Hajimehdipoor H, Kondori BM, Amin GR, Adib N, Rastegar H, Shekarchi M. (**2012**) Development of a validated HPLC method for the stimultaneous determination of flavonoids in *Cuscuta chinensis* Lam. by ultra-violet detection. *DARU Journal of Pharmaceutical Sciences*, ***20***, 1-6.

[12] Rahmatullah M, Sultan S, Toma TT, Lucky S-a-S, Chowdhury MH, Haque WM, Annay MEA, Jahan R. (**2010**) Effect of *Cuscuta reflexa* stem and *Calotropis procera* leaf extracts on glucose tolerance in glucose-induces hyperglycemic rats and mice. African Journal of Traditional, Complementary and Alternative Medicines, *7*, 109-112.

[13] Ardestani A, Yazdanparast R. (**2007**) Inhibitory effects of ethyl acetate extract of *Teucrium polium* on *in vitro* protein glycation. *Food and Chemical Toxicology*, ***45***, 2402-2411.

[14] Wang W, Yagiz Y, Buran TJ, Nunes CdN., Gu L. (**2011**) Phytochemicals from berries and grapes inhibited the formation of advanced glycation end-products by scavenging reactive carbonyls. *Food Research International*, ***44***, 2666-2673.

[15] Amol P, Vikas P, Kundan C, Vijay P, Rajesh C. (**2009**) *In vitro* free radicals scavenging activity of stems of *Cuscuta reflexa*. *Journal of Pharmacy Research*, ***2***, 58-61.

[16] Velioglu YS, Mazza G, Gao L, Oomah BD. (**1998**) Antioxidant activity and total phenolics in selected fruits, vegetables, and grain products. *Journal of Agricultural and Food Chemistry*, ***46***, 4113-4117.

[17] Proestos C, Chorianopoulos N, Nychas GJE, Komaitis M. (**2005**) RP-HPLC analysis of the phenolic compounds of plant extracts. Investigation of their antioxidant capacity and antimicrobial activity. *Journal of Agricultural and Food Chemistry*, ***53***, 1190-1195.

[18] Lee JC, Kim HR, Kim J, Jang YS. (**2002**) Antioxidant property of an ethanol extract of the stem of *Opuntiaficus indica* var. *saboten*. *Journal of Agriculture and Food Chemistry*, ***50***, 6490-6496.

[19] Ismail M, Mariod A, Bagalkotkar G, Ling HS. (**2010**) Fatty acid composition and antioxidant activity of oils from two cultivars of Cantaloupe extracted by supercritical fluid extraction. *Grasas y Aceites*, ***61***, 37-44.

[20] Karamać M, Buciński A, Pegg RB, Amarowicz R. (**2005**) Antioxidant and antiradical activity of ferulates. *Czech Journal of Food Sciences*, ***23***, 64-68.

[21] Sroynak R, Srikalong P, Raviyan P. (**2013**) Radical scavenging capacity and antioxidant activity of the vitamin E extracted from palm fatty acid distillate by sequential cooling hexane. *Journal of Agricultural Science*, ***5***, 224-237.

[22] Meyer AS, Heinonen M, Frankel EN. (**1998**) Antioxidant interactions of catechin, cyanidin, caffeic acid, quercetin, and ellagic acid on human LDL oxidation. *Food Chemistry, **61***, 71-75.

[23] Yang J, Guo J, Yuan J. (**2008**) *In vitro* antioxidant properties of rutin. *LWT-Food Science and Technology*, ***41***, 1060-1066.

[24] Kaewnarin K, Niamsup H, Shank L, Rakariyatham N. (**2014**) Antioxidant and antiglycation activities of some edible and medicinal plants. *Chiang Mai Journal of Science*, ***41***, 105-116.

[25] Thitilertdecha N, Teerawutgulrag A, Rakariyatham N. (**2008**) Antioxidant and antimicrobial activities of *Nephelium lappacium* L. extracts. *LWT Food Science and Technology*, ***41***, 2029-2035.

[26] Oki T, Matsui T, Osajima Y. (**1999**) Inhibitory effect of α-glucosidase inhibitors varies according to its origin. *Journal of Agricultural and Food Chemistry*, ***47***, 550-553.

[27] Adisakwattana S, Chantarasinlapin P, Thammarat H, Yibchok-Anun S. (**2009**) A series of cinnamic acid derivatives and their inhibitory activity on intestinal α-glucosidase. *Journal of Enzyme Inhibition and Medicinal Chemistry*, ***24***, 1194-1200.

[28] Gülçın İ, Oktay M, Kıreççı E, Küfrevıoğlu Öİ. (**2003**) Screening of antioxidant and antimicrobial activities of anise (*Pimpinella anisum* L.) seed extracts. *Food Chemistry*, ***83***, 371-382.

[29] Li Y, Guo C, Yang J, Wei J, Xu J, Cheng S. (**2006**) Evaluation of antioxidant properties of pomegranate peel extract in comparison with pomegranate pulp extract. *Food Chemistry*, ***96***, 254-260.

NPC Natural Product Communications

2017
Vol. 12
No. 1
55 - 56

Chemical Composition, Antioxidant and Anticholinesterase Activities of *Gentianella azurea* from Russian Federation

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Received: September 2nd, 2016; Accepted: November 2nd, 2016

Phytochemical study of *Gentianella azurea* (Bunge) Holub (Gentianaceae) collected in Buryatia Republic (Russian Federation) resulted in the isolation of twenty-one compounds including bellidifolin, bellidin, isobellidifolin, norswertianolin, isobellidifolin-8-*O*-β-D-glucopyranoside, orientin, cynaroside, cosmosiin, apigenin, 4′-*O*-caffeoylswertiamarin, swertiamarin-6′-*O*-β-D-glucopyranoside and sweroside, firstly detected in this species. The extracts and individual compounds were shown to possess antioxidant and anticholinesterase potential.

Keywords: *Gentianella azurea*, Gentianaceae, Xanthones, Flavones, Iridoids, Antioxidant activity, Anticholinesterase activity, HPLC.

Gentianella azurea (Bunge) Holub (azure little gentian) is a small annual herb, common in the forests of Eastern Siberia, China and Mongolia. The decoction of the total herb (local name *sum cu tig ta*) is used in traditional medicine of Buryatia as a choleretic and antidepressant [1]. Previously, some xanthones, flavonoids, and iridoids were reported [2]. All earlier studies were carried out on plant material collected in Mongolia [2a] and China [2b, c]. Hitherto, there is no data about the biological activities of *G. azurea*. As a part of our ongoing research of the chemical composition and bioactivities of bitter gentian teas [3] this study presents the results of a phytochemical and biological investigation of *G. azurea* growing in Buryatia Republic (Russian Federation).

The 60% MeOH-extract of the total herb was partitioned with $CHCl_3$, EtOAc, and *n*-BuOH to yield three fractions, which were separated to give twenty-one compounds including eight xanthones {bellidifolin (**1**), bellidin (**2**), isobellidifolin (**3**) [4], 1,3,5-trihydroxy-8-methoxyxanthone (**4**) [3a], swertianolin (**5**), norswertianolin (**6**), isobellidifolin-8-*O*-β-D-glucopyranoside (**7**) [4], 1,3,5-trihydroxy-8-methoxyxanthone-1-*O*-β-D-glucopyranoside (**8**) [3a]}, six flavones {orientin (**9**), isoorientin (**10**) [5], cynaroside (**11**), cosmosiin (**12**), luteolin (**13**), apigenin (**14**) [6]}, six iridoids {gentiopicroside (**15**), swertiamarin (**16**), 4′-*O*-caffeoylswertiamarin (**17**) [7], swertiamarin-6′-*O*-β-D-glucopyranoside (**18**) [8], sweroside (**19**), loganic acid (**20**) [9]}, and one triterpene oleanolic acid (**21**) [3b], which were identified by comparison of their m.ps, UV, FTIR, ^1H and ^{13}C NMR spectral, and MS data with those reported in the literature. Compounds **1**-**3**, **6**, **7**, **9**, **11**, **12**, **14**, **17**-**19** were isolated from *G. azurea* herb for the first time.

HPLC data showed that the flowers and leaves of *G. azurea* accumulated xanthones, flavonoids and iridoids (Table 1). The dominant xanthones of the flowers and leaves were **8** and **5**. The prevailing flavonoid (**9**) concentrated in the leaves. Iridoids were the most significant group of extracted compounds, and **16** was found in higher levels in the flowers and leaves. A characteristic feature of the roots was their ability to concentrate **15**, with small concentrations of xanthones and flavonoids. The total content of xanthones, flavonoids and iridoids in *G. azurea* herbs collected in three regions of Buryatia Republic were 15.4–21.8, 17.9–27.5 and 104.8–161.6 mg/g respectively (Table 1). Four components (**8**, **9**, **16** and **19**) were prevalent in all herb samples.

Table 1: Content of xanthones (Xt), flavonoids (Fl) and iridoids (Ir) in *G. azurea* organs[a], total herb (GaH1-GaH3) and MeOH-extract (GaM)[b].

Comps	Fl	L	St	R	GaH1	GaH2	GaH3	GaM
1	0.4	0.2	tr.[c]	0.1	0.6	0.3	0.4	0.9
2	1.5	0.3	0.3	tr.	2.9	4.6	0.7	12.5
3	0.1	0.1	n.d.[d]	n.d.	0.3	0.4	tr.	1.1
4	0.4	0.4	0.4	0.5	0.5	0.2	0.5	0.6
5	6.1	7.3	1.0	0.5	2.4	1.3	6.4	3.5
6	4.7	5.7	2.0	n.d.	5.1	2.9	5.6	8.0
8	7.1	6.7	3.1	0.9	7.1	5.7	8.2	15.3
9	14.6	20.7	4.3	n.d.	17.9	11.8	19.4	31.6
10	2.4	2.1	0.3	n.d.	3.3	2.7	5.0	7.3
11	7.3	2.4	0.6	n.d.	4.0	2.5	2.6	6.7
12	tr.	0.9	tr.	n.d.	0.6	0.9	0.5	2.4
15	tr.	2.2	3.1	16.0	2.8	2.5	5.2	6.7
16	154.8	121.5	38.2	2.4	107.6	73.6	113.5	197.8
19	31.7	3.8	6.1	0.5	27.6	24.1	37.0	62.7
20	4.0	2.4	4.3	0.6	4.6	4.6	5.9	11.9
ΣXt	20.3	20.7	6.8	2.0	18.9	15.4	21.8	41.9
ΣFl	24.3	26.1	5.2	n.d.	25.8	17.9	27.5	48.0
ΣIr	190.5	129.9	51.7	19.5	142.6	104.8	161.6	279.1

[a] Fl – flowers, L – leaves, St – stems, R – roots; [b]mg/g, dry plant (extract) weight; [c]traces (<LOQ); [d]not detected (<LOD).

Table 2: Radical-scavenging (DPPH) and anticholinesterase activity (ACE) of *G. azurea* MeOH-extract (GaM) and individual compounds [a].

Compds	DPPH[a]	ACE[b]	Compds	DPPH[a]	ACE[b]
GaM	39.8	22.9	**12**	>100	>100
1	5.5	1.2	**13**	10.6	4.96
2	3.4	5.3	**14**	>100	>100
3	6.3	2.9	**15**	>100	>100
4	7.1	44.2	**16**	>100	>100
5	14.1	9.2	**17**	52.4	>100
6	12.6	47.5	**18**	>100	>100
7	21.7	20.6	**19**	>100	>100
8	25.2	>100	**20**	>100	>100
9	13.8	6.8	**21**	>100	22.9
10	19.7	28.5	Trolox [b]	11.6	-
11	14.5	35.1	Neostigmine [b]	-	0.91

[a] IC_{50}, μg/mL; [b] reference compound.

The IC_{50} value for the radical-scavenging activity of the MeOH-extract (GaM) against DPPH$^{\bullet}$-radicals was 32.3 μg/mL. The most active compounds were xanthone aglycones (**1**-**4**) (Table 2). The acetylcholinesterase-inhibiting activity of GaM was characterized by an IC_{50} value 22.9 μg/mL. Comparative analysis of individual compounds showed that xanthone aglycones **1** and **3** with a methoxy-group in positions C-3 and C-5, respectively, were more effective inhibitors than **2** and **4** (Table 2). It should be noted that the activities of flavone **13** and *C*-glucoside **9** were close to that of xanthone aglycones.

In conclusion, the results obtained confirmed the data of the ethnopharmacological use of *G. azurea* as a natural phytotherapeutic agent.

Experimental

General experimental procedures: UV-Vis, SF-2000 UV-Vis-spectrophotometer (OKB Specter), Uniplan microplate UV-Vis-spectrophotometer (Pikon); Elemental composition, MAT 8200 spectrometer (Thermo Finnigan); IR, FT-801 FTIR spectrophotometer (Simex); Melting point, IA9100 micromelting point apparatus (Electrothermal Thermo Scientific); NMR, VXR 500S spectrometer (Varian); ESIMS (positive mode), Thermo Finnigan LCQ mass spectrometer; CC, Silica gel 60 (Merck, 70-230 mesh), Amberlite XAD7HP (Sigma), Woelm polyamide; GPC, Sephadex LH-20 (25-100 μm; Pharmacia); prep. HPLC-UV, Summit system with UV-Vis detector (Dionex), LiChrosorb RP-18 (4.6×250 mm, 5 μm, Merck), T 35ºC, flow rate 1 mL/min; solvent, linear gradient of 5-70% of B (MeCN) in A (H_2O) for 90 min; detector 254 nm; analytical HPLC, Milichrom A-02 system (Econova), as described previously [3b]; Biological assays, radical-scavenging activity – microplate assay [10], acetylcholinesterase inhibition activity – microplate assay [11].

Plant material: The samples of *G. azurea* were collected in the flowering period in the Buryatia Republic (Russian Federation), dried and stored at 4°C in the PlaMeta Group Plant Repository: Nur-Tukhum village (No GaH1; Dzhidinskii region, 15.VIII.2012, 50°73′31″ N, 106°25′98″ E; voucher specimen No Gn/h-31/01-64/0812); Tsakir village (No GaH2; Zakamenskii region, 20.VIII.2012, 50°49′26″ N, 103°57′36″ E; voucher specimen No Gn/h-36/06-82/0812); Turka village (No GaH3; and Pribaikal'skii region, 22.VIII.2012, 52°94′57″ N, 108°24′43″ E; voucher specimen No Gn/h-54/42-104/0812).

Extraction and isolation: Air-dried, ground herb of *G. azurea* (sample GaH3; 1.7 kg) was extracted with 70% EtOH (60°C, ×3), and the combined extracts were partitioned with $CHCl_3$ (Ga-F_1, 108.3 g), EtOAc (Ga-F_2, 159.8 g) and *n*-BuOH (Ga-F_3, 283.9 g) respectively. The Ga-F_1 fr. (60 g) was chromatographed using flash chromatography on a silica column [3×100 cm, $CHCl_3$-MeOH (100:0→0:100)], and by prep. HPLC to yield **1** (63 mg), **2** (82 mg), **3** (22 mg), **4** (18 mg), **13** (12 mg), **14** (8 mg) and **21** (3.35 g). The Ga-F_2 fr. (80 g) was loaded onto Amberlite XAD7HP (400 g) and eluted with a water-MeOH mixt. (100:0→0:100) to give 6 sub-fractions Ga-F_2-1–Ga-F_2-6. Subfr. Ga-F_2-1 was chromatographed by prep. HPLC to yield **17** (12 mg) and **18** (9 mg). Subfr. Ga-F_2-2, Ga-F_2-3 was chromatographed on a polyamide column [2×50 cm, H_2O-MeOH (100:0→0:100)] and a Sephadex LH-20 column [3×100 cm, MeOH-H_2O (100:0→50:50)] to yield **9** (512 mg), **10** (84 mg), **11** (16 mg) and **12** (6 mg). Subfr. Ga-F_2-4 – Ga-F_2-6 was chromatographed on a silica column [2×100 cm, $CHCl_3$-MeOH (100:0→60:40)], a polyamide column [2×50 cm, H_2O-MeOH (100:0→0:100)] and a Sephadex LH-20 column [3×100 cm, MeOH-H_2O (100:0→50:50)] to yield **5** (87 mg), **6** (245 mg), **7** (12 mg) and **8** (87 mg). The Ga-F_3 fr. (100 g) was loaded onto Amberlite XAD7HP (600 g) and eluted with a water-MeOH mixt. (100:0→0:100) to give 6 sub-fractions Ga-F_3-1–Ga-F_3-6. Subfr. Ga-F_3-1 was chromatographed on a polyamide column [2×70 cm, H_2O-MeOH (100:0→0:100)] and by prep. HPLC to yield **15** (15 mg), **16** (2.63 g), **19** (163 mg) and **20** (32 mg).

Acknowledgments – The author acknowledges the financial support provided by the Presidium of SD RAS, Project No VI.62.1.8.

References

[1] Aseeva TA, Dashiev DB, Dashiev AD, Nikolaev SM, Surkova NA, Chekhirova GV, Yurina TA. (**2008**) *Tibetan Medicine of Buryats.* SO RAN, Novosibirsk. 217–264. (in Russian).

[2] (a) Purev O, Oyun Kh, Odontuya G, Tankhaeva LM, Nikolaeva GG, Khan KM, Ali Shan ST, Voelter W. (**2002**) Isolation and structure elucidation of two xanthones from *Gentiana azurium* Bunge (Fam. Gentianaceae). *Zeitschrift für Naturforschung*, ***57B***, 331–334; (b) Zhang Y, Yang C. (**1994**) Chemical studies on *Gentianella azurea*, a Tibetan medicinal plant. *Acta Botanica Yunnanica*, ***16***, 401–406; (c) Shi K, Wang Y, Jiang Q, Liao Z. (**2010**) Chemical constituents of *Gentianella azurea*. *Chinese Journal of Natural Medicine*, ***8***, 425–427.

[3] (a) Olennikov DN. (**2013**) Chemical investigation of *Anagallidium dichotomum* and anticholinesterase activity of its constituents. *Chemistry of Natural Compounds*, ***49***, 1137–1139; (b) Olennikov DN, Kashchenko NI, Chirikova NK, Tankhaeva LM. (**2015**) Iridoids and flavonoids of four Siberian gentians: Chemical profile and gastric stimulatory effect. *Molecules*, ***20***, 19172–19188; (c) Olennikov DN, Kashchenko NI, Chirikova NK, Koryakina LP, Vladimirov LN. (**2015**) Bitter gentian teas: Nutritional and phytochemical profiles, polysaccharide characterisation and bioactivity. *Molecules*, ***20***, 20014–20030; (d) Olennikov DN, Chirikova NK. (**2016**) Algidisides I and II, new iridoids glycosides from *Gentiana algida*. *Chemistry of Natural Compounds*, ***52***, 637–641.

[4] Markham KR. (**1964**) Gentian pigments – I: Xanthones from *Gentiana bellidifolia*. *Tetrahedron*, ***20***, 991–997.

[5] Williams CA, Murray BG. (**1972**) Flavonoid variation in the genus *Briza*. *Phytochemistry*, ***11***, 2507–2512.

[6] Malikov VM, Yuldashev MP. (**2002**) Phenolic compounds of plants of the *Scutellaria* L. genus. *Chemistry of Natural Compounds*, ***38***, 358–406.

[7] Zhou Y, Di YT, Gesang S, Peng SL, Ding LS. (**2006**) Secoiridoid glucosides from *Swertia mileensis*. *Helvetica Chimica Acta*, ***89***, 94–102.

[8] Tan RX, Wolfender JL, Zhang LX, Ma WG, Fuzzati N, Marston A, Hostettmann K. (**1996**) Acyl secoiridoids and antifungal constituents from *Gentiana macrophylla*. *Phytochemistry*, ***42***, 1305–1313.

[9] El-Naggar LJ, Beal JL. (**1980**) Iridoids. A review. *Journal of Natural Products*, ***43***, 649–707.

[10] Zhang HM, Wang CF, Shen SM, Wang GL, Liu P, Liu ZM, Wang YY, Du SS, Liu ZL, Deng ZW. (**2012**) Antioxidant phenolic compounds from pu-erh tea. *Molecules*, ***17***, 14037–14045.

[11] Di Giovanni S, Borloz A, Urbain A, Marston A, Hostettmann K, Carrupt PA, Reist M. (**2008**) *In vitro* screening assays to identify natural or synthetic acetylcholinesterase inhibitors: Thin layer chromatography *versus* microplate methods. *European Journal of Pharmaceutical Sciences*, ***33***, 109–119.

NPC Natural Product Communications

2017
Vol. 12
No. 1
57 - 58

Constituents of Fermented Male Flowers of *Alnus sieboldiana* (Betulaceae)

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Received: August 21st, 2016; Accepted: September 20th, 2016

Fractionation of the ethyl acetate crude extract of the fermented male flowers of *Alnus sieboldiana* resulted in the isolation of two diarylheptanoids and six flavonoids. Yashabushidiol A, yashabushidiol B and naringenin were known constituents of the male flowers of *A. sieboldiana* while kaempferol, quercetin, pinocembrin dimethyl ether**,** 5,7-dimethoxy-3-hydroxyflavanone and 5,7-dimethoxy-3-hydroxyflavone were isolated for first time from the fermented male flowers of *A. sieboldiana*. The isolated compounds were characterized using NMR and HRMS and compared with available literature.

Keywords: *Alnus sieboldiana*, Betulaceae, Biotransformation, Flavones, Flavanones, 1,7-Diarylheptanoids.

The genus *Alnus* (family Betulaceae) contains 35 deciduous tree and shrub species. The plants are widely distributed in temperate regions of the northern hemisphere and can be found in wetlands, volcanic soils, glacial deposits and sand dunes [1].

A. sieboldiana Matsum. is a deciduous plant found on the south western coast of Japan. The roots contain *Azotobacter* species, which are involved in nitrogen fixation [2]. Therefore, this species can be planted in poorly nourished mountain and coastal areas to protect them from erosion. The male and female flowers emit a strong aromatic and resinous odor. *A. sieboldiana* has been of special interest to Asakawa's research group who studied the plant over three decades (1969-1995). The male flowers were reported to contain β-phenylethyl cinnamate, stilbene monomers, flavanones, flavones, 1,7-phenylheptanoids and some sesquiterpenoids [3]. The flavone galangin, isolated from the methanol extract of the leaves of *A. sieboldiana*, has been found to inhibit the A549 tumor cell line [4]. It is interesting to note that the whole plant, including the flowers and young leaves, even the cones, are easily infected by the white-colored fungus *Penicillium* sp. [5], causing the powerful aromatic odor of both male and female flowers to diminish dramatically and finally turn unpleasant. The powerful aromatic odor was due to the presence of 2-phenylethanol [6].

The present paper concerns a phytochemical study of fermented *A. sieboldiana* male flowers. From the ethyl acetate extract of the 6-month-old fermented male flowers, two diarylheptanoids and six flavonoids were successfully isolated. Of these isolated compounds, the diarylheptanoids yashabushidiol A (**1**) [7a] and yashabushidiol B (**2**) [7], and the flavonoid naringenin (**3**) [4a, 8] are known constituents of the original male flowers, while the other five isolated flavonoids kaempferol (**4**), quercetin (**5**), pinocembrin dimethyl ether (7), 5,7-dimethoxy-3-hydroxyflavanone (**8**) and 5,7-dimethoxy-3-hydroxyflavone (**9**) are biotransformation products, resulting from the enzymatic actions of the endophytic white-colour fungus.

Naringenin (**3**) was previously isolated from fresh male flowers, but in this experiment was only isolated in a minute amount. In addition, kaempferol (**4**) [9] and quercetin (**5**) [10] were successfully isolated from the fermented male flowers for the first time. Naringenin (**3**) is biosynthesized in the male flowers of *A. sieboldiana* through the shikimate acid pathway [11a]. Flavonoids **4** and **5** isolated from the fermented male flowers in this work are hydroxylated derivatives of naringenin (**3**), probably resulting from biotransformation by flavanone 3-hydroxylase (E1) and flavonoid 3'-hydroxylase (E2) enzymes [11b], as proposed in Figure 2.

Figure 1: Two diarylheptanoids from the male flowers of *A. sieboldiana*.

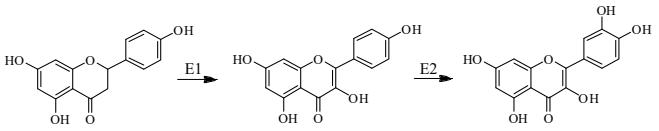
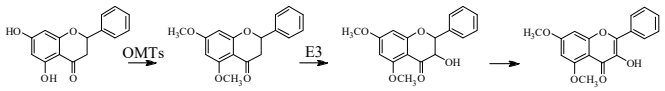


Figure 2: Metabolic pathway of **4** and **5**, from naringenin (**3**).

In addition, three 5,7-dimethoxyflavonoids, **7**, **8** and **9**, were also isolated from the ethyl acetate crude extract of the fermented male flowers. These compounds have never been reported in the original flowers. We herein report these compounds as biotransformation products in the fermented male flowers of *A. sieboldiana*.

Compound **7** may be produced from pinocembrin (**6**), which is a known constituent of the male flowers of *A. sieboldiana* [4c], through methylation by plant-*O*-methyltransferases (OMTs) [11c]. Compound **7** is converted to compound **8** by flavanone 3-hydroxylase (E3) [11d]. Further oxidation of compound **8** led to compound **9** as the final product. In this work, pinocembrin was not detected; presumable it was fully converted into the derivatives. The proposed metabolic pathway of compounds **7**, **8** and **9** are illustrated in Figure 3.



6 **7** **8** **9**

Figure 3: Metabolic pathway of **7**, **8** and **9**, from pinocembrin (**6**).

In conclusion, fermentation of *A. sieboldiana* male flowers by the white fungus *Penicillium* sp. caused hydroxylation at positions 3 and 3' of naringenin (**3**) to produce **4** and **5**, while methylation at positions 5 and 7 of pinocembrin (**6**) gave **7**. Further hydroxylation and oxidation of **7** produced **8** and **9**.

Experimental

Plant material: *Alnus sieboldiana* (male flowers) were collected in February 2015 at Naruto (Shikoku Island), Japan at the beginning of spring. The flowers were identified by YA and deposited at the herbarium of Tokushima Bunri University.

The 6-month-old fermented male flowers (0.97 kg) were mechanically crushed and extracted with ethyl acetate. The extract was evaporated to give a viscous extract (49.64 g), which The was subjected to VLC (8 cm x 12 cm) eluting with mixtures of *n*-hexane / ethyl acetate (10:0; 9:1; 8:2; 7:3; 6:4; 5:5; 7.5:2.5; 0:10) to obtain 12 fractions. Yashabushidiol B (**2**) was obtained as white crystals upon evaporation of fraction 7 (1.37 g). Fraction 8 was further separated using Sephadex LH-20 eluting with a mixture of dichloromethane:methanol (1:1) giving yashabushidiol A (**1**) as white-yellowish crystals (944.3 mg), and naringenin (**3**) (1.8 mg). Further treatment of fraction 9 using Sephadex, followed by normal phase silica gel column chromatography eluting with a combination of *n*-hexane and ethyl acetate gave kaempferol (**4**) (9.0 mg), pinocembrin dimethyl ether (**7**) (1.5 mg), 5,7-dimethoxy-3-hydroxyflavanone (**8**) (2.0 mg) and 5,7-dimethoxy-3-hydroxyflavone (**9**) (2.7 mg). Lastly, quercetin (**5**) (82.88 mg) was obtained as yellow powder isolated from the tenth fraction.

5,7-Dimethoxy-3-hydroxyflavanone (**8**)
Yellow amorphous.
^{1}H NMR ($CDCl_3$, 500 MHz): 4.45 (1H, dd, J = 0.8, 12.2 Hz, H-2), 5.04 (1H, d, J = 12.2 Hz, H-3), 6.13 (2H, dd, J = 1.5, 6.5 Hz, H-6, H-8), 7.57 (2H, dd, J = 1.5, 7.4 Hz, H-2′, H-6′), 7.44 (3H, m, H-3′, H-4′, H-5′), 3.82 (3H, s, OCH_3-7), 3.92 (3H, s, OCH_3-5).
^{13}C NMR (125 Hz): 72.7 (C-2), 83.3 (C-3), 190.8 (C-4), 162.1 (C-5), 93.3 (C-6), 167.0 (C-7), 93.8 (C-8), 164.9 (C-9), 102.9 (C-10), 136.5 (C-1′), 127.5 (C-2′, C-6′), 128.7 (C-3′, C-5′), 129.2 (C-4′), 56.2 (OCH_3-5), 55.7 (OCH_3-7).
MS *m/z*: 300.0997 $C_{17}H_{16}O_5$.

5,7-Dimethoxy-3-hydroxyflavone (**9**)
Light yellow powder.
^{1}H NMR ($CDCl_3$, 500 MHz): 6.36 (1H, *d, J* = 2.15 Hz, H-6), 6.58 (1H, *d, J* = 2.2 Hz, H-8), 8.20 (2H, *dd, J* = 1.35, 7.9 Hz, H-2′, H-6′), 7.52 (2H, *dd, J* = 7.9, 7.5 Hz, H-3′, H-5′), 7.45 (1H, *m*, H-4′), 3.93 (3H, *s*, OCH_3-5), 3.99 (3H, *s*, OCH_3-7).
^{13}C NMR (125 Hz): 141.8 (C-2), 138.3 (C-3), 172.1 (C-4), 160.6 (C-5), 95.7 (C-6), 164.5 (C-7), 92.4 (C-8), 159.1 (C-9), 106.3 (C-10), 131.1 (C-1′), 127.1 (C-2′, C-6′), 128.5 (C-3′, C-5′), 129.6 (C-4′), 55.8 (OCH_3-5), 56.4 (OCH_3-7).
MS *m/z* 298.0850 $C_{17}H_{14}O_5$.

Acknowledgments - The authors gratefully acknowledge Universiti Teknologi MARA and Tokushima Bunri University for research facilities and support for this project. Nurunajah thanks the Ministry of Education, Malaysia for the PhD scholarship.

References

[1] (a) Schwencke J, Caru M. (**2001**) Advances in actinorhizal symbiosis: Host plant-frankia interactions, biology and applications in arid and reclamations. A review. *Arid and Land Resources Management*, ***15***, 285-327; (b) Benson DR, Dawson JO. (**2007**) Recent advances in the biogeography and genecology of symbiotic frankia and its host plant. *Physiology of Plant*, ***130***, 318-330.

[2] Kitamura S, Murata G. (**1979**) *Colored illustration of woody plants of Japan.* Hoikusha, Osaka*,* ***2***.

[3] (a) Asakawa Y. (**1971**) Chemical constituents of *Alnus sieboldiana* (Betulaceae) II. The isolation and structure of flavonoids and stilbenes. *Bulletin of the Chemical Society of Japan,* ***44***, 2761-2766; (b) Asakawa Y. (**1971**) Four new flavonoids isolated from *Alnus sieboldiana. Bulletin of the Chemical Society of Japan*, ***44***, 297; (c) Asakawa Y. (**1972**) Chemical constituents of *Alnus sieboldiana* (Betulaceae). III. The synthesis and stereochemistry of yashabushiketols*. Bulletin of the Chemical Society of Japan*, ***45***, 1794-1797.

[4] Ludwiczuk A, Saha A, Kuzuhara T, Asakawa Y. (**2011**) Bioactivity guided isolation of anticancer constituents from leaves of *Alnus sieboldiana* (Betulaceae). *Phytomedicine*, ***18***, 491-498.

[5] Harinantenaina L, Noma Y, Asakawa Y. (**2005**) *Penicillium sclerotiorum* catalysed the conversion of herbertenediol into its dimers: mastigophorenes A and B. *Chemical and Pharmaceutical Bulletin,* ***53***, 256-257.

[6] Ghani NA, Ismail NH, Asakawa Y. (**2016**) Comparative study of the volatile components of the fresh and fermented male flowers of *Alnus sieboldiana* (Betulaceae). *Natural Product Communications*, ***11***, 265-266.

[7] (a) Hashimoto T, Tori M, Asakawa Y. (**1986**) Five new diarylheptanoids from the male flowers of *Alnus sieboldiana*. *Chemical and Pharmaceutical Bulletin*, ***34***, 1846-1849; (b) Pawar VU, Shinde VS. (**2011**) Chiron approach to the synthesis of yashabushidiol B (3*S*,5*S*)-1-(4′-hydroxyphenyl)-7-phenylheptane-3,5-diol, and its 4′-methoxy analogue. *Tetrahedron: Asymmetry*, ***22***, 8-11.

[8] Jeon SJ, Chun W, Choi YJ, Kwon YS. (**2008**) Cytotoxic constituents from the bark of *Salix hulteni*. *Archives of Pharmacal Research*, ***31***, 978-982.

[9] Ding HY, Lin HC, Teng CM, Wu YC. (**2000**) Phytochemical and pharmacological studies on Chinese *Paeonia* species. *Journal of the Chinese Chemical Society*, ***47***, 381-388.

[10] Min BS, Lee SY, Kim JH, Lee JK, Kim TJ, Kim DH, Kim YH, Joung H, Lee HK, Nakamura N, Miyashiro H, Hattori M. (**2003**) Anti-complement activity of constituents from the stem bark of *Juglans mandshurica. Biological and Pharmaceutical Bulletin*, ***26***, 1042-1044.

[11] (a) Winkel BS. (**2006**) The biosynthesis of flavonoids. In *The science of flavonoids*. Springer, USA 71-98; (b) Ueyama Y, Suzuki KI, Fukuchi-Mizutani M, Fukui Y, Miyazaki K, Ohkawa H, Kusumi T, Tanaka Y. (**2002**) Molecular and biochemical characterization of torenia flavonoid 3'-hydroxylase and flavone synthase II and modification of flower color by modulating the expression of these genes. *Plant Science*, ***163,*** 253-263; (c) Lam KC, Ibrahim RK, Behdad B, Dayanandan S. (**2007**) Structure, function and evolution of plant *O*-methyltransferases. *Genome*, ***50***, 1001-1013. (d) Holton TA, Cornish EC. (**1995**) Genetics and biochemistry of anthocyanin biosynthesis. *The Plant Cell*, *7*, 1071-1083.

[12] Hodgetts KJ (**2005**) Inter- and intramolecular mitsunobu reaction based approaches to 2-substituted chromans and chroman-4-ones. *Tetrahedron*, ***61***, 6860-6870.

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No. 1
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Secondary Metabolites from the Leaves of *Digitalis viridiflora*

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Received: September 11th, 2016; Accepted: November 4th, 2016

A new phenylethanoid glycoside, named digiviridifloroside (**1**), was isolated from the leaves of *Digitalis viridiflora* Lindley along with a known phenylethanoid glycoside, calceolarioside A (**2**), two flavonoid glycosides, scutellarein 7-*O*-β-D-glucopyranoside (**3**) and hispidulin 7-*O*-β-D-glucopyranoside (**4**), two cleroindicins, cleroindicins B (**5**) and F (**6**), a nucleoside, adenosine (**7**), as well as a mixture of β-glucopyranosyl-(1→6)-4-*O*-caffeoyl-α/β-glucopyranose and 3,4-dihydroxyphenylethanol. The structure of the new compound was established as 3,4-dihydroxy-β-phenylethoxy-6-*O*-(*E*)-feruloyl-β-glucopyranosyl-(1→6)-4-*O*-(*E*)-caffeoyl-β-glucopyranoside (**1**) based on extensive 1D- and 2D-NMR spectroscopy, as well as HR-ESI-MS. Digiviridifloroside represents a rare type of phenylethanoid glycoside which bears two aromatic acyl units in its structure. In addition to phytochemical studies, the isolates were evaluated for their *in vitro* antimicrobial activities against three pathogenic bacteria and three yeast strains using a microdilution method. Among the tested compounds, **5** exhibited moderate antibacterial activity against *Bacillus cereus* NRRLB 3711 with a MIC value of 25 µg/mL, whereas compounds **5** and **6** showed relatively high anticandidal activity against *Candida* strains with MIC values down to 12.5 µg/mL, in comparison to the standard antimicrobial compounds.

Keywords: *Digitalis viridiflora*, Plantaginaceae, Phenylethanoid glycoside, Digiviridifloroside, Antimicrobial activity.

The genus *Digitalis* (Plantaginaceae) contains biennial or perennial species. It is represented by nine species in the flora of Turkey including *D. viridiflora* Lindley [1]. Previous studies on the genus showed the presence of a wide range of secondary metabolites including phenylethanoid glycosides, cardiac glycosides, steroidal saponins, pregnane glycosides, cleroindicins, flavonoids and anthraquinones [2-6]. In continuation of our systematic survey on the phytochemical composition of *Digitalis* species from Turkey, five phenylethanoid glycosides were recently reported from the initial work on *D. viridiflora* [7]. Further detailed chromatographic studies on the chemical constituents of the leaves of *D. viridiflora* led to the isolation of one new (**1**) and one known phenylethanoid glycoside, two flavonoid glycosides, two cleroindicins and a nucleoside (Figure 1). This paper reports the isolation, structure elucidation and antimicrobial activities of these compounds.

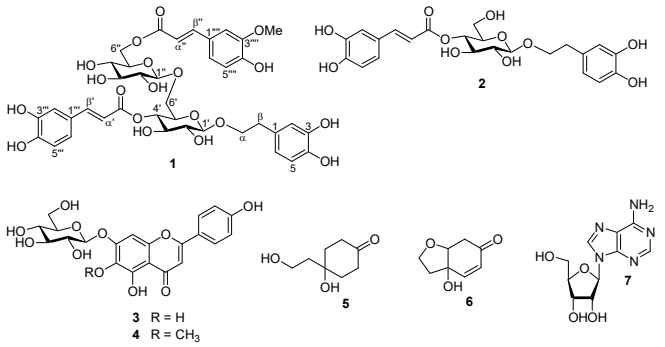

Figure 1: Chemical structures of compounds **1**-**7**.

Compound **1** was obtained as a yellowish amorphous powder. Its UV and IR spectra were characteristic for a phenylethanoid glycoside. It possesses a molecular formula of $C_{39}H_{44}O_{19}$, as determined by the analysis of its HRESIMS (*m/z* 839.2390 [M + Na]$^+$, calcd for $C_{39}H_{44}NaO_{19}$, 839.2375) and ^{13}C NMR data (Table 1). The ^{1}H NMR spectrum (Table 1) of **1** showed resonances at δ_H 7.55 and 6.25 (each d, J = 15.8 Hz) as an *AX* system as well as signals at δ_H 7.00 (d, J = 2.0 Hz), 6.86 (dd, J = 8.1, 2.0 Hz) and 6.74 (d, J = 8.1 Hz) as an *ABX* system attributable to an (*E*)-caffeoyl moiety. Moreover, the spectrum also contained three aromatic signals as an *ABX* system at δ_H 6.70 (d, J = 1.9 Hz), 6.67 (d, J = 8.0 Hz), and 6.55 (dd, J = 8.0 and 1.9 Hz), two geminal benzylic methylene signals at δ_H 2.78 (t, J = 8.0 Hz), and two nonequivalent oxymethylene signals δ_H 4.01 (m) and 3.71 arising from a 3,4-dihydroxyphenylethanol moiety. Furthermore, the presence of two anomeric signals at δ_H 4.37 (d, J = 7.8 Hz) and 4.35 (d, J = 7.7 Hz) revealed the diglycosidic structure of **1**, which was confirmed by the corresponding anomeric carbon resonances at δ_C 105.0 and 104.5 in the ^{13}C NMR spectrum. These findings taken together with 2D NMR experiments (COSY, HSQC and HMBC (Figure 2) revealed the presence of a lugrandoside [2] backbone in **1**. However, the ^{1}H NMR spectrum of **1** contained additional signals at δ_H 7.18 (d, J = 2.0 Hz), 7.05 (dd, J = 8.0, 2.0) and 6.80 (d, J = 8.0 Hz) as an *ABX* type, a pair of *trans*-coupled *AX* type signals at δ_H 7.62 and 6.37 (each d, J = 15.9 Hz) and a methoxy signal at δ_H 3.87 (s) suggesting the presence of a (*E*)-feruloyl unit in the structure of **1**.

The deshielded H_2-6″ (δ_H 4.53 and 4.26) and C-6 ″ (δ_C 64.6) signals of the terminal β-glucopyranose signals indicated that the (*E*)-feruloyl unit was located at C-6″(OH), which was further

Table 1. ^{13}C and ^{1}H NMR data[a] for digiviridifloroside (**1**) (CD_3OD, ^{13}C 125 MHz; ^{1}H 500 MHz).

Position	δ_C (ppm)	δ_H (ppm, *J* in Hz)
Aglycone		
1	131.6	-
2	117.2	6.70 (d, *J* = 1.9)
3	146.2	-
4	144.7	-
5	116.6	6.67 (d, *J* = 8.0)
6	121.5	6.55 (dd, *J* = 8.0, 1.9)
α	72.5	4.01 (m) / 3.71†
β	36.7	2.78 (t, *J* = 8.0)
Glucose		
1′	104.5	4.37 (d, *J* = 7.8)
2′	75.4	3.28 †
3′	75.8	3.62 (t, *J* = 8.0)
4′	72.8	4.83 †
5′	75.1	3.77 (m)
6′	70.3	3.85 †
		3.69 (dd, *J* = 11.5, 4.7)
Glucose		
1″	105.0	4.35 (d, *J* = 7.7)
2″	74.9	3.23 (t, *J* = 8.2)
3″	77.7	3.35†
4‴	77.4	3.33†
5″	75.7	3.48 (m)
6″	64.6	4.53 (dd, *J* = 11.7, 1.8)
		4.26 (dd, *J* = 11.7, 5.8)
Caffeoyl		
1‴	127.8	-
2‴	115.4	7.00 (d, *J* = 2.0)
3‴	146.9	-
4‴	149.8	-
5‴	116.5	6.74 (d, *J* = 8.1)
6‴	123.2	6.86 (dd, *J* = 8.1, 2.0)
α′	114.8	6.25 (d, *J* = 15.8)
β′	147.8	7.55 (d, *J* = 15.8)
C=O	168.7	-
Feruloyl		
1⁗	127.8	-
2⁗	111.8	7.18 (d, *J* = 2.0)
3⁗	150.8	-
4⁗	149.5	-
5⁗	116.8	6.80 (d, *J* = 8.0)
6⁗	124.5	7.05 (dd, *J* = 8.0, 2.0)
α″	115.4	6.37 (d, *J* = 15.9)
β″	147.3	7.62 (d, *J* = 15.9)
C=O	169.2	-
OMe	56.6	3.87 s

[a]Assignments are based on COSY, HSQC and HMBC experiments. † Overlapped.

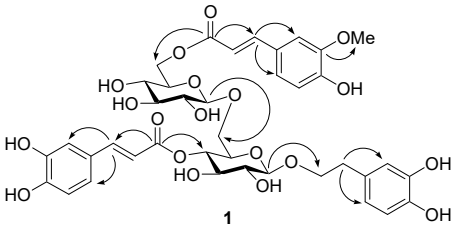

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Figure 2: Key HMBC (C→H) correlations for **1**.

confirmed by the long-range correlation of the carbonyl carbon (δ_C 169.2) of the (*E*)-feruloyl unit with H_2-6″ of the terminal glucopyranose unit in the HMBC spectrum (Figure 2). Based on these spectroscopic data, the structure of **1** was elucidated as 2-(3,4-dihydroxyphenyl)ethyl-*O*-6-*O*-(*E*)-feruloyl-β-glucopyranosyl-(1→6)-4-*O*-(*E*)-caffeoyl-β-glucopyranoside, and named digiviridifloroside.

The known compounds were characterized as calceolarioside A (**2**) [8], scutellarein 7-*O*-β-D-glucopyranoside (**3**) [9], hispidulin 7-*O*-β-D-glucopyranoside (**4**) [10], cleroindicins B (**5**) and F (**6**) [11], and adenosine (**7**) [12] by comparing their spectroscopic data with those published previously. Moreover, a mixture of β-glucopyranosyl-(1→6)-4-*O*-caffeoyl-α/β-glucopyranose and 3,4-dihydroxyphenyl-ethanol, which could be an artefact formed during the isolation procedure, was characterized.

To the best of our knowledge, digiviridifloroside (**1**) is the third example of a rare phenylethanoid glycoside obtained from the genus *Digitalis*, which contains two aromatic acyl units in its structure; the first two such compounds were reported from *D. lanata* [4]. In a very recent study by Skhirtladze *et al.* [13], another new phenylethanoid glycoside esterified with two aromatic acids was reported. Therefore, the occurrence of such rare phenylethanoid glycosides might possess significant chemotaxonomic importance for the genus *Digitalis* within its new family Plantaginaceae.

The *in vitro* antimicrobial activities of the isolates (except for the mixture) were evaluated against three pathogenic bacteria (*Bacillus cereus* NRRLB 3711, *Pseudomonas aeruginosa* ATCC 10145, *Staphylococcus aureus* ATCC 6538) and three yeast (*Candida albicans* ATCC 90028, *C. parapsilosis* ATCC 22019, and *C. krusei* ATCC 6258) strains using a microdilution method. Compounds **4** and **5** displayed moderate activity against *Bacillus cereus* NRRLB 3711 with MIC values 50 and 25 µg/mL respectively, while the rest were inactive against the tested bacteria (Table 2). Compounds **4 - 6** displayed moderate activity against all *Candida* strains tested with MIC values ranging from 12.5 to 100 µg/mL, being **6** the most potent one against *C. parapsilosis* ATCC 22019. To the best of our knowledge the antimicrobial activities of the cleroindicins (**5** and **6**) are being reported for the first time in this study.

Table 2: Antimicrobial activities (MIC, µg/mL) of compounds **1**-**7**.

Comp.	*B. cereus* NRRLB 3711	*P. aeruginosa* ATCC 10145	*S. aureus* ATCC 6538	*C. albicans* ATCC 90028	*C. parapsilosis* ATCC 22019	*C. krusei* ATCC 6258
1	>100	>100	>100	>100	>100	100
2	>100	100	>100	>100	>100	100
3	>100	>100	>100	>100	>100	>100
4	50	>100	>100	50	100	50
5	25	>100	>100	25	50	25
6	>100	>100	>100	>100	12.5	25
7	>100	>100	>100	>100	>100	>100
S1	-	-	-	0.031	0.062	0.125
S2	0.002	0.062	0.001	-	-	-

S1: Amphotericin B, S2: Chloramphenicol.

Experimental

General experimental procedures: Optical rotation ($[\alpha]_D^{26}$) was measured on a Perkin-Elmer 341 polarimeter. UV spectra and IR spectra were recorded on a HP Agilent 8453 spectrophotometer and a Perkin-Elmer 2000 FT-IR spectrometer, respectively. NMR experiments were performed on a Bruker Avance DRX 500 instrument. COSY, HSQC and HMBC experiments were run under standard conditions at 300 K, dissolving each sample in 550 µL of 99.8% D CD_3OD (VWR) (^{1}H, δ= 3.34 ppm; ^{13}C, δ= 49.0 ppm). A Q Exactive orbitrap from Thermo Scientific with a HESI ion source was used for HRMS analysis. TLC analyses were carried out on silica gel 60 F_{254} precoated plates (Merck, Darmstadt), and the compounds were stained with 1% vanillin/H_2SO_4 and heating at 105°C. for 1-2 min. For medium-pressure liquid chromatographic (MPLC) separations, Sepacore® Flash Systems X10 / X50 (Büchi) was used with Redi sep columns packed with LiChroprep C_{18} (13, 43 and 130 g, Teledyne Isco) and SiO_2 (40 g, Teledyne Isco). Open column chromatography (CC) was performed using Silica gel 60 (0.063-0.200 mm; Merck, Darmstadt), polyamide and Sephadex LH-20 (Sigma-Aldrich, St. Louis, MO, USA).

Plant material: The leaves of *Digitalis viridiflora* Lindley were collected from Demirköy, Kırklareli, Turkey, in July 2012 and authenticated by Dr. H. Kırmızıbekmez. A voucher specimen (YEF 12012) has been deposited at the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Yeditepe University, İstanbul, Turkey.

Extraction and isolation: The air-dried and powdered leaves of *D. viridiflora* (185 g) were extracted with MeOH (2 L x 2) at 45°C for 4 h. The solvent was evaporated *in vacuo* to afford the crude MeOH extract (45.1 g, yield 24.3%), which was suspended in H_2O (100 mL) and partitioned with $CHCl_3$ (100 mL x 3). The H_2O sub-extract (35 g) was subjected to a polyamide column (120 g) eluting with a gradient solvent system of H_2O/MeOH (100:0 to 0:100) to give 7 main fractions, A-G [7]. Fr. B (12.1 g, eluted with 20% MeOH) was applied to silica gel (150 g) CC eluting with a CH_2Cl_2/MeOH/H_2O gradient (90:10:1 to 50:40:10) to obtain 4 sub-fractions, $B_{1\text{-}4}$. Purification of sub-fraction B_1 (340 mg) by medium pressure liquid chromatography (SiO_2, 40 g) eluting with a stepwise CH_2Cl_2/MeOH gradient (100:0 to 70:30) gave compounds **6** (50 mg) and **5** (7 mg). Repeated chromatography of sub-fraction B_4 (610 mg) by C_{18}-medium pressure liquid chromatography (C_{18}-MPLC, 43 g, using a H_2O/MeOH gradient, 90:10 to 30:70) and Sephadex LH-20 CC (10 g, MeOH), respectively, gave **7** (2 mg). Fraction D (656 mg, eluted with 40% MeOH) was submitted to C_{18}-MPLC (130 g) eluting with a H_2O/MeOH gradient (85:15 to 35:65) to yield a mixture of β-glucopyranosyl-(1→6)-4-*O*-caffeoyl-α/β-glucopyranose and 3,4-dihydroxyphenylethanol (11 mg). Fraction G (380 mg, eluted with 100% MeOH) was applied to Sephadex LH-20 CC (60 g) eluting with MeOH to give sub-fraction G_1 as well as **3** (24 mg). Purification of sub-fraction G_1 (65 mg) by C_{18}-MPLC (13 g) eluting with H_2O/MeOH mixtures (85:15 to 0:100) yielded **2** (4 mg), **4** (4 mg) and **1** (15 mg).

Digiviridifloroside (1)

$[\alpha]_D^{26}$: -59 (*c* 0.1, MeOH).
IR (KBr): 3383, 1698, 1630, 1604, 1515, 1462 cm^{-1}.
UV/Vis λ_{max} (MeOH) nm: 219, 288 (sh), 328.
HR-MS-ESI: *m/z* [M + Na^+] calcd. for $C_{39}H_{44}NaO_{19}$: 839.2375; found: 839.2390.
^1H NMR (500 MHz, CD_3OD) and ^{13}C NMR (125 MHz, CD_3OD): Table 1.

Antimicrobial activity assay: A micro-dilution broth susceptibility assay was used, as previously described [14,15]. All microorganisms were stored at -85°C in 15% glycerol prior to the experiments. The bacteria were refreshed on Mueller Hinton agar (MHA, Mast Diagnostics, U.K.), whereas the *Candida* strains were refreshed on Potato Dextrose Agar (PDA, Merck) plates at 37°C. Thereafter, the bacterial suspensions were grown overnight in Mueller-Hinton broth (MHB, Merck, Germany) and were standardized to 1 x 10^8 CFU/mL versus McFarland No: 0.5 in Mueller-Hinton broth (MHB, Merck, Germany), turbidimetrically. Also, *Candida* strains were inoculated, and standardized in the same way, however in sterile saline (% 0.85) to 5 x 10^3 CFU/ per well in RPMI medium (Sigma-Aldrich). Stock solutions of the test samples were prepared in dimethylsulfoxide (DMSO). Dilution series were prepared from 0.6-100 µg/mL accordingly in Mueller Hinton Broth (MHB, Merck) for bacteria and RPMI medium for *Candida* strains in 96-well microtiter plates. Each bacterial (10 µL) and fungal suspension (100 µL) was then added to each well. The last row containing medium with microorganism was used as negative control and medium served as a positive growth control. After incubation at 37°C for 24 h, for staining of viable microorganisms, 0.01% resazurin (20 µL) was added to all of the plates. The first blue well was determined as the minimal inhibitory concentration (MIC, µg/mL). Amphotericin B and chloramphenicol (Sigma, Germany) were used as standard antimicrobial agents at a concentration range of 0.125-64 µg/mL. All experiments were repeated in triplicate and average MICs are given in Table 2.

Supplementary data: HR-MS, ^1H and ^{13}C NMR, COSY, HSQC, HMBC spectra of the new compound **1**.

Acknowledgments - The authors thank Attila Csorba (Department of Pharmacognosy, University of Szeged) for the HRMS measurements.

References

[1] Davis PH. (**1978**) *Digitalis* L. In *Flora of Turkey and the East Aegean Islands*, Vol. ***6***, Davis PH. (Ed.), University Press, Edinburgh, 680-697.
[2] Çalış İ, Taşdemir D, Sticher O, Nishibe S. (**1999**) Phenylethanoid glycosides from *Digitalis ferruginea* subsp. *ferruginea* (= *D. aurea*). *Chemical and Pharmaceutical Bulletin*, ***47***, 1305-1307.
[3] Kırmızıbekmez H, Taşdemir D, Ersöz T, Ireland CM, Çalış İ. (**2002**) A new pregnane glycoside and a furostanol glycoside from *Digitalis cariensis*. *Pharmazie*, ***57***, 716–720.
[4] Kırmızıbekmez H, Celep E, Masullo M, Bassarello C, Yeşilada E, Piacente S. (**2009**) Phenylethyl glycosides from *Digitalis lanata*. *Helvetica Chimica Acta*, ***92***, 1845–1852.
[5] Kırmızıbekmez H, Masullo M, Festa M, Capasso A, Piacente S. (**2014**) Steroidal glycosides with antiproliferative activities from *Digitalis trojana*. *Phytotherapy Research*, ***28***, 534-538.
[6] Imre S. (**1972**) Flavone and anthraquinone pigments of *Digitalis* species. III. Anthraquinone and flavone compounds from the leaves of *Digitalis viridifloria*. *Planta Medica*, ***21***, 274-278.
[7] Kırmızıbekmez H. (**2015**) Phenylethanoid glycosides from *Digitalis viridiflora*. *Records of Natural Products*, ***9***, 369-373.
[8] Nicoletti M, Galeffi C, Messana I, Garbarino JA, Gambaro V, Nyandat E, Marini-Bettola GB. (**1986**) New phenylpropanoid glucosides from *Calceolaria hypericina*, *Gazzetta Chimica Italiana*, ***116***, 431-433.
[9] Peng ZF, Strack D, Baumert A, Subramaniam R, Goh N., Chia TF, Tan SN, Chia, LS. (**2003**) Antioxidant flavonoids from leaves of *Polygonum hydropiper* L. *Phytochemistry*, ***62***, 219-228.
[10] Hajdú Z, Martins A, Orbán-Gyapai O, Forgo P, Jedlinszki N, Máthé I, Hohmann J. (**2014**) Xanthine oxidase-inhibitory activity and antioxidant properties of the methanol extract and flavonoids of *Artemisia asiatica*, *Records of Natural Products*, ***8***, 299-302.
[11] Tian J, Zhao QS, Zhang HJ, Lin ZW, Sun HD. (**1997**) New cleroindicins from *Clerodendrum indicum*. *Journal of Natural Products*, ***60***, 766–769.
[12] Ciuffreda P, Casati S, Manzocchi A. (**2007**) Complete ^1H and ^{13}C NMR spectral assignment of α- and β-adenosine, 2′-deoxyadenosine and their acetate derivatives. *Magnetic Resonance in Chemistry*, ***45***, 781-784.
[13] Skhirtladze A, Kemertelidze E, Nebieridze V, Ganzera M. **(2016**) Phenylethanoid glycosides from the roots of *Digitalis ciliata* TRAUTV. *Helvetica Chimica Acta*, ***99***, 241-245.
[14] Kırmızıbekmez H, Demirci B, Yeşilada E, Başer KHC, Demirci, F. (**2009**) Chemical composition and antimicrobial activity of the essential oils of *Lavandula stoechas* L. ssp. *stoechas* growing wild in Turkey, *Natural Product Communications*, ***4***,1001-1006.
[15] Iscan G, Kırımer N, Kürkcüoğlu M, Baser KHC, Demirci F. (**2002**) Antimicrobial screening of *Mentha piperita* essential oils. *Journal of Agricultural and Food Chemistry*, ***50***, 3943-3946.

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(±)-Perforison A, A Pair of New Chromone Enantiomers from *Harrisonia perforata*

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Received: October 1st, 2016; Accepted: November 4th, 2016

Dedicated to Prof. Lisheng Ding on the occasion of his 60th birthday.

(+)-Perforison A and (−)-perforison A, a new pair of chromone enantiomers, along with four known compounds, were isolated from the leaves and stems of *Harrisonia perforata.* Their structures and absolute configurations were determined on the basis of extensive analysis of spectroscopic data and electronic circular dichroism (ECD) calculations. The cytotoxic activities *in vitro* of these compounds were evaluated, but none showed significant activity.

Keywords: *Harrisonia perforata*, Simaroubaceae, Chromone, Electronic circular dichroism, Perforison A.

The genus *Harrisonia* (Simaroubaceae) comprises four species and is mainly distributed in south-east Asia, Africa and Oceania [1]. *H. perforata* (Blanko) Merr. is the only species growing in China [1], and has been used as a folk medicine against malaria and boils [2]. Several chromones [3-6], limonoids [4, 7-11], quassinoids [12, 13], and polyketides [14] have been isolated from *H. perforata*. As part of our effort to search for biologically active constituents of this species [7, 12, 15], a new pair of chromone enantiomers, perforisone A, along with four known compounds, peucenin-7-methyl ether (**2**) [16], umtatin (**3**) [17], greveichromenol (**4**) [17], and saikochromone A (**5**) [18], were isolated from the leaves and stems of the title plant. Perforisone A was resolved to (+)-perforisone A (**1a**) and (−)-perforisone A (**1b**) by HPLC using a chiral column, and their absolute configurations were determined by computational methods via calculation of their electronic circular dichroism (ECD) spectra. Herein, we describe the structural elucidation and biological evaluation of the isolated compounds.

Perforisone A (**1**) was obtained as a colorless solid and had the molecular formula $C_{16}H_{18}O_6$ by the positive HRESIMS (*m/z* 329.0996 $[M + Na]^+$, calcd: 329.0996), requiring 8 degrees of unsaturation. Its IR spectrum showed absorption bands for hydroxyl (3435 cm^{-1}), carbonyl (1653 cm^{-1}), and aromatic (1582, 1559, 1490 and 1457 cm^{-1}) groups. In the ^1H NMR spectrum, two aromatic H-atoms (δ_H 6.32, s, 6.58, s), one spin system (δ_H 2.95, dd, J = 13.1, 7.6 Hz, δ_H 2.86, dd, J = 13.1, 6.8 Hz, δ_H 4.34, t, J = 7.1 Hz), one singlet methyl (δ_H 1.80, s), together with one methoxyl (δ_H 3.89, s) were observed (Table 1). The ^{13}C NMR (DEPT) spectrum exhibited 16 carbon resonances, assigned to one aryl ketocarbonyl (δc 184.2), seven sp^2 quaternary carbons (δc 171.6, 165.5, 160.0, 158.2, 148.7, 111.1, 106.1), one sp^2 methylene (δ_C 111.3), two sp^2 methines (δ_C 106.9, 91.0), two sp^3 methylenes (δ_C 61.4, 29.4), one methoxy (δ_C 56.6), and one methyl (δ_C 17.3). These groups accounted for six out of eight indices of unsaturation.

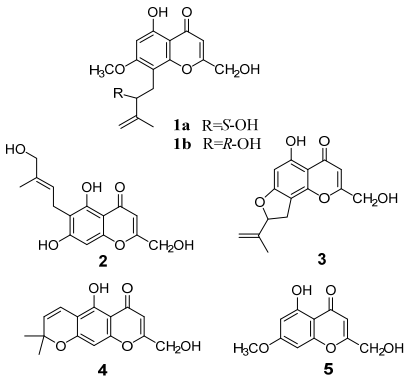


Figure 1: Structures of compounds **1-5**.

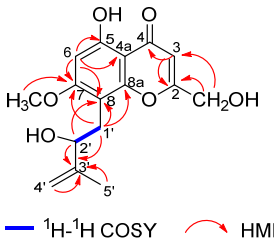


Figure 2: Selected ^{1}H-^{1}H COSY (bold) and key HMBC correlations (Arrow, H→C) of **1**.

From the ^{1}H-^{1}H COSY correlation of H_2-1′ and H-2′, combined with the HMBC correlations of Me-5′/C-3′, H-2′/C-3′, H_2-1′/C-3′, and H-2′/C-3′, the side chain was assigned as a 2-hydroxy-3-methyl-3-butenyl group (Figure 2). The attachment of this unit at C-8 was confirmed by HMBC correlations from H_2-1′ to C-7, C-8, and C-8a. The NMR data of **1** were similar to those of perforamone C [6], except for the presence of a hydroxymethyl group instead of a methyl group in the latter. The position of the hydroxymethyl group

Table 1: ^{1}H and ^{13}C NMR data (600 and 125 MHz, resp.) of compound **1** in MeOD.

No	^{13}C	^{1}H
2	171.6	
3	106.9	6.32 (1H, *s*)
4	184.2	
4a	106.1	
5	158.2	
6	91.0	6.58 (1H, *s*)
7	165.5	
8	111.1	
8a	160.0	
1′	29.4	2.95 (1H, dd, *J* = 13.1, 7.6) 2.86 (1H, dd, *J* = 13.1, 6.8)
2′	76.0	4.34 (1H, t, *J* = 7.1)
3′	148.7	
4′	111.3	4.61 (1H, br s) 4.60 (1H, br s)
5′	17.3	1.80 (3H, s)
2-CH_2OH	61.4	4.48 (2H, s)
7-OCH_3	56.6	3.89 (3H, s)

at C-2 was supported by HMBC correlations of 2-CH_2OH (δ_H 4.48) to C-2 (δ_C 171.6) and C-3 (δ_C 106.9). Thus, the structure of **1** was established as 5-hydroxy-7-methoxy-2-hydroxymethyl-8-(2-hydroxy-3-methyl-3-butenyl)chromone. It is worth noting that the CD spectrum of **1** is almost a straight line, implying a racemic nature of **1**, which was in accordance with its optical activity (**1**: $[\alpha]^{25}_D$ = 0). Chiral resolution of **1** was achieved on a CHIRALPAK IC column; the enantiomers **1a** and **1b** were well separated.

Compounds **1a** and **1b** had opposite optical rotations (**1a**: $[\alpha]^{25}_D$= + 21.5; **1b**: $[\alpha]^{25}_D$= − 24.4) and Cotton effects in their CD spectra. The final assignment of absolute configurations of **1a** and **1b** was achieved by comparison of the calculated electronic circular dichroisms (ECDs) with experimental data [19]. In detail, their ECDs calculated at the b3lyp/6-311++g(d,p)//b3lyp/6-311++g(d,p) level showed good agreement with the experimental spectra (Figure 3).

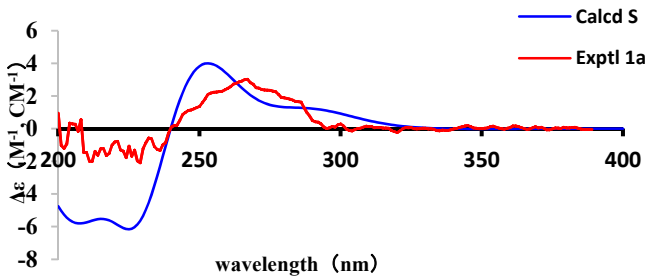
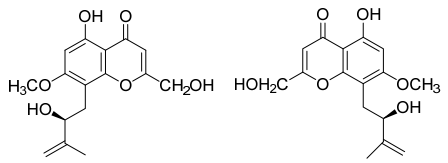


Figure 3: Calculated and experimental ECD spectra of **1a** (blue, at the B3LYP-SCRF (PCM)/6-311+G(2d,p)//B3LYP/6-311+G(2d,p) level in MeOH; red, experimentally observed in MeOH).



1a(2'S) **1b(2'R)**

Figure 4: Absolute configuration for **1a** (2'S) and **1b** (2'R).

From the above evidence, the absolute stereochemistry for **1a** (2'S) and **1b** (2'R) were unambiguously determined as shown (Figure 4).

All compounds were evaluated for their *in vitro* growth inhibitory effects against five human cancer cell lines, namely, HL-60 (human promyelocytic leukemia cell line), SMMC-7721 (human hepatocellular carcinoma cell line), A-549 (human lung cancer cell line), MCF-7 (human breast cancer cell line), and SW480 (colorectal cancer cell line) using MTS method [20]. However, none of them exhibited significant bioactivity (IC_{50} > 40 μM).

Experimental

General experimental procedures: Optical rotations were measured with a Jasco P-1020 polarimeter. UV spectra were obtained using a Shimadzu UV-2401A spectrophotometer. A Tenor 27 spectrophotometer was used for IR spectra as KBr pellets. 1D and 2D NMR spectra were recorded on a Bruker AV-500 spectrometer with TMS as internal standard. HRESIMS were obtained on a triple quadrupole mass spectrometer. Semi-preparative HPLC was performed on an Agilent 1100 liquid chromatograph with either a Waters X-Bridge Prep Shield RP18 (10 × 150 mm) column or a CHIRALPAK IC (10 × 250 mm) column. Column chromatography (CC) was performed using silica gel (100-200 mesh and 300-400 mesh, Qingdao Marine Chemical, Inc., Qingdao, P. R. China) and Sephadex LH-20 (40-70 μm, Amersham Pharmacia Biotech AB, Uppsala, Sweden). MCI gel CHP 20P (75-150 μm, Mitsubishi Chemical Industries, Tokyo, Japan)

***Plant material*:** The dried leaves and stems of *H. perforata,* collected in Hainan Province, P. R. China, in October 2014, were identified by Prof. Yanhui Fu (Hainan Normal University). A voucher specimen (HXJ20141208) was deposited at the State Key Laboratory of Phytochemistry and Plant Resource in West China, Kunming Institute of Botany, Chinese Academy of Science (CAS).

Isolation and purification: The air-dried powdered leaves and stems (19.0 kg) were extracted 3 times (4, 3, and 3 h) with 95% ethanol (20 L × 3) at room temperature and concentrated *in vacuo* at 60ºC to give a residue (2.5 kg). This was dispersed in water (4.0 L) and extracted with light petroleum and ethyl acetate in turn. The light petroleum and ethyl acetate portions (1 kg) were combined and then passed over a silica gel column, eluting with a gradient of light petroleum-acetone (from 1:0 to 0:1), to yield 5 major fractions (1-5). Subsequently, Fr.3 (200 g) was separated over an MCI column (MeOH-H_2O from 3:7 to 10:0) to obtain 4 sub-fractions (3A-3D). Fr.3B (20 g) was then chromatographed on a silica gel column eluted with chloroform-acetone (100:1 to 5:1), to afford 5 sub-fractions (3B1-3B5). Fr3B2 (2.2 g) was purified by Sephadex LH-20 (methanol) to obtain **4** (55 mg) and a major fraction (Fr.3B21). Fr.3B21 (200 mg) was separated by semi-preparative HPLC (CH_3CN-H_2O, 4:6) to give **2** (3 mg) and **3** (5 mg). Fr3B2 (2.2 g) was purified by Sephadex LH-20 (methanol) to obtain Fr3B22 and Fr3B24. Fr3B22 (500 mg) was then chromatographed on a silica gel column eluted with chloroform-acetone (25:1) to obtain **1** (6 mg). Compound **1** was finally subjected to chiral HPLC to afford **1a** ((+)-perforisone A, 1.1 mg) and **1b** ((−)-perforisone A, 2.3 mg) (*n*-hexane/isopropanol, 85:15). Fr3B24 (200 mg) was separated by semi-preparative HPLC (CH_3CN-H_2O, 5:5) to give **5** (10 mg). The purities of compounds **1-5** were 95%, as determined by TLC and HPLC.

Calculation methodology: Calculations were performed using the Gaussian 03 program package. Geometries were fully optimized with the density functional theory methods of B3LYP at the 6-31+G* level. Harmonic vibrational frequency of the conformation was then calculated at the same level to confirm their stability. Lastly, the "self-consistent reaction field" method (SCRF) was employed to perform the ECD calculation of compounds **1a** and **1b** in methanol at the B3LYP-SCRF/6-311++G (d, p) level.

(±)-Perforison A
Colourless solid.
{$[\alpha]^{25}_D$: + 21.5 (*c* 0.18, MeOH); **1a**}; {$[\alpha]^{25}_D$: −24.4 (*c* 0.16, MeOH); **1b**}
UV (MeOH) λ_{max} (log ε): 312 (3.78), 292 (3.91), 258 (4.16), 232 (4.24) nm
IR (KBr) ν_{max}: 3435, 1669, 1653, 1624, 1582, 1559, 1540, 1490, 1457, 1411 cm^{-1}.
^1H and ^{13}C NMR: Table 1.
ESI-MS (*m/z*): 329 $[M + Na]^+$, 635 $[2M + Na]^+$;
HR-ESI-MS: *m/z*: 329.0996, $[M + Na]^+$ (calcd for $C_{16}H_{18}O_6Na$, 329.0996).

Supplementary data: ^1H NMR, ^{13}C NMR, 2D NMR spectra data of new compounds can be found in the online version.

Acknowledgments - This research was supported financially by grants from the National Science Foundation of China (21432010, 21372228, and 81573323), Technological leading talent project of Yunnan (2015HA020), Central Asian Drug Discovery and Development Center of Chinese Academy of Sciences (CAM201402, CAM201302), the Science Foundation of Yunnan (2014A050), and the Xibuzhiguang Project (grant to Ying-Tong Di).

References

[1] Hannan Institute of Botany. (**1974**) *Hannan Flora (Hainan Zhiwu Zhi)*. Science, Beijing, ***3***, 54-55.
[2] White TC. (**1973**) *Tree Flora of Malaya*. Longman, London. Vol.***II***, 349
[3] Tanaka T, Koike K, Mitsunaga K, Narita K, Takano S, Kamioka A, Sase E, Ouyang Y, Ohmoto T. (**1995**) Chromones from *Harrisonia perforata*. *Phytochemistry*, ***40***, 1787-1790.
[4] Choodej S, Sommit D, Pudhom K. (**2013**) Rearranged limonoids and chromones from *Harrisonia perforata* and their anti-inflammatory activity. *Bioorganic & Medicinal Chemistry Letters*, ***23***, 3896-3900.
[5] Liu G, Zheng RR, Liu ZW, Wang WJ, Li GQ, Fan CL, Zhang XQ, Ye WC, Che CT. (**2014**) Enantiomeric chromones from *Harrisonia perforata*. *Phytochemistry Letters*, ***10***, 295-299.
[6] Tuntiwachwuttikul P, Phansa P, Pootaengon Y, Taylor, WC. (**2006**) Chromones from the branches of *Harrisonia perforata. Chemical & Pharmaceutical Bulletin,* ***54***, 44-47.
[7] Yan XH, Di YT, Fang X, Yang SY, He HP, Li SL, Lu Y, Hao XJ. (**2011**) Chemical constituents from fruits of *Harrisonia perforata. Phytochemistry*, ***72***, 508-513.
[8] Thu HDT, Tri MV, Ngoc NB, Sevenet T, Pais M, Martin MT. (**2006**) Foritin, a new limonoid from *Harrisonia perforata. Natural Product Letters*, ***14***, 191-195.
[9] Khuong-Huu Q, Chiaroni A, Riche C, Nguyen-Ngoc H, Nguyen-Viet K, Khuong-Huu F. (**2001**) New rearranged limonoids from *Harrisonia perforata.* III. *Journal of Natural Products*, ***64***, 634-637.
[10] Khuong-Huu Q, Chiaroni A, Riche C, Nguyen-Ngoc H, Nguyen-Viet K, Khuong-Huu F. (**2000**) New rearranged limonoids from *Harrisonia perforata. Journal of Natural Products*, ***63***, 1015-1018.
[11] Sung TV, Phung NM, Kamperdick C, Adam G. (**1995**) Perforatinolone, a limonoid from *Harrisonia perforata. Phytochemistry*, ***38***, 213-215.
[12] Fang X, Di YT, Zhang Y, Xu ZP, Lu Y, Chen QQ, Zheng QT, Hao XJ. (**2015**) Unprecedented quassinoids with promising biological activity from *Harrisonia perforata. Angewandte Chemie International Edition*, ***54***, 5592-5595.
[13] Kamiuchi K, Mitsunaga K, Koike K, Ouyang Y, Ohmoto T, Nikaido. (**1996**) Quassinoids and limonoids from *Harrisonia perforata. Heterocycles*, ***43***, 653-664.
[14] Yin S, Chen X, Su ZS, Yang SP, Fan CQ, Ding J, Yue JM. (**2009**) Harrisotones A-E, five novel prenylated polyketides with a rare spirocyclic skeleton from *Harrisonia perforata. Tetrahedron*, ***65***, 1147-1152.
[15] Lv C, Yan XH, Tu Q, Di YT, Yuan CM, Fang X, David YB, Xia L, Gong JX, Shen YM, Yang Z, Hao XJ. (**2016**) Isolation and asymmetric total synthesis of perforaniod A. *Angewandte Chemie International Edition*, ***55***, 7539-7543.
[16] Thadaniti S, Archakunakorn W, Tuntiwachwuttikul P, Bremner. (**1994**) Chromones from *Harrisonia perforata* (Blanco.) Merr. *Journal of the Science Society of Thailand*, ***20***, 183-187.
[17] Dean FM, Robinson ML. (**1971**) Heartwood chromones of *Cedrelopsis grevei*. *Phytochemistry*, ***10***, 3221-3227.
[18] Kobayashi M, Tawara T, Tsuchida T, Mitsuhashi H. (**1990**) Studies on the constituents of Umbelliferae plants. XVIII. Minor constituents of Bupleuri radix: occurrence of saikogenins, polyhydroxysterols, a trihydroxy C_{18} fatty acid, a lignan and a new chromone. *Chemical & Pharmaceutical Bulletin*, ***38***, 3169-3171.
[19] Frisch MJ, Trucks GW, Schlegel HB, Scuseria GE, Robb MA, Cheeseman JR, Scalmani G, Barone V, Mennucci B, Petersson GA, Nakatsuji H, Caricato M, Li X, Hratchian HP, Izmaylov AF, Bloino J, Zheng G, Sonnenberg JL, Hada M, Ehara M, Toyota K, Fukuda R, Hasegawa J, Ishida M, Nakajima T, Honda Y, Kitao O, Nakai H, Vreven T, Montgomery Jr JA, Peralta JE, Ogliaro F, Bearpark M, Heyd JJ, Brothers E, Kudin KN, Staroverov VN, Keith T, Kobayashi R, Normand J, Raghavachari K, Rendell A,Burant JC, Iyengar SS, Tomasi J, Cossi M, Rega N, Millam JM, Klene M, Knox JE, Cross JB, Bakken V, Adamo C, Jaramillo J, Gomperts R, Stratmann RE, Yazyev O, Austin AJ, Cammi R, Pomelli C, Ochterski JW, Martin RL, Morokuma K, Zakrzewski VG, Voth GA, Salvador P, Dannenberg JJ, Dapprich S, Daniels AD, Farkas O, Foresman JB, Ortiz JV, Cioslowski J, Fox DJ. (**2010**) Gaussian 09, Revision C.01, Gaussian, Inc., Wallingford CT.
[20] Wang PW, Henning SM, Herbe, D. (**2010**) Limitations of MTT and MTS-based assays for measurement of antiproliferative activity of green tea polyphenols. *PLoS ONE*, **5**, e10202.

NPC Natural Product Communications

2017
Vol. 12
No. 1
67 - 72

Antiinflammatory and Analgesic Effects in Rodent Models of Ethanol Extract of *Clausena anisata* Roots and their Chemical Constituents

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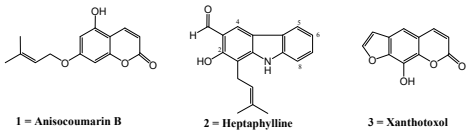
Received: July 14th, 2016; Accepted: September 10th, 2016

The *in vivo* antiinflammatory and analgesic activities of the crude ethanol extract and chemical constituents of *Clausena anisata* roots were investigated. The crude extract, which was devoid of any visible acute toxicity, displayed significant antiinflammatory effect at the dose of 1000 mg/kg (p.o.) when assessed using the carrageenan-induced oedema model. In the acetic acid-induced writhing and hot plate tests, it produced a very significant ($p < 0.001$), dose-dependent analgesic effect, with maximum analgesic activity of 72.1% at 1000 mg/kg (p.o.). Phytochemical analysis of the crude extract resulted in the isolation of four coumarins (anisocoumarin B, osthol, imperatorin and xanthotoxol) and a carbazole alkaloid, heptaphylline. Among the isolated compounds, osthol and anisocoumarin B produced the highest antiinflammatory activity at 9 mg/kg (p.o.): slightly better than the positive control, indomethacin. Except for xanthotoxol, all the isolated compounds administered at 6 mg/kg (p.o.) produced significant analgesic activity and higher than diclofenac; with heptaphylline being the most potent (48.7%). The analgesic activity of anisocoumarin B (50.4%) was the highest among the isolates tested and the standard, tramadol, in the hot plate test. The nonselective opioid receptor antagonist, naloxone, abolished the analgesic effect of the crude extract and the tested isolates (anisocoumarin B and xanthotoxol) in the hot plate test suggesting an effect *via* the central opioidergic system. These findings provide the scientific basis for the use of *C. anisata* roots in traditional medicine as antiinflammatory and analgesic agents.

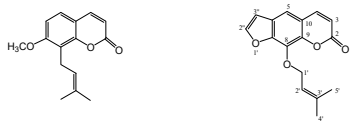
Keywords: *Clausena anisata* roots, Antiinflammatory, Analgesic, Coumarins, Carbazole alkaloid, Antinociception, Opioid antagonist.

While self-limiting acute inflammatory response is critical for maintaining homeostasis and defense against a range of pathogens, unregulated inflammation is a feature of many disease conditions from inflammatory pain and allergies to cancer, cardiovascular diseases, and autoimmune disorders. To date, a number of drugs belonging both to the small molecular weight and protein-based modulators are available to combat inflammation and the associated diseases. Due to various limitations (e.g. poor efficacy and unwanted side effects), especially for chronic diseases such as arthritis and inflammatory bowel diseases, the world-wide effort on the search for safe and effective drugs continues in many academic and pharmaceutical industry laboratories [1a,b]. In addition to suppression of inflammatory responses through action like the steroidal or non-steroidal mechanisms, drug candidates targeting inflammatory pain through the opiate mechanism of action, like that of tramadol, are highly valuable.

Clausena anisata (Wild.) Hook f. ex. Benth (Rutaceae) is an evergreen shrub or tree that grows up to 10 m high. The plant is widely distributed in higher-rainfall regions of tropical West Africa, as well as Asia and Australia [2a]. The plant is characterised by its distinctive pinnate compound leaves, and by flowers which are highly scented [2a]. In West Africa, the leaves, stems and roots are used to treat countless diseases such as oral candidiasis, fungal infections, epilepsy, convulsion, toothache, gut disturbances, malaria and high blood pressure [2b-f]. Furthermore, the roots serve as major ingredients in a decoction known as 'Asena' in Ghana, which is commonly used to treat arthritis, general bodily pains and other inflammatory conditions at the WHO collaborative Centre for Plant Medicine Research (CPMR) at Mampong-Akwapim. Antiinflammatory, antipyretic, anti-plasmodial and analgesic activities of its leaves extracts and hypoglycemic effect of the roots have been previously reported [3a,3b]. Even though various classes of secondary metabolites (e.g carbazole alkaloids, coumarins and limonoid) have been isolated from the plant [3c-3g], the antiinflammatory and analgesic activities of the roots so far have not been investigated. This work was therefore designed to investigate these pharmacological properties and isolate and characterize the active principles of *C. anisata* roots (**1**-**5**).



1 = Anisocoumarin B **2 = Heptaphylline** **3 = Xanthotoxol**



4 = Osthole **5 = Imperatorin**

The acute toxicity study of the crude ethanoic extract of *C. anisata* roots over a period of 12 days was conducted at exceedingly high doses of either 2500 or 5000 mg/kg p.o, which are 2.5 and 5-times higher than the maximum therapeutic concentrations used in the study respectively. All extract-treated animals neither showed any sign of toxicity nor died during the observation period.

The carrageenan-induced oedema in rat's paw that serves as the gold standard *in vivo* model of antiinflammatory evaluations was employed. The dose regime and protocols were based on our preliminary experiments and previous reports where we have employed the method for the identification of several novel anti-inflammatory agents from medicinal plants [4a-c]. As shown in Figure 1, the maximum carrageenan-induced oedema was attained within 3 h of injection and was sustained during the 5 h observation period. As expected, and in line with our previous report [4c], the positive control indomethacin displayed a time- and dose-dependent antiinflammatory effect within the dose range of 9 and 30 mg/kg (p.o.) (Figure 1).

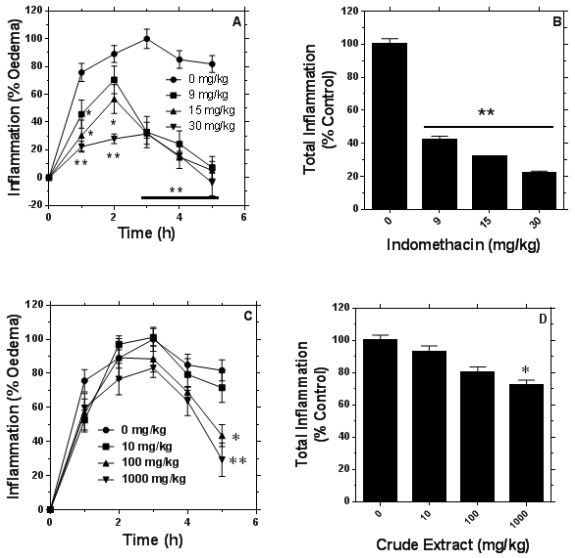


Figure 1: The effect of indomethacin and crude ethanolic extract of *C. anisata* roots on the time-course and overall carrageenan-induced oedema formation in rats paw. * and ** represent significant differences at the level of $p < 0.01$ and $p < 0.001$, respectively. All data represent means and SEM (n = 5) values.

The time-course curve and total mean oedema of the crude extract on the carrageenan-induced inflammation are shown in Figure 1c and 1d, respectively. The dose dependent effect of the crude extract was evident though a significant ($p < 0.01$) inhibition of inflammation was observed at the highest dose (1000 mg/kg p.o.) tested. At this dose, the overall antiinflammatory activity of 27.5% ($p < 0.05$) was recorded. As compared with the maximum anti-inflammatory activity of indomethacin (78.2% at 30 mg/kg p.o. ($p < 0.001$), the activity of the crude extract appears to be somehow weaker. As the crude extract is a mixture of several hundreds of potentially therapeutically useful and non-useful pharmacological agents, the isolation and identification of the active principles is well justified. On the basis of these observations, our study seems to provide the scientific evidence for the traditional medicinal uses of *C. anisata* roots for inflammatory disorders. It is also worth noting that the carrageenan-induced oedema in rat paws [5a] is an acute inflammatory model that is attributed to three distinct phases marked by sequential release of several mediators [5b]. The 0-1.5 h period after carrageenan injection is known to involve the release of histamine and serotonin and constitutes Phase 1. This is followed by Phase 2 mediated by the liberation of bradykinin from 1.5 to 2.5 h [5c]; the final phase is mediated by the release of prostaglandins and slow reaction agents from 2.5 to 6 hours [5d]. The fact that the crude extract of *C. anisata* roots significantly inhibited the oedema at 5 h, which corresponds to the 3rd phase, suggests a mechanism of action through inhibition of prostaglandin synthesis and/or actions.

Even though the analgesic activities of *C. anisata* leaves were demonstrated previously [3a], the roots have not yet been studied for such activities. The acetic acid-induced writhing response in animals is one of the commonest methods of analgesic activity evaluation for experimental agents. As expected, the standard positive control, diclofenac, caused a dose-dependent analgesic effect, as evidenced by the reduction in the righting response (Figure 2). Similarly, the crude extract displayed dose-dependent analgesic effects, with all the tested concentrations (10, 100 and 1000 mg//kg, p.o.) inducing significant activities. Hence, the analgesic activity of the crude extract appears to be more pronounced than the antiinflammatory activity as only the 1000 mg/kg dose produced a suppressive effect against the carrageenan-induced oedema (Figure 1).

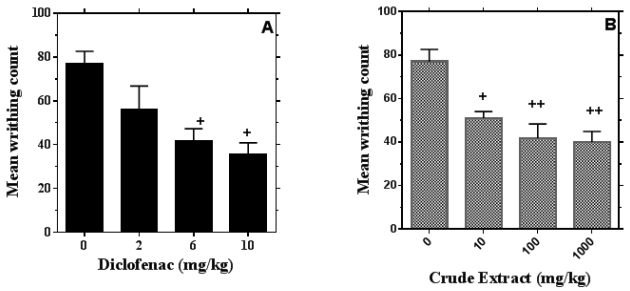


Figure 2: Analgesic effect of the crude ethanolic extract of *C. anisata* and diclofenac on acetic acid-induced pain in mice. + and ++ represent significant differences at the level of $p < 0.05$ and $p < 0.01$, respectively when compared with the vehicle treated control group.

The acetic acid-induced writhing test is a visceral pain model [6a] used in evaluating both central and peripheral analgesic activities of substances [6b]. Intraperitoneal injection of acetic acid into the abdominal cavity of rats or mice results in elevated levels of prostaglandins in the peritoneal exudates after about 30 min [6c]. The stretching and abdominal contractions obtained in the acetic acid-induced writhing test therefore correlates with sensitization of nociceptors to the production of prostaglandins [6b]. Our data on the analgesic effect of the crude extract of *C. anisata* roots are therefore consistent with those obtained in the carrageenan-induced oedema assay, where possible inhibition of prostaglandins production and/or action could serve as the main mechanism of action.

The analgesic activity of the crude extract was further assayed using the hot plate method where the standard tramadol was employed as a positive control. In untreated animals, there was a tendency for the percent mean PTI to reduce over time as the animals appear to be more sensitive to the hot plate stimuli. This sensation was however suppressed in a time-dependent manner when animals were treated by the positive control, tramadol (Figure 3a). The overall analgesic effect, as evidenced from the percent OPTI of over 20% (Figure 3b), was recorded for the two doses of tramadol. As shown in Figure 3c, the crude extract also showed a dose-dependent analgesic effect that appears to be gradually reversed over a period of 5 h. Furthermore, the percent OPTI response showed a remarkable dose-dependent analgesic effect that ranged from about 25 to just over 75% (far superior to that of the positive control) for the

concentration range tested (Figure 3d). It is worth noting that the hot plate test, as a thermally-induced nociception model, is routinely employed to evaluate the central analgesic effect of pharmacological agents [6b]. The dose-dependent antinociception of the crude extract in the hot plate test, similar to that of a known analgesic with an opioidergic mechanism of action, tramadol ($p < 0.001$), may suggest a central mechanism of action.

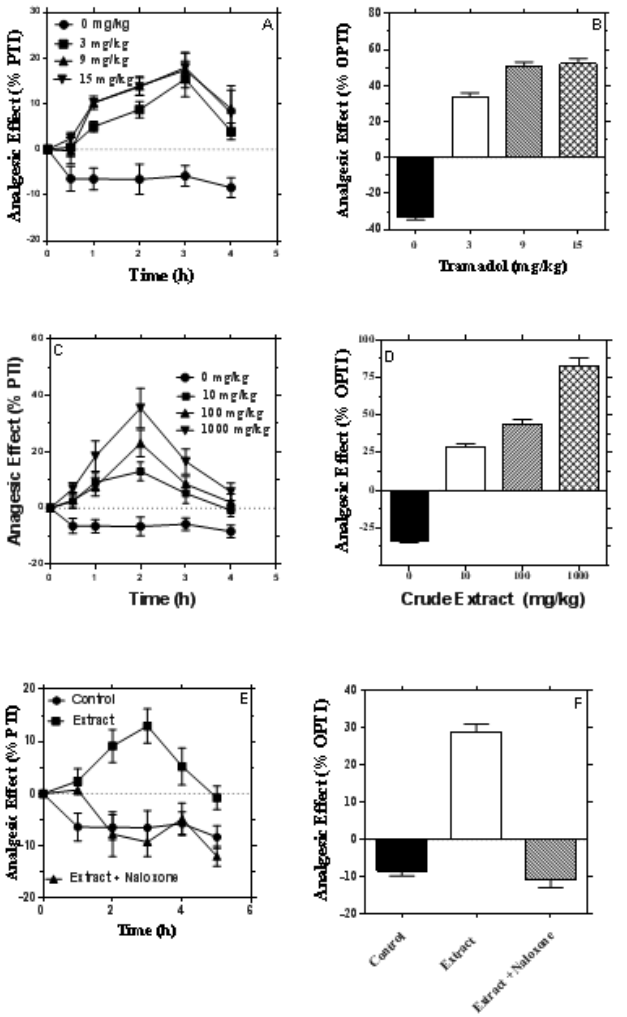


Figure 3: Analgesic effect of crude extract of Tramadol and *C. anisata* roots on the time course curves (left panel) and % overall analgesic response, % OPTI, (right panel) on thermally-induced pain in mice. Effect of naloxone (2 mg/kg, i.p.) on the crude extract (10 mg/kg, p.o.), on thermal mediated antinociception on time course curve (E) and % OPTI (F) are also shown. All data are expressed as means ± SEM and the % OPTI data for the drug treated groups, except for the naloxone group were significantly different (at least at $p < 0.05$) when compared with the vehicle-treated control group.

In order to establish the possible central mechanism of the crude extract in the observed analgesic/antinociceptive effect, the hot plate assay was further reassessed with naloxone co-administration. As shown in Figure 3 (e,f), the activity of the crude extract was completely abolished by the non-selective opioid antagonist, naloxone. This indicates that the analgesic effect of the crude extract was likely to be mediated *via* the central opioidergic system.

Having the antiinflammatory and analgesic activity of the crude extract of *C. anisata* roots established, it was worthwhile to investigate the possible active principles of the roots. Preliminary phytochemical screening of the crude extract using the standard procedure [7a] revealed the presence of coumarins, alkaloids, terpenoids and free reducing sugars. When the crude extract was suspended in aqueous medium and re-extracted with light petroleum, the presence of coumarins, alkaloids and terpenoids was still evident. Hence, the light petroleum fraction was taken for further purification through repeated column chromatography over silica gel and recrystallization to yield five compounds (**1-5**). The detection and identification of all of the isolated compounds in the various parts of *C. anisata* have been described previously [3c-g, 7b-c]. In the present study, comprehensive spectroscopic analysis including 2D NMR studies (COSY, HMQC, HMBC and NOESY) and accurate mass analyses were employed for unambiguous chemical shift assignments and structural analysis.

As with the crude extract, the isolated compounds were tested for their *in vivo* antiinflammatory activities using the same carrageenan-induced oedema model. The dose-dependent effect of these compounds in an overall total inflammatory effect analysis during the entire observation period is shown in Figure **4**. All compounds showed significant antiinflammatory activity at 9 mg/kg (p.o.) suggesting their potent antiinflammatory effects *in vivo*. Furthermore, all compounds, except for compound **2** (heptaphylline) and **5** (imperatorin) showed comparable activity with the positive control indomethacin when tested at the dose of 9 mg/kg ($p > 0.05$).

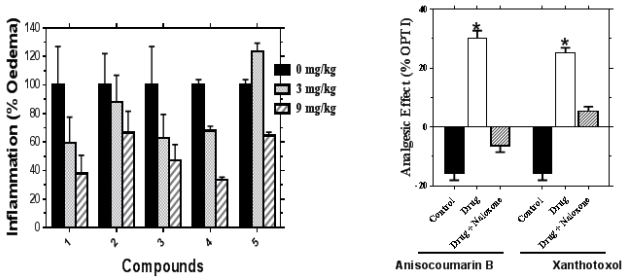


Figure 4: Antiinflammatory and analgesic activity of the isolated compounds. The overall carrageenan-induced oedema formation in rats paw (left) and thermal antinociception effect (right panel) are shown. *, indicate significant difference ($p < 0.001$) *vs* either the vehicle treated control group or the drug plus naloxone group. Significant antiinflammatory activity (at least $p < 0.05$) was observed at the dose of 9 mg/kg. All data are means ± SEM (n = 5).

Osthole (**4**) is one the best characterised anti-inflammatory natural products with known effect as an inhibitor of the carrageenan-induced hind paw edema in rats [8a]; carrageenan-induced lung inflammation in rats [8b]; hepatic injury in a rodent model of trauma-hemorrhage [8c]; inhibition of inflammatory reaction following permanent middle cerebral artery occlusion in rats [8d]; and iNOS protein expression at 10 μM [8e], among others. Imperatorin (**5**) has been shown to suppress the degranulation and eicosanoid generation in activated bone marrow-derived mast cells [8f]; inhibit the release of pro-inflammatory cytokines production, including interleukin (IL)-6 and tumor necrosis factor (TNF)-α in LPS-stimulated RAW 264.7 cells [8g-8h]; block the protein expression of iNOS and cyclooxygenase-2 (COX-2) in LPS-stimulated RAW264.7 macrophages [8i]; inhibit acute lung injury induced by lipopolysaccharide in mice [8j] and anti-inflammatory activity on TPA (12-*O*-tetradecanoylphorbol-13-acetate) mice ear model [8k]. While xanthotoxol (**3**) is known to exert neuroprotective effects *via* suppression of the inflammatory response in a rat model of focal cerebral ischemia [8l] its antiinflammatory effect is largely unknown. On the other hand, heptaphylline (**2**) has been known for some time to have an *in vitro* antiinflammatory effect such as suppression of inflammation mediators (iNOS, TNF-alpha, and COX-2) expression in the mouse macrophage RAW 264.7 cell line [8f], while anisocoumarin B (**1**) has not previously been shown to display anti-inflammatory

activity. The identification of compounds **1-5** from the roots of *C. anisata* is, therefore, in good agreement with the observed *in vivo* antiinflammatory effect of the crude extract and the proposed mechanism of action, including the prostaglandins system.

Since the yield of the isolated compounds did not permit the undertaking of a comprehensive dose range analysis, they, along with the positive control, were screened for analgesic activity at one fixed dose of 6 mg/kg (p.o.). As shown in Table 1, only compound **3** (xanthotoxol) failed to show analgesic activity at the tested dose. The observed analgesic activity of the active compounds, which was comparable in many cases with the standard diclofenac, was in good agreement with the activity profile of our data for the crude extract in the same assay. It is, therefore, reasonable to conclude that the isolated active compounds as active principles could contribute to the analgesic activity of the acclaimed *C. anisata* roots in the traditional medicine.

Table 1: Analgesic activity of the isolated compounds and diclofenac on acetic acid - induced writhing pain in mice.*

Compounds	% Righting Response Vs Control	% Analgesic Activity
1 (Anisocoumarin B)	61.4 ± 16.0	38.6
2 (Heptaphylline)	49.1 ± 12.6	50.9
3 (Xanthotoxol)	112.1 ± 3.3	No effect
4 (Osthol)	53.6 ± 6.7	46.4
5 (Imperatorin)	61.9 ± 12.7	38.1
Diclofenac	53.9 ± 7.5	46.1

*Tested at 6 mg/kg (p.o.)

The analgesic activity of osthole (**4**) was reported before [8l], including in sciatica induced by the lumber disc herniation model [9a-b], while the rest of the isolated compounds appear to be reported here for the first time. Due to the small yields of the compounds, it was not possible to repeat all of the experiments in the hot plate assay and hence data were provided in Figure 4 only for anisocoumarin B (**1**) and xanthotoxol (**3**). While both compounds appear to show significant activity, the effect of anisocoumarin B (**1**) was higher than that of the positive control, tramadol. As with the analgesic effect of the crude extract, the antinociceptive effects of these compounds were suppressed by the non-selective opioid antagonist, naloxone, suggesting a similar central effect (Figure 4).

In conclusion, the present study established that the roots of *C. anisata* possess significant antiinflammatory and analgesic effects similar to NSAIDs and/or opioids and could be safely used to manage some inflammatory and painful disorders. This study also validates the use of *C. anisata* roots in traditional medicine as an anti-inflammatory and analgesic agent. Phytochemical analysis of the crude extract resulted in the isolation of prenyl derivative compounds: four coumarins (anisocoumarin B, osthol, imperatorin and xanthotoxol) and a carbazole alkaloid, heptaphylline. In a further validation of the traditional uses of the plant, the isolated compounds also possess potent antiinflammatory and analgesic activities.

Experimental

Materials: Carrageenan, naloxone hydrochloride dehydrate, and acetic acid were products of Sigma Chemical Co. (St. Louis USA). Indomethacin, diclofenac sodium, tramadol hydrochloride and Tween 80 were purchased from Cayman Chemical Company, Bliss GVS (India), Bristol Laboratories Ltd and VWR International, PROLABO (CE) respectively. Sodium chloride was from Timstar Laboratory Suppliers Ltd., Cheshire, England. All organic solvents and other reagents used for extraction, isolation and purification of the compounds were either analytical or HPLC grade.

Plant material: The roots of *C. anisata* were harvested from the arboretum of CPMR at Ayikumah in the Eastern Region of Ghana in March, 2012. After authentication by Mr H. R. Blaggoge, a voucher specimen (CPM0312) was deposited at the herbarium of CPMR.

Extraction and isolation: The crude extract (140 g) was suspended in 80% ethanol-water (2.4 L) and extracted 4-times with light petroleum (2.4 L). The combined fractions were dried in a rotary evaporator at 40ºC to obtain 16.8 g of a bark brown syrup. A portion of this fraction (13.0 g) was subjected to column chromatography (CC) over normal phase silica gel (400 g) and elution made with light petroleum, light petroleum containing increasing amount of ethyl acetate, and finally a gradient of EtOAc containing ethanol. Aliquots of fractions from the column were collected and grouped by similarity in their TLC profiles to give 7 main fractions (P1-P7). Fraction P1 eluted with light petroleum /ethyl acetate (95:5) gave colorless crystals, which were filtered, washed (light petroleum /chloroform 6:1) and recrystallized from light petroleum /ethyl acetate (70:30) to give compound **1** (40.5 mg). Fraction P2 (light petroleum /ethyl acetate 80:20) gave a yellow ppt, which appeared to be a mixture when assessed by TLC (solvent, light petroleum/chloroform (2:3); anisaldehyde spray reagent). Further CC over 40 g of silica and elution with light petroleum /chloroform gave 3 sub-fractions. The sub-fraction eluted with light petroleum /chloroform (90:10) gave compound **2** (10 mg) as bright yellow crystals. Fraction P4 (solvent, eluent, light petroleum /ethyl acetate 60:40) was subjected to further fractionation with a stepwise gradient elution with light petroleum /chloroform/ ethyl acetate. A total of 87 fractions of 30 mL aliquots was obtained and grouped into 4 sub-fractions. Compound **3** (35.0 mg) was obtained from the fraction eluted with light petroleum /chloroform/ethyl acetate (40:40:20) as brown solid particles. Fraction P5, eluted with light petroleum /ethyl acetate (60:40) gave a colorless ppt, which was subjected to repeated recrystallization from light petroleum /chloroform (4:1) to obtain compound **5** (20.5 mg). Fraction P6, eluted with light petroleum /ethyl acetate (30:70) yielded white crystals, which were recrystallized from light petroleum /ethyl acetate (5:1) to obtain compound **6** (30.0 mg).

Animals: All animals used in the study were bred at the Animal House Unit of CPMR, Ghana. The animals were fed on palette feed purchased from Agricare Limited located in Kumasi, Ghana; and were allowed free access to sterile water and feed *ad libitum.* Animals were housed in aluminum cages under standard temperature and pressure. Animals were cared for and handled according to the guidelines and procedures of the Foundation for Biomedical Research on the use of animals in research (F.B.R., 1987).

Acute toxicity studies: The acute toxicity of the crude extract was assessed in groups of 6 male Sprague-Dawley rats, Swiss albino mice and C57/BL6 mice. For each animal group, 2 doses of the crude extract (2500 and 5000 mg/kg p.o.) and a vehicle control (2% Tween 80 aqueous solutions) were administered at 10 mL/kg body weight per animal. They were observed for signs of toxicity such as pilo-erection, motor impairment, sedation, salivation, hyperexcitability and death within 24 h and for an extra 16 days.

Antiinflammatory activity assay: Our carrageenan-induced paw oedema model of antiinflammatory study [5a] has been described previously [4a-c]. Briefly, Sprague–Dawley rats of either sex were divided into groups and received orally either the vehicle (2% Tween 80 in water) or various doses of test agents (extracts, isolated

compounds or the reference drug, indomethacin). After 1 h of drug treatment, inflammation was induced by injection of 0.1 mL of 1%, w/v, carrageenan in 0.9% normal saline into the sub plantar area of the right hind paw of rats. Paw volumes were measured by volume displacement using a Plethysmometer (Ugo Basile 7140) before and at hourly intervals from 1-5 h after carrageenan injection. The mean hourly oedema for each group and the total mean oedema for the 5 h observation period were calculated as described previously [4b].

Analgesic activity assay: Acetic acid-induced writhing assay: The analgesic effect of the test agents was assessed using the acetic acid-induced writhing model as described previously [10a]. Swiss albino mice were divided into 7 groups (n = 5) and treated (p.o.) with various doses of the crude extract, isolated compounds and the standard, diclofenac sodium. Animals receiving 2% Tween 80 in water served as the untreated vehicle control group. Following drug or vehicle treatment (45 min), each mouse was injected with aqueous acetic acid (1%, v/v) at 1 mL/100 g (i.p) and isolated into separate plastic cages. The number of writhing movements and stomach contractions was counted for 20 min. The inhibition of writhing movements in the treated group(s) compared with the control group was taken as the percentage analgesic effect (% AE), which was calculated using the formula:

$$\% \text{ Analgesic effect} = (MRc - MRt)/MRC \times 100$$

Where: MRc = mean writhing count of the control; MRt = mean writhing count of extract or other drug treated group.

Analgesic activity assay: Hot plate assay: The mouse hot plate analgesic activity evaluations were performed as described previously [10b]. C57/BL6 mice of either sex were divided into 7 groups, each containing 6 animals. The mice were separately placed on an electric hot plate (Ugo Basile hot/cold plate 35 100) maintained at 55 ± 0.5°C and the time taken to lick, lift, shake or stamp any of the hind limbs or jump constitute latency time and was recorded. Baseline latencies (T_o) were obtained as the mean of 2 determinations prior to any treatment. Only mice with baseline latency of 3.5–9 s were used. Test samples of either the crude extract, purified compounds or standard reference, tramadol (3, 9 and 15 mg/kg, Group 4-6) or vehicle control (2% Tween 80 in water control) were then administered orally. Latency of each mouse was measured at 1 h (T_t) intervals for 5 h after each treatment. The analgesic activity was expressed as percentage of pain threshold inhibition (%PTI) [6b] calculated as:

$$\%PTI = (Tt - T0)/T0 \times 100$$

Where Tt and T0 refer to treatment time and time zero respectively.

Overall analgesic response (% OPTI) for each group was obtained as the sum of mean % PTI of all animals per group over the experimental period.

Mechanism of antinociceptive action: The effect of the nonselective opioid antagonist, naloxone, on the extract or purified compounds- induced analgesia was evaluated in the hot plate assay using 3 groups (n=6) of C57BL/6 mice. Drug administration was made as follows: CRE (at 10 mg/kg p.o.) was administered and 15 min later naloxone 2 mg/kg (i.p) (in distilled water) at 10 mL/100 g was given (Group 1). The procedure was repeated for CRE (10 mg/kg p.o.) alone (Group 2) and 2% Tween 80 aqueous solution as vehicle control (Group 3). The analgesic activity was then assessed as described above in the hot plate test. The same procedure was repeated for compounds 1 and 3 at 9 mg/kg p.o. each.

Statistical analysis: All statistical analyses were performed using Graph Pad Prism Version 6 with the level of significance set at 95 % confidence interval of difference. The overall mean oedema and overall analgesic response (%OPTI) were calculated as sum of the oedema volume or latency in each group.

Spectroscopic analysis: ^{1}H NMR, ^{13}C NMR and 2D-NMR (COSY, NOESY, HMQC and HMBC) spectra were obtained on a JEOL 500 MHz instrument, as described previously [10c].

Acknowledgments – The excellent support from the EPSRC National Mass Spectrometry Facility (Singleton Park, Swansea, UK) and the technical assistance of staff at the Greenwich NMR Laboratory is greatly appreciated.

References

[1] (a) Habtemariam S. (**2013**) Targeting the production of monocytes/macrophages-derived cytokines by anti-inflammatory herbal drugs. *Phytopharmacolology*, ***4***, 131–148; (b) Habtemariam S. (**2010**) Applying new science for old medicines: targeting leukocyte-endothelial adhesions by antiiflammatory herbal drugs. *Natural Products Communications*, ***5***, 1329-1336.

[2] (a) Protobase Record Display. *Clausena anisata*. https://translate.google.co.uk/translate?hl=en&sl=fr&u=http://uses.plantnet-project.org/fr/Clausena_anisata_(PROTA)&prev=search (Accessed, 5 July 2016); (b) Uwaifo, AO. (**1984**) The mutagenicities of seven coumarin derivatives and a furan derivative (nimbolide) isolated from three medicinal plants. *Journal of Toxicology and Environmental Health*, ***13***, 521-530; (c) Hamza OJ, Van Den Bout-Van Den Beukel CJ, Matee MIEA. (**2006**) Antifungal activity of some Tanzanian plants for the treatment of fungal infections. *Journal of Ethnopharmacolology*, ***108***, 124-132; (d) Mshana NR, Abbiw DK, Addae-Mensah I, Adjanouhoun E, Ahyi MRA, Ekpere JA, Enow-Orock EG, Gbile ZO, Noamesi GK, Odei MA, Odunlami,H, Oteng-Yeboah AA, Sarpong K, Soforowa A, Tackie AN. (**2000**) *Traditional Medicine and Pharmacopoiea: Contribution to the Revision of Ethnobotanical and Floristic Studies in Ghana*, Organisation of Africa Unity/ Scientific, Technical and Reseach Commission (OAU/STRC), pp 535; (e) Moshi MJ, Kagashe GA, Mbwambo ZH. (**2005**) Plants used to treat epilepsy by Tanzanian traditional healers. *Journal of Ethnopharmacology*, ***97***, 327-363; (f) Okunade AL. (**1987**) Estragole: An acute toxic principle from the volatile oil of the leaves of *Clausena anisata*. *Journal of Natural Products*, ***50***, 990-991.

[3] (a) Okokon JA, Udoh AE, Andrew UE, Amazu LU. (**2012**) Antiinflammatory and antipyretic activities of *Clausena anisata*. *Molecular and Clinical Pharmacology*, ***3***, 47-54; (b) Ojewole JAO. (2002) Hypoglycaemic effect of *Clausena anisata* (Willd) Hook methanolic root extract in rats. *Journal of Ethnopharmacology*, ***81***, 231-237; (c) Ngadjui BT,. Ayafor JF, Sondengam BL, Connolly DJ. (**1989**) Coumarins from *Clausena anisata*. *Phytochemistry*, ***28***, 585-589; (d) Ngadjui BT, Ayafor JF, Sondengam BL, Connolly JD. (**1989**) Quinolone and carbazole alkaloids from *Clausena anisata. Phytochemistry*, ***28***, 1517-1519; (e) Ngadjui BT, Ayafor JF, Sondengam BL, Connolly DJ. (**1989**) Limonoids from *Clausena anisata. Journal of Natural Products*, ***52***, 832-836; (f) Ito C, Itoigawa M, Aizawa K, Yoshida K, Ruangrungsi N, Furukawa H. (**2009**) γ-Lactone carbazoles from *Clausena anisata. Journal of Natural Products*, ***72***, 1202–1204; (g) Ito C, Katsuno S, Itoigawa M, Ruangrungsi N, Mukainaka T, Okuda M, Kitagawa Y, Tokuda H, Nishino H, Furukawa H. (**2000**) New carbazole alkaloids from *Clausena anisata* with antitumor promoting activity. *Journal of Natural Products*, ***63***, 125-128.

[4] Armah FA, Annan K, Mensah AY, Amponsah IK, Tocher DA, Habtemariam S. (**2015**) Erythroivorensin A: a novel antiinflammatory diterpene from the root-bark of *Erythrophleum ivorense* (A Chev.). *Fitoterapia*, ***105*,** 37-42*;* (b) Mireku EA, Mensah AY, Mensah MLK, Tocher DA, Habtemariam S. (**2014**) Anti-inflammatory properties of the stem-bark of *Anopyxis kaline*ana and its major constituent, methyl angolensate. *Phytotherapy Research*, ***28*,** 1855–1860; (c) Habtemariam S. (**1998**) Cistifolin, an integrin-dependent cell adhesion blocker from the anti-rheumatic herbal drug, gravel root (rhizome of *Eupatorium purpureum*). *Planta Medica*, ***64***, 683–685.

[5] (a) Winter C, Riselay EA, Nuss GW. (**1962**) Carrageenan induced oedema in the hind paw of the rats as an assay for anti-inflammatory drugs. *Proceedings of the Society for Experimental Biology and Medicine*, ***111***, 544-547; (b) Di Rosa M. (**1972**) Biological properties of carrageenin. *Journal of Pharmacy and Pharmacology **24***, 89–102; (c) Antonio MA, Brito ARMS. (**1998**) Oral anti-inflammatory and antiulcerogenic activities of hydroalcoholic extract and partitioned fractions of *Turnera ulmifolia* (Turneraceae). *Journal of Ethnopharmacology*, ***61***, 215–228; (d) Spector WG, Willoghby DA. (**1963**) The inflammation response. *Bacteriological Reviews*, ***84***, 117-154.

[6] (a) Vyklicky L. (**1979**) Techniques for the study of pain in animals. In *Advances in Pain Research and Therapy.* Bonica JJ, Liebeskind JC, Albe-Fessard DG. (Eds). Raven Press, New York; (b) Ye Y, Guo Y, Luo YT. (**2012**) Anti-inflammatory and analgesic activities of a novel biflavonoid from shells of *Camellia oleifera. International Journal of Molecular Sciences*, ***13***, 12401-1124; (c) Derardt R, Jougney S, Delevalcee F, Falhout M. (**1980**) Release of prostaglandins E and F in analgogenic reaction and its inhibition. *European Journal of Pharmacology*, ***61***, 17-24.

[7] (a) Farnsworth NR, Dobberstein RH. (**1977**) *Phytochemical Screening Methods.* College of Pharmacy, University of Illinois, Chicago, Illinois, pp 6-85; (b) Lakshmi V, Prakash D, Raj K, Kapil RS, Popli SP. (**1984**) Monoterpenoid furanocoumarin lactones from *Clausena anisata**.*** *Phytochemistry **23***, 2629-2631; (c) Okorie DA. (**1975**) A new carbazole alkaloid and coumarins from roots of *Clausena anisata*. *Phytochemistry*, ***14***, 2720-2721

[8] (a) Liu J, Zhang W, Zhou L, Wang X, Lian Q. (**2005**) Anti-inflammatory effect and mechanism of osthole in rats. *Zhong Yao Cai*, ***28***, 1002-1006; (b) Li Z, Ji H, Song X, Hu J, Han N, Chen N. (**2014**) Osthole attenuates the development of carrageenan-induced lung inflammation in rats. *International Immunopharmacology*, ***20***, 33-36; (c) Yu HP, Liu FC, Tsai YF, Hwang TL. (**2013**) Osthole attenuates hepatic injury in a rodent model of trauma-hemorrhage. *PLoS One **8:***e65916; (d) Li F, Gong Q, Wang L, Shi J. (**2012**) Osthole attenuates focal inflammatory reaction following permanent middle cerebral artery occlusion in rats. *Biological and Pharmaceutical Bulletin*, ***35***, 1686-1690; (e) Nakamura T, Kodama N, Arai Y, Kumamoto T, Higuchi Y, Chaichantipyuth C, Ishikawa T, Ueno K, Yano S. (**2009**) Inhibitory effect of oxycoumarins isolated from the Thai medicinal plant *Clausena guillauminii* on the inflammation mediators, iNOS, TNF-alpha, and COX-2 expression in mouse macrophage RAW 264.7. *Journal of Natural Medicine*, ***63***, 21-27; (f) Jeong KT, Lee E, Park NY, Kim SG, Park HH, Lee J, Lee YJ, Lee E. (**2015**) Imperatorin suppresses degranulation and eicosanoid generation in activated bone marrow-derived mast cells. *Biomolecules and Therapeutics* (Seoul), ***23***, 421-427; (g) Guo W, Sun J, Jiang L, Duan L, Huo M, Chen N, Zhong W, Wassy L, Yang Z, Feng H. (**2012**) Imperatorin attenuates LPS-induced inflammation by suppressing NF-κB and MAPKs activation in RAW 264.7 macrophages. *Inflammation*, ***35***, 1764-1772; (h) Yang IJ, Lee DU, Shin HM. (**2015**) Anti-inflammatory and antioxidant effects of coumarins isolated from *Foeniculum vulgare* in lipopolysaccharide-stimulated macrophages and 12-*O*-tetradecanoylphorbol-13-acetate-stimulated mice. *Immunopharmacology and Immunotoxicology*, ***37***, 308-317; (i) Huang GJ, Deng JS, Liao JC, Hou WC, Wang SY, Sung PJ, Kuo YH. (**2012**) Inducible nitric oxide synthase and cyclooxygenase-2 participate in anti-inflammatory activity of imperatorin from *Glehnia littoralis*. *Journal of Agricultural and Food Chemistry*, ***60***, 1673-1681; (j) Sun J, Chi G, Soromou LW, Chen N, Guan M, Wu Q, Wang D, Li H. (**2012**) Preventive effect of Imperatorin on acute lung injury induced by lipopolysaccharide in mice. *International Immunopharmacology*, ***14***, 369-374; (k) García-Argáez AN, Ramírez-Apan TO, Parra-Delgado H, Velázquez G, Martínez-Vázquez M. (**2000**) Anti-inflammatory activity of coumarins from *Decatropis bicolor* on TPA ear mice model. *Planta Medica*, ***66***, 279-281; (l) He W, Chen W, Zhou Y, Tian Y, Liao F. (**2013**) Xanthotoxol exerts neuroprotective effects *via* suppression of the inflammatory response in a rat model of focal cerebral ischemia. *Cellular and Molecular Neurobiology*, ***33***, 715-722.

[9] (a) Chen YF, Tsai HY, Wu TS. (**1995**) Anti-inflammatory and analgesic activities from roots of *Angelica pubescens*. *Planta Medica*, ***61***, 2-8; (b) Wei M, Zhang JJ, He QL, Wang L, Ren ZH, Sun LB, Liu XG. (**2011**) Effects and mechanisms of osthole on sciatica induced by lumber disc herniation. *Zhong Yao Cai*, ***34***, 746-750.

[10] (a) Koster R, Anderson M, De Beer MJ. (**1959**) Acetic acid for analgesic screening. *Federation Proceedings*, ***18***, 412–417; (b) Eddy NB, Leimbach D. (**1953**) Synthetic analgesics. II. Dithienylbutenyl - and dithienylbutylamines. *Journal of Pharmacology and Experimewntal Therapeutics*, ***107***, 385–393; (c) Jackson N, Annan K, Mensah AY, Ekuadzi E, Mensah MLK, Habtemariam S. (**2015**) A novel triterpene from the roots of *Paullinia pinnata*: 6a-(3'-methoxy-4'-hydroxybenzoyl)-lup-20(29)-ene-3-one. *Natural Product Communications*, ***10***, 563-566.

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2017
Vol. 12
No. 1
73 - 78

Preventive Effects of Resveratrol-enriched Extract of Peanut Sprout on Bacteria- and Estradiol-induced Prostatitis in Mice

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Received: September 23rd, 2016; Accepted: November 7th, 2016

The present study investigated the effect of peanut sprout extract (PSE) as a natural resveratrol supplement on chronic bacterial prostatitis (CBP) and estradiol-induced benign prostatic hyperplasia (BPH). PSE contained a high level of resveratrol (148.51 ± 3.05 μg/g), and was tested on the mouse models of CBP (induced by *Escherichia coli* 292 infection) and BPH (induced by treatment with β-estradiol and dihydrotestosterone). PSE toxicity was assessed on the basis of changes in body weight, alanine aminotransferase activity (an indicator of hepatotoxicity), and expression of the kidney injury marker KIM-1. The effects of PSE on the histopathology of prostate tissue, the proportion of neutrophils, and immune cell profiles in the blood and spleen were examined. PSE administration did not result in any toxicity but reduced the bacterial burden and histopathological changes in the prostate. In addition, lymphocytes ($CD4^+$, $CD8^+$, and $CD19^+$) in the spleen were significantly increased after PSE administration in CBP mice, suggesting immune enhancement. PSE treatment of bone marrow–derived macrophages increased the expression of CD40, which is related to the pro-inflammatory function and host defense against pathogens. It is concluded that PSE would be a good supplement for the mitigation of prostate hyperplasia and prostatitis.

Keywords: Peanut sprout extract, Resveratrol, Chronic bacterial prostatitis, Benign prostate hyperplasia, Anti-inflammation, Immune enhancement.

Prostatitis and prostate hyperplasia are diseases that commonly occur during men's lifespan all over the world, and 35–50% of men have prostatitis at some time in their lives [1, 2]. Prostate diseases include chronic bacterial prostatitis (CBP), benign prostatic hyperplasia (BPH), and prostate cancer [2-5]. Prostatitis is frequently present in aging men, and many patients are suffering from chronic prostatitis syndrome [1, 5, 6]. Chronic prostatitis of bacterial and nonbacterial origin is a very common disease in men; nevertheless, there are no established management strategies in everyday life. After the onset of symptoms, anti-inflammatory drugs, α-adrenergic blocking agents, and 5α-reductase inhibitors are the best remedies [2, 6]. However, in recent years, phytomedicines are becoming popular all over the world for management of prostate diseases [4, 5, 7-9]. The major infection routes in chronic bacterial prostatitis are through the urinary tract, rectum, and blood [10-12]. CBP treatment with antibiotics is difficult because of biofilm formation by bacteria, especially *E. coli* spp. [10]. If the symptoms persist, chronic prostatitis can progress to bacterial cystitis, pelvic pain, and acute pyelonephritis [1, 4]. BPH commonly occurs in aged men, accordingly general preventive and therapeutic measures have to be planned for a long time [5]. Complementary alternative medicine (CAM) is becoming popular all over the world for treatment of various diseases. Among prostate diseases, the usefulness of CAM has been mainly reported for prostate cancer, CBP, and BPH [4, 5, 13]. The small polyphenol compound resveratrol is a natural ingredient of herbal remedies and has received widespread attention as a potential therapy or preventive agent for numerous diseases [14]. The scientific interest to resveratrol is related to its use for prevention of carcinogenesis and cardiovascular disease, and to its anti-inflammatory and anti-oxidant properties [14]. Resveratrol also exerts anti-inflammatory effects and enhances multiple immune functions of human T-cells in vitro, suggesting its ability to regulate the immune response; it also inhibits proliferation of human prostate cancer cell lines [15, 16]. However, the in vivo and in vitro effects of resveratrol on the prostatitis are not well understood. Resveratrol (3,5,4′-*trans*-trihydroxystilbene) is mainly found in grapes, a variety of other berries, and peanuts [14]. The most important dietary source of resveratrol is red wine; however, peanut (*Arachis hypogaea* L.), which has a high content of resveratrol, has been recently highlighted as a next-generation alternative dietary source of resveratrol [14, 17]. In particular, the content of resveratrol in peanut sprouts (germinated peanut seeds) is higher than in wines or peanut plants [14, 17]. To take advantage of the high resveratrol content in this dietary source, peanut sprouts are processed by drying and extraction of the sprout powder; these extracts (PSE) have been tested for the biological effects of resveratrol in various disease models [17-24]. In this respect, the present study examined the concentration of resveratrol in PSE, assessed the effect of PSE on prostate inflammation in the mouse models of CBP and BPH, evaluated whether administration of PSE as a dietary source of resveratrol causes toxicity to the liver or kidneys, and finally examined the possible role of the immune-stimulating effect of PSE in its ability to alleviate prostatitis.

The composition of PSE used in the present study is shown in Table 1. The major components were proteins, carbohydrates, lipids, and water (25.6, 20.4, 0.3, and 49.1 g per 100 g of PSE, respectively). Among other nutrients shown in Table 1, *trans*-resveratrol was present at 14.851 mg per 100g of PSE (Table 1). *Trans* fat and cholesterol were not detected (data not shown).

Table 1: Composition of the peanut sprout extract (PSE).

Ingredient	Content (per 100 g of PSE)	Nutrient	Content (per 100 g of PSE)
Lipids (g)	0.3	Dietary fiber (g)	5.9
Proteins (g)	25.6	Vt. B1/B2/B3/B6 (mg)	3.1/1.8/30.9/0.5
Carbohydrates (g)	20.4	Ca/Na/P/Zn (mg)	223.5/52.3/833.2/1.8
Ash (g)	4.6	Fe/Mg/K/Mn (mg)	1.9/366.1/1169.7/2.0
Water (g)	49.1	*Trans*-resveratrol (mg)	14.851 ± 0.305

Before the experiments in prostatitis mouse models, the toxicity of PSE was examined in control BALB/c mice (Figure 1). ALT activity and the expression level of the Kim-1 gene were used as indicators of toxicity to the liver and kidney, respectively. No toxicity was found even after administration of a high dose of PSE (200 µL of PSE prepared in 100 mg/mL) (Figure 1). Therefore, this dose was used in further experiments. The concentration of resveratrol contained in this dose is about 2.97 µg (per 200 µL of PSE prepared in 100 mg/mL), and the injection dose is 0.119 mg/kg.

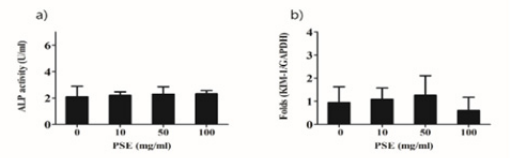

Figure 1: Evaluation of toxicity markers in the liver and kidney after PSE administration (n=3). PSE toxicity was evaluated by serum ALP activity for the liver (a) and Kim-1 gene expression for the kidney (b). ALP activity was evaluated using colorimetric assay kit. Kim-1 gene expression was analyzed by RT-PCR and normalized to that of GAPDH.

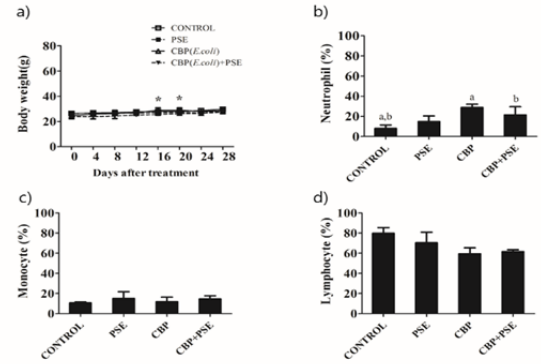

Figure 2: Changes in body weight and in blood cell profiles after PSE administration in CBP mice (n=5). Body weight was measured every 4 days (a). Changes (%) in neutrophils (b), monocytes (c), and lymphocytes (d) were examined by microscopic observation. *A statistical significant difference between CBP and CBP+PSE ($p < 0.05$). [a, b]A statistical significant results ($p < 0.05$) based on Scheffe *post hoc* one-way ANOVA analysis (each letter should be compared separately with the letters of other bars).

The mice were divided into four groups (n=5): control, CBP (*E. coli*), PSE, and CBP+PSE. The amount of resveratrol in 200 µL of PSE (100 mg/mL) was calculated as 2.97 µg. To investigate the effect of PSE, body weight and blood neutrophils were examined in the four groups. A significant difference in body weight between *E. coli* inoculation (CBP) with or without PSE administration was shown at day 16 and 20 after treatment (Figure 2a, $p < 0.05$). Unless, there was no significant change in body weight of all experimental groups (Figure 2a). The population of inflammatory cells predominantly increased upon the induction of prostatitis-was neutrophils. *E. coli* inoculation (CBP induction) significantly increased the proportion of blood neutrophils (%) compared to that in control mice (28.8 ± 3.4 vs. 8.0 ± 3.4, respectively, $p < 0.05$) (Figure 2b). PSE administration in CBP mice resulted in a reduction of neutrophils compared to that in CBP mice although it is not statistically different (25.4 ± 11.2 (%) vs. 28.8 ± 3.4 (%)). In contrast, the proportion of blood monocytes was slightly increased in the CBP+PSE group compared to that in the CBP group although the difference is not statistically (14.0 ± 2.9 (%) vs. 11.8 ± 4.6 (%)) (Figure 2c). Increase in neutrophils and monocytes conversely caused the decrease in lymphocytes proportion (Figure 2d).

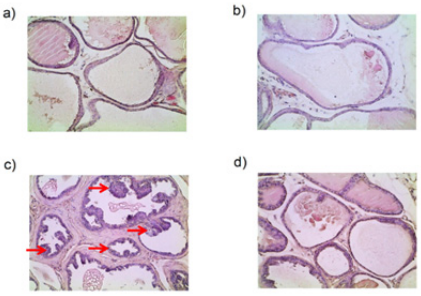

Figure 3: Effect of PSE on histopathology of the prostate in CBP mice. The pathologic changes were examined by H&E staining. Experimental groups were as follows: Control (a); PSE (b); CBP (c); CBP + PSE (d). Arrows in (c) show fibrotic features with enlarged and edematous stroma and tufting of the mucin-secreting epithelium in the granules. PSE administration resulted in a recovery of tissue pathology (d). Tissue sections were examined under ×200 magnification.

Histologically, normal prostate is composed of glandular and stromal tissue (Figure 3a). The prostate of rodents contains 3 separate lobes: dorsal, ventral, and lateral (frequently combined with dorsal as the dorsolateral lobe). Glands (tubules) are lined by two cell layers: the outer layer and the inner layer of columnar mucin-secreting epithelium. The stroma is the fibromuscular layer. The fibromuscular stroma between glandular tissues had an intact muscle layer, and the columnar mucin-secreting epithelium of glands was intact in both control and PSE groups (Figure 3a, b). In contrast, edema was detected in the prostate stroma in CBP mice (2.4×10^5 cfu per gram of prostate tissue, data not shown); in these mice, the prostate was enlarged and had fibrotic stroma suggesting prostatic hypertrophy (Figure 3c). In addition, mucosal epithelial hyperplasia and tufting of the mucin-secreting epithelium in the inner layer of granules were found in CBP mice (Figure 3c). However, these abnormalities disappeared after PSE administration (Figure 3d). In this experiment, the presence of *E. coli* in the prostate was reduced to 0.6×10^5 cfu per gram of prostate tissue (data not shown). In general, PSE administration in CBP mice reduced *E. coli* proliferation to 25% of that without PSE.

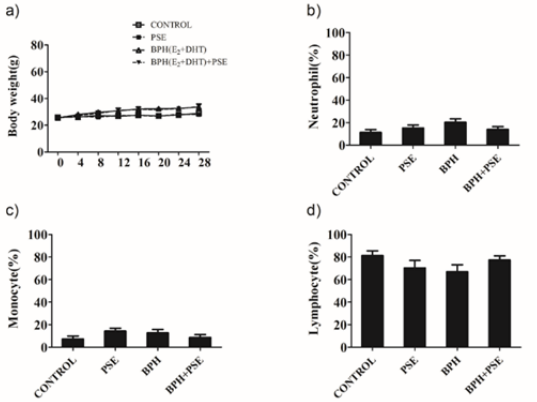

Figure 4: Changes in body weight and blood cell profiles after PSE administration in BPH (E2 + DHT) mice (n=5). Body weight was measured every 4 days (a). Changes (%) in neutrophils (b), monocytes (c), and lymphocytes (d) were examined by microscopic observation.

To investigate the effect of PSE on non-bacterial prostate hyperplasia, we used E2 + DHT to induce estradiol-induced prostatitis with BPH. The mice were divided into four groups (n=5): control, BPH, PSE, and BPH+PSE. There was no significant increase in body weights although BPH mice was increased from day 12 to day 28 (from 30.8 ± 0.7 g to 33.6 ± 1.4 g) in comparison with control mice (from 26.8 ± 0.6 g to 29.0 ± 1.1 g) (Figure 4a). During this period, PSE did not affect the body weight both in the control and E2 + DHT groups. To examine the induction of prostatitis in BPH mice, blood neutrophils were differentially counted as for CBP mice (Figure 4b). The proportion of neutrophils

(%) increased from 11.3 ± 2.1 in the control group to 18.3 ± 5.9 in the BPH group, suggesting the induction of prostatitis. In contrast, in the BPH+PSE group the proportion of neutrophils was reduced to 14.0 ± 2.6, i.e. by 4.3 percentage points in comparison with the BPH group although it is not statistically different (Figure 4b). The proportion of blood monocytes (%) was also reduced in the BPH+PSE group in comparison with the BPH-group (8.7 ± 2.5 and 12.0 ± 2.8, respectively) although it is not statistically different (Figure 4c).

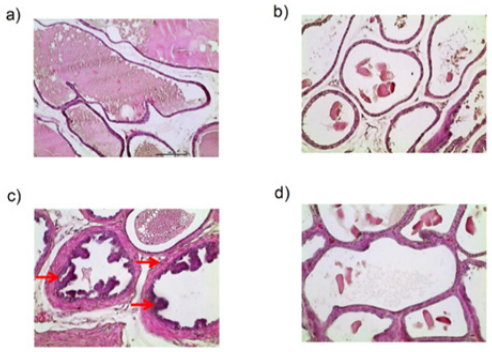

Figure 5: Effect of PSE on histopathology of the prostate in BPH mice. The pathologic changes were examined by H&E staining. Experimental groups were as follows: Control (a); PSE (b); BPH (c); BPH + PSE (d). Arrows in (c) show fibrotic features with enlarged and edematous stroma and tufting of the mucin-secreting epithelium in the granules. PSE administration resulted in a recovery of tissue pathology (d). Tissue sections were examined under ×200 magnification.

In the dorsal lobes of prostate glands, intact muscle layers in the fibromuscular stroma were found both in the control and PSE groups, and glands also had an intact epithelium in the columnar mucin-secreting epithelium of granules (Figure 5a, b). In contrast, epithelial cell hyperplasia and swollen epithelial layers were observed in the prostate glands of BPH mice (Figure 5c). In addition, the stroma in BPH mice was thicker than in control mice, suggesting prostatic hypertrophy (Figure 5c). PSE administration remarkably reduced the pathological signs (edema with enlarged tissue and fibrotic features in the stroma as well as mucosal epithelial hyperplasia in the granules) in the prostates of BPH mice (Figure 5d). Macrophages (BMMs) were isolated from the bone marrow. To investigate the possibility of immune activation after PSE treatment, we incubated BMMs with PSE, *E. coli* LPS, or both, and analyzed the expression of the macrophage activation marker $CD11b^+$ and the antigen presenting cell activation marker $CD40^+$ (Figure 6).

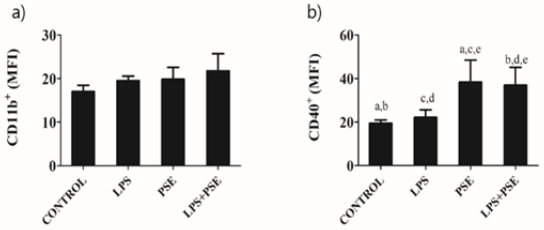

Figure 6: Effect of PSE on the CD11b and CD40 expression. BMMs were incubated with PSE (5 mg/mL), and examined for CD11b (a) or CD40 (b) by FACS analysis. The expression of CD40 was increased in PSE-treated BMMs both at 24 h post-treatment. [a, b, c, d, e] A statistical significant results ($p < 0.05$) based on Scheffe *post hoc* one-way ANOVA analysis (each letter should be compared separately with the letters of other bars).

The expression of $CD11b^+$ was not significantly increased under any experimental conditions (Figure 6a). However, the expression of the $CD40^+$ costimulatory molecule for antigen presentation was significantly increased in PSE-treated cells, suggested that the effect of PSE may be related to the M1 macrophage phenotype and T cell immunity for anti-pathogenic immunity (Figure 6b). Accordingly, our results strongly suggest that the reduction of *E. coli* burden in the prostate of CBP mice may be related to the PSE treatment for 4 weeks.

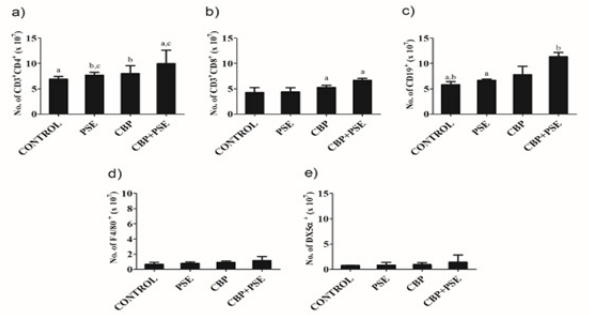

Figure 7: Changes in immune cell phenotypes in the spleens of CBP mice. PSE administration induced the increase in lymphocytes: $CD3^+CD4^+$-T cells (a), $CD3^+CD8^+$-T cells (b), and $CD19^+$-B cells (c). Macrophages (F4/80) (d), NK cells (DX5α) (e). [a, b, c] A statistical significant results ($p < 0.05$) based on Scheffe *post hoc* one-way ANOVA analysis (each letter should be compared separately with the letters of other bars).

To investigate whether the reduction of bacterial burden in the CBP+PSE-group was related to immune enhancement, the phenotypes of splenocytes were examined by FACS analysis (Figure 7). PSE administration in non-infected mice resulted in a slight increase in $CD4^+$ T cells, $CD8^+$ T cells, and $CD19^+$ B cells in comparison with control mice ($CD3^+CD4^+$: $7.7 \pm 0.57 \times 10^7$ vs. $6.9 \pm 0.53 \times 10^7$; $CD3^+CD8^+$: $4.4 \pm 0.85 \times 10^7$ vs. $4.3 \pm 0.95 \times 10^7$; $CD19^+$: $6.7 \pm 0.002 \times 10^7$ vs. $5.8 \pm 0.58 \times 10^7$) (Figure 7a,b,c). The increase in lymphocytes was stronger in the CBP+PSE group in comparison with the CBP group ($CD3^+CD4^+$: $9.97 \pm 0.27 \times 10^7$ vs. $8.01 \pm 0.15 \times 10^7$; $CD3^+CD8^+$: $6.68 \pm 0.37 \times 10^7$ vs. $5.28 \pm 0.4 \times 10^7$; $CD19^+$: $11.35 \pm 0.08 \times 10^7$ vs. $7.78 \pm 0.17 \times 10^7$) (Figure 7a,b,c). The number of F4/80-macrophages was also increased in CBP+PSE mice in comparison with CBP mice without a statistical significance ($1.16 \pm 0.52 \times 10^7$ vs. $0.92 \pm 0.16 \times 10^7$) (Figure 7d). In addition, the numbers of DX5a-NK cells were increased in CBP+PSE mice in comparison with CBP mice although there was no statistical significance ($1.41 \pm 1.41 \times 10^7$ vs. $0.92 \pm 0.16 \times 10^7$) (Figure 7e). The increased numbers of lymphocytes in PSE-administered CBP mice were likely related to the anti-bacterial immune response and helped to relieve inflammation by decreasing the *E. coli* burden in the prostate.

Prostate diseases include bacterial (CBP) and non-bacterial prostatitis (BPH) [1]. The therapeutic measures for CBP are mainly antibiotic prescription [6], whereas BPH is treated with α-adrenergic blocking agents and 5-α-reductase inhibitors [2], or often by phytotherapy using phytochemicals such as a polyphenolic flavonoid [4]. BPH therapy by using herbal remedies was attempted with the expectation of their diverse favorable effects such as antibacterial, anti-inflammatory and antioxidant activity [13]. In particular, because the BPH and prostate cancer occurrence in aged men cannot be predicted, general phytomedicines for the prostate continue to attract attention and are becoming more popular all over the world [5]. Resveratrol was introduced as a candidate nutritional substance for prostate cancer prevention [9]; recently, it was suggested to have many beneficial health effects including anti-oxidant, anti-inflammatory, anti-proliferative, pro-apoptotic, and anti-angiogenic [8]. Those health effects of resveratrol were first recognized when it was realized that red wine consumption results in a lower incidence of cardiovascular diseases in the French population [8, 15]. *Trans*-resveratrol is the biologically active form of resveratrol, and is present in the skin of grapes and in other fruits (such as raspberries, blueberries, and mulberries), as well as in Scots pine, Eastern white pine, and knotweed [8]. Particularly high concentrations of *trans*-resveratrol were found in peanuts and grapes [9]; resveratrol content is 3 μg/g, 0.6 μg/g, and 110.3 μg/g in grapes, wine, and peanut sprouts, respectively [17]. According to a recent study that used PSE (38.17 mg/mL, 0.05% resveratrol), in which rats were fed a high-fat diet with the addition of PSE (30

mg/kg), PSE had an anti-obesity effect because it controlled the levels PPARγ and adiponectin proteins in adipose tissue [17]. In another study, *trans*-resveratrol content in PSE obtained by dissolving peanut sprout powder with water was 54.2 μg/g [20]. PSE processed by hot water extraction and concentration in the present study was rich in *trans*-resveratrol (148.51 ± 3.05 μg/g). In the present study, we were interested in the beneficial health effect of PSE on bacterial and nonbacterial prostatitis because PSE is a natural source of resveratrol [8, 9]. PSE administration reduced bacterial proliferation to one-quarter in of that in CBP mice. Although PSE is not an antibiotic, highly concentrated resveratrol in PSE may have a role in restoration of prostate tissue through its anti-inflammatory and immune-enhancing effect [8, 15]. Biomedical effects of resveratrol observed in multiple biological systems include those related to immunity, for example the anti-inflammatory effect and effects on multiple immune functions of T cells [15]. The present study has also shown an increase in the immune cells, $CD4^+$-, $CD8^+$-, and $CD19^+$-lymphocytes, in PSE-treated mice. This suggests that the administration of PSE induces immune enhancement due to the presence of resveratrol. Under the same conditions, PSE had no toxicity as assessed by measuring body weight as well as liver and kidney toxicity.

BPH is also an important prostate disease and represents hormone-induced chronic inflammation that results from the infiltration of inflammatory cells; it may show no distinct clinical signs and generally lead to urination problems resulting from urinary tract obstruction by an enlarged prostate [13]. With respect to BPH, the usefulness of phytotherapy and complementary alternative medicine has been suggested with the expectation of the reduction in the risk of death and long-term morbidity [4, 5]. Experimentally, BPH can be induced by E2 and DHT treatment [2, 13, 25]. Fortunately, BPH can be relieved by herbal remedies due to their ability to inhibit cyclooxygenases 1 and 2 (COX-1 and COX-2) [13]. The resveratrol content in PSE is very high compared with a natural phytomedicine [17]. Similar to CBP mice, blood neutrophils were increased in BPH mice; PSE administration reduced the blood neutrophil population by approximately 4.3 percentage points compared to that in untreated BPH mice. At the same time, PSE-administered mice showed a reduction in epithelial cell hyperplasia and swelling in epithelium of the glandular tissues as well as the thickening and fibrous features in the stroma related to prostate hypertrophy. These results are in line with those of a recent study that investigated the effect of resveratrol on BPH induced by testosterone propionate injection [25]. Resveratrol (1 mg/kg) introduced into BPH rats showed favorable effects such as a decrease of prostate weight and DHT production [26]. In the present study, mice were treated with a much lower resveratrol concentration, 0.119 mg/kg, but it still showed the preventive and therapeutic effect in CBP and BPH-induced prostatitis.

The anti-prostatitis effect of PSE is likely to result in part from immune enhancement, because our ex vivo experiment showed activation of 95% pure BMMs by PSE. Activation of these antigen-presenting cells suggests the induction of further immune responses. CD40 is an M1 macrophage costimulatory marker, and M1 macrophages produce considerable amounts of nitric oxide in response to *E. coli* and have important roles in chronic inflammatory diseases [26]. In contrast, CD11b is a monocyte marker and a marker of CD8+ cytotoxic T cell activation during virus infection [27]. We found that PSE treatment increased CD40 expression on BMMs. This result agrees with the cell phenotype increased in the spleen. The numbers of T cells ($CD4^+$ and $CD8^+$), B cells ($CD19^+$), and macrophages were increased more in the CBP+PSE group than in the CBP group; a decrease in *E. coli* burden in the prostate in the CBP+PSE group suggests the induction of anti-bacterial immune response. Therefore, it seems that the increase in lymphocytes in PSE-administered mice with prostatitis enhanced the anti-bacterial immune response and helped to relieve inflammation.

Since PSE used in the present study was a mixture of natural compounds, it is impossible to definitely point out the ingredient that had the favorable effect in the prostatitis mouse model. However, peanut contains a high level of resveratrol, and resveratrol biosynthesis in peanut is enhanced by germination [24]; moreover, the extraction of peanut sprouts using steaming and ethanol is a processing method that yields highly concentrated resveratrol [19]. Recently, resveratrol has been reported to have an effect on BPH via regulation of inflammatory and apoptotic proteins [25]. On the basis of our results and the previous studies, we speculate that the effect of PSE on both CBP and BPH prostatitis may be due primarily to the presence of resveratrol.

Experimental

General: Peanut kernel, used for producing peanut sprout in this experiment, was purchased from Laixi city shunxiang peanut product's Co., LTD (shandong, china) and its scientific name is "*Arachis hypogaea* L." (HS code: 12024200). *Arachis hypogaea* L. is registered in "Bio Resource Information Service center (Sejong-si, Republic of Korea) and its code is "MANUN1201292056". PSE was prepared by the established method with some modifications [17]. Briefly, peanut seeds were germinated by spraying and watering them dropwise for 7 to 8 days in the dark at 24–25ºC and 85% humidity, and then processed at a local plant (Jangsuchae Co., Seoul, Korea) for further the preparation of liquid PSE. Liquid PSE was extracted by vacuum distillation and condensation with hot water extraction (100ºC for 7 h), concentrated under reduced pressure (95ºC for 48 h), diluted 10 times with 10 mM PBS (pH 7.2), filtered through a 0.22-μm PVDF syringe filter (Millipore, Billerica, MA), and then used for in vitro and animal experiment. The final PSE samples were orally introduced by 200 μL of PSE prepared in 100 mg/mL using gavage needle every day into CBP or BPH mice for 4 weeks. The composition of PSE was analyzed by the Korea Advanced Food Research Institute (KAFRI, Seoul, Korea).

Lipid, protein, ash, and moisture contents of PSE were determined using the standard methods of the Association of Official Analytical Chemists (AOAC, 2000; methods 960.39, 981.10, 920.153, and 950.46B, respectively). The total dietary fiber content was measured using a total dietary fiber assay kit (Sigma, St. Louis, MO, USA) according to the method of Prosky-AOAC (1985). Water-soluble vitamins (B1, B2, B3, and B6) were determined using HPLC according to the method of Ji et al. [28]. The contents of Ca, Na, P, Zn, Fe, Mg, K, and Mn were quantified by inductively coupled plasma atomic emission spectrometry (ICP-AES; Spectro Analytical Instruments, Kleve, Germany) according to the method of the Korean Food Standards Codex (Korea Food and Drug Administration, 2009). Resveratrol content of PSE were analyzed using 2695 HPLC system (Waters, Milford, MA) equipped with photodiode array detector. PSE was diluted in 80% MeOH and separated on a YMC-triart C18 column (4.6 x 250mm, 5um particle size; YMC, Kyoto, Japan) at 25˚C. The flow rate was 1.0 mL/min and the injection volume was 10 μL. The gradient mobile phases were used with solvent (A) water:acetic acid (97.5:2.5 v/v) and solvent (B) acetonitrile:water:acetic acid (77.5:20:2.5 v/v/v). The gradient condition of the mobile phase was as follows: 0-5 min; solvent (B) 18%, 15 min; solvent (B) 30%, 25 min; solvent (B) 45%, 30 min; solvent (B) 50%, 35 min; solvent (B) 100%, 37min; solvent (B) 18%. The absorbance was measured at 308 nm and trans-resveratrol (Sigma) was used as standard. Identification of resveratrol in PSE chromatogram was based on comparison with

spectra and retention time of standards. Trans-resveratrol concentration in PSE used in present study was 148.51 ± 3.05 μg/g.

Eleven-week-old BALB/c male mice purchased from Orient Bio (Seongnam, Korea) were housed in a specific pathogen-free barrier zone of Seoul National University College of Medicine animal facilities, and treated in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) at Seoul National University (IACUC No: SNU-140127-4-1) under approval for the use of animals for the present study. Mice were housed at 25 °C, 50% relative humidity and 12-h light/dark cycles, and sacrificed by CO_2 inhalation in a chamber after the experiment was finished. For the experiments on CBP and BPH, four experimental groups (5 mice per group) were used: control (no treatment), CBP (or BPH), PSE, and CBP+PSE (or BPH+PSE). PSE solution (200 μL, 100 mg/mL) was administered daily for 4 weeks from following day after E. coli was injected (or from that day when β-estradiol dissolved in sesame oil was treated). Body weight was examined every 4 days during the period of PSE administration.

E. coli 292 isolated from humans was kindly donated by Korea Prostate Bank (KPB, Seoul, Korea), and grown overnight in LB broth (Difco, Franklin Lakes, NJ). The genotype of the bacteria was confirmed by sequencing bacteria-specific 16S rRNA using universal 16S rRNA primers (forward primer: 5′-AGA GTT TGA TCM TGG CTC AG-3′, reverse primer: 5′-ATT ACC GCG GCT GCT GG-3′). Sequencing was performed by SolGent Co., Ltd (Daejeon, Korea), and the results were analyzed using the Geneious Pro 5.5 software (Biomatters, Auckland, New Zealand).

CBP was induced as follows. 2×10^6 cfu of *E. coli* 292 per 20 μL was slowly injected into the urinary tract over 1 min using a catheter (20 mm in length, 0.61 mm in diameter) with 10-μL Hamilton syringe (Hamilton #80300, Reno, NV) under anesthesia with 30 mg/kg of Zoletil (Virbac Korea Co. Ltd., Seoul, Korea) plus 10 mg/kg of Xylazine (Rumpun; Bayer Korea Co. Ltd., Seoul, Korea) [6, 10]. Then the penis was tied with a sterilized string for 30 min to prevent the backflow of injected bacteria. The success of bacterial infection was confirmed by E. coli colony (cfu) counting in the prostate tissue of sacrificed mice at the end of the experiment. Briefly, tissues were removed from the ventral and dorsal lobes of the prostate, weighted, and ground in PBS using a blender in 50-mL conical tubes. The samples were spread on LB agar plates and cultivated at 37°C for 12 h. The bacterial colonies were counted by cfu $\times 10^5$ per 1 g of tissue. The colony number was approximately 2.4×10^5 cfu per gram of prostate tissue, indicating successful infection.

Prostate hyperplasia was induced by subcutaneous injection of β-estradiol (E2; Sigma-Aldrich) and 5α-androstan-17β-ol-3-one (dihydrotestosterone, DHT; Sigma-Aldrich) for 4 weeks [16]. For the first 2 weeks, β-estradiol dissolved in sesame oil (0.1 mL; 0.25 mg/kg/day) was injected. For the next 2 weeks, a mixture of β-estradiol and DHT dissolved in sesame oil (0.1 mL; 0.25 mg/kg/day each hormone) was injected.

To examine the safety of PSE as a dietary supplement, we examined the body weight, hepatotoxicity and renal toxicity. PSE solution (200 μL, 100 mg/mL) was administered for 2 weeks for three mice. Serum alkaline phosphatase (ALP) was measured using an ALP colorimetric assay kit (Abcam, Cambridge, UK) according to manufacturer's instructions. Kidneys were isolated from PSE-administered mice, and Kim-1 gene expression was examined by PCR amplification using specific KIM-1 gene primers (forward primer: 5′-GAG ATA CCT GGA GTA ATC ACA CTG AA-3′, reverse primer: 5′-TGA TAG CCA CGG TGC TCA-3′). For PCR reaction, total RNA was extracted using RNease kit (Qiagen, Hilder, Germany), and synthesized cDNA using oligo d(T)15 primer (EBT-1515, ELPIS biotech, Daejeon, Korea). 200 ng of cDNA synthesized was applied to PCR reaction. The PCR amplification was performed as follows: 2 min at 95 C, (20 sec at 95 ºC for denaturing, 40 sec at 64 ºC for annealing, 30 sec at 72 ºC for elongation) x 30 cycles, and 5 min at 72 ºC. For PCR reaction on house-keeping gene (GAPDH), specific primers were used (forward: 5′-AGGTCGGTGTGAACGGATTTG-3′, reverse: 5′-TGTAGACC ATGTAGTTGAGGTCA′). The result was captured as gel image (DNR bio imaging, Jerusalem, Israel) after 2 % agarose gel electrophoresis and, and expressed as a fold-value comparing with GAPDH expression using Image J software (NIH, Bethesda, MD).

The prostate was isolated after the end of the experiment, fixed in 10% neutral formaldehyde for 3 days, and washed with tap water for 1 h. Tissues were embedded in paraffin (Leica TP1020; Leica Biosystems, Buffalo Grove, IL). Paraffin blocks were sliced into 7-μm sections on a microtome (Leica RM2145) and stained with H&E staining. Slides were observed and photos were taken using an optical microscope (Olympus, Tokyo, Japan).

Mice were treated orally with PSE (200 μL, 100 mg/mL) for 28 days. At the end of the experiment, mice were euthanized, and whole blood was collected before sacrifice into K2-EDTA tubes (BD Vacutainer, Franklin Lakes, NJ). Blood was thinly smeared on glass slides and stained using Diff-Quik (DQ) staining solution. A total of 100 white blood cells were counted per mouse by microscopic observation, and each cell population (neutrophils, monocytes, and lymphocytes) was counted separately.

Lipopolysaccharide (LPS) was extracted from *E. coli* 292 using an LPS extraction kit (Intron Biotechnology, Seoul, Korea). LPS from *E. coli* 292 was dissolved in HBSS to be concentration of 200 μg/mL, and stored until use. For in vitro cell cultures, LPS was used as a positive control at a final concentration of 1 μg/mL.

Bone marrow–derived macrophages (BMMs) were collected from the femur and tibia. Briefly, cells were collected by flushing the bone marrow with cold complete RPMI medium (Welgene Inc., Daegu, Korea) containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and 1% antibiotic–antimycotic (Invitrogen), and then cultured in 30% L929-conditioned medium (M-CSF–containing medium) in a 100-mm dish (SPL, Pocheon, Korea) for 2 weeks with subculturing every 3 days. The purity of harvested BMMs was measured by using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) after immunostaining with the pan-macrophage marker F4/80; the purity was confirmed to exceed 95%. For further in vitro culture, 2×10^5 BMMs were cultured with either PSE (5 mg/mL) or *E. coli* LPS (1 μg/mL) in a 6-well plate (SPL) for 24 h. CD11b- and CD40-expression level were determined by FACS analysis using anti-CD11b-FITC and anti-CD40-PE antibodies (both 1:100, eBioscience, San Diego, CA), respectively.

For investigating the inflammatory cell population, spleens were collected from CBP mice (n=5) at the end of the experiment, dissociated into single-cell suspensions, and washed with RPMI. Red blood cells (RBCs) in the spleen were destroyed by hypotonic shock using RBC lysis buffer containing 0.15 M NH_4Cl, 1 M $KHCO_3$, and 0.1 mM Na_2EDTA (pH 7.4). Splenocytes were counted by Trypan blue exclusion test, and were pooled in equal amount for further FACS analysis; 2×10^6 splenocytes for each cell population were analyzed in duplicate. The cells were incubated with rat anti-mouse CD16/32 (Fcr III/II receptor) antibody (1:100

dilution, eBioscience) to block nonspecific binding before staining with specific antibodies, washed and stained with the following FACS antibodies (all from eBioscience; 1:100, 20 μL of each antibody for 10 min at room temperature in the dark): anti-CD19, phycoerythrin (PE)-conjugated; anti-F4/80, PE-conjugated; anti-DX5a, fluorescein isothiocyanate (FITC)-conjugated; anti-CD3, allophycocyanin (APC)-conjugated; anti-CD4, PE-conjugated; and anti-CD8, PE-cy5–conjugated. After washing, labelled cells were fixed in 20 μL of 4% paraformaldehyde solution. Fluorescence was quantified using a FACSCalibur flow cytometer (BD Biosciences). Data, which were originally expressed as percentage of splenocytes, were recalculated as cell number in each stained cell phenotype relative to the total spleen cell number.

The results are presented as means ± SD. Statistical analysis was performed by a one-way ANOVA followed by Scheffe *post hoc* comparison. A statistical significance is represented by same lowercase letter. $P < 0.05$ was set as the level of statistical significance. The graphics program used in the present study is GraphPad Prism 5.0a.

Acknowledgments - This work was supported by Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry and Fisheries (IPET) through 'High Value-added Food Technology Development Program' funded by Ministry of Agriculture, Food and Rural Affairs (MAFRA) (grant number: 112118-2).

References

[1] Pavone-Macaluso M. (**2007**) Chronic prostatitis syndrome: A common, but poorly understood condition. Part II. *EAU-EBU Update Series*, ***5***, 16-25.
[2] Thorpe A, Neal D. (**2003**) Benign prostatic hyperplasia. *Lancet*, ***361***, 1359-1367.
[3] Suzuki K. (**2009**) Epidemiology of prostate cancer and benign prostatic hyperplasia. *Japan Medical Association Journal*, ***52***, 478-483
[4] Shoskes DA. (**2002**) Phytotherapy in chronic prostatitis. *Urology*, ***60***, 35-37; discussion 37.
[5] Steenkamp V. (**2003**) Phytomedicines for the prostate. *Fitoterapia*, ***74***, 545-552.
[6] Wong L, Hutson PR, Bushman W. (**2015**) Resolution of chronic bacterial-induced prostatic inflammation reverses established fibrosis. *Prostate*, ***75***, 23-32.
[7] Feifer AH, Fleshner NE, Klotz L. (**2002**) Analytical accuracy and reliability of commonly used nutritional supplements in prostate disease. *Journal of Urology*, ***168***, 150-154; discussion 154.
[8] Jasinski M, Jasinska L, Ogrodowczyk M. (**2013**) Resveratrol in prostate diseases - a short review. *Central European Journal of Urology*, ***66***, 144-149.
[9] Stewart JR, Artime MC, O'Brian CA. (**2003**) Resveratrol: a candidate nutritional substance for prostate cancer prevention. *Journal of Nutrition*, ***133***, 2440S-2443S.
[10] Kanamaru S, Kurazono H, Terai A, Monden K, Kumon H, Mizunoe Y, Ogawa O, Yamamoto S. (**2006**) Increased biofilm formation in *Escherichia coli* isolated from acute prostatitis. *International Journal of Antimicrobial Agents*, ***28* Suppl 1**, S21-25.
[11] La Vignera S, Condorelli RA, Vicari E, Salmeri M, Morgia G, Favilla V, Cimino S, Calogero AE. (**2014**) Microbiological investigation in male infertility: a practical overview. *Journal of Medical Microbiology*, ***63***, 1-14.
[12] Rudick CN, Berry RE, Johnson JR, Johnston B, Klumpp DJ, Schaeffer AJ, Thumbikat P. (**2011**) Uropathogenic *Escherichia coli* induces chronic pelvic pain. *Infection and Immunity*, ***79***, 628-635.
[13] Steenkamp V, Gouws MC, Gulumian M, Elgorashi EE, van Staden J. (**2006**) Studies on antibacterial, anti-inflammatory and antioxidant activity of herbal remedies used in the treatment of benign prostatic hyperplasia and prostatitis. *Journal of Ethnopharmacology*, ***103***, 71-75.
[14] Smoliga JM, Baur JA, Hausenblas HA. (**2011**) Resveratrol and health--a comprehensive review of human clinical trials. *Molecular Nutrition & Food Research*, ***55***, 1129-1141.
[15] Calabrese EJ, Mattson MP, Calabrese V. (**2010**) Resveratrol commonly displays hormesis: occurrence and biomedical significance. *Human & Experimental Toxicology*, ***29***, 980-1015.
[16] Nicholson TM, Ricke WA. (**2011**) Androgens and estrogens in benign prostatic hyperplasia: past, present and future. *Differentiation*, ***82***, 184-199.
[17] Kang NE, Ha AW, Woo HW, Kim WK. (**2014**) Peanut sprouts extract (*Arachis hypogaea* L.) has anti-obesity effects by controlling the protein expressions of PPARgamma and adiponectin of adipose tissue in rats fed high-fat diet. *Nutrition Research and Practice*, ***8***, 158-164.
[18] Chang JC, Lai YH, Djoko B, Wu PL, Liu CD, Liu YW, Chiou RY. (**2006**) Biosynthesis enhancement and antioxidant and anti-inflammatory activities of peanut (*Arachis hypogaea* L.) arachidin-1, arachidin-3, and isopentadienylresveratrol. *Journal of Agricultural and Food Chemistry*, ***54***, 10281-10287.
[19] Choi DI, Choi JY, Kim YJ, Lee JB, Kim SO, Shin HT, Lee SC. (**2015**) Ethanol extract of peanut sprout exhibits a potent anti-inflammatory activity in both an oxazolone-induced contact dermatitis mouse model and compound 48/80-treated HaCaT cells. *Annals of Dermatology*, ***27***, 142-151.
[20] Choi JY, Choi DI, Lee JB, Yun SJ, Lee DH, Eun JB, Lee SC. (**2013**) Ethanol extract of peanut sprout induces Nrf2 activation and expression of antioxidant and detoxifying enzymes in human dermal fibroblasts: implication for its protection against UVB-irradiated oxidative stress. *Photochemistry and Photobiology*, ***89***, 453-460.
[21] Kim SS, Seo JY, Kim BR, Kim HJ, Lee H-Y, Kim J-S. (**2014**) Anti-obesity activity of peanut sprout extract. *Food Science and Biotechnology*, ***23***, 601-607.
[22] Kim WK, Kang NE, Kim MH, Ha AW. (**2013**) Peanut sprout ethanol extract inhibits the adipocyte proliferation, differentiation, and matrix metalloproteinases activities in mouse fibroblast 3T3-L1 preadipocytes. *Nutrition Research and Practice*, ***7***, 160-165.
[23] Seo JY, Kim SS, Kim HJ, Liu KH, Lee HY, Kim JS. (**2013**) Laxative effect of peanut sprout extract. *Nutrition Research and Practice*, ***7***, 262-266.
[24] Wang KH, Lai YH, Chang JC, Ko TF, Shyu SL, Chiou RY. (**2005**) Germination of peanut kernels to enhance resveratrol biosynthesis and prepare sprouts as a functional vegetable. *Journal of Agricultural and Food Chemistry*, ***53***, 242-246.
[25] Chung KS, Cheon SY, An HJ. (**2015**) Effects of resveratrol on benign prostatic hyperplasia by the regulation of inflammatory and apoptotic proteins. *Journal of Natural Products*, ***78***, 689-694.
[26] Holden JA, Attard TJ, Laughton KM, Mansell A, O'Brien-Simpson NM, Reynolds EC. (**2014**) Porphyromonas gingivalis lipopolysaccharide weakly activates M1 and M2 polarized mouse macrophages but induces inflammatory cytokines. *Infection and Immunity*, ***82***, 4190-4203.
[27] Lin Y, Roberts TJ, Sriram V, Cho S, Brutkiewicz RR. (**2003**) Myeloid marker expression on antiviral CD8+ T cells following an acute virus infection. *European Journal of Immunology*, ***33***, 2736-2743.
[28] Ji C-M, Shin J-A, Cho J-W, Lee K-T. (**2013**) Nutritional evaluation of immature grains in two Korean rice cultivars during maturation. *Food Science and Biotechnology*, ***22***, 903-908.

NPC Natural Product Communications

2017
Vol. 12
No. 1
79 - 82

Phenanthrenes from *Eulophia macrobulbon* as Novel Phosphodiesterase-5 Inhibitors

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Received: September 29th, 2016; Accepted: October 26th, 2016

Phosphodiesterase 5 (PDE5) inhibitors can be used for the treatment of erectile dysfunction and pulmonary hypertension. In order to search for new leads of PDE5 inhibitors, we investigated the chemical constituents of the tubers of *Eulophia macrobulbon* (E.C. Parish & Rchb. f.) Hook. f. A new phenanthrene, 9,10-dihydro-4-(4′-hydroxybenzyl)-2,5-dimethoxyphenanthrene-1,7-diol (**1**) and three known phenanthrenes i.e., 1-(4′-hydroxybenzyl)-4,8-dimethoxyphenanthrene-2,7-diol (**2**), (9,10-dihydro-2,5-dimethoxyphenanthrene-1,7-diol (**3**) and 1,5,7-trimethoxyphenanthrene-2,6-diol) (**4**) were isolated. Among these, **2** was the most potent PDE5 inhibitor (IC_{50} =1.67±0.54 μM**)** evaluated by the [^{3}H]cGMP radioassay method, whereas **1** showed mild activity (IC_{50} = 62.3±3.3 μM). Their inhibitory selectivities against PDE5 over PDE6 were also studied. This study suggests phenanthrenes as a new class of PDE5 inhibitors.

Keywords: *Eulophia macrobulbon*, Phenanthrenes, Phosphodiesterase-5 inhibitors, Erectile dysfunction.

Erectile dysfunction (ED) is the inability to achieve or sustain penile erection [1-4] and causes low self-esteem. Its prevalence is associated with aging and in the US 18 million men are affected [5] and the number is predicted to grow to 300 million globally by 2025 [6]. In Brazil, 45% of the male population over 18 years have some degree of ED [7]. PDE5 inhibitors, sildenafil (Viagra®), vardenafil (Levirate®), tadalafil (Cialis®) and Avanafil (Stendra®) have become first-line treatments [8-11]. The most important side-effects of PDE5 inhibitors is retinal defects via PDE6 inhibition [12-14]. Consequently, the search continues for both synthetic and herbal PDE5 inhibitors having high selectivity. We have screened Thai medicinal plants for PDE5 inhibitory activity, and *Eulophia macrobulbon* (E.C. Parish & Rchb. f.) Hook. f., a plant belonging to the Orchidaceae family, showed promising PDE5 inhibition. The present study aimed to isolate the biologically active phytochemicals of this plant, which hitherto have not been reported.

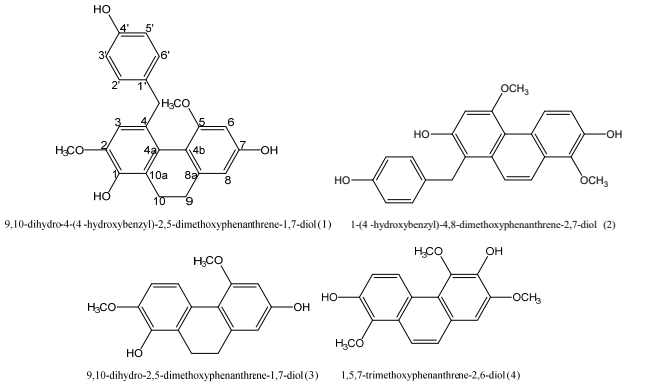

Figure 1: Structures of phenanthrenes isolated from *E. macrobulbon* extract.

The separation of *E. macrobulbon* tuber extracts yielded four compounds (Figure 1). The HREIMS data of compound **1** exhibited a molecular ion peak at *m/z* 378.1478, which represented $[MH]^+$ for $C_{23}H_{22}O_5$. Its IR spectrum showed a typical characteristic band of a hydroxyl group at 3338 (O-H stretching) and aromatic bands at 1614, and 1516 cm^{-1} (C=C stretching). Its ^{13}C NMR spectrum in MeOH-d_4 showed 21 resonances, which represented 23 carbon atoms. These were determined as two methyls, three methylenes, seven aromatic methines and eleven aromatic quaternary carbons by DEPT and HSQC experiments. The ^{1}H NMR spectrum indicated resonances for four protons at δ 2.04, 3.20, 2.38, and 2.61 (ddd), which are typical for methylene protons of a 9,10-dihydro-phenanthrene derivative. Two methoxyl resonances were observed at δ 3.62 and 3.69. One singlet aromatic proton signal at δ 6.47 was found and a pair of doublets at δ 6.36 and 6.38 with a coupling constant of 2.0 Hz (*meta* coupling of H-6 and H-8, respectively). The presence of two doublets for a benzylic-CH_2 at δ 3.72 and 3.81 (J = 15.0 Hz) indicated a benzyl substitution. Two doublets (2H) at δ 6.81 and 6.61 with *ortho* coupling constants of 8.4 Hz were assigned to H-2′/H-6′ and H-3′/H-5′, respectively. These data suggested a *para*-disubstituted benzene. The ^{1}H and ^{13}C NMR assignments were based on HMBC and NOESY experiments (Figure 2). The presence of a cross peak between H-3 and the benzylic-CH_2 in the HBMC experiment supported the connectivity between the 4′ hydroxybenzyl group and the phenanthrene structure at C-4. The methoxy group positions in the structure were assigned using NOESY. The methoxy at δ 3.62 was located at C-5 since it displayed cross peaks with H-6, while the other methoxy (δ 3.69) showed a cross peak with H-3 in the NOESY experiment, suggesting that it was located at C-2. Finally, compound **1** was unambiguously identified as 9,10-dihydro-4-(4′-hydroxybenzyl)-

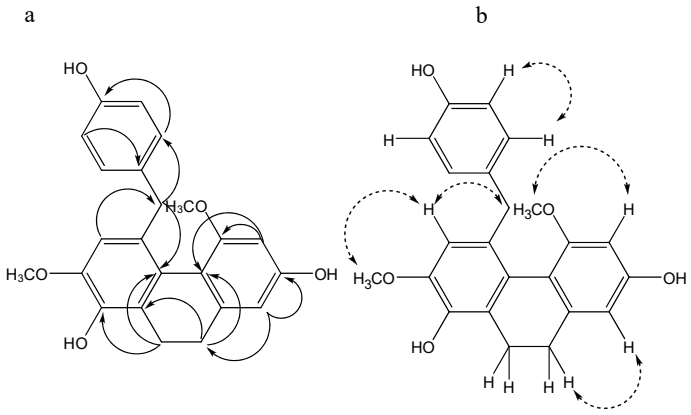

Figure 2: Diagnostic HMBC (a) and NOESY (b) correlations of 9,10-dihydro-4-(4′-hydroxybenzyl)-2,5-dimethoxyphenanthrene-1,7-diol (**1**).

2,5-dimethoxyphenanthrene-1,7-diol, a new 9,10-dihydro-phenanthrene derivative. The structures of **2, 3** and **4** were identified by spectroscopy and by comparing with published spectroscopic data [15-18].

Phenanthrene compounds **1**, **2** and **4** showed >70% inhibition against both PDE5 and PDE6 at 50 µg/mL (Table 1). Compound **2**, the main constituent, was the most potent PDE5 and PDE6 inhibitor, but less than sildenafil (Table 2). Even though PDE5/PDE6 selectivities were poor, they showed high selectivity over PDE1, which is abundant in the heart [19]. This appears to be the first study reporting phenanthrenes inhibiting PDEs and pharmacologically-active constituents of *E. macrobulbon*. Phenanthrenes may offer leads to synthesize more potent and selective PDE5 inhibitors. The limited number of compounds restricted interpretation of structure activity relationships. Nevertheless, the aromaticity of these compounds might be important for PDE5 and PDE6 inhibitory activities. Other *Eulophia* spp. studied have been *E. nuda* [15-16, 20-22]*, E. petersii* [23] and *E. ochreata* [24-25], which also contain phenanthrenes. Their pharmacological properties include tumor cytotoxicity, and platelet anti-aggregation, anti-microbial, anti-inflammatory, and spasmolytic actions [22, 26]. PDE inhibition might underlie some actions, particularly those on smooth muscle.

Table 1: % Inhibitory activity of isolated phenanthrenes from *E. macrobulbon* on PDE5, PDE6 and PDE1

Compds	%Inhibitory activity at 50 µg/mL[a]		
	PDE-5	PDE-6	PDE-1
1	78.5 ± 3.6	70.1 ± 4.3	25.5 ± 1.3
2	99.2 ± 0.5	104.2 ± 0.2	0.6 ± 2.8
3	44.6 ± 4.1	66.2 ± 8.7	6.2 ± 3.8
4	72.3 ±6.8	87.2 ± 4.8	5.8 ± 3.2

[a] Data are reported as the mean ± 1 S.D and are derived from three repeats.

Table 2: IC_{50} values of isolated phenanthrenes from *E. macrobulbon* on PDE5 and PDE6

Compds	IC_{50} (µM)[a]	
	PDE-5	PDE-6
1	62.3 ± 3.3	94.1 ± 3.9
2	1.7 ± 0.5	1.8 ± 0.07
4	98.1 ± 13.3	51.0 ± 3.8
Sildenafil	0.03 ± 0.01	0.05 ±0.02

[a] Data are reported as the mean ± 1 S.D and are derived from three repeats.

Experimental

General: ^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR) spectra were recorded in acetone-d_6, MeOH-d_4 and dimethylsulfoxide-d_6 on a Bruker AV400 (USA) spectrometer at 400 and 100 MHz, respectively**.** Mass spectra were obtained using an electron ionization mass spectrometer (EI-MS) (MAT 95 XL, Thermo-Finnigan, Germany). Infrared (IR) absorption spectra were recorded using the KBr technique on a Fourier Transform Infrared Spectrometer (Spectrum GX Series, PerkinElmer, USA**).** Optical rotations were measured in MeOH solution at the sodium D-line (589 nm) (Parameter 341, PerkinElmer). PDE inhibition was measured using a beta counter (TopCount NXT, (Perkin-Elmer, USA)

Plant material: *E. macrobulbon* was collected from Prachinburi Province, Thailand and identified by Asst. Prof. Dr Anupan Kongbangkerd, Faculty of Sciences, Naresuan University. The herbarium specimen (No. 002716) is kept in the Biology Department, Faculty of Science, Naresuan University, Thailand.

Chemicals: cGMP, crude snake venom (*Crotalus atrox*), histone from calf thymus, bovine serum albumin (BSA), ethylene glycol tetra-acetic acid (EGTA), imidazole, Tris, $MgCl_2$, DEAE-Sephadex, and bovine heart PDE1 were purchased from Sigma Chemical (St. Louis, MO, USA). [^{3}H]cGMP was obtained from Perkin Elmer (Boston, MA, USA). Sildenafil was a gift from Bioequivalence Unit, Department of Pharmacology, Faculty of Medicine, Khon Kaen University, Thailand. The organic solvents **(**analytical grade**)** were purchased from Burdick & Jackson **(**B&J**) (**UK**).** All other reagents were bought from LabScan **(**Bangkok, Thailand**).** TLC aluminum sheets and silica gel 60 F254 were purchased from Merck (Darmstadt, Germany)**.**

Extraction and isolation: The fresh tubers of *E. macrobulbon* were chopped and dried at 55°C. The dried material (2 kg) was ground into a fine powder, macerated in 95% EtOH (14 L) twice (3 days each time), and the solvent removed under reduced pressure to afford a brown syrupy EtOH extract (225 g). This **(**200 g**)** was dissolved in MeOH **(**200 mL**)** then partitioned with *n*-hexane **(**6 x 200 mL**)**, CH_2Cl_2 **(**8 x 400 mL**)**, EtOAc **(**8 x 400 mL**)** and *n*-BuOH **(**5 x 400 mL**).** All fractions were then dried under vacuum**.** PDE5 inhibitory activity was detected in the CH_2Cl_2 fraction, part of which **(**50 g**)** was loaded onto a column of SiO_2 and eluted with increasing proportions of MeOH in CH_2Cl_2**,** followed by elution with 1**%** 1N HCl in MeOH**.** Fractions were monitored by TLC **(**CH_2Cl_2/MeOH**) (**98**:**2**)** and light petroleum/EtOAc **(**1**:**1**)** and the fractions with similar chemical profiles were pooled**.** Thirteen pooled fractions **(**ED-Q1 - ED-Q13**)** were obtained**.** Fraction ED-Q4 **(**1.2 g**)** was further fractionated by CC over SiO_2, yielding 6 pooled fractions**.** ED-Q4-2 **(**37 mg**)** was subjected to SiO_2 preparative TLC **(**prep-TLC**)** with light petroleum/EtOAc **(**27**:**13**)** as a solvent system to afford **3 (**24 mg**).** Fraction ED-Q6 **(**1 g**)** was separated by Sephadex LH-20 with MeOH to yield fraction ED-Q6-3 **(**100 mg**)** and ED-Q6-5 **(**200 mg**).** Fraction ED-Q6-3 was further purified by CC using SiO_2 and gradient elution with light petroleum and EtOAc to give 10 fractions**.** ED-Q6-3-4 **(**40 mg**)** was isolated by Sephadex LH-20 with MeOH to give fraction ED-Q6-3-4-3, which was finally purified by SiO_2 prep-TLC with *n*-hexane-EtOAc **(**3**:**7**)** as mobile phase yielding **1 (**8 mg**).** Fraction ED-Q6-5 **(**200 mg**)** was further separated on a SiO_2 column using gradient elution with light petroleum and EtOAc to give **2 (**110 mg**).** Fraction ED-Q2 **(**50 mg**)** was chromatographed over SiO_2 using *n*-hexane/EtOAc as mobile phase **(**7**:**3, isocratic system**)** to provide fractions ED-Q2-1 to 8**.** Fraction ED-Q2-8 was further purified by prep-TLC on silica gel using *n*-hexane/EtOAc **(**6**:**4**)** to afford **4** (2 mg).

9,10-Dihydro-4-(4′-hydroxybenzyl)-2,5-imethoxyphenanthrene-1,7-diol（1）

Pale yellow amorphous.

$[\alpha]_D^{20}$: 0 (*c* 0.1, MeOH).

IR (KBr): ν_{max} 3412, 1612, 1512, 1463 cm^{-1}.
UV (MeOH) λ_{max} (log ε): 273 (5.48).
^{1}H NMR (400 MHz, in methanol-d_4): 2.04 (H, ddd, J = 14.5, 14.7, 4.0 Hz, H-10′), 2.38 (H, ddd, J = 14.7, 14.0, 4.0 Hz, H-9′), 2.61 (H, ddd, J = 14.0, 4.0, 2.4 Hz, H-9″), 3.20 (H, ddd, J = 14.5, 4.0, 2.4 Hz, H-10″), 3.62 (3H, s, C-5-OCH_3), 3.69 (3H, s, C-2-OCH_3), 3.73 (1H, d, J = 15.0 Hz, benzylic-CH_2), 3.81 (1H, d, J = 15.0 Hz, benzylic-CH_2), 6.36 (H, d, $J_{6,8}$ = 2.0 Hz, H-6), 6.38 (H, d, $J_{8,6}$ = 2.0 Hz, H-8), 6.47 (H, s, H-3), 6.61 (2H, d, $J_{3',2'}$ or $J_{5',6'}$ = 8.4 Hz, H-3′ and H-5′), 6.81 (2H, d, $J_{2',3'}$ or $J_{6',5'}$ = 8.4 Hz, H-2′ and H-6′).
^{13}C NMR (100 MHz, methanol-d_4): 24.4 (CH_2, C-10), 32.2 (CH_2, C-9), 40.4 (benzylic-CH_2), 55.3 (C, C-5-OCH_3), 56.2 (C, C-2-OCH_3), 98.6 (CH, C-6), 107.7 (CH, C8), 112.1 (CH, C-3), 115.8 (CH, C-3′and C-5′), 117.3 (C, C-4b), 127.0 (C, C-10a), 127.4. (C, C-8a), 131.0 (CH, C-2′ and C-6′), 132.7 (C, C-4a), 135.5 (C, C-1′), 141.0 (C, C-4), 144.4 (C, C-1), 147.1 (C, C-2), 156.0 (C, C-4′), 158.1 (C, C-7), 158.3 (C, C-5).
EIMS *m/z* (relative intensity): 378 $[M]^+$ (100), 363 $[M\text{-}15]^+$ (23), 347[M-31$^+$] (22), 335 [M-43$^+$] (11), 272 [M-106$^+$] (11), 107 [M-271$^+$] (19).
HREIMS *m/z*: 378.1478 (calcd. for $C_{23}H_{22}O_5$).

Enzyme preparation: PDE5 was obtained from rat lung tissues. Briefly, fresh tissue was cut into small sections and homogenized in 1 mL of Tris buffer (50 mM Tris, pH 7.5, 2 mM EDTA, 1 mM DTT and 1:100 of 100 mM phenylmethylsulfonyl fluoride). The homogenate was centrifuged at 14,000 rpm for 20 min at 4°C and the supernatant was used as a source of PDE5. The PDE5 inhibitor, sildenafil, was used to confirm that the supernatant contained abundant PDE5. All of the tissues were obtained from animals for which we received approval from the Institutional Animal Care and Use Committee (IACUC), Naresuan University (NU-AE570408). PDE6 was a gift from Prof. Dr Joseph A. Beavo, Department of Pharmacology, University of Washington, USA.

PDE inhibition assay: The method of Sonnenburg *et al*. [27] was used to assess PDE5 inhibition, with some modification. In brief, the reaction mixture comprised 20 µL of Reagent A (100 mM Tris-HCl (pH 7.5), 100 mM imidazole, 15 mM $MgCl_2$, 1.0 mg/mL BSA and 2.5 mg/mL snake venom), 20 µL of 10 mM EGTA, 20 µL of PDE5 solution, and 20 µL of test sample, or only solvent (5% DMSO) as a control. The reaction was started by adding 20 µL of 5 µM [^{3}H]cGMP (~50,000 cpm) and performed at 30°C for 40 min**.** Then, 100 µL of 50**%** DEAE resin was added to the reaction**.** After shaking for 10 min, the resin was allowed to settle **(**20 min**).** The supernatant was transferred to a fresh 100 µL of 50**%** DEAE resin, shaken for 10 min and the resin allowed to settle again**.** The supernatant **(**100 µL**)** was shaken with 200 µL of Microscint® 20 and tritium counted on a TopCount NXT **(**PerkinElmer, USA**)** for 2 h**.** The amount of PDE5 used hydrolyzed 20–25% of the substrate. Each experiment was duplicated in a 96-well plate. Inhibition of calmodulin-dependent PDE1 and PDE6 assays were modified from Sonnenburg *et al*. [27] and Huang *et al*. [28], respectively, similar to that for PDE5, but with Ca-calmodulin and histone in Reagent A. The PDE1 assay needed $CaCl_2$ (0.8 mM*)* and calmodulin *(*4 µg/mL) while histone *(*0.5 mg/mL*)* was needed for PDE6. In preliminary screening, 50 µg/mL of plant extracts was tested and pure compounds tested at 10 µM final conc. All samples were dissolved in DMSO and diluted with water. DMSO was limited to 1% in the final assay medium. In the case of > 80% PDE inhibition, the IC_{50} values were measured by the determinations of serial concentrations of the samples. The IC_{50} values were calculated using Prism software (Graph Pad Inc., San Diego, CA). Sildenafil was the positive control**.**

Acknowledgments - We thank Asst. Prof. Dr Anupan Kongbungkerd for *E. macrobulbon* identification and Dr Norman Scholfield for his help in manuscript preparation. This work was supported by Thailand Research Fund (TRF), National Research Council of Thailand (NRCT), the Royal Golden Jubilee Ph.D. Program and the Center of Excellence for Innovation in Chemistry (PERCH-CIC), Commission on Higher Education, Ministry of Education and Naresuan University.

References

[1] Diaz VA, Close JD. (**2010**) Male sexual dysfunction. *Primary Care: Clinics in Office Practice*, ***37***, 473-489.
[2] Hatzimouratidis K, Amar E, Eardley I, Giuliano F, Hatzichristou D, Montorsi F, Vardi Y, Wespes E. (**2010**) Guidelines on male sexual dysfunction: erectile dysfunction and premature ejaculation. *European Urology*, ***57***, 804-814.
[3] Wylie K, Kenney G. (**2010**) Sexual dysfunction and the ageing male. *Maturitas*, ***65***, 23-27.
[4] Shamloul R, Ghanem H. (**2013**) Erectile dysfunction. *The Lancet*, ***381***, 153-165.
[5] Selvin E, Burnett AL, Platz EA. (**2007**) Prevalence and risk factors for erectile dysfunction in the US. *The American Journal of Medicine*, ***120***, 151-157.
[6] Aytac I, McKinlay J, Krane R. (**1999**) The likely worldwide increase in erectile dysfunction between 1995 and 2025 and some possible policy consequences. *BJU International*, ***84***, 50-56.
[7] de Jesus C, Passos XS, Santos FB, Faria EB, Maciel T. (**2016**) The prevalence of erectile dysfunction in the Brazilian male population. *World Journal of Pharmacy and Pharmaceutical Sciences*, ***5***, 1903-1914.
[8] Brock G. (**2002**) Oral agents: first-line therapy for erectile dysfunction. *European Urology Supplements*, ***1***, 12-18.
[9] Montorsi F, Briganti A, Salonia A, Rigatti P, Burnett AL. (**2006**) Can phosphodiesterase type 5 inhibitors cure erectile dysfunction? *European Urology*, ***49***, 979-986.
[1] Diaz VA, Close JD. (**2010**) Male sexual dysfunction. *Primary Care: Clinics in Office Practice*, ***37***, 473-489.
[2] Hatzimouratidis K, Amar E, Eardley I, Giuliano F, Hatzichristou D, Montorsi F, Vardi Y, Wespes E. (**2010**) Guidelines on male sexual dysfunction: erectile dysfunction and premature ejaculation. *European Urology*, ***57***, 804-814.
[3] Wylie K, Kenney G. (**2010**) Sexual dysfunction and the ageing male. *Maturitas*, ***65***, 23-27.
[4] Shamloul R, Ghanem H. (**2013**) Erectile dysfunction. *The Lancet*, ***381***, 153-165.
[5] Selvin E, Burnett AL, Platz EA. (**2007**) Prevalence and risk factors for erectile dysfunction in the US. *The American Journal of Medicine*, ***120***, 151-157.
[6] Aytac I, McKinlay J, Krane R. (**1999**) The likely worldwide increase in erectile dysfunction between 1995 and 2025 and some possible policy consequences. *BJU International*, ***84***, 50-56.
[7] de Jesus C, Passos XS, Santos FB, Faria EB, Maciel T. (**2016**) The prevalence of erectile dysfunction in the Brazilian male population. *World Journal of Pharmacy and Pharmaceutical Sciences*, ***5***, 1903-1914.
[8] Brock G. (**2002**) Oral agents: first-line therapy for erectile dysfunction. *European Urology Supplements*, ***1***, 12-18.
[9] Montorsi F, Briganti A, Salonia A, Rigatti P, Burnett AL. (**2006**) Can phosphodiesterase type 5 inhibitors cure erectile dysfunction? *European Urology*, ***49***, 979-986.

[10] Moreland RB, Goldstein I, Kim NN, Traish A. (**1999**) Sildenafil citrate, a selective phosphodiesterase type 5 inhibitor: research and clinical implications in erectile dysfunction. *Trends in Endocrinology & Metabolism*, ***10***, 97-104.
[11] Burke RM, Evans JD. (**2012**) Avanafil for treatment of erectile dysfunction: review of its potential. *Vascular Health and Risk Management*, ***8***, 517-523.
[12] Rashid A. (**2005**) The efficacy and safety of PDE5 inhibitors. *Clinical Cornerstone*, ***7***, 47-55.
[13] Goldenberg MM. (**1998**) Safety and efficacy of sildenafil citrate in the treatment of male erectile dysfunction. *Clinical Therapeutics*, ***20***, 1033-1048.
[14] Cote R. (**2004**) Characteristics of photoreceptor PDE (PDE6): similarities and differences to PDE5. *International Journal of Impotence Research*, ***16***, S28-S33.
[15] Tuchinda P, Udchachon J, Khumtayeeporn K, Taylor WC. (**1989**) Benzylated phenanthrenes from *Eulophia nuda*. *Phytochemistry*, ***28***, 2463-2466.
[16] Tuchinda P, Udchachon J, Khumtaveeporn K, Taylor WC, Engelhardt LM, White AH. (**1988**) Phenanthrenes of *Eulophia nuda*. *Phytochemistry*, ***27***, 3267-3271.
[17] Fan C, Wang W, Wang Y, Qin G, Zhao W. (**2001**) Chemical constituents from *Dendrobium densiflorum*. *Phytochemistry*, ***57***, 1255-1258.
[18] Zhang G-N, Zhong L-Y, Bligh SA, Guo Y-L, Zhang C-F, Zhang M, Wang Z-T, Xu L-S. (**2005**) Bi-bicyclic and bi-tricyclic compounds from *Dendrobium thyrsiflorum*. *Phytochemistry*, ***66***, 1113-1120.
[19] Das SB, Dinh C, Shah S, Olson D, Ross A, Selvakumar P, Sharma RK. (**2007**) Calmodulin-dependent cyclic nucleotide phosphodiesterase (PDE1) splice variants from bovine cardiac muscle. *Gene*, ***396***, 283-292.
[20] Bhandari S, Kapadi A. (**1983**) A 9, 10-dihydrophenanthrene from tubers of *Eulophia nuda*. *Phytochemistry*, ***22***, 747-748.
[21] Bhandari S, Kapadi A, Mujumder P, Joardar M, Shoolery J. (**1985**) Nudol, a phenanthrene of the orchids *Eulophia nuda*, *Eria carinata* and *Eria stricta*. *Phytochemistry*, ***24***, 801-804.
[22] Shriram V, Kumar V, Kishor PK, Suryawanshi SB, Upadhyay AK, Bhat MK. (**2010**) Cytotoxic activity of 9, 10-dihydro-2, 5-dimethoxyphenanthrene-1, 7-diol from *Eulophia nuda* against human cancer cells. *Journal of Ethnopharmacology*, ***128***, 251-253.
[23] Blitzke T, Masaoud M, Schmidt J. (**2000**) Constituents of *Eulophia petersii*. *Fitoterapia*, ***71***, 593-594.
[24] Kshirsagar R, Kanekar Y, Jagtap S, Upadhyay S, Rao R, Bhujbal S, Kedia J. (**2010**) Phenanthrenes of *Eulophia ochreata* Lindl. *International Journal of Green Pharmacy*, ***4***, 147-152.
[25] Datla P, Kalluri MD, Basha K, Bellary A, Kshirsagar R, Kanekar Y, Upadhyay S, Singh S, Rajagopal V. (**2010**) 9, 9,10-Dihydro-2,5-dimethoxyphenanthrene-1,7-diol, from *Eulophia ochreata*, inhibits inflammatory signalling mediated by Toll-like receptors. *British Journal of Pharmacology*, ***160***, 1158-1170.
[26] Kovács A, Vasas A, Hohmann J. (**2008**) Natural phenanthrenes and their biological activity. *Phytochemistry*, ***69***, 1084-1110.
[27] Sonnenburg WK, Rybalkin SD, Bornfeldt KE, Kwak KS, Rybalkina IG, Beavo JA. (**1998**) Identification, quantitation, and cellular localization of PDE1 calmodulin-stimulated cyclic nucleotide phosphodiesterases. *Methods*, ***14***, 3-19.
[28] Huang D, Hinds TR, Martinez SE, Doneanu C, Beavo JA. (**2004**) Molecular determinants of cGMP binding to chicken cone photoreceptor phosphodiesterase. *Journal of Biological Chemistry*, ***279***, 48143-48151.

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Vol. 12
No. 1
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Bioactive Glucitol-Core Containing Gallotannins and other Phytochemicals from Silver Maple (*Acer saccharinum*) Leaves

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Received: October 27th, 2016; Accepted: November 21st, 2016

In the course of our group's investigation of members of the maple (*Acer*) genus, a series of glucitol-core containing gallotannins (GCGs) were isolated and identified (by NMR and HREISMS). Among higher plants, only certain maple species are known to produce GCGs, compounds with potential nutraceutical and cosmetic applications due to their reported antioxidant, antidiabetic, anti-α-glucosidase, anti-glycation, anticancer, and skin health promoting effects. Herein, we sought to investigate whether the previously un-investigated silver maple (*Acer saccharinum*) species was also a source of GCGs. Nine phenolic compounds, including six GCGs, were identified (by HPLC-DAD analyses using previously isolated standards) as ginnalins A-C (**1-3**), maplexins B, D, and F (**4-6**), methyl syringate (**7**), methyl gallate (**8**), and 3-methoxy-4-hydroxyphenol-1-β-D-(6-galloyl)-glucopyranoside (**9**). In addition, one sesquiterpenoid, namely, pubineroid A (**10**), was isolated and identified (by NMR).

Keywords: Silver maple, *Acer saccharinum*, Phenolic, Glucitol-core containing gallotannins (GCGs).

The maple (*Acer*) genus contains thirteen species which are native to North America including the sugar (*A. saccharum*), red (*A. rubrum*), and silver (*A. saccharinum*) maples. Apart from sap which is primarily consumed as maple syrup [1], other maple plant parts (leaves, bark, etc.) have also been used as traditional medicines by the indigenous people of North America [2].

Our group has reported on the isolation and structure elucidation (by NMR and HREISMS) of over 70 phytochemicals, from the sugar, red, and sycamore (*A. pseudoplatanus*) maples [3-7]. Among these species, only the red maple yielded glucitol-core containing gallotannins (GCGs) [3, 4, 6]. These compounds have been reported, by our group [8-15], and others [16-20], to show a wide range of *in vitro* and *in vivo* biological effects including, antioxidant, anti-diabetic, anti-α-glucosidase, anti-glycation, anticancer, and skin-health promoting effects. Given the potential nutraceutical and cosmetic applications of these compounds, as well as our access to the aforementioned maple-derived phytochemical standards, herein, we sought to investigate whether the silver maple was a source of GCGs.

Using authentic standards (previously isolated by our laboratory), nine phenolics, including six GCGs, were identified from silver maple leaves as ginnalins A-C (**1-3**), maplexins B, D, and F (**4-6**), methyl syringate (**7**), methyl gallate (**8**), and 3-methoxy-4-hydroxyphenol-1-β-D-(6-galloyl)-glucopyranoside (**9**) (by HPLC-DAD analyses, see Supplementary data, Table S1) [6, 7]. In addition, one sesquiterpenoid, namely, pubinernoid A (**10**), was isolated and identified (by NMR; see Supplementary data, Figures S1 and S2). Pubinernoid A was obtained as a white amorphous powder and its ^{1}H and ^{13}C NMR data were consistent with the literature [7]. The chemical structures of the compounds identified from silver maple leaves are shown in Figure 1.

In summary, this is the first reported phytochemical investigation of the silver maple which led to the identification of nine phenolic compounds (six GCGs and three phenolics) and a sesquiterpenoid. Our findings add to the growing body of data of phytochemicals identified from the maple (*Acer*) genus, and more importantly, about which maple species produce GCGs, a promising class of bioactive plant polyphenols with nutraceutical and cosmetic applications.

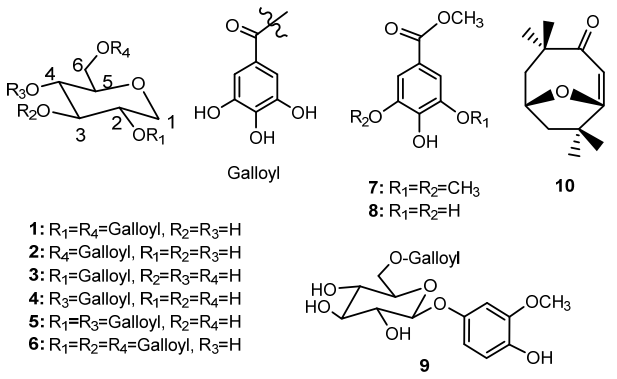

Figure 1: Chemical structures of compounds **1-10**.

Experimental

General: High performance liquid chromatography (HPLC) analyses were performed on a Hitachi Elite LaChrom system consisting of a L-2130 pump, a L-2200 autosampler, and a L-2455 Diode Array Detector. All ^{1}H and ^{13}C NMR spectra were acquired on a Bruker 300 MHz spectrometer using $MeOD_4$ as solvent.

Plant material: Silver maple (*Acer saccharinum*) leaves were collected on the Kingston campus of the University of Rhode Island (Kingston, RI, USA) in the summer of 2010 and were botanically authenticated by Mr. J. Peter Morgan. Voucher specimens (16JPM1-APS6310) are deposited in the Heber-Youngken Garden and Greenhouse at the College of Pharmacy, University of Rhode Island.

Extraction of plant material: Air-dried silver maple leaves (1007.8 g) were macerated for 9 days with MeOH (5.5 L× 3). The filtrate

was concentrated under reduced pressure to afford a dried methanol extract (250 g). This was suspended in distilled water and successively partitioned to yield *n*-hexanes (67.5 g), ethyl acetate (EtOAc; 28.7 g), and *n*-butanol (37.5 g) extracts after solvent removal *in vacuo*. The EtOAc extract, which contained the highest total polyphenol content (58.8% GAEs based on the Folin-Ciocalteau assay, see Supplementary data, Table S2), was selected for further phytochemical investigation (described below).

Identification of compounds 1-9 in the EtOAc extract of silver maple leaves by HPLC-DAD analyses: The EtOAc extract (5 mg) was analyzed on a Phenomenex C_{18} column (250 × 4.6 mm, i.d. 5 μm). The injection volume was 10 μL and the flow rate was 0.75 mL/min. A gradient mobile phase solvent system consisting of solvent A (0.1% aqueous trifluoroacetic acid) and solvent B (MeOH) was used as follows: 0-50 min, 5%-46% B. The HPLC profile was monitored at 220 nm and compounds **1-9** were identified by comparison of their retention times to authentic standards previously isolated by our laboratory from red maple [3, 4, 6].

Isolation of compound 10 from the EtOAc extract of silver maple leaves: Having identified the major constituents in the EtOAc extract which were phenolics (**1-9**), we attempted to isolate the (minor) non-phenolic constituent/s (based on UV absorbance; see Supplementary Data, Table S1) present in the extract. Briefly, the EtOAc extract (28.2 g) was purified on a silica gel column eluted with a gradient solvent system of chloroform/MeOH (50:1 - 1:1; v/v) to afford 11 fractions (F1 - F11). Fraction F3 (440 mg) was chromatographed on a Sephadex LH-60 column eluting with MeOH to afford 6 sub-fractions (13A - 13F). Fraction 13B (340 mg) was chromatographed on a silica gel column eluting with a gradient solvent system of hexane/dichloromethane (10:0 - 0:10; v/v) to afford 5 fractions (16A - 17C). Fraction 16C (15 mg) was further purified by C_{18} reversed-phase semi-preparative HPLC with a mobile phase consisting of MeOH and 0.1% aqueous trifluoroacetic acid (60:40; v/v) to afford compound **10** (3.4 mg, tR =12.2 min).

Supplementary data: Supplementary data are available for this article and available free of charge.

References

[1] Ball DW. (**2007**) The chemical composition of maple syrup. *Journal of Chemical Education*, ***84***, 1647-1650.

[2] Arnason T, Hebda RJ, Johns T. (**1981**) Use of plants for food and medicine by Native peoples of eastern Canada. *Canadian Journal of Botany*, ***59***, 2189-2325.

[3] Wan C, Yuan T, Li L, Kandhi V, Cech NB, Xie M, Seeram NP. (**2012**) Maplexins, new α-glucosidase inhibitors from red maple (*Acer rubrum*) stems. *Bioorganic Medicinal Chemistry Letters*, ***22***, 597-600.

[4] Yuan T, Wan C, Liu K, Seeram NP. (**2012**) New maplexins F-I and phenolic glycosides from red maple (*Acer rubrum*) bark. *Tetrahedron*, ***68***, 959-964.

[5] Yuan T, Wan C, González-Sarrías A, Kandhi V, Cech NB, Seeram NP. (**2011**) Phenolic glycosides from sugar maple (*Acer saccharum*) bark. *Journal of Natural Products*, ***74***, 2472-2476.

[6] Zhang Y, Ma H, Yuan T, Seeram NP. (**2015**) Red maple (*Acer rubrum*) aerial parts as a source of bioactive phenolics. *Natural Product Communications*, ***10***, 1409-1412.

[7] Zhang L, Tu Z, Yuan T, Ma H, Niesen DB, Wang H, Seeram NP. (**2015**) New gallotannin and other phytochemicals from sycamore maple (*Acer pseudoplatanus*) leaves. *Natural Product Communications*, ***10***, 1977-1980.

[8] Seeram NP, Xu J, Li L, Slitt A. (**2012**) Mining red maple (*Acer rubrum*) trees for novel therapeutics to manage diabetes. *Medicine and Health, Rhode Island*, ***95***, 283-284.

[9] Ma H, Wang L, Niesen DB, Cai A, Cho BP, Tan W, Gu Q, Xu J, Seeram NP. (**2015**) Structure activity related, mechanistic, and modeling studies of gallotannins containing a glucitol-core and α-glucosidase. *RSC Advances*, ***5***, 107904-107915.

[10] Ma H, Liu W, Frost L, Kirschenbaum LJ, Dain JA, Seeram NP. (**2016**) Glucitol-core containing gallotannins inhibit the formation of advanced glycation end-products mediated by their antioxidant potential. *Food & Function*, ***7***, 2213-2222.

[11] Ma H, Xu J, Guo L, Lu W, Seeram N. (**2016**) Bioactive glucitol-core containing gallotannins from red maple (*Acer rubrum*) inhibit melanogenesis via down-regulation of tyrosinase and melanogenic gene expression in B16F10 melanoma cells. American Chemical Society, 252nd ACS National Meeting & Exposition, Philadelphia, PA, United States, August 21-25, 2016, AGFD-12.

[12] González-Sarrías A, Ma H, Edmonds ME, Seeram NP. (**2013**) Maple polyphenols, ginnalins A-C, induce S-and G2/M-cell cycle arrest in colon and breast cancer cells mediated by decreasing cyclins A and D1 levels. *Food Chemistry*, ***136***, 636-642.

[13] González-Sarrías A, Li L, Seeram NP. (**2012**) Effects of maple (*Acer*) plant part extracts on proliferation, apoptosis and cell cycle arrest of human tumorigenic and non-tumorigenic colon cells. *Phytotherapy Research*, ***26***, 995-1002.

[14] Apostolidis E, Li L, Kang B, Lee CM, Seeram NP. (**2012**) Seasonal influence on phenolic-mediated antihyperglycemic properties of Canadian sugar and red maple leaves using *in vitro* assay models. *Food Science and Biotechnology*, ***21***, 753-760.

[15] González-Sarrías A, Yuan T, Seeram NP. (**2012**) Cytotoxicity and structure activity relationship studies of maplexins A-I, gallotannins from red maple (*Acer rubrum*). *Food and Chemical Toxicology*, ***50***, 1369-1376.

[16] Honma A, Koyama T, Yazawa K. (**2011**) Anti-hyperglycaemic effects of the Japanese red maple *Acer pycnanthum* and its constituents the ginnalins B and C. *Journal of Enzyme Inhibition and Medicinal Chemistry*, ***26***, 176-180.

[17] Bi W, Shen J, Gao Y, He C, Peng Y, Xiao P. (**2016**) Ku-jin tea (*Acer tataricum* subsp. *ginnala* or *A. tataricum* subsp. *theiferum*), an underestimated functional beverage rich in antioxidant phenolics. *Journal of Functional Foods*, ***24***, 75-84.

[18] Kamori A, Kato A, Miyawaki S, Koyama J, Nash RJ, Fleet GW, Miura D, Ishikawa F, Adachi I. (**2016**) Dual action of acertannins as potential regulators of intracellular ceramide levels. *Tetrahedron: Asymmetry*. doi.org/10.1016/j.tetasy.2016.09.006

[19] Lu RL, Hu FL, Xia T. (**2010**) Activity-guided isolation and identification of radical scavenging components in Gao-Cha tea. *Journal of Food Science*, ***75***, H239-H243.

[20] Honma A, Koyama T, Yazawa K. (**2010**) Anti-hyperglycemic effects of sugar maple *Acer saccharum* and its constituent acertannin. *Food Chemistry*, ***123***, 390-394.

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Pterostilbene and Its Glucoside Induce Type XVII Collagen Expression

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Received: October 28th, 2016; Accepted: November 9th, 2016

The glycosylation of pterostilbene by cultured plant cells of *Phytolacca americana* gave pterostilbene 4'-*O*-β-D-glucoside. Both pterostilbene and its 4'-*O*-β-D-glucoside induced type XVII collagen expression in the EpiDermFT EFT-400 human skin cell model. Pterostilbene 4'-*O*-β-D-glucoside strongly induced type XVII collagen expression rather than pterostilbene.

Keywords: Glycosylation, *Phytolacca americana*, Pterostilbene, Collagen expression.

Recently, it has been reported that the aging of hair follicle stem cells is triggered by DNA damage induced type XVII collagen (COL17A1) proteolysis [1]. Once aged hair follicle stem cells are activated during the hair cycle, they leave the niche and terminally differentiate into epidermal keratinocytes and are then eliminated from the skin surface.

Glycosylation occurs readily in plant cells, i.e., many kinds of secondary metabolites such as saponins and anthocyanins are produced in the form of glycosides in higher plants. Many of these secondary metabolites exert specific physiological activities and have been widely used in folk medicines [2]. Therefore, the glycosides of natural compounds are of pharmaceutical importance. Although studies on the production of glycosides of stilbenes have been reported [3-5], there are few reports on their physiological activities.

Herein, we report, for the first time, the rapid induction of type XVII collagen (COL17A1) expression in hair follicle stem cells by a pterostilbene glucoside.

The level of type XVII collagen (COL17A1) was examined in the cells of the EpiDermFT EFT-400 human skin model treated with pterostilbene (**1**) and pterostilbene 4'-*O*-β-D-glucoside (**2**) (Figure 1), which had been prepared by glycosylation of pterostilbene with cultured cells of *P. americana*. One week after treatment of the cells of the EpiDermFT EFT-400 human skin model with pterostilbene (**1**), the COL17A1 level was increased in the cells (Figure 2). The level was enhanced dose dependently; the COL17A1 level of the 50 μM pterostilbene-treated cells (1.35) was higher than that of the 25 μM pterostilbene-treated cells (1.13). The level of the 50 μM pterostilbene-treated cells was elevated in the cells by ca. 1.35-fold, compared with that of control cells, but the 100 μM pterostilbene-treatment significantly reduced the COL17A1 level (0.69), probably due to the cytotoxicity of the pterostilbene.

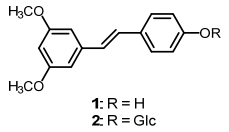

Figure 1: Chemical structures of pterostilbene (**1**) and pterostilbene 4'-β-D-glucoside (**2**).

A similar tendency was found in the case of pterostilbene 4'-*O*-β-D-glucoside (**2**) treatment of the EpiDermFT EFT-400 human skin cell model. At one week after treatment of the cells with pterostilbene 4'-*O*-β-D-glucoside (**2**), the COL17A1 level was elevated (Figure 3). The enhancement of the COL17A1 level was dose dependent; the COL17A1 level of the 50 μM treated cells (1.61) was higher than that of the 25 μM pterostilbene 4'-*O*-β-D-glucoside-treated cells (1.36). The COL17A1 level of the 50 μM pterostilbene 4'-*O*-β-D-glucoside-treated cells was elevated by ca. 1.6-fold, compared

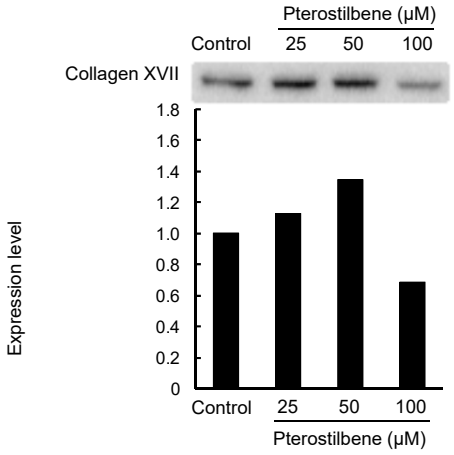

Figure 2: Effect of pterostilbene (**1**) on the expression of COL17A1 in EFT-400 3D human skin cell model.

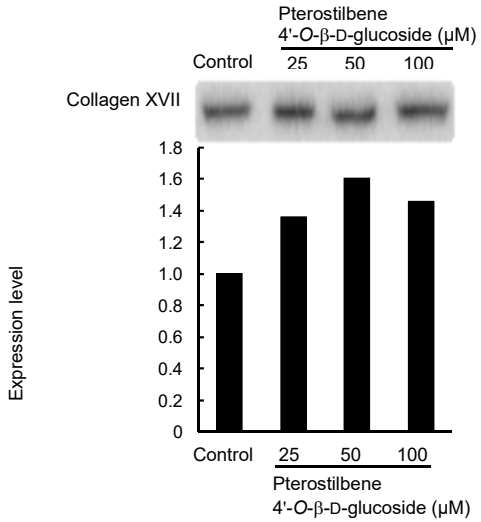

Figure 3: Effect of pterostilbene 4'-*O*-β-D-glucoside (**2**) on the expression of COL17A1 in EFT-400 3D human skin cell model.

with that of the control cells. However, the COL17A1 level of the 100 μM treated cells (1.46) was slightly decreased, but was higher than that of the 25 μM pterostilbene 4'-*O*-β-D-glucoside-treated cells.

Thus, both pterostilbene and pterostilbene 4'-*O*-β-D-glucoside, which had been prepared by glucosylation of pterostilbene with cultured cells of *P. americana*, quickly induced type XVII collagen (COL17A1) expression in cells of the EpiDermFT EFT-400 human skin model. This is the first report on the induction of COL17A1 expression by plant polyphenols. The COL17A1 level of pterostilbene 4'-*O*-β-D-glucoside-treated cells was higher than that of pterostilbene-treated cells. Stilbenes and, particularly, their glucosides may be useful for treatment of hair thinning.

Experimental

General: Pterostilbene used as a substrate was purchased from Wako Pure Chemicals Co. and was used without further purification. The cultured plant cells of *Phytolacca americana* were sub-cultured at 4-week intervals on a solid medium containing 2% glucose, 1 ppm 2,4-dichlorophenoxyacetic acid, and 1% agar (adjusted to pH 5.7) in the dark. A suspension culture was started by transferring 20 g of the cultured cells into 300 mL of liquid Murashige and Skoog medium in a 500 mL-conical flask.

Biotransformation procedure: The suspension plant cells (20 g) were incubated in conical flasks for 2 days. The cultured cells in the stationary growth phase were used for the experiments. After the cultivation period, 10 mg of the substrate was added. The biotransformation was performed by incubating the mixture at 25°C on a rotary shaker for 2 days. The culture medium and cells were separated by filtration. The culture medium was extracted with ethyl acetate. The cells were extracted (x3) by homogenization with methanol. The methanol fraction was concentrated and partitioned between H_2O and ethyl acetate. The ethyl acetate fractions were concentrated and analyzed by HPLC. The yield of product was determined on the basis of the peak area from HPLC using a calibration curve prepared with authentic glycosides.

Western blot analyses: EpiDermFT EFT-400 human skin culture cells (MatTek Corp., Ashland, MA, USA) were cultivated at 37°C in a 10% CO_2 atmosphere for 24 h before being mixed with 25, 50, and 100 μM of either pterostilbene or pterostilbene 4'-*O*-β-D-glucoside. After one week of incubation with the compounds, the cells were lysed, sonicated, and analyzed by Western blot analysis using anti-collagen XVII antibody (Abcam, Cambridge, MA, USA) and anti-GAPDH antibody (standard) (Sigma-Aldrich, St. Louis, MO, USA). The protein solution was fractionated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. Membranes containing fractionated proteins were blotted with anti-collagen XVII antibody. Membranes were then blotted with horseradish peroxidase conjugated second antibody and the immunoreactive protein bands were visualized by enhanced chemiluminescence.

References

[1] Matsumura H, Mohri Y, Binh NT, Morinaga H, Fukuda M, Ito M, Kurata S, Hoejimakers J, Nishimura EK. (**2016**) Hair follicle aging is driven by transepidermal elimination of stem cells via COL17A1 proteolysis. *Science*, ***351***, aad4395-1-13.

[2] Mastelic J, Jerkovic I, Vinkovic M, Dzolic Z, Vikic-Topic D. (**2004**) Synthesis of selected naturally occurring glucosides of volatile compounds. Their chromatographic and spectroscopic properties. *Croatica Chemica Acta*, ***77***, 491-500.

[3] Sato D, Shimizu N, Shimizu Y, Akagi M, Eshita Y, Ozaki S, Nakajima N, Ishihara K, Masuoka N, Hamada H, Shimoda K, Kubota N. (**2014**) Synthesis of glycosides of resveratrol, pterostilbene, and piceatannol, and their anti-oxidant, anti-allergic, and neuroprotective activities. *Bioscience Biotechnology and Biochemistry*, ***78***, 1123–1128.

[4] Shimoda K, Kubota N, Uesugi D, Tanigawa M, Hamada H. (**2016**) Hydroxylation and glycosylation of phenylpropanoids by cultured cells of *Phytolacca americana*. *Natural Product Communications*, ***11***, 197-198.

[5] Uesugi D, Hamada H, Shimoda K. (**2016**) Glycosylation of *trans*-resveratrol by cultured plant cells under illumination of LEDs. *Natural Product Communications*, ***11***, 199-200.

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Metabolites from *Penicillium* sp. Associated with *Paris polyphylla*

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Received: October 4th, 2016; Accepted: November 15th, 2016

One new gentisyl alcohol derivative and seven known compounds were isolated from the culture of *Penicillium* sp. Their chemical structures were elucidated by extensive spectroscopic analysis. Compounds **1, 4,** and **6** inhibited *Bacillus subtilis*, *Staphylococcus aureus*, and *Escherichia coli* with MICs of 32-128 μg/mL.

Keywords: *Penicillium* sp., Metabolite, Endophyte, Antimicrobial activity.

Paris polyphylla Sm., family Trilliaceae, a perennial herb mainly distributed in the Provinces of Yunnan, Sichuan and Guizhou in southwest China, has been used as an important and traditional Chinese medicine (TCM) for the treatment of injuries from falls, fractures, contusions, bleeding and immunity adjustment [1,2]. Endophytic micro-organisms live inside plants without causing them any apparent damage. Recently, endophytic microorganisms have attracted attention because they can produce bioactive compounds of biotechnological interest. The endophytic microorganisms in *P. polyphylla* are under scrutiny because they may be responsible for producing the bioactive metabolites associated with the plant [3]. As part of our program to discover structurally unique and biologically active secondary metabolites from fungi associated with medicinal plants, a *Penicillium* sp. isolated from the rhizosphere soil of *P. polyphylla* from Mile, Yunnan Province, China has drawn our interest. One new metabolite along with seven known compounds were isolated and determined as terrestrol I (**1**), (*E*)-ascladiol (**2**) [4], (*Z*)-ascladiol (**3**) [4], gentisyl alcohol (**4**) [5], 3-hydroxybenzyl alcohol (**5**) [6], (22*E*,24*R*)-ergosta-5α,6α-epoxide-8,22-diene-3β,7α-diol (**6**) [7], 5α,8α-epidioxy-ergosta-6,22-dien-3β-ol (**7**) [7], and 7-hydroxy-4,6-dimethylphthalide (**8**) [8].

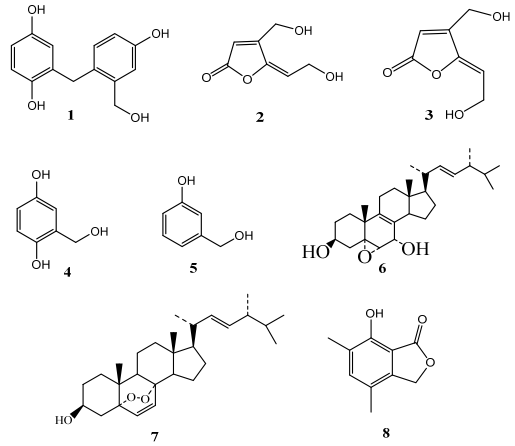

Figure 1: Structures of compounds **1**-**8**.

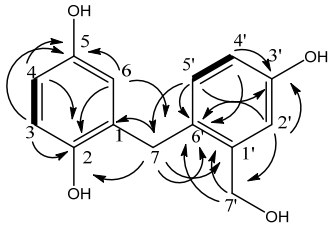

Figure 2: ^{1}H-^{1}H COSY correlations and the selected HMBC correlations of compound **1**.

Table 1: ^{13}C NMR (125 MHz) and ^{1}H NMR (500 MHz) data for compound **1** in $CDCl_3$.

Pos.	δ_C	δ_H	Pos	δ_C	δ_H
1	130.7		1′	130.8	
2	149.7		2′	116.3	6.93 (s, H-2′)
3	117.4	6.63 (d, *J*=8.4 Hz)	3′	157.6	
4	115.1	6.48 (dd, *J*=8.4, 3.2 Hz)	4′	115.8	6.63 (d, *J*=8.8 Hz)
5	151.9		5′	133.1	6.93(d, *J*=8.8 Hz)
6	118.3	6.28 (d, *J*=3.2 Hz, H-6)	6′	142.5	
7	33.0	3.83 (s)	7′	63.7	4.53 (s)

The HR-ESI-MS analysis of compound **1** revealed a sodium adduct at *m/z* 269.0791[M+Na]$^{+}$ (calcd for $C_{14}H_{14}O_4Na$ 269.0790). The molecular formula was determined as $C_{14}H_{14}O_4$, which was further corroborated by the NMR data. The δ 6.0-7.0 region of the ^{1}H NMR spectrum of **1** (Table 1) showed resonances for three aromatic protons of the 1, 2, 5-trisubstituted phenyl ring A, at δ 6.28 (d, *J*=3.2 Hz, H-6), 6.48 (dd, *J*=8.4, 3.2 Hz, H-4), and 6.63 (d, *J*=8.4 Hz, H-3), as well as three aromatic protons of 1′, 3′, 6′-trisubstituted phenyl ring B at δ 6.63 (d, *J*=8.8 Hz, H-4′), 6.93 (d, *J*=8.8 Hz, H-5′), and 6.93 (s, H-2′). In addition, two singlets were observed at δ 3.83 and 4.53 for CH_2 at H-7 and H-7′. By comparing NMR data, the structure of compound **1** was shown to be similar to the gentisyl alcohol derivatives isolated previously from *Penicillium* [9]. The connection of ring A to ring B through C-7 was confirmed by the HMBC correlations from H-7 to C-1, 2, 1′, and 6′, and from H-5′ and H-6 to C-7; C-7′ was connected to C-1′ based on the HMBC correlations from H-7′ to C-1′ and C-6′, and from H-2′ to C-7′. Three hydroxyls connected to C-2, 5, and 3′ were determined by the HMBC correlations from H-3, 4, and 6 to C-2, and from H-2′, H-4′ and H-5′ to C-3. Other HMBC correlations also determined the structure of the gentisyl alcohol derivative **1** (Figure 2).

Table 2: Antimicrobial activities (MICs, μg/mL) of compounds **1**, **4**, and **6** from *Penicillium* sp.

Strain	**1**	**4**	**6**	N	K
B. subtilis	64	32	64		8
F. oxysporum	—	—	—	1	
F. solani	—	—	—	1	
S. aureus	64	64	—		16
M. albicans	—	—	—	1	
E. coli	—	128	—		64

—: MICs > 512 μg/mL; N: nystatin; K: kanamycin.

The antimicrobial activities (*Bacillus subtilis*, *Fusarium oxysporum*, *Fusarium solani*, *Staphylococcus aureus*, *Monilia albicans*, *Escherichia coli*) of compounds **1**, **4**, and **6** were evaluated. The MICs for these compounds are listed in Table 2.

Experimental

General experimental procedures: Silica gel (200–300 mesh; Qingdao Marine Chemical Group Co., Qingdao, China), and Sephadex LH-20 (GE Healthcare Co., Buckinghamshire, UK) were used for column chromatography (CC). 1D and 2D NMR spectra were obtained on a Bruker AVANCE 500 MHz NMR instrument (Bruker, Karlsruhe, Germany). MS were recorded with an Agilent G3250AA (Agilent, Santa Clara, USA).

Biological material and cultivation of strain Y-12: The endophytic fungus was isolated by using PDA medium from *Paris polyphylla* collected at Mile, Yunnan, China, and was identified as *Penicillium* sp. by ITS gene sequence. The strain is preserved at the School of Chemical Science and Technology, Yunnan University, Kunming, China. The strain was cultured in 500 mL Erlenmeyer flasks each containing 200 mL potato dextrose broth (PDB) seed culture (1000 mL water, 20 g glucose, 200 g potato). After 3 days of incubation at 28°C on a rotary shaker (125 r.p.m.), 10 mL portions of the culture were inoculated into 1000 mL Erlenmeyer flasks, each containing 250 mL PDB fermentation medium for 7 days at 28°C on a rotary shaker (150 r.p.m.). The fermented whole broth (30 L) was filtered through hospital gauze to separate the culture broth and mycelia.

Antibacterial assays: For the *in vitro* antimicrobial test, PDB was used as an incubation medium for fungi and LB medium for bacteria. The final volume of each well was 100 μL. Aliquots (5 μL) of the metabolite solutions in dimethyl sulfoxide (DMSO) were added into the 96-well sterilized microplates, and their final concentrations ranged from 512 to 1 μg/mL using a twofold serial dilution method. Wells containing kanamycin and nystatin (Taicheng Pharmaceutical Co., Ltd, Guangdong, China) were introduced as the positive controls. The minimum inhibitory concentrations of the metabolites that completely inhibited pathogenic bacteria growth were defined as the MICs.

Extraction and isolation of compounds: The fermentation broth was extracted 3 times with EtOAc to give a crude residue (25 g). This was separated by silica gel CC with $CHCl_3$/MeOH (1:0-0:1) to give 5 fractions (Fr. 1 to Fr. 5). In the further purification, silica gel CC with light petroleum/ethyl acetate, and $CHCl_3$/MeOH were used. Finally, compound **1** (14.5 mg) was obtained from Fr. 2. The mycelium extract (12.8 g) was separated by silica gel CC with $CHCl_3$/MeOH to give 5 fractions (Fr. 1 to Fr. 5), Compound **8** (3.4 mg) was isolated from Fr. 2-1 by silica gel CC with light petroleum/ethyl acetate, and $CHCl_3$/MeOH. Compounds **2** (2 mg), **3** (2 mg), **4** (9.2 mg), and **5** (12.4 mg) were isolated from Fr. 2-3 using silica gel CC with $CHCl_3$/MeOH and Sephadex LH-20 (MeOH). Compounds **6** (6.7 mg), and **7** (10.3 mg) were obtained from Fr. 2-2 using silica gel CC with $CHCl_3$/MeOH and Sephadex LH-20 (MeOH).

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^1H and ^{13}C NMR: Table 1.

HR-ESIMS *m/z*: 269.0791 $[M+Na]^+$ (calcd for $C_{14}H_{14}O_4Na$ at 269.0790).

Acknowledgments - This project was supported by the National Natural Science Foundation of China (81360480, 81460536, 81560571), and Yunnan Provincial Department of Education Science Research Fund (No. 2015Y011, 2016ZZX003).

References

[1] Chen CX, Zhou J, Zhang YT, Zhao YY. (**1990**) Steroids saponins of aerial parts of *Paris polyphylla* var. yunnanensis. *Acta Botanica Yunnanica*, ***12***, 323-329.

[2] Matsuda H, Pongpiriyadacha Y, Morikawa T, Kishi A, Kataoka S, Yoshikawa M. (**2003**) Protective effects of steroid saponins from *Paris polyphylla* var. *yunnanensi* on ethanol- or indomethacin-induced gastric mucosal lesions in rats: structural requirement for activity and mode of action. *Bioorganic & Medicinal Chemistry Letters*, ***13***, 1101-1106.

[3] (a) Huang Y, Zhao J, Zhou L, Wang M, Wang J, Li X, Chen Q. (**2009**) Antimicrobial compounds from the endophytic fungus *Fusarium* sp. Ppf4 isolated from the medicinal plant *Paris polyphylla* var. *yunnanensis. Natural Product Communications*, ***4***, 1455-1458; (b) Zhao J, Shan T, Huang Y, Liu X, Gao X, Wang M, Jiang W, Zhou L. (**2009**) Chemical composition and *in vitro* antimicrobial activity of the volatile oils from *Gliomastix murorum* and *Pichia guilliermondii*, two endophytic fungi in *Paris polyphylla* var. *yunnanensis. Natural Product Communications*, ***4***, 1491-1496; (c) Zhang Y, Zhao J, Wang J, Shan T, Mou Y, Zhou L, Wang J. (**2011**) Chemical composition and antimicrobial activity of the volatile oil from *Fusarium tricinctum*, the endophytic fungus in *Paris polyphylla* var. *yunnanensis*. *Natural Product Communications*, ***6***, 1759-1762; (d) Yang Y, Yang SC, Zhao J, Udikeri S, Liu T. (**2015**) Microbial diversity in *Paris polyphylla* var. *yunnanensis* rhizomes of varying ages. *Genetics and Molecular Research: GMR*, ***14***, 17612-17621.

[4] Sekiguchi J, Shimamoto T, Yamada Y, Gaucher GM. (**1983**) Patulin biosynthesis: enzymatic and nonenzymatic transformations of the mycotoxin (*E*)-ascladiol. *Applied and Environmental Microbiology*, ***45***, 1939-1942.

[5] Liu YF, Gan ML, Yang YH, Liu YS, Guan Y, Xiao CL. (**2014**) Antibacterial metabolites from *Penicillium* sp. I09F 484. *Chinese Pharmaceutical Journal*, ***49***, 632-635.

[6] Chen LJ, Shi HZ, Hu KF. (**2010**) Antifungal activity and chemical component analysis of volatile compounds from *Penicillium vulpinum*. *Journal of Fungal Research*, ***8***, 231-233, 244.

[7] Li W, Yang XQ, Yang YB, Qin SH, Li QL, Zhao LX, Ding ZT. (**2015**) A new natural nucleotide and other antibacterial metabolites from an endophytic *Nocardia* sp. *Natural Product Research*, ***29***, 132-136.

[8] Miao CP, Hu J, Zhai YZ, Song F, Xuan QC, Chen YW, Wu SH. (**2012**) Identification and secondary metabolites of endophytic fungus PR20 from *Paeonia delavayi*. *Natural Product Research and Development*, ***24***, 1339-1342.

[9] Chen L, Fang YC, Zhu TJ, Gu QQ, Zhu WM. (**2008**) Gentisyl alcohol derivatives from the marine-derived fungus *Penicillium terrestre. Journal of Natural Products*, ***71***, 66-70.

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Ferulic Acid Esters of Oligo-glucose from *Allium macrostemon*

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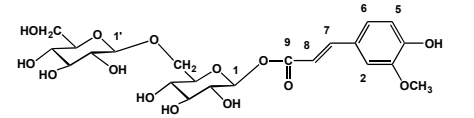
Received: September 9th, 2016; Accepted: November 1st, 2016

Two new ferulic acid esters of oligo-glucose, 1-*O*-*(E)*-feruloyl-β-D-gentiobioside (**1**) and 1-*O*-(*E*)-feruloyl-{β-D-glucopyranosyl (1→6)-[β-D-glucopyranosyl (1→2)]}-β-D-glucopyranoside (allimacronoid D, **2**) were isolated together with 1-*O*-(*E*)-feruloyl-β-D-glucopyranoside (**3**) and *trans*-ferulic acid (**4**) from the leaves of *Allium macrostemon* Bunge. The chemical structures were elucidated based on the analyses of the spectroscopic and chemical data.

Keywords: Amaryllidaceae, *Allium macrostemon*, Allimacronoid, Ferulic acid, Glucoside.

Allium macrostemon Bunge (Amaryllidaceae), known as wild onion, is widely distributed in East Asian countries. Some steroidal saponins [1] in the bulb showed various activities with the potential for treatment of acute myocardial ischemia, hyperglycemia, hyperlipidemia and visceral obesity [2-4]. An antidepressant-like activity of the aqueous extract of the plant was also reported [5]. Nakane *et. al.* reported the isolation and HPLC analysis of the flavonoids in the leaves of *A. macrostemon* [6]. Recently, Usui *et al*. also isolated ferulic acid esters with oligo-glucose (allimacronoids A-C) [7], 1-*O*-(*E*)-caffeoyl-β-D-sophoroside [8] and kaempferol glycosides [8] from the leaves of this species. In the present study of the phenolic compounds of the leaves of *A. macrostemon*, two new ferulic acid esters with oligo-glucose (**1** and **2**) were isolated, together with two known phenolics (**3** and **4**). The chemical structures of these compounds were elucidated from their spectroscopic and chemical data. The known phenolics **3** and **4**, were identified as 1-*O*-(*E*)-feruloyl-β-D-glucopyranoside (**3**) [9] and *trans*-ferulic acid (**4**) [10], by comparison of their spectroscopic data (Figure 1).



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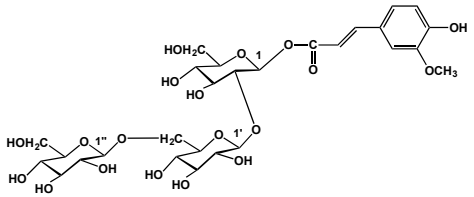

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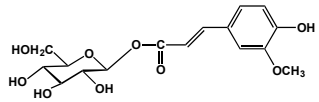

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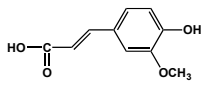

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Figure 1: Chemical structures of **1**-**4**.

Compound **1** gave a quasi-molecular ion peak at *m/z* 517.1584 $[M-H]^-$, which corresponded to the molecular formula $C_{22}H_{30}O_{14}$. The ^{1}H NMR spectrum (Table 1) showed ABX-type (δ 6.81, *J*=8.4 Hz, 1H; δ 7.10 *J*=1.8, 8.4 Hz, 1H and δ 7.21, *J*=1.8 Hz, 1H) aromatic proton signals, together with *trans*-olefinic (δ 6.40 and 7.73, *J*=15.6 Hz, each 1H), methoxyl (δ 3.89, 3H), and two sugar anomeric (δ 5.55, *J*=7.6 Hz and δ 4.33, *J*=7.6 Hz) proton signals. In the ^{13}C-NMR spectrum of **1** (Table 1), 9 carbon signals of an aglycone (δ 112.0, 114.8, 116.6, 124.5, 127.7, 148.5, 149.6, 151.1 and 167.8) with a methoxyl signal (δ 56.5), which were attributable to ferulic acid [9, 10], were observed, as well as other sugar signals (12 carbons), which indicated the presence of two hexose moieties. Acid hydrolysis of **1** with 0.5 N HCl yielded an aglycone component, which was identified as *trans*-ferulic acid (**4**) by HPLC analysis. The hexose units of **1** were also identified as D-glucose by the method of Tanaka *et al*. [11]. Therefore, **1** was supposed to be a diglucoside of *trans*-ferulic acid. The NMR data of **1** were very similar to those of phenylpropanoid esters of gentiobioside such as 1-*O*-(*E*)-caffeoyl-β-D-gentiobioside [12], 1-*O*-(*E*)-cinnamoyl-β-D-gentiobioside [13] and 1-(3", 4"-dihydroxy-5"-methoxyl)-*O*-(*E*)-cinnamoyl-β-D-gentiobioside [14], with the only difference being the existence of ferulic acid as an aglycone moiety. The large *J* values (7.6 Hz) of the two anomeric proton signals in the ^{1}H NMR spectrum of **1** (Table 1) also confirmed the β configurations. From the data mentioned, **1** was concluded to be as 1-*O*-(*E*)-feruloyl-β-D-gentiobioside (Figure 1).

Table 1: ^{13}C- and ^{1}H-NMR data for **1** and **2** (δ, CD_3OD).

Compound Position	**1** ^{13}C	**1** ^{1}H	**2** ^{13}C	**2** ^{1}H
Ferulic acid				
1	127.7		127.5	
2	112.0	7.21 d (1.8)	112.0	7.23 d (1.9)
3	149.6		149.5	
4	151.1		151.0	
5	116.6	6.81 d (8.4)	116.6	6.83 d (8.2)
6	124.5	7.10 dd (1.8, 8.4)	124.5	7.14 dd (1.9, 8.2)
7	148.5	7.73 d (15.6)	148.2	7.71 d (15.9)
8	114.8	6.40 d (15.6)	115.1	6.41 d (15.9)
9	167.8		167.4	
OMe	56.5	3.89 s	56.6	3.91 s
Glucose-1				
1	95.9	5.55 d (7.6)	94.5	5.73 d (8.1)
2	74.0	3.42 m	81.8	3.78 m
3	78.0	3.31 m	77.6	3.66 m
4	71.0	3.44 m	70.8	3.46 t (8.0)
5	78.0	3.58 m	78.8	3.41 m
6	69.6	3.77 dd (11.6, 5.2)	62.3	3.71 m
		4.17 br. d (11.6)		3.85 dd (11.8, 2.0)
Glucose-2				
1'	104.6	4.33 d (7.6)	104.9	4.60 d (7.8)
2'	75.1	3.20 t (7.6)	75.8	3.18 m
3'	77.8	3.30 m	77.7*	3.36 m
4'	71.5	3.46 m	71.5	3.26 m
5'	77.9	3.25 m	77.3	3.40 m
6'	62.7	3.64 dd (12.0, 4.8)	69.4	3.71 m
		3.83 dd (12.0, 1.6)		4.00 dd (11.7, 2.0)
Glucose-3				
1''			104.5	4.42 d (7.8)
2''			75.0	3.16 m
3''			77.6*	3.34 m
4''			71.3	3.30 m
5''			77.8	3.24 m
6''			62.7	3.62 m
				3.78 m

The numbers in parentheses are *J* values in Hz. * assignments may be interchangeable.

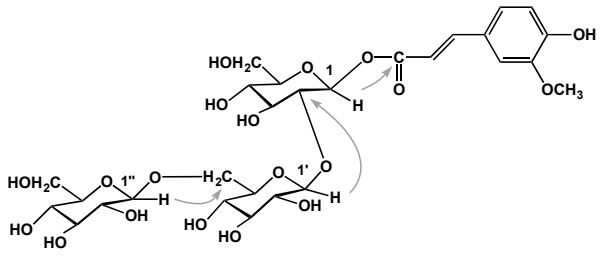

Figure 2: The selected HMBC (^{1}H-^{13}C) correlations of **2**.

Compound **2** showed a quasi-molecular ion peak at *m/z* 679.2130 $[M-H]^-$ (corresponded to the molecular formula $C_{28}H_{40}O_{19}$). The ^{1}H- and ^{13}C-NMR spectral data of **2** (Table 1), the assignments of which were also certified by COSY, HSQC and HMBC spectra, were similar to those of **1**, with the difference being the signals of an additional hexose moiety, suggesting **2** to be a ferulic acid ester of a triglycoside. Acid hydrolysis of **2** yielded *trans*-ferulic acid (**4**), which was identified by HPLC analysis. The sugar components of **2** were also identified as D-glucose by HPLC analysis [11]. The positions of the glucoses were confirmed by the HMBC correlations between glucose H-1 to ferulic acid C-9, glucose H-1' to glucose C-2 and glucose H-1'' to glucose C-6' (Figure 2). The *J* values of the three anomeric proton signals in the ^{1}H NMR spectrum of **2** (Table 1) indicated β configurations of the anomeric carbons. From the data described above, **2** was concluded to be 1-*O*-(*E*)-feruloyl-{β-D-glucopyranosyl (1→6)-[β-D-glucopyranosyl (1→2)]}-β-D-glucopyranoside, and named as allimacronoid D (Figure 1).

Although ferulic acid is commonly distributed in *Allium* species [15], the ester derivatives with oligo-glucose are rarely found. Han *et al*. reported tuberonoid A [1-*O*-(*E*)-feruloyl-β-D-sophoroside] and tuberonoid B [1-*O*-(*Z*)-feruloyl-β-D-sophoroside] from *A. tuberosum* [16]. Including the reported compounds allimacronoids A-C [7], it is very noteworthy that *A. macrostemon* contains various types of ferulic acid esters of oligo-glucose.

Experimental

General: Nuclear magnetic resonance (NMR) spectra were recorded in CD_3OD using JEOL JNM-A500 (500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR) and JEOL JNM-A400 spectrometers (400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR). Optical rotation was measured with a JASCO DIP-1000 Digital Polarimeter, and UV spectra using a JASCO V-530 UV/VIS spectrophotometer. Negative-ion HRESI-TOF-MS were recorded on an Agilent Technologies 6540 Accurate-Mass Q-TOF LC/MS spectrometer. For column chromatography, DIAION HP20SS (Mitsubishi Chemical Corporation), Sephadex LH20 (Pharmacia Corporation), TOYOPEARL HW-40F (Tosoh Corporation) and Cosmosil 140C18-OPN (Nacalai Tesque Inc.) were used. Preparative HPLC was performed on a JASCO preparative HPLC system (Pump: PU-2086 Plus, Detector: UV-2075 Plus, detected at 254 nm) equipped with a COSMOSIL Cholester (10 mm *i. d.* x 250 mm, Nacalai Tesque Inc.) column.

Plant material: The leaves of *Allium macrostemon*, grown in the campus of Saga University, Japan, were collected in October 2015. The plant material was identified by one of the authors (K. Ohshima, professor of the laboratory of Plant Virology, Saga University). A voucher sample is deposited in the laboratory of Analysis of Plant Metabolism at the Faculty of Agriculture, Saga University.

Extraction and isolation: The fresh leaves (1800 g) of *Allium macrostemon* were extracted with 60% aqueous EtOH (7 L) for 2 days. The solvent was concentrated *in vacuo* and subjected to a column of Cosmosil 140C18-OPN, which was eluted with H_2O containing increasing amounts of MeOH to afford 6 fractions {Fr. 1 (20 g), Fr. 2 (25 g), Fr. 3 (10 g), Fr. 4 (8 g), Fr. 5 (8 g) and Fr. 6 (10 g)}. Fr. 1 was purified by DIAION HP20SS (stepwise elution with H_2O and MeOH) CC to afford 3 fractions (Fr. 1-1 and Fr. 1-3). Fr. 1-2 (1 g) was applied to a column of TOYOPEARL HW-40F (stepwise elution with H_2O and MeOH), followed by preparative HPLC separation on COSMOSIL Cholester (elution with 30% aqueous MeOH, 3 mL/ min) to give **1** (2 mg, Rt=13.5 min). Fr. 1-3 (2 g) was purified by Sephadex LH-20 (80% aqueous EtOH) and DIAION HP-20SS (stepwise elution with H_2O and MeOH) CC to give **4** (14 mg). Fr. 2, on DIAION HP-20SS (stepwise elution with H_2O and MeOH) CC, afforded 3 fractions (Fr. 2-1 to Fr. 2-3). Fr.2-1 (0.5 g) was purified by TOYOPEARL HW-40F (stepwise elution with H_2O and MeOH) CC, followed by preparative HPLC separation with COSMOSIL Cholester (elution with 12% aqueous CH_3CN, 3 mL/ min) to give **2** (2 mg, Rt=13.5 min). Fr. 2-3 (0.5 g) was purified by TOYOPEARL HW-40F (stepwise elution with H_2O and MeOH) CC, followed by preparative HPLC separation with COSMOSIL Cholester (elution with 15% aqueous CH_3CN, 3 mL/ min) to give **3** (2 mg, Rt=15.0 min).

HPLC-QTOF-MS analysis of 1 and 2: Negative-ion HRESI-TOF-MS of **1** and **2** were obtained in the HPLC-QTOF-MS analysis using the following conditions. HPLC system (Agilent 1100 series); Column: Agilent Eclipse Plus C18 (2.1 mm *i. d.* x 100 mm, 3.5 μm), Mobile phase: 0.1% formic acid with 2.5 mM $AcONH_4$-CH_3CN [100 : 0 (0 min)→0 : 100 (20 min)], Flow rate : 0.2 mL/min, Column temperature : 40°C, Detection : 280 nm (UV). Retention times (min): **2** (3.9) and **1** (4.2). TOFMS system (Agilent

G1969A); Ionization: ESI (negative), Drying gas: N_2, 350°C, 10 L/ min, Nebulizer gas: N_2, 50 psig, Capillary: 4000 V, Fragmentor: 150 V, Mass range: 50-1200 *m/z*.

1-*O*-(*E*)-feruloyl-β-D-gentiobioside (**1**)
Off-white amorphous powder.
$[\alpha]_D$: – 28.6 (*c* 0.09, MeOH).
$UV_{\lambda max}$ (MeOH) nm (log ε): 330 (4.29).
^1H NMR (400 MHz, CD_3OD): Table 1
^{13}C NMR (100 MHz, CD_3OD): Table 1
HRESI-TOF-MS (negative-ion mode) *m/z:* 517.1584 $[M-H]^-$ (Calcd for $C_{22}H_{29}O_{14}$: 517.1557).

Allimacronoid D (**2**)
Off-white amorphous powder.
$UV_{\lambda max}$ (MeOH) nm (log ε): 330 (4.18).
$[\alpha]_D$: – 30.1 (*c* 0.22, MeOH).
^1H NMR (500 MHz, CD_3OD): Table 1
^{13}C NMR (125 MHz, CD_3OD): Table 1
HRESI-TOF-MS (negative-ion mode) *m/z*: 679.2130 $[M-H]^-$ (Calcd for $C_{28}H_{39}O_{19}$: 679.2086).

Identification of the sugar moieties of 1 and 2: Identification of the sugar moieties of **1** and **2** was made by the following analytical method of Tanaka *et. al*. [11]. Solutions of **1** and **2** (0.5 mg, respectively) in 0.5 M HCl (0.1 mL) were heated at 95°C in screw-capped vials for 2 h. The mixtures were neutralized by addition of Amberlite IRA400 (OH- form) and filtered. The filtrates were dried *in vacuo*, dissolved in 0.1 mL of pyridine containing L-cysteine methyl ester (5 mg/mL) and reacted at 60°C for 1 h. To the mixtures a solution (0.1 mL) of *o*-torylisothiocyanate in pyridine (5 mg/mL) was added and they were heated at 60°C for 1 h. The final mixtures were directly analyzed by HPLC [COSMOSIL Cholester (4.6 mm *i. d.* x 250 mm, Nacalai Tesque Inc.); mobile phase, 0.1% formic acid – CH_3CN (9:1 to 1:4 in 30 min); flow rate, 0.6 mL/min; column temperature, 40°C; detection, 250 nm]. The tRs of the peaks at 28.65 min (from **1**) and at 28.60 min (from **2**) coincided with that of D-glucose (28.58 min, *cf.* tR of the peak for L-glucose was 28.23 min).

Acid hydrolysis of 1 and 2: Solutions of **1** and **2** (0.1 mg, respectively) in 0.5 M HCl (0.1 mL) were heated at 90°C in screw-capped vials for 1 h. The mixtures were directly analyzed by HPLC [COSMOSIL Cholester (4.6 mm *i. d.* x 250 mm, Nacalai Tesque Inc.); mobile phase, 0.1% formic acid – CH_3CN (9:1 to 1:4 in 30 min); flow rate, 0.6 mL/min; column temperature, 40°C; detection, 280 nm]. The tRs of the peaks at 25.50 min (from **1**) and at 25.49 min (from **2**) coincided with that of *trans*-ferulic acid (**4**, 25.24 min).

Supplementary data: ^1H and ^{13}C NMR spectral data for compounds **1**-**2**, COSY, HSQC and HMBC NMR for compound **2** and TOFMS for compounds **1** and **2** are also available.

References

[1] Peng J, Yao X, Okada Y, Okuyama T. (**1994**) Further studies on new furostanol saponins from the bulbs of *Allium macrostemon*. *Chemical and Pharmaceutical Bulletin*, ***42***, 2180-2182.
[2] Li F, Xu Q, Zheng T, Huang F, Han L. (**2014**) Metabonomic analysis of *Allium macrostemon* Bunge as a treatment for acute myocardial ischemia in rats. *Journal of Pharmaceutical and Biomedical Analysis*, ***88***, 225-234.
[3] Xie W, Zhang Y, Wang N, Zhou H, Du L, Ma X, Shi X, Cai G. (**2008**) Novel effects of macrostemonoside A, a compound from *Allium macrostemon* Bunge, on hyperglycemia, hyperlipidemia, and visceral obesity in high-fat diet-fed C57BL/6 mice. *European Journal of Pharmacology*, ***599***, 159-165.
[4] Chen HF, Wang GH, Luo Q, Wang NL, Yao XS. (**2009**) Two new steroidal saponins from *Allium macrostemon* Bunge and their cytotoxicity on different cancer cell lines. *Molecules*, ***14***, 2246-2253.
[5] Lee S, Kim DH, Lee CH, Jung JW, Seo YT, Jang YP, Ryu JH. (**2010**) Antidepressant-like activity of the aqueous extract of *Allium macrostemon* in mice. *Journal of Ethnopharmacology*, ***131***, 386-395.
[6] Nakane R, Iwashina T. (**2015**) Flavonol glycosides from the leaves of *Allium macrostemon*. *Natural Product Communications*, ***10***, 1381-1382.
[7] Usui A, Matsuo Y, Tanaka T, Ohshima K, Fukuda S, Mine T, Yakashiro I, Ishimaru K. (**2016**) Ferulic acid esters of glucosylglucose from *Allium macrostemon* Bunge. *Journal of Asian Natural Products Research*, DOI:10.1080/10286020.2016.1213722.
[8] Usui A, Matsuo Y, Tanaka T, Ohshima K, Fukuda S, Ishimaru K. (**2017**) Phenolic constituents from *Allium macrostemon* Bunge. *The Japanese Journal of Pharmacognosy*, in press.
[9] Katsuragi H, Shimoda K, Yamamoto R, Ishihara K, Hamada H. (**2011**) Glycosylation of capsaicin derivatives and phenylpropanoid derivatives using cultured plant cells. *Biochemistry Insights*, ***4***, 1-12.
[10] Park HB, Lee KH, Kim KH, Lee IK, Noh HJ, Choi SU, Lee KR. (**2009**) Lignans from the roots of *Berberis amurensis*. *Natural Product Sciences*, ***15***, 17-20.
[11] Tanaka T, Nakashima T, Ueda T, Tomii K, Kouno I. (**2007**) Facile discrimination of aldose enantiomers by reversed-phase HPLC. *Chemical and Pharmaceutical Bulletin*, ***55***, 899-901.
[12] Jiang Z-H, Hirose Y, Iwata H, Sakamoto S, Tanaka T, Kouno I. (**2001**) Caffeoyl, coumaroyl, galloyl, and hexahydroxydiphenoyl glucoses from *Balanophora japonica*. *Chemical & Pharmaceutical Bulletin*, ***49***, 887-892.
[13] Latza S, Ganber D, Berger RG. (**1996**) Carbohydrate esters of cinnamic acid from fruits of *Physalis perviana*, *Psidium guajava* and *Vaccinium vitis-idaea*. *Phytochemistry*, ***43***, 481-485.
[14] Hosoya T, Yun YS, Kunugi A. (**2008**) Antioxidant phenylpropanoid glycosides from the leaves of *Wasabia japonica*. *Phytochemistry*, ***69***, 827-832.
[15] Vlase L, Parvu M, Parvu EA, Toiu A. (**2013**) Chemical constituents of three *Allium* species from Romania. *Molecules*, ***18***, 114-127.
[16] Han SH, Suh WS, Park KJ, Kim KH, Lee KR. (**2015**) Two new phenylpropane glycosides from *Allium tuberosum* Rottler. *Archives of Pharmacal Research*, ***38***, 1312-1316.

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No. 1
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Quantitative Analysis of Phenolic Acids and Antiplatelet Activity of *Melissa officinalis* Leaf Extracts

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Received: August 12th, 2016; Accepted: September 29th, 2016

The contents of total phenolic acids and rosmarinic acid were determined and the antiplatelet potential assessed of extracts of lemon balm (*Melissa officinalis* L., Lamiaceae) leaf samples collected during 2014 and 2015. The quantity of total phenolic acids ranged from 4.8 to 8.5%, while the rosmarinic acid content ranged from 1.78 to 4.35%. Results of statistical analysis showed that the undiluted extracts have antiplatelet activity in the ADP test (final concentration 31.25 mg/L). While there are reports on antiplatelet activity of lemon balm essential oil, to the best of our knowledge, this is the first report on antiplatelet activity of lemon balm leaf extracts.

Keywords: *Melissa officinalis*, Polyphenols, HPLC, Spectrophotometry, Antiplatelet activity.

Lemon balm (*Melissa officinalis* L., Lamiaceae) is an endogenous plant of western Asian regions and the Eastern Mediterranean, and is cultivated in almost all of Europe and North America [1]. It grows as a shrub, 0.3 to 0.9 m high, usually with several stems. The major chemical constituents are polyphenolic acids namely rosmarinic (most abundant), *p*-coumaric, caffeic and chlorogenic acids. Lemon balm essential oil (0.02-0.37%) is composed of more than 40% monoterpenes and more than 35% sesquiterpenes [2]. Other constituents determined in leaf samples include flavonoids, tannins and triterpenes [3]. Externally, lemon balm extracts are used for symptomatic treatment of herpes labialis while orally applied extracts act as a carminative and a sedative [1].

Antiplatelet activity, i.e. prevention of platelet aggregation, of polyphenols is of interest as it has been shown that they can act in submicromolar concentrations on platelet aggregation. This is a useful characteristic as polyphenols could serve as new sources of antiaggregatory drugs in prevention of cardiovascular diseases (e.g. prevention of thrombosis, heart attack or stroke). On the other hand, polyphenols from everyday diet could interfere with platelet functional tests and subsequently hinder diagnosis of platelet related disorders [4]. It has been shown that lemon balm essential oil has antiplatelet activity [5] that can be attributed to major terpene constituents [6,7]. As major constituents of lemon balm leaves are phenolic acids, primarily rosmarinic acid, the objective of this work was to determine the contents of total phenolic acids and rosmarinic acid, and the antiplatelet activity of ethanolic lemon balm leaf extracts.

Total phenolic acid concentrations ranged from 4.8 to 8.5% (Table 1) fulfilling the requirements of European Pharmacopeia (more than 4% of phenolic acids). Rosmarinic acid, as a major constituent, was determined by HPLC and its content ranged from 1.78 to 4.35% (Table 1).

Lemon balm ethanolic extracts (non-diluted, 10%, 1%) were tested for antiaggregatory activity in a thrombocyte functional test induced with adenosine diphosphate (ADP). Statistical analysis (one-way ANOVA with post hoc Tukey test) of the results showed that only the undiluted extracts had antiplatelet activity in the test (Figure 1). Standard rosmarinic acid was analyzed in concentrations of 0.09, 0.9, 9 and 90 μM. Only 90 μM rosmarinic acid solution showed statistically significant reduction of platelet aggregation.

Table 1: Content of total phenolic acids and rosmarinic acid.*

Sample	Total phenolic acids	Rosmarinic acid
1	6.5 ± 0.5	1.78 ± 0.02
2	7.1 ± 0.1	2.14 ± 0.02
3	4.8 ± 0.1	1.99 ± 0.01
4	8.5 ± 0.2	4.35 ± 0.02
5	6.5 ± 0.1	2.30 ± 0.02

*Results are expressed as percentage (± SD) of dry mass of leaf powder.

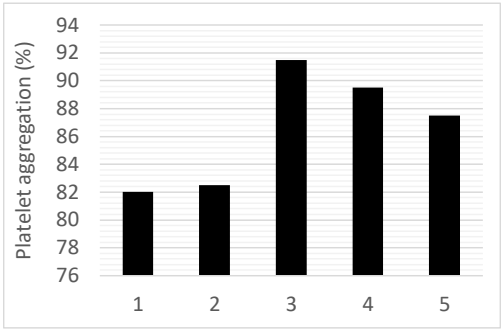

Figure 1: Antiaggregatory effect of *Melissa officinalis* leaf extracts (1-5) (see Table 1 for concentrations). Platelet aggregation is expressed as percentage to the solvent control.

To the best of our knowledge this is the first report of antiplatelet effect of lemon balm leaf extracts. This activity can be attributed mainly to rosmarinic acid as all extracts showed antiplatelet activity at concentrations of 50-120 μM (based on HPLC results, % rosmarinic acid {(see Table 1) *x* concentration of extract (1 mg/mL) / *M* (rosmarinic acid, 360.3 g/mol)}, which is comparable with 90 μM concentration of rosmarinic acid in the antiplatelet test.

Previously, lemon balm essential oil was shown to have an antiplatelet effect at a concentration of 4 µg/mL using collagen and thrombin as inducers of aggregation that was attributed to terpene constituents [5]. The lower results that we have observed can be attributed to the different chemical composition of the leaf extract compared with the essential oil. The advantage of our approach is that the analysis was on whole blood rather than platelet rich plasma used in the previous study [8].

Experimental

Plant material: Some of the leaf samples collected in 2014 and 2015 (samples 1 and 2) were cultivated in the Pharmaceutical Botanical Garden "Fran Kušan", Faculty of Pharmacy and Biochemistry, University of Zagreb, while the others (samples 3-5) were obtained from Suban (Strmec, Croatia). Voucher specimens are deposited in the Herbarium of the Department of Pharmaceutical Botany, University of Zagreb.

Determination of total phenolic acids: The content of phenolic acids in the plant samples was determined by the official Eur. Ph. method [3]. Extracts were prepared by reflux extraction of 0.2 g of the powdered drug with 190 mL of ethanol (50%, v/v) for 30 min. Filtrate and washings were collected and diluted to 200 mL with diluted ethanol. Total phenolic acid content was determined by measuring the absorbance of the complex formed between phenolic acids and sodium nitrite – sodium molybdate at 505 nm [9]. The measurements were carried out using a Varian Cary 50 Bio spectrophotometer (Varian Inc., USA). The content of phenolic acids was expressed as the equivalent of rosmarinic acid based on 3 independent analyses.

HPLC analysis of rosmarinic acid: Rosmarinic acid content was determined by reverse phase high performance liquid chromatography coupled with a diode array detector on an Agilent 1100 system (Agilent, USA) using the method described by Medić-Šarić *et al*. [10]. Extracts used for determination of phenolic acids were filtered (Whatman 0.22 µm syringe filter, Sigma Aldrich, USA). A Zorbax SB-C18 (250 mm x 4.6 mm, particle size 5 µm) column with precolumn (12.5 mm x 4.6 mm, particle size 5 µm) was used as the stationary phase. Elution was achieved with a gradient of mobile phases A (water, methanol and formic acid in ratio 93:5:2) and B (water, methanol and formic acid in ratio 3:95:2) following the timetable (t/min, %B): (0, 20), (10, 40), (35, 50), (47, 50), (70, 80), (80, 20). Detection was based on retention time and UV spectra compared with standard; quantification was based on calibration curves recorded at 254 nm.

Determination of antiplatelet activity: Platelet aggregation was analyzed in whole blood utilizing a Multiplate® impedance analyzer (Roche, Switzerland). Blood samples were obtained from 9 healthy volunteers with their written consent. This study has been approved by the ethical committee of the Croatian Institute of Transfusion Medicine. The following procedure was used for all analyses: 300 µL of blood was incubated at 37°C for 6 min with 20 µL of lemon balm extract solution and 300 µL of saline with $CaCl_2$. Aggregation cascade was induced according to protocol by adding 20 µL of agonist ADP reagent (6.3 µM) [3]. Aggregation was measured for 6 min and expressed as area under the curve in arbitrary units (AU). Untreated samples (negative control, 100% of aggregation) were analyzed with 20 µL of solvent. All measurements were performed in triplicate. Final results for extracts were expressed as percentage of residual platelet aggregation compared with the untreated sample. Rosmarinic acid standard was used as positive control. Statistical analysis was performed using the Microsoft Office Excel 2003 (Microsoft Corporation, USA) program; p values lower than $\alpha = 0.05$ were regarded as statistically significant.

Acknowledgments – We would like to thank V. Suban Jakuš for providing some of the lemon balms leaf samples. This work was in part supported by University of Zagreb (BM088-2015) and Croatian Science Foundation (UIP-2014-09-5704).

References

[1] *WHO Monographs on Selected Medicinal Plants - Volume 2*. (**2004**) World Health Organization, Geneva, CH, 180-187.
[2] Nurzyńska-Wierdak R, Bogucka-Kocka A, Szymczak G. (**2014**) Volatile constituents of *Melissa officinalis* leaves determined by plant age. *Natural Product Communications*, ***9***, 703-706.
[3] *European Pharmacopoeia*. (**2005**) 5th Ed. Council of Europe, Strasbourg Cedex, 01/2005, 1447.
[4] (a) Bojić M, Debeljak Z, Tomičić M, Medić-Šarić M, Tomić S. (**2011**) Evaluation of antiaggregatory activity of flavonoid aglycone series. *Nutrition Journal*, ***10***, 73, 1-8; (b) Bojić M, Debeljak Ž, Medić-Šarić M, Tomičić M. (**2012**) Interference of selected flavonoid aglycons in platelet aggregation assays. *Clinical Chemistry and Laboratory Medicine*, ***50***, 1403-1408.
[5] Yen HF, Wang SY, Wu CC, Lin WY, Wu TY, Chang FR, Wang CK. (**2012**) Cytotoxicity, anti-platelet aggregation assay and chemical components analysis of thirty-eight kinds of essential oils. *Journal of Food and Drug Analysis*, ***20***, 478-483.
[6] Suschke U, Sporer F, Schneele J, Geiss HK, Reichling J. (**2007**) Antibacterial and cytotoxic activity of *Nepeta cataria* L., *N. cataria* var. *citriodora* (Beck.) Balb. and *Melissa officinalis* L. essential oils. *Natural Product Communications*, ***2***, 1277-1286.
[7] D'Angiolillo F, Tonelli M, Pellegrini E, Nali C, Lorenzini G, Pistelli L, Pistelli L. (**2015**) Can ozone alter the terpenoid composition and membrane integrity of *in vitro Melissa officinalis* shoots? *Natural Product Communications*, ***10***, 1055-1058.
[8] Chapado L, Linares-Palomino PJ, Salido S, Altarejos J, Rosado JA, Salido GM. (**2010**) Synthesis and evaluation of the platelet antiaggregant properties of phenolic antioxidants structurally related to rosmarinic acid. *Bioorganic Chemistry*, ***38***, 108-114.
[9] Medić-Šarić M, Rastija V, Bojić M, Maleš Ž. (**2009**) From functional food to medicinal product: systematic approach in analysis of polyphenolics from propolis and wine. *Nutrition Journal*, ***8***, 33, 1-18.
[10] Medić-Šarić M, Rastija V, Bojić M. (**2011**) Recent advances in the application of high-performance liquid chromatography in the analysis of polyphenols in wine and propolis. *Journal of AOAC International*, ***94***, 32-42.

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A Concise Synthesis of Rhodanthpyrone A and B, Natural 4-(Hydroxyphenyl)-substituted α-Pyrones

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Received: June 28th, 2016; Accepted: November 3rd, 2016

A concise synthesis of rhodanthpyrone A and B was accomplished *via* a Suzuki coupling reaction. To find the conditions appropriate to install hydroxyphenyl moieties to the α-pyrone skeleton, a model study was conducted using commercially available boronic acids. It was revealed that the hydroxy moiety of the phenylboronic acids should be concealed when reacted with labile 4-tosyl α-pyrone. Consequently, rhodanthpyrone A and B could be synthesized in high yields by Suzuki reaction using TIPS-protected arylboronic acids. This procedure provided a concise and versatile route for the synthesis of rhodanthpyrones and their 4-aryl substituted α-pyrone analogs.

Keywords: Synthesis, Rhodanthpyrones, α-Pyrone, Suzuki coupling reaction.

α-Pyrone (=2*H*-pyran-2-one) is a six-membered cyclic unsaturated ester found in numerous biologically active natural products, which exhibit strong and a broad variety of biological efficacies such as antimalarial, antimicrobial, and anti-cancer [1-3]. The pyrone moiety and its fused derivatives such as coumarin and chromone structures have been recognized as a privileged scaffold [4]. In this regard, these natural α-pyrones and their derivatives have been considered as an attractive target in terms of both synthetic and medicinal chemistry [5]. Recently, rhodanthpyrone A (**1**) and B (**2**), rare 4-aryl substituted α-pyrones, were isolated by Wang *et al*. from *Gentiana rhodantha*, a plant used as a traditional medicine in China for the treatment of inflammation, cholecystitis, and tuberculosis [6-7]. Considering not only the therapeutic properties of *G. rhodantha*, but also the usefulness of the privileged α-pyrone structure in medicinal chemistry, it is believed that these rhodanthpyrones can exhibit therapeutically useful biological activities, and the 4-aryl substituted α-pyrone structure may be a novel scaffold for further medicinal chemistry research.

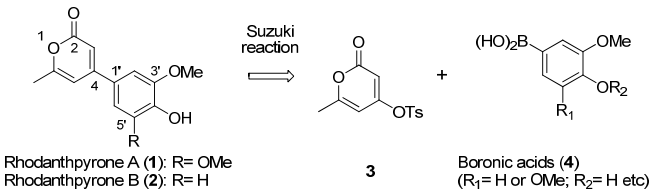
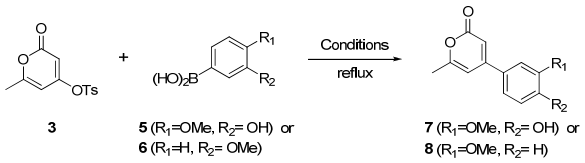


Figure 1: Structure of rhodanthpyrones and retrosynthetic analysis.

Palladium catalyzed cross-coupling reactions such as the Suzuki reaction have become a general and versatile method in organic synthesis for the introduction of aryl and heteroaryl substituents into diverse molecules. For instance, Rossi and his colleagues reported palladium-catalyzed Suzuki reactions between known 4-tosyloxy-pyranone **3** [8] and several substituted arylboronic acids in high yields using $Pd(OAc)_2$ with tricyclohexyphosphine (PCy_3) in methanol as an optimized catalyst system [9]. Based on this previous study, it was supposed that a concise synthetic method for rhodanthpyrones could be established by the Suzuki reaction between 4-tosylate (**3**) and suitable 4-hydroxyphenyl boronic acids (**4**), as shown in Figure 1.



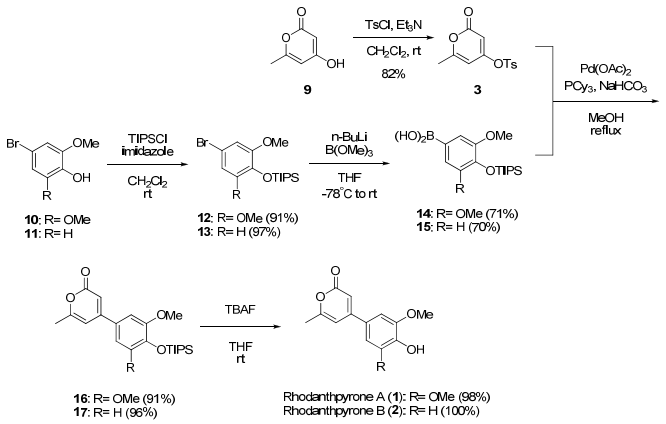
3 **5** (R_1=OMe, R_2= OH) or **6** (R_1=H, R_2= OMe) **7** (R_1=OMe, R_2= OH) or **8** (R_1=OMe, R_2= H)

Table 1: Model study of Suzuki reaction

Entry	Condition	Result
1	**5**, $Pd(OAc)_2$ (5%), PCy_3 (10%), $NaHCO_3$, MeOH	
2	**5**, $Pd(OAc)_2$ (5%), PCy_3 (10%), $NaHCO_3$, toluene/H_2O	
3	**5**, $Pd(OAc)_2$ (5%), PCy_3 (10%), $NaHCO_3$, dioxane	mixture of
4	**5**, $Pd(OAc)_2$ (10%), PCy_3 (20%), $NaHCO_3$, MeOH	unidentifiable
5	**5**, $Pd(OAc)_2$ (5%), PCy_3 (10%), K_2HPO_4, MeOH	side products
6	**5**, $Pd(PPh_3)_4$ (5%), CuI, $NaHCO_3$, benzene/EtOH	
7	**6**, $Pd(OAc)_2$ (5%), PCy_3 (10%), $NaHCO_3$, MeOH	**8** (95%)

Initially, as shown in Table 1, the Suzuki reactions between 4-toxylate (**3**) and commercially available boronic acids such as regioisomeric 3-hydroxy-4-methoxyphenylboronic acid (**5**) were carried out as model studies. The Suzuki coupling reaction of the phenylboronic acid (**5**) with 4-tosyloxypyranone (**3**) using $Pd(OAc)_2$ with tricyclohexyphosphine in methanol, reported in entry 1, resulted in only a mixture of unidentifiable side products. Several modifications of the reaction conditions, such as solvent (entry 2 and 3), loading amount of catalyst (entry 4), and base (entry 5), were tried, but 4-aryl α-pyrone (**7**) could not be observed. Moreover, copper-facilitated Suzuki reaction (entry 6), used for the synthesis of 4-arylcoumarin analogues [10], also did not afford the 4-aryl α-pyrone (**7**). On the other hand, the Suzuki coupling reaction using 3-methoxyphenylboronic acid (**6**) (entry 7) afforded 4-aryl α-pyrone (**8**) in a high yield. Based on these observations, it was supposed that the electrophilic α-pyrone structure might be labile to the nucleophilic phenol of the boronic acid **5**, enough to lead to various undesired reactions [11]. Based on this idea, introduction of a protecting group to the phenol of boronic acid was decided. Among the various protecting groups for phenol, the triisopropylsilyl (TIPS) group was selected as it is known to be a stable and easily removable silyl protecting group in substrates bearing reducible olefin and labile ester.

As shown in Scheme 1, syntheses of rhodanthpyrone A and B commenced with TIPS-protection of bromophenols (**10** [12] and **11**).



Scheme 1: Synthesis of rhodanthpyrone A and B

Bromobenzenes (**12** and **13**) were converted to arylboronic acids (**14** and **15**) in moderate yields by treatment with *n*-butyllithium, followed by addition of trimethylborate and hydrolysis using 3N hydrochloric acid. Suzuki reaction using $Pd(OAc)_2$ with tricyclohexyphosphine in methanol between TIPS protected arylboronic acids (**14** and **15**) and 4-toxylate (**3**), prepared from 4-hydroxypyranone (**9**) [8], readily afforded 4-aryl α-pyrones (**16** and **17**), respectively. Rhodanthpyrone A (**1**) and B (**2**) could be obtained from 4-aryl α-pyrones (**16** and **17**) by deprotection using tetrabutylammonium fluoride (TBAF), without any side product. The spectral data of synthetic rhodanthpyrone A (**1**) and B (**2**) were identical to those of the reported data [6].

In summary, this article describes a concise synthesis of natural rhodanthpyrones via the Suzuki coupling reaction of labile 4-tosyl α-pyrone using silyl protected boronic acids. To find the Suzuki reaction conditions appropriate to install hydroxyphenyl moieties at the 4-position of the α-pyrone, model, a study was conducted using commercially available boronic acids. It was supposed that the hydroxy moiety of phenylboronic acids should be concealed. In this connection, the TIPS group, stable in the Suzuki reaction and easily removable without side-reactions, was introduced to protect the 4-hydroxy moiety of the arylboronic acids. Rhodanthpyrone A and B could be synthesized in high yields by the Suzuki reaction using TIPS-protected arylboronic acids, followed by deprotection. To my best knowledge, this study is the first practical example of the synthesis of 4-aryl substituted α-pyrones bearing a hydroxy moiety. This will be helpful in the synthesis of derivatives of rhodanthpyrone and construction of a chemical library of 4-aryl substituted α-pyrones.

Experimental

General: Unless noted otherwise, all starting materials and reagents were obtained commercially and were used without further purification. All solvents utilized for routine product isolation and chromatography were of reagent grade and glass-distilled, and reaction flasks were dried at 100°C before use. Flash column chromatography was performed using silica gel 60 (230-400 mesh, Merck) with the indicated solvents. Thin-layer chromatography (TLC) was performed using 0.25-mm silica gel plates (Merck). Mass spectra were obtained using a VG Trio-2 GC-MS instrument, and high-resolution mass spectra were obtained using a JEOL JMS-AX 505WA unit. Infrared spectra were recorded on a FT-IR spectrometer. ^{1}H and ^{13}C spectra were recorded on a Bruker Avance III 700 (700 MHz) spectrometer in either deuteriochloroform ($CDCl_3$), or deuteriomethanol (CD_3OD). Chemical shifts are expressed in parts per million (ppm, δ) downfield from tetramethylsilane and are referenced to the deuterated solvent. ^{1}H NMR data are reported in the order of chemical shift, multiplicity (s, singlet; bs, broad singlet; d, doublet; t, triplet; q, quartet; m, multiplet; and/or multiple resonances), number of protons, and coupling constant in Hertz (Hz).

6-Methyl-2-oxo-2*H*-pyran-4-yl tosylate (3): Tosylated compound **3** was obtained in 82% yield (915 mg) as a white solid from 500 mg (3.97 mmol) of **9** *via* the reported procedure, and the NMR spectra data were identical with those reported [8].

(4-Bromo-2,6-dimethoxyphenoxy)triisopropylsilane (12): To a solution of 4-bromo-2,6-dimethoxyphenol (**10**) [11] (350 mg, 1.50 mmol) and imidazole (153 mg, 2.25 mmol) in CH_2Cl_2 (20 mL) was added triisopropylsilyl chloride (0.42 mL, 1.95 mmol) at ambient temperature. The reaction mixture was stirred for 48 h at the same temperature and then quenched with H_2O. The reaction mixture was diluted with CH_2Cl_2. The organic layer was washed with water and brine, dried over $MgSO_4$, and concentrated *in vacuo*. Purification of the residue via flash column chromatography on silica gel (EtOAC/*n*-hexane = 1 : 70) afforded 532 mg (91%) of **12** as a colorless oil.
IR (thin film, neat): 2943 (m), 2866 (m), 1587 (m), 1504 (s), 1131 (s), 822 (m) cm^{-1}.
^{1}H NMR (700 MHz, $CDCl_3$) δ: 6.65 (s, 2H, Ph-H), 3.77 (s, 6H, -OCH_3), 1.26-1.21 (m, 3H, -Si-C*H*-), 1.08 (d, 18 H, *J* = 7.7 Hz, -CH_3).
^{13}C NMR ($CDCl_3$, 175 MHz) δ: 152.0 (2C, C), 134.3 (1C, C), 112.3 (1C, C), 108.5 (2C, CH), 55.7 (2C, CH_3), 17.9 (3C, CH), 13.4 (6C, CH_3).
LR-MS: (ESI) *m/z*: 411 ($M+Na^+$).
HR-MS (FAB+): Calcd for $C_{17}H_{30}BrO_3Si^+$ ($M+H^+$): 389.1142. Found: 389.1148

(4-Bromo-2-methoxyphenoxy)triisopropylsilane (13): Silyloxy ether **13** was prepared by the procedure for **12**, using **11** (530 mg, 2.61 mmol) instead of **10**. Purification of the residue via flash column chromatography on silica gel (EtOAC/*n*-hexane = 1 : 70) afforded 909 mg (97%) of **13** as a colorless oil.
IR (thin film, neat): 2943 (s), 2867 (s), 1584 (s), 1496 (s), 1227 (s), 835 (s) cm^{-1}.
^{1}H NMR (700 MHz, $CDCl_3$) δ: 6.94 (d, 1H, *J* = 2.1 Hz, Ph-H), 6.91 (dd, 1H, *J* = 2.1, 8.4 Hz, Ph-H), 6.73 (d, 1H, *J* = 8.4 Hz, Ph-H), 1.26-1.20 (m, 3H, -Si-C*H*-), 1.08 (d, 18H, *J* = 7.0 Hz, -CH_3).
^{13}C NMR ($CDCl_3$, 175 MHz) δ: 151.9 (1C, C), 145.1 (1C, C), 123.7 (1C, CH), 121.7 (1C, CH), 115.6 (1C, HC), 113.2 (1C, C), 55.8 (1C, CH_3), 18.1 (3C, CH), 13.0 (6C, CH_3).
LR-MS (ESI): *m/z* 359 ($M+H^+$).
HR-MS (FAB+): Calcd for $C_{16}H_{28}BrO_2Si^+$ ($M+H^+$): 359.1036.

3,5-Dimethoxy-4-(triisopropylsilyloxy)phenylboronic acid (14): To a solution of bromobenzene **12** (330 mg, 0.85 mmol) in fresh distilled THF (10 mL) was added slowly 2.5 M *n*-BuLi in *n*-hexane (0.44 mL, 1.10 mmol) at -78°C. After 30 min, to the reaction mixture was added trimethyl borate (0.19 mL, 1.70 mmol) and the mixture was stirred for 1 h before warming to ambient temperature. After 1 h, the reaction mixture was quenched with 3N HCl, and diluted with EtOAc. The organic layer was washed with water and brine, dried over $MgSO_4$, and concentrated *in vacuo*. Purification of the residue via flash column chromatography on silica gel (EtOAC/*n*-hexane = 1 : 2) afforded 213 mg (71%) of **14** as a white solid.
MP: 185 ~ 187°C.
IR (thin film, neat): 2944 (m), 2867 (m), 1129 (w), 1414 (m), 1343 (s), 1129 (m), 886 (m) cm^{-1}.

^{1}H NMR (700 MHz, $CDCl_3$) δ: 7.43 (s, 2H, Ph-H), 3.93 (s, 6H, -OCH_3), 1.34-1.28 (m, 3H, -Si-C*H*-), 1.12 (d, 18 H, *J* = 7.0 Hz, -CH_3).
^{13}C NMR ($CDCl_3$, 175 MHz) δ: 151.3 (2C, C), 140.0 (1C, C), 112.2 (1C, C), 110.1 (1C, CH), 55.6 (2C, CH_3), 17.9 (3C, CH), 13.4 (6 C, CH_3).
LR-MS (ESI): *m/z* 355 ($M+H^+$).
HR-MS (FAB+): Calcd for $C_{17}H_{32}BO_5Si^+$ ($M+H^+$): 355.2107. Found: 355.2113

3-Methoxy-4-(triisopropylsilyloxy)phenylboronic acid (15): Boronic acid **15** was prepared by the procedure for **14**, using **13** (350 mg, 0.97 mmol) instead of **12**. Purification of the residue via flash column chromatography on silica gel (EtOAC/*n*-hexane = 1 : 5) afforded 220 mg (70%) of **15** as a white solid.
MP: 150 ~ 151°C.
IR (thin film, neat): 2945 (m), 2868 (m), 1595 (m), 1413 (s), 1371 (m), 1274 (m), 881 (m) cm^{-1}.
^{1}H NMR (700 MHz, $CDCl_3$) δ: 7.73 (dd, 1H, *J* = 1.4, 7.7 Hz, Ph-H), 7.67 (d, 1H, *J* = 1.4 Hz, Ph-H), 7.00 (d, 1H, *J* = 7.7 Hz, Ph-H), 3.93 (s, 3H, -OCH_3), 1.32-1.26 (m, 3H, -Si-C*H*-), 1.11 (d, 18 H, *J* = 7.7 Hz, CH_3).
^{13}C NMR ($CDCl_3$, 175 MHz) δ: 150.9 (1C, C), 150.2 (1C, C), 129.7 (1C, CH), 126.8 (1C, C), 118.9 (1C, CH), 117.3 (1C, CH), 55.7 (1C, CH_3), 18.1 (3C, CH), 13.1 (6C, CH_3).
LR-MS (ESI) *m/z*: 347 ($M+Na^+$).
HR-MS (FAB+): Calcd for $C_{16}H_{30}BO_4Si^+$ ($M+H^+$): 325.2001.

General procedure for Suzuki coupling reaction: To a solution of 4-tosyloxypyranone (1 equiv.), $NaHCO_3$ (3 equiv), and arylboronic acid (1.2 equiv.) in methanol (0.05 M) under an argon atmosphere, were added a solution of tricyclohexylphosphine in toluene (0.60 M, 0.1 equiv.) and $Pd(OAc)_2$ (0.05 equiv.). The reaction mixture was heated to reflux for 3 h, and then cooled to ambient temperature. The reaction mixture was concentrated *in vacuo*, and diluted with EtOAc and H_2O. The organic layer was washed with water and brine, dried over $MgSO_4$, and concentrated *in vacuo*. Purification of the residue via flash column chromatography on silica gel (EtOAC/*n*-hexane = 1 : 5~10) afforded the corresponding 4-aryl substituted α-pyrones.

4-(3-Methoxyphenyl)-6-methyl-2*H*-pyran-2-one (8): 4-Aryl α-pyrone **8** was obtained in 95% yield (37 mg) as a white solid from 50 mg (0.18 mmol) of **3** *via* the general procedure.
MP: 86 ~ 87°C.
IR (thin film, neat): 2349 (w), 1714 (s), 1549 (m), 1290 (w), 1039 (w) cm^{-1}.
^{1}H NMR (700 MHz, $CDCl_3$) δ: 7.41 (t, 1H, *J* = 8.4 Hz, Ph-H), 7.17 (qd, 1H, *J* = 0.7, 7.7 Hz, Ph-H), 7.09 (t, 1H, *J* = 2.1 Hz, Ph-H) 7.04 (ddd, 1H, *J* = 0.7, 1.4, 7.7 Hz, Ph-H), 6.37 (s, 1H, H-5), 6.31 (s, 1H, H-3), 3.88 (s, 3H, -OCH_3), 2.35 (s, 3H, -CH_3).
^{13}C NMR ($CDCl_3$, 175 MHz) δ: 163.5 (1C, C), 162.1 (1C, C), 160.1 (1C, C), 155.5 (1C, C), 137.3 (1C, CH), 130.2 (1C, C), 119.0 (1C, CH), 116.0 (1C, CH), 112.3 (1C, CH), 108.3 (1C, CH), 103.6 (1C, CH), 55.4 (1C, CH_3), 20.2 (1C, CH_3).
LR-MS (ESI) *m/z*: 217 ($M+H^+$).
HR-MS (FAB+): Calcd for $C_{13}H_{13}O_3^+$ ($M+H^+$): 217.0859. Found: 217.0860

4-(3,5-Dimethoxy-4-(triisopropylsilyloxy)phenyl)-6-methyl-2*H*-pyran-2-one (16): 4-Aryl α-pyrone **16** was obtained in 91% yield (179 mg) as a white solid from 132 mg (0.47 mmol) of **3** and 200 mg of boronic acid **14** (0.56 mmol) *via* the general procedure.
MP: 152 ~ 153°C,
IR (thin film, neat): 2943 (w), 2865 (w), 1700 (s), 1516 (m), 1129 (m) cm^{-1}.
^{1}H NMR (700 MHz, $CDCl_3$) δ: 6.75 (s, 2H, Ph-H), 6.32 (s, 1H, H-5), 6.31 (s, 1H, H-3), 3.85 (s, 6H, -OCH_3), 2.32 (s, 3H, CH_3), 1.29-1.24 (m, 3H, -Si-C*H*-), 1.07 (d, 18 H, *J* = 7.7 Hz, CH_3).
^{13}C NMR ($CDCl_3$, 175 MHz) δ: 163.7 (1C, C), 161.7 (1C, C), 155.6 (1C, C), 151.8 (2C, C), 137.8 (1C, C), 127.5 (1C, C), 106.8 (1C, CH), 103.7 (2C, CH), 103.4 (1C, CH), 55.8 (2C, CH_3), 20.2 (1C, CH_3), 17.9 (3C, CH), 13.4 (6C, CH_3).
LR-MS (ESI) *m/z*: 419 ($M+H^+$).
HR-MS (FAB+): Calcd for $C_{23}H_{35}O_5Si^+$ ($M+H^+$): 419.2248.

4-(3-Methoxy-4-(triisopropylsilyloxy)phenyl)-6-methyl-2*H*-pyran-2-one (17): 4-Aryl α-pyrone **17** was obtained in 96% yield (152 mg) as a white solid from 116 mg (0.41 mmol) of **3** and 200 mg of boronic acid **14** (0.62 mmol) *via* the general procedure.
MP: 145 ~ 146°C.
IR (thin film, neat): 2944 (w), 2868 (w), 1767 (s), 1521 (m), 1296 (m), 1034 (m), 774 (m) cm^{-1}.
^{1}H NMR (700 MHz, $CDCl_3$) δ: 7.10 (dd, 1H, *J* = 2.1, 8.4 Hz, Ph-H), 7.07 (d, 1H, *J* = 2.1 Hz, Ph-H), 6.95 (d, 1H, *J* = 8.4 Hz, Ph-H), 6.33 (s, 1H, H-5), 6.32 (s, 1H, H-3), 3.89 (s, 3H, -OCH_3), 2.33 (s, 3H, -CH_3), 1.31-1.28 (d, 3H, -Si-C*H*-), 1.11 (d, 18H, *J* = 7.7 Hz, -CH_3).
^{13}C NMR ($CDCl_3$, 175 MHz) δ: 163.8 (1C, C), 161.7 (1C, C), 155.2 (1C, C), 151.3 (1C, C), 148.4 (1C, C), 128.6 (1C, C), 120.7 (1C, CH), 120.0 (1C, CH), 110.2 (1C, CH), 106.6 (1C, CH), 103.3 (1C, CH), 55.6 (1C, CH_3), 20.2 (1C, CH_3), 17.9 (3C, CH), 12.9 (6C, CH_3).
LR-MS (ESI) *m/z*: 411 ($M+Na^+$).
HR-MS (FAB+): Calcd for $C_{22}H_{33}O_4Si^+$ ($M+H^+$): 389.2143.

Rhodanthpyrone A (1): To a solution of silyloxy ether (**16**) (100 mg, 0.24 mmol) in THF (6 mL) was added 1.0 M solution of TBAF in THF (0.36 mL, 0.36 mmol) at ambient temperature. The reaction mixture was stirred for 30 min at the same temperature and then quenched with H_2O. The reaction mixture was diluted with EtOAc. The organic layer was washed with water and brine, dried over $MgSO_4$, and concentrated *in vacuo*. Purification of the residue via flash column chromatography on silica gel (EtOAC/*n*-hexane = 1 : 1) afforded 62 mg (98%) of rhodanthpyrone A (**1**) as a yellow solid.
MP: 214 ~ 215°C.
IR (thin film, neat): 3377 (w), 2992 (w), 2947 (w), 2838 (w), 1688 (m), 1633 (m), 1549 (m), 1517 (m), 1454 (m), 1326 (m), 1214 (m), 1119 (m), 847 (m), 718 (m) cm^{-1}.
^{1}H NMR (700 MHz, CD_3OD) δ: 7.03 (s, 2H, H-6'), 6.69 (d, 1H, *J* = 1.6 Hz, H-5), 6.41 (d, 2H, *J* = 1.6 Hz, H-3), 3.94 (s, 6H, -OCH_3), 2.35 (s, 3H, CH_3).
^{13}C NMR ($CDCl_3$, 175 MHz) δ: 166.3 (1C, C), 163.5 (1C, C), 158.1 (1C, C), 149.7 (2C, C), 140.2 (1C, C), 126.9 (1C, C), 106.3 (1C, CH), 105.7 (2C, CH), 104.8 (1C, CH), 57.0 (2C, CH_3), 19.9 (1C, CH_3).
LR-MS (FAB+) *m/z*: 263 ($M+H^+$).
HR-MS (FAB+): Calcd for $C_{14}H_{15}O_5$ ($M+H^+$): 263.0914. Found: 263.0914.

Rhodanthpyrone B (2): Rhodanthopyrone B (**2**) was prepared by the procedure for rhodanthopyrone A (**1**) using **17** (30 mg, 0.077 mmol) instead of **16**. Purification of the residue via flash column chromatography on silica gel (EtOAC/*n*-hexane = 1 : 2) afforded 18 mg (100%) of rhodanthopyrone B (**2**) as a yellowish solid.
MP: 138 ~ 140°C.
IR (thin film, neat): 2926 (w), 1708 (s), 1632 (m), 1594 (m), 1516 (m), 1321 (w), 1286 (m), 1210 (m), 1131 (w), 1033 (w), 819 (w), 769 (w), 627 (w) cm^{-1}.

^{1}H NMR (700 MHz, CD_3OD) δ: 7.26 (d, 1H, *J* = 2.1 Hz, H-2'), 7.24 (dd, 1H, *J* = 2.1, 8.4 Hz, H-6'), 6.89 (d, 1H, *J* = 8.4 Hz, H-5'), 6.64 (s, 1H, H-5), 6.36 (s, 1H, H-3), 3.93 (s, 3H, -OCH_3), 2.32 (s, 3H, -CH_3).
^{13}C NMR ($CDCl_3$, 175 MHz) δ: 166.4 (1C, C), 163.5 (1C, C), 157.9 (1C, C), 151.2 (1C, C), 149.6 (1C, C), 127.8 (1C, C), 121.9 (1C, CH), 116.8 (1C, CH), 111.2 (1C, CH), 105.9 (1C, CH), 104.6 (1C, CH), 56.6 (1C, CH_3), 19.9 (1C, CH_3).
LR-MS (FAB+) *m/z*: 233 ($M+H^+$).
HR-MS (FAB+): Calcd for $C_{13}H_{13}O_4$ ($M+H^+$): 233.0808. Found: 233.0809.

References

[1] Liu Y, Rakotondraibe LH, Brodie PJ, Wiley JD, Cassera MB, Miller JS, Ratovoson F, Rakotobe E, Rasamison VE, Kingston DG. (**2015**) Antimalarial 5,6-dihydro-α-pyrones from *Cryptocarya rigidifolia*: related bicyclic tetrahydro-α-pyrones are artifacts. *Journal of Natural Products*, ***78***, 1330-1338.

[2] Zhang J, Jiang Y, Cao Y, Liu J, Zheng D, Chen X, Han L, Jiang C, Huang X. (**2013**) Violapyrones A-G, α-pyrone derivatives from *Streptomyces violascens* isolated from *Hylobates hoolock* feces. *Journal of Natural Products*, ***76***, 2126-2130.

[3] Kim RK, Suh Y, Lim EJ, Yoo KC, Lee GH, Cui YH, Son A, Hwang E, Udiin N, Yi JM, Kang SG, Lee SJ. (**2013**) A novel 2-pyrone derivative, BHP, impedes oncogenic KRAS-driven malignant progression in breast cancer. *Cancer Letters*, ***337***, 49-59.

[4] Goel A, Taneja G, Raqhuvanshi A, Kant R, Maulik PR. (**2013**) Diversity-oriented general protocol for the synthesis of privileged oxygen scaffolds: pyrones, coumarins, benzocoumarins and naphthocoumarins. *Organic & Biomolecular Chemistry*, ***11***, 5239-5253.

[5] Dias LC, Kuroishi PK, de Lucca EC, Jr. (**2015**) The total synthesis of (-)-cryptocaryol A. *Organic & Biomolecular Chemistry*, ***13***, 3575-3584.

[6] Yao HQ, Wu LH, Chou GX, Liu Q, Wang ZT. (**2015**) Two rare α-pyrone (=2H-pyran-2-one) derivatives from *Gentiana rthodantha* Franchet. *Helvetica Chimica Acta*, ***98***, 657-662.

[7] Xu M, Zhang M, Wang D, Yang CR, Zhang YJ. (**2011**) Phenolic compounds from the whole plants of *Gentiana rhodantha* (Gentianaceae). *Chemistry & Biodiversity*, ***8***, 1891-1900.

[8] Hansen AL, Skrydstrup T. (**2005**) Regioselective Heck couplings of alpha, beta-unsaturated tosylates and mesylates with electron-rich olefins. *Organic Letters*, ***7***, 5585-5587.

[9] Bellina F, Marchetti C, Rossi R. (**2009**) An economical access to 3,4-diaryl-2(5*H*)-furanones and 4-aryl-6-methyl-2(2*H*)-pyranones by Pd-catalyzed Suzuki-type arylation of 3-aryl-4-tosyloxy-2(5*H*)-furanones and 6-methyl-4-tosyloxy-2(2*H*)-pyranones, respectively. *European Journal of Organic Chemistry*, ***27***, 4685-4690.

[10] Combes S, Barbier P, Douillard S, McLeer-Florin A, Bourgarel-Rey V, Pierson JT, Fedorov AY, Finet JP, Boutonnat J. Peyrot V. (**2011**) Synthesis and biological evaluation of 4-arylcoumarin analogues of combretastatins. Part 2. *Journal of Medicinal Chemistry*, ***54***, 3153-3162.

[11] Lee JH, Kim WS, Lee YY, Cho CG. (**2002**) Stille couplings of 3-(trimethylstannyl)-5-bromo-2-pyrone for the syntheses of 3-aryl-5-bromo-2-pyrones and their ambident dienyl characters. *Tetrahedron Letters*, ***43***, 5779-5782.

[12] Zhang JJ, Yan CS, Peng Y, Luo ZB, Xu XB, Wang YW. (**2013**) Total synthesis of (±)-sacidumlignans D and A through Ueno-Stork radical cyclization reaction. *Organic & Biomolecular Chemistry*, ***11***, 2498-2513.

[13] Djakovitch L, Rollet P. (**2004**) Sonogashira cross-coupling reactions catalysed by copper-free palladium zeolites. *Advanced Synthesis & Catalysis*, ***346***, 1782-1792.

NPC Natural Product Communications

2017
Vol. 12
No. 1
99 - 100

A New Lignan from the Leaves of *Zanthoxylum armatum*

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[†]Deceased 28 March 2016

Received: August 2nd, 2016; Accepted: October 20th, 2016

A new furofuran lignan, zanthonin (**1**) together with 13 known compounds including seven furofuran lignans (**2-8**), one isobutyl amide (**9**), a furanocoumarin (**10**) and four flavonoids (**11-14**) have been isolated from the leaves of *Zanthoxylum armatum*. The chemical structures of these compounds were elucidated mainly on the basis of NMR (1D and 2D) and MS data. This is the first report on the isolation of methylxanthoxylol (**4**) from *Z. armatum*.

Keywords: *Zanthoxylum armatum*, Rutaceae, Furofuran lignan, Zanthonin.

Zanthoxylum armatum DC [syn. *Z. alatum* Roxb.] (Rutaceae), is a deciduous aromatic tree distributed in tropical and subtropical regions of India at altitudes of 600-2100 m. It is commonly known as Indian prickly ash, Nepal pepper and toothache tree. The bark, seeds and fruit have been widely used in traditional medicine for the treatment of stomach ache, fever and dyspepsia, and is also used for catching fish (piscicidal). The fruit pericarp is used as a flavoring agent and spice in certain traditional dishes because of its pleasant flavor and delectable pungency. The fruit, bark and leaves exhibit anti-inflammatory, hepatoprotective, anti-fungal and anti-microbial activities [1]. Previous phytochemical studies on *Z. armatum* showed the presence of alkaloids, amides, lignans, flavonoids and terpenoids. Among these, lignans and amides are the main active constituents [1,2]. As a part of our search for bioactive compounds from Western Himalayan medicinal plants [3], herein we report the isolation and characterization of a new furofuran lignan (**1**), along with 13 known compounds from the leaves of *Z. armatum*.

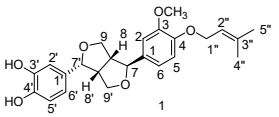

Table 1: ^{13}C (150 MHz) and ^{1}H (600 MHz) NMR data of **1** in $CDCl_3$. δ in ppm, *J* in Hz.

Position	δ_C	δ_H	Position	δ_C	δ_H
1	133.2	-	4′	143.1	-
2	109.4	6.90 (1H, s)	5′	112.8	6.83-6.88 (1H,m)
3	149.5	-	6′	117.9	6.69-6.78 (1H, m)
4	147.9	-	7′	82.1	4.81 (1H, d, *J* = 6.0)
5	112.8	6.83-6.88 (1H, m)	8′	49.9	3.29 (1H, m)
6	118.1	6.83-6.88 (1H, m)	9′a	69.6	3.83 (1H, m)
7	87.7	4.48 (1H, d, *J* = 6.9)	9′b		3.35 (1H, t, *J* = 8.5)
8	54.1	2.94 (1H, q, *J* = 7.3)	1″	65.8	4.55 (2H, d, *J* = 6.6)
9a	71.0	4.13 (1H, m)	2″	119.7	5.48 (1H, t, *J* = 6.6)
9b		3.88 (1H, m)	3″	137.8	-
1′	130.5	-	4″	18.2	1.71 (3H, s)
2′	115.1	6.69-6.78 (1H, m)	5″	25.8	1.74 (3H, s)
3′	143.7	-	OCH_3	55.9	3.85 (3H, s)

Compound **1** was isolated as colorless oil with optical rotation $[\alpha]^{25}_D$ = -116 (*c* 0.12, MeOH) and its molecular formula was deduced to be $C_{24}H_{28}O_6$ by positive HR-ESI-QTOF-MS, exhibiting a protonated ion at *m/z* 413.1964 $[M+H]^+$ (calcd. for $C_{24}H_{29}O_6$ 413.1951 $[M+H]^+$). The IR spectrum indicated the presence of a hydroxyl group (3537 cm^{-1}) and an olefinic double bond (1606 cm^{-1}). The UV spectrum in methanol showed absorption maxima at λ_{max} 211, 231 and 281 nm, suggesting the presence of aromatic and unsaturated moieties. The ^{1}H NMR data displayed resonances for two oxymethylene groups at δ_H 3.83 (1H, m, H-9′a) and 3.35 (1H, t, *J* = 8.5 Hz, H-9′b) and at δ_H 4.13 (1H, m, H-9a) and 3.88 (1H, m, H-9b), two oxymethine groups at δ_H 4.48 (1H, d, *J* = 6.9 Hz, H-7) and 4.81 (1H, d, *J* = 6.0 Hz, H-7′) and two methine groups at δ_H 2.94 (1H, q, *J* = 7.3 Hz, H-8) and 3.29 (1H, m, H-8′), indicating the presence of a furofuran moiety. The ^{1}H NMR spectrum also showed resonances for six aromatic protons as two ABX systems at δ_H 6.90 (1H, s, H-2), 6.83-6.88 (3H, m, H-5, H-6 and H-5′), and 6.69-6.78 (2H, m, H-2′ and H-6′), representing the presence of 1,3,4-trisubstituted benzene rings, one prenyloxy group at δ_H 5.48 (1H, t, *J* = 6.6 Hz, H-2″), 4.55 (2H, d, *J* = 6.6 Hz, H-1″), 1.71 (3H, s, H-4″), 1.74 (3H, s, H-5″) and one methoxy group at δ_H 3.85 (3H, s). The ^{13}C and DEPT NMR spectroscopic data showed twenty-four signals. Except for the resonances for a methoxy group and one prenyloxy group, the remaining eighteen carbon signals were consistent with a furofuran lignan derivative. The ^{1}H and ^{13}C NMR spectra of **1** were similar to those of planispine-A (**8**) with the major difference being that the methoxy group of **8** was replaced by a hydroxyl group in **1**.

The position of these groups was established from 2D NMR (HMQC, HMBC and NOESY) data. In the HMBC spectra, the correlations of -OCH_3 (δ_H 3.85) to C-3, C-4; H-2 (δ_H 6.90) to C-3, C-4, C-6, and H-1″ (δ_H 4.56) to C-4, C-3″, C-2″ indicated that one methoxy and one prenyloxy group were located at the C-3 and C-4 positions, respectively. The positions of the methoxy and prenyloxy groups were also confirmed by NOESY experiments, which showed the correlation of -OCH_3 (δ_H 3.85) to H-2 (δ_H 6.90) and H-1″ (δ_H 4.55) to H-5 (δ_H 6.83). The two hydroxyl groups were connected at C-3′ (δc 143.7) and C-4′ (δ_H 143.1), supported by the HMBC correlations of H-2′ to C-1′, C-3′ and H-6′ to C-5′, C-4′. The NOESY correlations of Hα-7 to Hα-9′ and Hβ-8′ to Hβ-7′ showed that the proton of Hα-7 and Hα-9′ were on the same face and had α orientations, whereas Hβ-8′ and Hβ-7′ had β orientations. This observation was further supported by the levorotatory optical rotation, which was similar to that of **8** ($[\alpha]^{25}_D$ = -41, *c* 0.12, MeOH). Thus, based on the above analysis and literature survey [4] the structure of zanthonin (**1**) was established as (-)-(7α, 7′β, 8β, 8′β)-3-methoxy-4-prenyloxy-7,9′: 7′,9-diepoxylignan-3′,4′-diol.

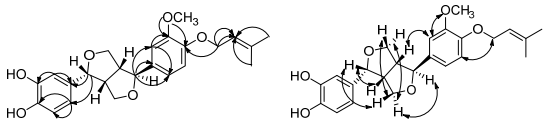

Figure 2: HMBC (⟶) and NOESY (⟷) correlations of compound **1**.

Along with the new compound (**1**), 13 known compounds were also isolated and identified as seven furofuran lignans: asarinin (**2**) [5], sesamin (**3**) [6], methylxanthoxylol (**4**) [7], kobusin (**5**) [8], fargesin (**6**) [9], eudesmin (**7**) [10] and planispine-A (**8**) [4]; isobutyl amide: α-sanshool (**9**) [11], a furanocoumarin: psoralin (**10**) [12] and four flavonoids: catechin (**11**) [13], vitexin (**12**) [14], isovitexin (**13**) [14] and hesperidin (**14**) [15] by comparing their spectroscopic data with those published. Among the known compounds, **4** was isolated for the first time from *Z. armatum*.

Experimental

General: Optical rotation, HORIBA SEPA-300 digital polarimeter; IR, Shimadzu IR Prestige-21 FT-IR spectrophotometer; UV, Shimadzu UV-2600; NMR, Bruker Avance-600; HR-ESI-MS, Waters QTOF Micro mass; TLC, silica gel 60 F254 plates and the compounds were visualized with iodine vapor and 20% H_2SO_4 in ethanol, followed by heating at 110°C. Normal phase (Merck 60-120 and 230-400 mesh) and C18-RP, 23% C (Acros organis, 40-63 micron) silica gel was used for column chromatography.

Plant material: The leaves of *Z. armatum* were collected from Palampur, Himachal Pradesh (alt. 1220 m) in June 2014. A voucher specimen (PLP-16528) was deposited in the Herbarium of CSIR-Institute of Himalayan Bioresource Technology, Palampur, Himachal Pradesh-176061, India.

Extraction and isolation*:* Air dried and powdered leaves of *Z. armatum* (3.5 kg) were extracted with methanol (3 x 10 L) for 24 h at room temperature by percolation and the percolate was evaporated under reduced pressure at 50°C to give 530 g of methanol extract. This was suspended in water (1000 mL) and subsequently partitioned with *n*-hexane, chloroform, EtOAc and *n*-butanol to obtain *n*-hexane (116.7 g), chloroform (34.2 g), EtOAc (30.7 g) and *n*-butanol (88.5 g) fractions. The water insoluble part was crystallized in methanol to yield **12** (8.617 g). The $CHCl_3$ fraction (34.2 g) was chromatographed over silica-gel (60-120 mesh) and eluted using *n*-hexane: EtOAc mixtures (10:0 to 0:10). A total of 46 fractions (250 mL each) were collected. Fractions 13-22 were subjected to silica gel column chromatography (CC) with a gradient of increasing EtOAc in *n*-hexane (8.5:1.5-8:2) to afford **2** (0.984 g) and **3** (0.735 g), which were further purified by crystallization. Fractions 26-38 were subjected to silica gel CC using *n*-hexane: EtOAc (8:2) to furnish **10** (0.047 g), **9** (0.423 g), **6** (0.981 g) and **5** (0.826 g). Fraction 40-42 was subjected to RP C18 CC using MeOH: H_2O (8:2) as eluent to yield **7** (0.220 g). The EtOAc fraction (30.7 g) was separated by CC over silica gel by eluting with a gradient of *n*-hexane: EtOAc (10: 0 to 0: 10) and EtOAc: MeOH (9.5:0.5 to 0:10) yielding 33 fractions (250 mL each). Fractions 18-21 were subjected to CC with EtOAc: *n*-hexane (1:3) resulting in the isolation of **11** (2.141 g). Similarly fractions 24-28 afforded **13** (2.537 g) using EtOAc: MeOH (9.5: 0.5) as eluent. Fraction 12 (7.0: 3.0) was subjected to RP C18 CC using MeOH: H_2O (8:2) to afford **4** (0.030 g). Fractions 14-16 were chromatographed over silica gel CC (230-400 mesh) using *n*-hexane: EtOAc (1:1) yielding 5 fractions (B-1 to B-5). B-3 was subjected to RP C18 CC using MeOH: H_2O (8:2) to yield **8** (0.070 g). Likewise, B-5 yielded **1** (0.030) using MeOH: H_2O (8:2) as eluent. The *n*-butanol fraction (88.5 g) was chromatographed over HP-20 diaion resin and eluted with H_2O: MeOH (10:0 to 0:10) to give 6 fractions. Fraction 3 was subjected to silica gel CC using $CHCl_3$: MeOH: H_2O (7.8: 2.0: 0.2) to give **14** (0.128 g).

Zanthonin (**1**)
Colorless oil
$[\alpha]_D^{25}$: -116 (*c* 0.12, MeOH)
IR (neat): 3537, 1514, 1377, 1259, 1031 cm^{-1}.
UV/Vis λ_{max} (MeOH) nm (log ε): 211 (3.344), 231 (2.820), 281 (1.136).
^1H and ^{13}C NMR ($CDCl_3$): Table 1.

Acknowledgments - We are thankful to the Director of the Institute for providing the necessary facility, CSIR, India for financial support (PROMOTE, BSC-0213) and UGC, India for granting senior research fellowships to Mr VB and Ms SS.

References

[1] The Wealth of India. (**1976**) Raw Materials; PID, Council of Scientific and Industrial Research (CSIR), New Delhi, ***2***, 18-19; (b) Gaur RD. (**1999**) *Flora of district Garhwal, North Western Himalaya (with ethno botanical notes)*. Transmedia, Srinagar, 811.
[2] Kalia NK, Singh B, Sood RP. (**1999**) A new amide from *Zanthoxylum armatum*. *Journal of Natural Products*, ***62***, 311-312.
[3] Kumar V, Kumar S, Singh B, Kumar N. (**2014**) Quantitative and structural analysis of amides and lignans in *Zanthoxylum armatum* by UPLC-DAD-ESI-QTOF–MS/MS. *Journal of Pharmaceutical and Biomedical Analysis*, ***94***, 23-29; (b) Bhatt V, Kumar V, Singh B, Kumar N. (**2015**) A new geranylbenzofuranone from *Zanthoxylum armatum. Natural Product Communications*, ***10***, 313-314.
[4] Hu Y, Chen Y, Shang Y, Yang G. (**2009**) Two new lignans from the bark of *Zanthoxylum planispinum*. *Bulletin of the Korean Chemical Society*, ***30***, 1884-1886.
[5] Yang GZ, Hu Y, Yang B, Chen Y. (**2009**) Lignans from the bark of *Zanthoxylum planispinum*. *Helvetica Chimica Acta*, ***92***, 1657-1633.
[6] Li XM, Li Z, Zhen QA, Cui TY, Zhu W, Tu ZB. (**1996**) Studies on the chemical constituents of *Zanthoxylum armatum* DC. *Natural Product Research and Development*, ***8***, 24-27.
[7] Biavatti MW, Vieira PC, da Silva MF, Fernandes JB, Degani AL, Cass QB, Ferreira AG. (**2001**) Separation and NMR studies on lignans of *Raulinoa echinata*. *Phytochemical Analysis*, ***12,*** 64-68.
[8] Latip J, Hartley TG, Waterman PG. (**1999**) Lignans and coumarins metabolites from *Melicope hayesii*. *Phytochemistry*, ***51***, 107-110.
[9] Kakisawa H, Chen YP, Hsu HY. (**1972**) Lignans in flower buds of *Magnolia fargesii*. *Phytochemistry*, ***11***, 2289-2293.
[10] Parhoodeh P, Rahmani M, Hashim NM, Sukari MA, Lian GEC. (**2011**) Lignans and other constituents from aerial parts of *Haplophyllum villosum Molecules*, ***16***, 2268-2273.
[11] Yasuda I, Takeda K, Itokawa H. (**1982**) Distribution of unsaturated aliphatic acid amides in Japanese *Zanthoxylum* species. *Phytochemistry*, ***21***, 1295-1298.
[12] Saqib QN, Hui Y-H, Anderson JE, McLaughlin JL. (**1990**) Bioactive furanocoumarins from the berries of *Zanthoxylum americanum*. *Phytotherapy Research*, ***4***, 216-219.
[13] Davis, LA, Cai Y, Davies PA, Lewis RJ. (**1996**) ^1H and ^{13}C NMR assignments of some green tea polyphenols. *Magnetic Resonance in Chemistry*, ***34,*** 887-890.
[14] Choo CY, Sulong NY, Man F, Wong TW. (**2012**) Vitexin and isovitexin from the leaves of *Ficus deltoidea* with *in-vivo* α-glucosidase inhibition. *Journal of Ethnopharmacology*, ***142***, 776-781.
[15] Markham KR. (**1976**) ^{13}C NMR of flavonoids-II: Flavonoids other than flavone and flavonol aglycones. *Tetrahedron*, ***32***, 2607-2612.

NPC Natural Product Communications

2017
Vol. 12
No. 1
101 - 103

Production of Podophyllotoxin by Plant Tissue Cultures of *Juniperus virginiana*

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Received: June 15th, 2016; Accepted: November 18th, 2016

Plant tissue cultures are a potential source of secondary metabolites. However, their production, when compared with intact plants, is usually lower. Phenylalanine, a biogenetic precursor of podophyllotoxin, was used to stimulate podophyllotoxin production in callus and suspension cultures of *Juniperus virginiana* L. The best phenylalanine effect on podophyllotoxin production was manifested in three-years-old callus cultures after a 21-days application of a 10 mmol/L concentration. A podophyllotoxin content of 0.15 mg/g DW was determined, which was about 400% higher in comparison with the control. The maximum content (0.48 mg/g DW) in newly derived suspension cultures (the 4th passage) was induced by 14-days application of a 1 mmol/L concentration; this was about 243% higher than the control. In one-year-old suspension cultures the highest podophyllotoxin content (0.56 mg/g DW) was recorded also after 14-days application of a 1 mmol/L concentration; this was about 211% higher than in the control cultures.

Keywords: *Juniperus virginiana*, Callus cultures, Suspension cultures, Podophyllotoxin production.

A natural lignan, podophyllotoxin, is the preferred precursor for the semi-synthesis of the anticancer drugs etoposide, teniposide and etopophose [1-5]. The traditional plant sources of podophyllotoxin are *Podophylum* species, but it has also been detected in other plant genera, such as *Linum*, *Juniperus*, *Hyptis*, *Teucrium*, *Nepeta*, *Dysosma*, *Jeffersonia*, *Thymus* and *Thuja*, yet the availability of this lignan remains limited [6,7]. Chemical synthesis of podophyllotoxin is also an expensive process, and so the biotechnological production of this compound using plant cell and tissue cultures can be considered as a promising alternative procedure.

Callus cultures of *Juniperus virginiana* L. (varieties 'Hetzii', 'Glauca', 'Grey Owl') were derived from leaves on Schenk and Hildebrandt medium supplemented with α-naphthaleneacetic acid, kinetin and ascorbic acid. *J. virginiana* suspension cultures (varieties 'Hetzii', 'Glauca', 'Grey Owl') were derived from two-years-old callus cultures on the same medium and under the same conditions as callus cultures. The callus and suspension cultures of the variety 'Glauca' had the best viability and thus provided the most biomass [8].

Plant tissue cultures are a potential source of secondary metabolites, but their production, when compared with intact plants, is usually lower and this is the reason why we are looking for different methods and approaches to increase the production. Phenylalanine, a podophyllotoxin biogenetic precursor [5], was used to stimulate podophyllotoxin production in callus and suspension cultures of *J. virginiana* (varieta 'Glauca').

The results proved that podophyllotoxin production in three-years-old callus cultures of *J. virginiana* is low (Figure 1). The best effect of phenylalanine on the production was manifested after a 21-days application of a 10 mmol/L concentration; a maximum podophyllotoxin content of 0.15 mg/g DW was determined, which is about 400% higher than that of the control.

Production of podophyllotoxin in suspension cultures of *J. virginiana* Glauca was larger. The maximum content (0.48 mg/g DW) in newly derived suspension cultures (4th passage) was induced by 14-days application of phenylalanine (concentration 1 mmol/L). Production was increased by about 243% in comparison with the control (Figure 2). Increased production (about 213%) was also recorded after 1-day application of the strongest (100 mmol/L) concentration (0.47 mg/g DW).

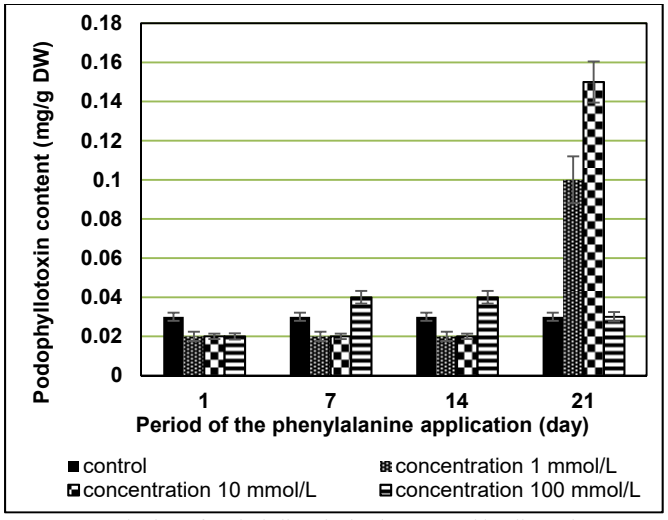

Figure 1: Production of podophyllotoxin in three-years-old callus cultures. Data represent the average values from 3 separate experiments ± SD.

In the one-year-old suspension cultures of *J. virginiana* (Figure 3), the best effect of phenylalanine was recorded also at the concentration of 1 mmol/L. The highest podophyllotoxin content (0.56 mg/g DW) was also recorded after 14-days application. This production was about 211% higher than that in the control. A positive phenylalanine effect was detected after the shortest, 1- day, application of the strongest concentration (100 mmol/L); podophyllotoxin production (0.55 mg/g DW) was increased by about 150%.

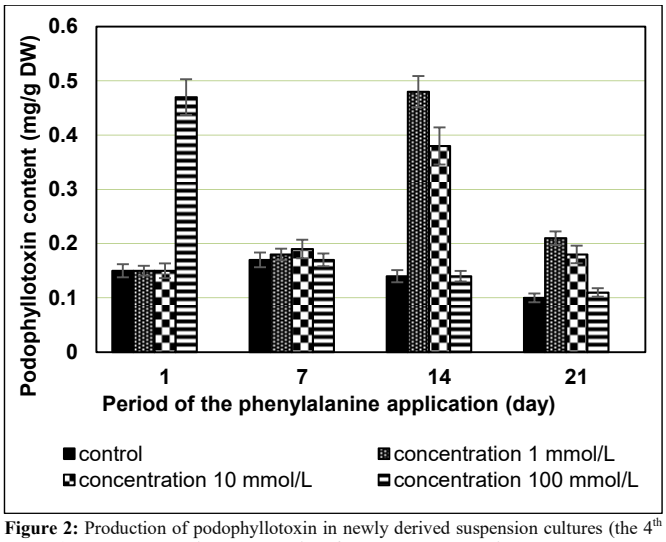

Figure 2: Production of podophyllotoxin in newly derived suspension cultures (the 4th passage). Data represent the average values from 3 separate experiments ± SD.

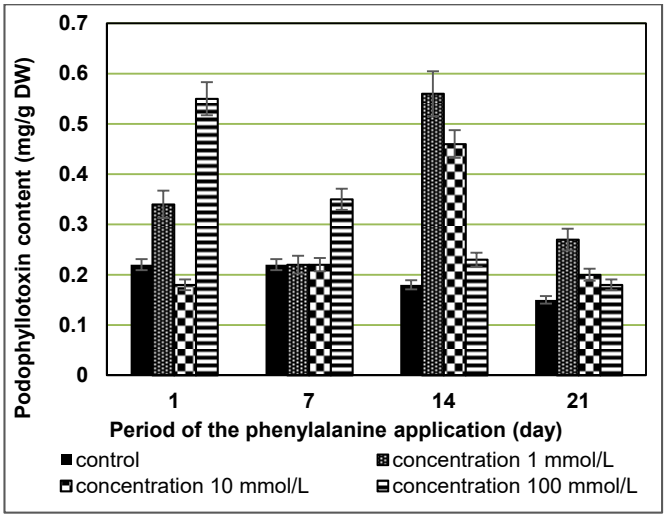

Figure 3: Production of podophyllotoxin in one-year-old suspension cultures. Data represent the average values from 3 separate experiments ± SD.

Comparison of podophyllotoxin production in the phenylalanine-treated *J. virginiana* suspension cultures (the 4th passage and one-year-old suspension cultures) confirms that the maximum increase in both cases was induced during the 14-days application of the 1 mmol/L concentration. Longer application of this concentration did not increase these values. In contrast, the results show that in three-years-old callus cultures of *J. virginiana* the podophyllotoxin content was the best stimulated after 21-days phenylalanine application (concentration 10 mmol/L). The higher production in the suspension cultures can be explained by the direct contact of cells with nutrient medium, which guarantees easier access of nutrients and the exchange of respiratory gases.

Stimulated podophyllotoxin production is documented also in the callus cultures of *J. chinensis* [9]. Production in the control cultures is low (0.05 mg/g DW), with the highest content being recorded (nearly 0.16 mg/g DW) 15 days after the addition of phenylalanine (concentration 0.5 mg/mL), similarly as in the callus cultures of *J. virginiana*.

The accumulation of lignans in plant tissue and organ cultures has been discussed based on the work of different authors [4,10-12]. It has been shown that production of podophyllotoxin and related lignans in cell cultures is possible. The newly derived plant tissue cultures as a source of anticancer lignans can play an important role in this respect.

Experimental

Plant material and growth conditions: The three-years-old callus cultures of *J. virginiana* (varieta 'Glauca'), the newly derived suspension cultures in the 4th passage and the one-year-old suspension cultures of *J. virginiana* (varieta 'Glauca') were cultivated in SH medium [6], supplemented with 3.0 mg/L of NAA, 0.2 mg/L of kinetin and 15 mg/L of ascorbic acid at 25°C and with a 16-h light/8-h dark photoperiod. A sub-cultivation interval of 28 days lasted for callus cultures and 21 days for suspension cultures.

Effect of a biogenetic precursor on production of podophyllotoxin: On the 7th day of cultivation, the callus and suspension cultures were treated with phenylalanine. One mL of an aqueous phenylalanine solution (concentrations of 1 mmol/L, 10 mmol/L and 100 mmol/L) was added to the SH medium (30 mL) and the cultures were cultivated for 1, 7, 14 and 21 days. The control cultures received 1.0 mL of distilled water. The cells were separated from the media by vacuuming, rinsed in distilled water and dried at room temperature.

Determination of podophyllotoxin: Preparation of the samples: 500 mg of control and treated cultures were extracted with 10.0 mL of methanol (100%) using ultrasound for 1 h at 30°C. The supernatant after centrifugation (at 4500 rpm for 5 min) was collected in vials and analyzed by HPLC-DAD. A JASCO 2000-Plus series liquid chromatograph (Jasco, Tokyo, Japan) equipped with diode array was used.

The HPLC conditions were as follows: the main wavelength was set at 280 nm and reference wavelengths for better peak identification were 270 and 290 nm. An RP-18 Lichrospher column (250 x 4 mm, particles size 5 µm) with a precolumn made of the same material was used. The injected volume of the samples and standard solutions was 20 µL and the column was operated at 25 °C.

The mobile phase consisted of (A) methanol/water/phosphoric acid (60:39.7:0.3; v/v/v) and (B) methanol/phosphoric acid (99.7:0.3; v/v). Gradient elution was designed as follows: 0 – 8 min, 0 – 80%; 8 – 9 min, 80 – 100%; 9 – 10 min, 100%; 10 – 11 min, 100% - 0%; 11 – 13 min, 0% of solvent B. The flow rate of the mobile phase was 1 mL/min.

For the podophyllotoxin quantification, a six-point calibration curve was obtained with a pure standard.

All the listed results are averages calculated from the results of 3 independent determination processes.

Acknowledgment - The study was supported by the research programs PRVOUK P40 and SVV 260294.

References

[1] Cantrell ChL, Zheljazkov VD, Osbrink WLA, Castro A, Maddox V, Craker LE, Astatkie T. (**2013**) Podophyllotoxin and essential oil profile of *Juniperus* and related species. *Industrial Crops and Products*, ***43***, 668-676.

[2] Guerram M, Jiang Z, Zhang L. (**2012**) Podophyllotoxin, a medicinal agent of plant origin: past, present and future. Chinese *Journal of Natural Medicines*, ***10***, 161-169.
[3] Yousefzadi M, Sharifi M, Behmanesh M, Moyano E, Bonfill M, Cusido RM, Palazon J. (**2010**) Podophyllotoxin: Current approaches to its biotechnological production and future challenges. *Engineering in Life Sciences*, ***10***, 281-292.
[4] Arroo RRJ, Alfermann AW, Medarde M, Petersen M, Pras N, Woolley JG. (**2002**) Plant cell factories as a source for anti-cancer lignans. *Phytochemistry Reviews*, ***1***, 27–35.
[5] Lv M, Xu H. (**2011**) Recent advances in semisynthesis, biosynthesis, biological activities, mode of action, and structure-activity relationship of podophyllotoxin. Mini-*Reviews in* Medicinal Chemistry, ***11***, 901-909.
[6] Ionkova I. (**2007**) Biotechnological approaches for the production of lignans. *Pharmacognosy Reviews*, ***1***, 57-68.
[7] Ionkova I. (**2010**) Biotechnology and modern production of plant made pharmaceuticals: anticancer compounds. *International Journal of Current Research in Chemistry*, ***1***, 199-209.
[8] Kašparová M, Spilková J, Cvak L, Siatka T, Martin J. (**2016**) Plant tissue cultures of *Juniperus virginiana*. *Natural Product Communications*, ***11***, 681-683.
[9] Muranaka T, Miyata M, Ito K, Tachibana S. (**1998**) Production of podophyllotoxin in *Juniperus chinensis* callus cultures treated with oligosaccharides and a biogenetic precursor. *Phytochemistry*, ***49***, 491-496.
[10] Ionkova I. (**2011**) Anticancer lignans - from discovery to biotechnology. Mini-*Reviews in* Medicinal Chemistry, ***11***, 843-856.
[11] Ramirez-Estrada K, Vidal-Limon H, Hidalgo D, Moyano E, Golenioswki M, Cusidó RM, Palazon J. (**2016**) Elicitation, an effective strategy for the biotechnological production of bioactive high-added value compounds in plant cell factories. *Molecules*, ***21***, 182-205.
[12] Bahadi SE, Sharifi M, Behmanesh M, Safaie N, Murata J, Araki R, Yamagaki T, Satake H. (**2012**) Time-course changes in fungal elicitor-induced lignan synthesis and expression of the relevant genes in cell cultures of *Linum album*. *Journal of Plant Physiology*, ***169***, 487-491.

NPC Natural Product Communications

2017
Vol. 12
No. 1
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New Data on Vanillin-Based Isothiazolic Insecticide Synergists

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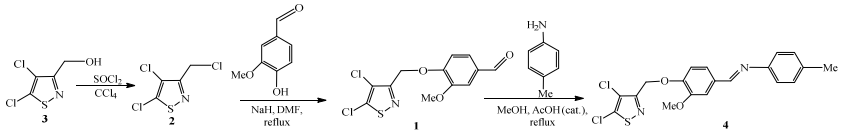
Received: August 9th, 2016; Accepted: September 28th, 2016

By alkylation of vanillin with 4,5-dichloro-3-chloromethylisothiazole the corresponding ether was synthesized. The latter was then reacted with *p*-toluidine to afford the corresponding azomethine. During the bioassays of synthesized isothiazolic derivatives of vanillin in mixtures with insecticides (imidacloprid and α-cypermethrin) a strong synergetic effect was observed.



Keywords: Vanillin, Isothiazole, Ether, Azomethine, Synergistic effect, Insecticide.

The development of new effective insecticides is one of the key tasks of agrochemistry. The constantly developing resistance of insects to the known insecticides and the aim to reduce the harmful impact on the environment are the main driving forces of this process [1,2]. Among insects with constantly growing resistance to insecticides, the Colorado potato beetle (CPB) is one of the highly notable examples as a serious threat to potato production. Since the middle of the last century, the beetle has developed resistance to 52 different compounds belonging to all major insecticide classes [3]. There are promising results on the creation of transgenic potato lines to control the damage and spread of CPB [4]. However, the development of new substances for chemical protection of potato does not lose its relevance.

Vanillin, a natural aldehydophenol, is widely used in the food industry, medicine and cosmetics. Previously we had reported a series of azomethins based on the vanillin ester of 4,5-dichlorisothiazolic acid, which showed high synergistic effects against CPB in the presence of the neonicotinoid insecticide Kerber® (imidacloprid) [5], whereas the vanillin ester of 4,5-dichlorisothiazolic acid itself showed a potentiating effect with the pyrethroid insecticide *Vitan® (*α-cypermethrin) [6]. During subsequent studies we found that reduction of the corresponding vanillin azomethins iminic fragment did not lead to significant change in their synergetic activity whereas substitution of chlorine in the 5th position of the isothiazolic ring for bulky fragments, except for the benzylthio fragment, results in a decrease of the isothiazolic vanillin derivatives potentiating activity [7]. Related compounds based on *iso*-vanillin also did not show a noticeable synergetic effect when mixed with the insecticides. For further study of structure activity relationships of isothiazolic vanillin conjugates we decided to synthesize isosters of initially tested compounds by changing the linker between the isothiazole and vanillin fragments from ester to ether. Ether **1** was synthesized by reaction of vanillin with 4,5-dichloro-3-chloromethylisothiazole (**2**)

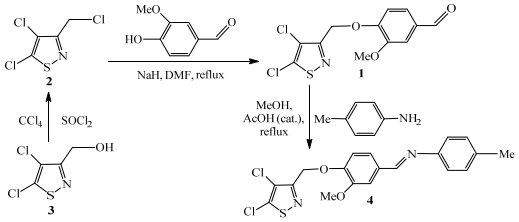


Figure 1: Preparation of vanillin and isothiazole conjugates.

Table 1: Insecticidal activity of insecticides Vitan®, Kerber®, and their mixtures with new vanillin and isothiazole conjugates against potato beetle larvae.

Entry	Active ingredient	a.i. concentration, mg/ha*	Average number of larvae after 24 h, % control	Average number of larvae after 72 h, % control
1	1	200	98.0	98.0
2	4	200	99.0	98.0
3	Vitan	160	83.3	36.7
4	Vitan	80	90.0	76.7
5	Kerber	200	43.3	33.3
6	Kerber	100	86.7	73.3
7	Vitan + 1	80	60.0	60.0
8	Vitan + 4	80	83.3	76.7
9	Kerber + 1	100	46.7	46.7
10	Kerber + 4	100	3.3	0.0

* a.i. means active ingredient, in entry 3-10 – concentration of basic insecticides.

in the presence of sodium hydride in refluxing DMF for 5 h. Chloromethyl derivative **2** was obtained by treatment of previously synthesized (4,5-dichloroisothiazol-3-yl)methanol (**3**) [8] with thionyl chloride. Condensation of ether **1** with *p*-toluidine in boiling methanol for 4 h in the presence of a catalytic amount of AcOH led to azomethine **4**, which was precipitated from the solution by cooling to 0°C. Product **4** was filtered, washed with cold methanol, dried in vacuum over P_2O_5 and further used without purification. The synthesized compounds were identified on the base of IR and ^{1}H- and ^{13}C-NMR spectra data. Azomethin **4** relates to aldimines, for which the *E*-form is preferable, as was established in the results of spectral studies of this group of compounds [9].

The synergetic activity of the synthesized isosters **1** and **4** in mixtures with Vitan® and Kerber® was evaluated by the greenhouse bioassay. Potato beetle larvae were treated with insecticide containing 5%, w/w, of either ether **1** or azomethine **4** with respect to the insecticide. The results of the bioassays are shown in Table 1. Individual compounds **1** and **4** had no insecticidal activity. However, their addition to the insecticides led to promotion of the insecticidal activity of both Vitan® and Kerber®. Vanillin ether **1** showed higher potentiating activity with Vitan® (α-cypermethrin), whereas azomethine **4** showed higher activity in composition with Kerber® (imidacloprid). The revealed potentiating activity of the tested compounds is similar to the activity of the initially tested vanillin ester of 4,5-dichlorisothiazolic acid and *p*-toluidine azomethine on its base. The obtained results, as well as our previous ones, indicate the crucial significance of the vanillin-isothiazolic scaffold for demonstration of biological activity in combination with α-cypermethrin and imidacloprid, though the linker nature between isothiazole and vanillin seems to be irrelevant. We assume this information will be useful for further research in this domain.

Experimental

General: Melting points, Boetius heating table; IR, Nicolet Protégé spectrometer; NMR, Brucker Avance-500 spectrometer.

4,5-Dichloro-3-(chloromethyl)isothiazole (2): A solution of 1.84 g (0.01 mol) of (4,5-dichloroisothiazol-3-yl)methanol (**3**) and 2.4 g (0.02 mol) of thionyl chloride in 15 mL dry tetrachloromethane was stirred for 10 h at room temperature, and then refluxed for 8 h. The reaction mixture was cooled, solvent and excess thionyl chloride were removed under vacuum, and 30 mL of *n*-hexane and 0.56 g (2 mmol) of phosphorous pentoxide were added to the crude product. The obtained suspension was stirred at room temperature for 1 h and filtered through a thin layer of silica gel. *n*-Hexane was evaporated under reduced pressure to give the product as a yellow oil. Yield 1.34 g (66%).
IR (KBr): 3022, 2964, 2851, 1507, 1435, 1391, 1375, 1310, 1262, 1213, 1154, 1100, 980, 925, 830, 807, 751, 675, 596, 523 cm^{-1}.
^1H NMR (500 MHz, $CDCl_3$): 4.63 (2H, s, CH_2).
^{13}C NMR (126 MHz, $CDCl_3$): 39.5 (CH_2), 123.2 (C), 149.1(C), 161.9 (C).
Anal. Calcd for $C_4H_2Cl_3NS$: C, 23.73; H, 1.00; Cl, 52.52; N 6.92; S 15.83. Found C, 23.82; H 1.18; Cl 52.60; N 6.99; S 15.97.

4-{(4,5-Dichloroisothiazol-3-yl)methoxy}-3-methoxybenzaldehyde (1): 0.3 g (1.85 mmol) of vanillin and 0.04 g (1.81 mmol) of sodium hydride (as 60% suspension in mineral oil) was stirred in 10 mL of DMF for 5 min. After that, 0.25 g (1.23 mmol) of 4,5-dichloro-3-(chloromethyl)isothiazole **2** was added, and the reaction mixture was refluxed for 5 h. The reaction mixture was cooled down, and quenched with 50 mL of sodium bicarbonate saturated solution, and stirred for 20 min. The precipitate was filtered off, dried under vacuum and purified by column chromatography on silica gel using benzene as the eluting solvent. Yield 0.28 g (71%).
MP 127-128°C.
IR (KBr): 3080, 3007, 2961, 2931, 2852, 2831, 1682, 1588, 1508, 1461, 1422, 1395, 1377, 1350, 1280, 1264, 1232, 1140, 1105, 1033, 1013, 1004, 859, 817, 734, 654 cm^{-1}.
^1H NMR (500 MHz, $CDCl_3$): 3.91 (3H, s, OCH_3), 5.27 (2H, s, CH_2), 7.09 (1H, d, *J*=8.7, $CH_{arom.}$), 7.39-7.42 (2H, m, $CH_{arom.}$), 9.84 (1H, s, HC=O).
^{13}C NMR (126 MHz, $CDCl_3$): 56.2 (OCH_3), 66.3 (CH_2), 109.9 ($CH_{arom.}$), 113.0 ($CH_{arom.}$), 123.2 (C), 126.3 ($CH_{arom.}$), 131.2 (C), 149.0 (C), 150.3 (C), 152.9 (C), 161.2 (C), 191.0 (CH=O).
Anal. Calcd for $C_{12}H_9Cl_2NO_3S$: C, 45.30; H, 2.85; Cl, 22.28; N 4.40; S 10.08. Found C, 45.41; H 2.99; Cl 22.35; N 4.43; S 10.14.

(*E*)-1-(4-((4,5-Dichloroisothiazol-3-yl)methoxy)-3-methoxyphenyl)-*N*-(*p*-tolyl)methanimine (4). To the solution of 0.32 g (1 mmol) of 4-((4,5-dichloroisothiazol-3-yl)methoxy)-3-methoxybenzaldehyde (**1**) and 0.11 g (1 mmol) of *p*-toluidine in 5 mL dry methanol, 1 drop of acetic acid was added and the mixture was refluxed for 4 h. Then the reaction mixture was cooled down to 0°C, the precipitate was filtered, washed with 1 mL of cooled methanol, and dried under vacuum over P_2O_5. Yield 0.30 g (72%).
MP 122-123°C.
IR (KBr): 3080, 3038, 3029, 3008, 1625, 1595, 1581, 1512, 1460, 1417, 1377, 1275, 1232, 1215, 1138, 1107, 1025, 1011, 808 cm^{-1}.
^1H NMR (500 MHz, $CDCl_3$): 2.41 (3H, s, Me), 4.01 (3H, s, OMe), 5.30 (2H, s, CH_2), 7.08 (1H, d, *J*=8.2, $CH_{arom.}$), 7.16 (2H, d, *J*=8.3, $2CH_{arom.}$), 7.23 (2H, d, *J*=8.1, $2CH_{arom.}$), 7.28-7.33 (1H, m, $CH_{arom.}$), 7.66 (1H, d, *J*=1.8, $CH_{arom.}$), 8.40 (1H, s, CH=O).
^{13}C NMR (126 MHz, $CDCl_3$): 21.1 (CH_3), 56.3 (OCH_3), 66.7 (CH_2), 110.0 ($CH_{arom.}$), 114.0 ($CH_{arom.}$), 120.9 ($2CH_{arom.}$), 123.3 (C), 123.9 ($CH_{arom.}$), 129.9 ($2CH_{arom.}$), 131.2 (C), 135.7 (C), 148.8 (C), 149.7 (C), 150.5 (C), 158.0 (C), 159.0 (C), 161.9 (CH=O).
Anal. Calcd for $C_{19}H_{16}Cl_2N_2O_2S$: C, 56.03; H, 3.96; Cl, 17.41; N 6.88; S 7.87. Found C, 56.15; H 4.07; Cl 17.39; N 6.78; S 7.92.

References

[1] Sarwar M, Salman M. (**2015**) Insecticides resistance in insect pests or vectors and development of novel strategies to combat its evolution. *International Journal of Bioinformatics and Biomedical Engineering*, ***1***, 344–351.

[2] Kaur H, Garg H. (**2014**) Pesticides*:* Environmental impacts and management strategies, pesticides - Toxic aspects. Soloneski S. (Ed.). InTech, DOI: 10.5772/57399. Available from: http://www.intechopen.com/books/pesticides-toxic-aspects/pesticides-environmental-impacts-and-management-strategies Accessed in 20/06/2016.

[3] Alyokhin A, Baker M, Mota-Sanchez D, Dively G, Grafius E. (**2008**) Colorado potato beetle resistance to insecticides. *American Journal of Potato Research*, ***85***, 395–413.

[4] Guo W, Wang Z, Luo X, Jin X, Chang J, He J, Tu E, Tian Y, Si H, Wu J. (**2016**) Development of selectable marker-free transgenic potato plants expressing cry3A against the Colorado potato beetle (*Leptinotarsa decemlineata* Say). *Pest Management Sciences*, ***72***, 497–504.

[5] Potkin V, Zubenko Yu, Bykhovetz A, Zolotar R, Goncharuk V. (**2009**) Synthesis of novel vanillin derivatives containing isothiazole moieties and its synergistic effect in mixtures with insecticides. *Natural Product Communications*, ***4***, 1205-1208.

[6] Potkin VI, Bykhovetz AI, Lakhvich FA, Nechai NI, Zolotar RM, Goncharuk VM, Zubenko YuS, Vashkevich EV. (**2006**) Insecticidal composition. Belarus patent 1239.

[7] Potkin VI, Kletskov AV, Zubenko YuS, Dikusar EA, Petkevich SK, Zvereva TD, Zolotar RM, Chepikn OP. (**2013**) Synthesis and the potentiating action of the vanillin derivatives containing the isothiazole heterocycle in compositions with insecticides. *Doklady Natsionalnoi Akademii Nauk Belarusi*, ***57***, 76–83.

[8] Potkin VI, Petkevich SK, Kletskov AV, Dikusar EA, Zubenko Yu S, Zhukovskaya NA, Kazbanov VV, Pashkevich SG. (**2013**) Synthesis of functionally substituted isoxazole and isothiazole derivatives. *Russian Journal of Organic Chemistry,* ***49***, 1523–1533.

[9] Saleem LMN. (**1982**) *Trans-cis* isomerization of Schiff's bases (*N*-benzylideneanilines) on addition of lanthanide shift reagents. *Organic Magnetic Resonance*, ***19***, 176–180.

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Phileucin - A Cyclic Dipeptide Similar to Phevalin (Aureusimine B) from *Streptomyces coelicolor* M1146

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Received: June 29th, 2016; Accepted: November 3rd, 2016

Overexpression of a putative type III polyketide synthase (PKSIII) from the marine myxobacterium *Enhygromyxa salina* SWB007 in *Streptomyces coelicolor* M1146 led to the accumulation of a novel monoketopiperazine consisting of phenylalanine and isoleucine. This compound was named phileucin and shows high structural similarity to phevalin (aureusimine B). The protease inhibiting activity was tested against human cathepsin L, human leukocyte elastase, bovine trypsin and bovine chymotrypsin. In contrast to phevalin, no protease inhibition was observed.

Keywords: Myxobacteria, Streptomyces, Monoketopiperazine.

Marine myxobacteria, e.g. of the genus *Enhygromyxa*, have the potential to biosynthesize so far unknown natural products [1]. For *E. salina* SWB007 production of enhygrolides and of salimabromide (**1**) was shown (Figure 1) [2, 3]. Compound **1** is a brominated polyketide with an unprecedented carbon skeleton, which exhibited moderate antibiotic activity against Gram-positive bacteria. However, the biosynthesis of salimabromide (**1**) remained elusive up to now. The structure of **1** gives hints towards the involvement of a polyketide synthase (PKS), a terpene synthase, and a halogenase. Thus, a putative type III PKS identified within the genome of *E. salina* SWB007, which is clustered with a putative halogenase, should be tested towards its involvement in the biosynthesis. For this purpose, the corresponding gene named *ks007* was heterologously expressed in *Streptomyces coelicolor* M1146 [4]. Using the vector pGM1202 (kindly provided by Dr Günther Muth, University of Tübingen, Germany), the expression of *ks007* was inducible under the control of the *tipA* promoter. The identity of the construct pGM1202-ks007 was verified by sequencing, and it was transferred to *S. coelicolor* M1146 by bi-parental conjugation, yielding strain *S. coelicolor* M1146 pGM1202-ks007. Cells of this strain were grown in ISP2 medium and the expression of the gene *ks007* was induced by adding thiostreptone to the culture. After seven days of incubation, the cultures were extracted using ethyl acetate. Analysis of the resulting crude extract by chromatographic methods revealed one additional peak in comparison to the host strain. To elucidate the identity of the metabolite causing the additional peak, the compound was purified by vacuum liquid chromatography (VLC) and high performance liquid chromatography (HPLC).

Following purification, the molecular formula of compound **2** (Figure 2) was deduced by high resolution mass measurement (HRESIMS *m/z* 265.1311 $[M+Na]^+$) to be $C_{15}H_{18}N_2O$ (calculated m/z 265.1316 $[M+Na]^+$). The ^{13}C NMR spectrum (Table 1) disclosed 15 resonances resulting from two methyl groups (δ_c 12.3 for C-1, δ_c 18.3 for C-4), two sp^3 methylene groups (δ_c 28.7 for C-2, δ_c 37.1 for C-9), six sp^2 methine groups (δ_c 123.5 for C-8, δ_c 129.9 for C-11, δ_c 129.9 for C-12, δ_c 128.2 for C-13, δ_c 129.9 for C-14, δ_c 129.9 for C-15), one sp^3 methine group (δ_c 37.6 for C-3) and four sp^2 quaternary carbon atoms (δ_c 161.5 for C-5, δ_c 158.5 for C-6, δ_c 139.9 for C-7, δ_c 138.1 for C-10). The ^1H NMR spectrum (Table 1) exposed two signals for methyl groups (δ_H 0.90 for H_3-1, δ_H 1.20 for H_3-4), one for a methine group (δ_H 3.22 for H-3) and six protons resonating in the olefinic region (δ_H 7.17 for H-8, δ_H 7.37 for H-11, δ_H 7.31 for H-12, δ_H 7.30 for H-13, δ_H 7.31 for H-14, δ_H 7.37 for H-15). H-8 is incorporated into the double bond $\Delta^{7,8}$ and H-11, H-12, H-13, H-14 and H-15 are incorporated into an aromatic ring system. The aromatic ring system was indicated by the UV spectrum, revealing an absorption band with a maximum at 328 nm (Figure S1).

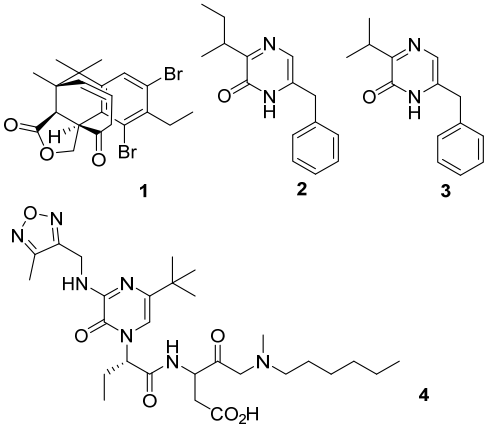

Figure 1: Structures of salimabromide (**1**), phileucin (**2**), phevalin (**3**) and M826 (**4**).

The planar structure of compound **2** as shown in Figure 1 was deduced from ^1H-^1H COSY and ^1H-^{13}C HMBC measurements. The COSY spectrum showed two independent spin systems: (i) one aromatic part from H-11 to H-15, and (ii) one smaller alkyl part from H_3-1 to H_3-4. The HMBC spectrum further reveals the coupling between H-3 and both C-5 and C-6, indicating a branched alkyl moiety. HMBC correlations between H_2-9 and all C-11, C-15, C-7 and C-8 delineated the presence of a phenylalanine derived moiety. However, the connection between both moieties could not be easily observed. A closer look at the HMBC spectrum revealed a very weak coupling between H-8 and C-5, indicating their connection via a nitrogen atom, evidenced by the chemical shift of

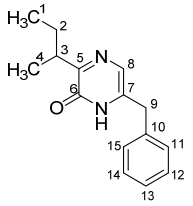

Figure 2: Structure of phileucin.

Table 1: ^{1}H and ^{13}C NMR spectral data of compound **2** in chloroform-d_1.

No.	^{13}C [ppm]	mult.	^{1}H [ppm]/*J*	^{1}H-^{1}H COSY	^{1}H-^{13}C HMBC
1	12.3	CH_3	0.9 (t 7.5)	2a;2b	2;3
2	28.7	CH_2	a: 1.81 (m) b: 1.55 (m)	1;3	1;3;4;5
3	37.6	CH	3.22 (m)		1;2;4;5;6
4	18.3	CH_3	1.2 (d 7.0)		2;3;5
5	161.5	C			
6	158.5	C			
7	139.9	C			
8	123.5	CH	7.17 (s)		5 (w)
9	37.1	CH_2	3.88 (s)	8;11;15	7;8;11;15
10	138.1	C			
11	129.9	CH	7.37 (m)	12	13
12	129.9	CH	7.31 (m)	11;13	10
13	128.2	CH	7.30 (m)	12;14	11;15
14	129.9	CH	7.31 (m)	13;15	10
15	129.9	CH	7.37 (m)	14	13

carbon C-5 (Table 1). Taking the molecular formula and the chemical shifts of C-6 and C-7 into account, their connection via a peptide bond was deduced, completing the planar structure of compound **2**. Due to the low amount of isolated substance, it was not possible to identify the stereochemistry of phileucin at C-3.

Unexpectedly, the isolated compound was not a polyketide, and thus not the product of the heterologously expressed PKSIII. Instead, structural analysis revealed a 3,6-disubstituted 2(1*H*)-pyrazinone, a compound which can be considered as dehydrated diketopiperazine, deduced from the amino acids phenylalanine and isoleucine. We suggest the name phileucin (**2**). The mechanism underlying the production of **2**, due to the expression of the putative PKSIII gene from *E. salina* SWB007, is unknown. Compound **2** bears strong resemblance to phevalin (**3**, also known as aureusimine B), which was reported to have calpain inhibiting properties (IC_{50} 1.3 µM) [5]. However, this finding was not confirmed with a synthetic sample of phevalin [6]. Phevalin and the structurally related tyrvalin were found in *Staphylococcus aureus* strains and are suspected to be involved in biofilm formation [7].

Moreover, the structurally related 2(1*H*)-pyrazinone derivative M926 (**4**) (Figure 1) potently inhibited caspase-3 [8]. In the light of the reported protease inhibiting properties of **3** and **4**, compound **2** was evaluated for inhibiting activities towards clinically relevant proteases. The selected enzymes were human cathepsin L, a cysteine protease, as well as the serine proteases human leukocyte elastase, bovine trypsin and bovine chymotrypsin. However, phileucin (**2**) showed no significant inhibitory activity towards any of the enzymes tested; all IC_{50} values were above 100 µM.

Experimental

Construction of strain* S. coelicolor *M1146 pGM1202-KS007: The putative ketosynthase gene *ks007* was amplified by colony-PCR, using *E. salina* SWB007 cells with the following primers: forward-primer containing an *Nde*I restriction-site (KS007-for: GCGCATATGTCAACCGTCATCCCCGTTGGTATT), and reverse-primer containing a *Hin*dIII restriction-site (KS007-rev: GGCAAGCTTCAGCGGCTCGAAGAAAAGAAGTAG). PCR reactions were analysed by agarose gel electrophoresis on a 1% agarose gel. The expected band with a size of 1139 bp was excised and the DNA was purified using the Promega SV Gel and PCR clean up system, and ligated into the pGEM-T vector (Promega). The construct was transferred into chemically competent *E. coli* XL1 Blue cells by heat-shock transformation. Candidate colonies were grown in liquid LB_{amp} medium for 16 h at 37°C and plasmids were isolated using Promega Pure yield Miniprep Kit. Restriction of the plasmids with *Nde*I and *Hin*dIII verified the presence of the target gene *ks007*. A plasmid harbouring the *ks007* was chosen and the identity of the insert was verified by sequencing with T7 and SP6 primers (GATC Biotech). Subsequently, the *ks007* gene was transferred into pGM1202 (*Nde*I, *Hin*dIII) by ligation for 16 h at 4°C. The resulting plasmid pGM1202-*ks007* was transferred into competent *E. coli* ET12567 pUZ8002 cells using heat-shock transformation, resulting in the strain *E. coli* ET12567 pUZ8002 pGM1202-*ks007*.

The pGM1202-*ks007* plasmid was transferred to *S. coelicolor* M1146 by conjugation between *E. coli* ET12567 pUZ8002 pGM1202-*ks007* and *S. coelicolor* M1146. For the conjugation, ET12567 pUZ8002 pGM1202-*ks007* cells were grown in 100 mL $LB_{apra/kan}$-medium at 37°C up to an OD_{600} of 0.5. The cells were then precipitated at 10000 rcf for 10 min and the resulting cell pellet washed twice in 10 mL LB-medium. The pellet was resuspended in 1 mL LB-medium. Meanwhile, 10 µL of *S. coelicolor* M1146 spore solution was added to 500 µL 2xYT broth (16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl, pH 7.0) and heat shocked at 50°C for 10 min. After cooling to room temperature, 500 µL of *E. coli* ET12567 pUZ8002 pGM1202-*ks007* suspension was added. The mixture was precipitated at 10000 rcf for 3 min and resuspended in the return flow. The resulting suspension was transferred to Cullum-medium agar plates (mannitol 20 g/L, soy-flour 20 g/L, agar 20 g/L, pH 7.3) containing 10 mM $MgCl_2$ and incubated at 30°C for 16 h. Subsequently, 1 mL nalidixic-acid solution (60 mg/mL in CH_3Cl) and 1 mL apramycin solution (100 mg/mL) were applied to the plates and allowed to set into the agar. The plates were incubated at 30°C for one week until colonies became visible. The visible colonies were transferred to Cullum-plates containing 60 µg/mL nalidixic acid and 100 µg/mL apramycin for selection, and were incubated for 5 days at 30°C. The presence of the *ks007* gene was verified by colony-PCR of the candidate colonies using the KS007-for and KS007-rev primers.

Isolation and purification of phileucin: *S. coelicolor* M1146 pGM1202-*ks007* was cultivated on a larger scale using 1 L of ISP2 medium (10 g/L malt extract, 4 g/L yeast extract, 4 g/L glucose, pH 7.5) containing 0.5 mg/L apramycin at 30°C, with a 120 rpm shaking speed. The expression of the gene was induced twice, once after 3 days and then after 5 days of incubation, by the addition of 0.05 mg/L thiostreptone. After 7 days the culture was extracted 2 times by liquid/liquid phase extraction using an equal amount of ethyl acetate. The ethyl acetate was evaporated and the crude extract dissolved in 10 mg/mL methanol/water (50/50 v/v). This extract was fractionated using Bakerbond™ SPE Octadecyl (C18) 2000 MG/6mL columns (Avantor Performance Materials, Center Valley, USA), resulting in a (50/50, v/v) MeOH/H_2O fraction and a 100% MeOH fraction. The 100% MeOH fraction was further processed by HPLC using a NUCLEOSHELL RP 18plus, 5 µm column and 65%/35% methanol/water (v/v) as mobile phase, with a flow rate of 0.8 mL/min. The chromatogram showed a peak at 8 min with high absorption at 400 nm, which was roughly collected in multiple runs. For the final purification of the compound, the

composition of the mobile phase was altered to 63%/37% methanol/water (v/v) and the flow rate decreased to 0.7 mL/min.

Structure elucidation of phileucin: High-resolution mass spectrometry was performed using an ESI-MS micrOTOF spectrometer (Bruker Daltonik, Bremen, Germany). The structure elucidation was performed by NMR spectroscopic analysis of the purified compound. The measurements were performed using a Bruker Avance 500 DRX (Bruker, Billerica, USA) spectrometer operating at 500 MHz (^{1}H) and 125 MHz (^{13}C). Processing of the collected data was performed using Bruker XWIN-NMR 3.5 (Bruker). The signals were referenced to the respective solvent signal for CD_3OD (σH/C 3.35/49.0). The molecular structure was elucidated, based on ^{1}H, ^{13}C, DEPT135, ^{1}H-^{1}H COSY, ^{1}H-^{13}C direct correlation (HSQC), and ^{1}H-^{13}C long-range correlation (HMBC). The resulting signals were compared with values calculated by Advanced Chemistry Development (ACD/Labs) Software V11.02 (ACD/Labs).

Enzymatic assays: The proteases were assayed as described [9,10].

Phileucin

$[\alpha]_D$: +0.015 (*c* 1.00, acetone).

IR (KBr): 2924.0, 1634.3, 1494.2, 1453.6, 960.0, 762.5, 717.6, 697.1 cm^{-1}.

UV/Vis λ_{max} (MeOH) nm (log ε): 206 (3.80), 328 (3.20)

^{1}H NMR (400 MHz, $CDCl_3$): 0.9 (3H, t, *J* = 7.5 Hz, H-1), 1.2 (1H, d, *J* = 7.0 Hz, H-4), 1.55 (1H, m, H-2b), 1.81 (1H, m, H-2a), 3.22 (1H, m, H-3), 3.88 (2H, s, H-9), 7.17 (1H, s, H-8), 7.30 (1H, m, H-13), 7.31 (1H, m, H-12), 7.31 (1H, m, H-14), 7.37 (1H, m, H-11), 7.37 (1H, m, H-115

^{13}C NMR (100 MHz $CDCl_3$): 12.3 (CH_3), 18.3 (CH_3)28.7 (CH_2), 37.1 (CH_2), 37.6 (CH), 123.5 (CH), 128.2 (CH), 129.9 (CH), 129.9 (CH), 129.9 (CH), 129.9 (CH) 138.1 (C), 139.9 (C), 158.5 (C), 161.5 (C).

HRESI-MS, *m/z*: measured, 265.1311 [M + Na^+]; calcd. for $C_{15}H_{18}N_2ONa$: 265.1316.

Supplementary data: The UV and IR spectra for phileucin are given.

Acknowledgments - The authors are grateful to the German Federal Ministry of Education and Research (BMBF) for funding this work. The authors thank Anna-Christina Schulz-Fincke for performing the enzyme assays.

References

[1] Dávila-Céspedes A, Hufendiek P, Crüsemann M, Schäberle TF, König GM. (**2016**) Marine-derived myxobacteria of the suborder Nannocystineae: An underexplored source of structurally intriguing and biologically active metabolites. *Beilstein Journal of Organic Chemistry*, ***12***, 969–984.

[2] Felder S, Dreisigacker S, Kehraus S, Neu E, Bierbaum G, Wright PR, Menche D, Schäberle TF, König GM. (**2013**) Salimabromide: Unexpected chemistry from the obligate marine myxobacterium *Enhygromyxa salina*. *Chemistry - A European Journal*, ***19***, 9319-9324.

[3] Felder S, Kehraus S, Neu E, Bierbaum G, Schäberle TF, König GM. (**2013**) Salimyxins and enhygrolides: Antibiotic, sponge-related metabolites from the obligate marine myxobacterium *Enhygromyxa salina*. *ChemBioChem*, ***14***, 1363-1371.

[4] Gomez-Escribano JP, Bibb MJ. (**2011**) Engineering *Streptomyces coelicolor* for heterologous expression of secondary metabolite gene clusters. *Microbial Biotechnology*, ***4***, 207-215

[5] Alvarez ME, White CB, Gregory J, Kydd GC, Harris A, Sun HH, Gillum AM, Cooper R. **(1995)** Phevalin, a new calpain inhibitor, from a *Streptomyces* sp. *The Journal of Antibiotics*, ***48***, 1165-1167.

[6] Zeng Y, Li Q, Hanzlik RP, Aubé J. (**2005**) Synthesis of a small library of diketopiperazines as potential inhibitors of calpain. *Bioorganic & Medicinal Chemistry Letters*, ***15***, 3034-3038.

[7] Secor PR, Jennings LK, James GA, Kirker KR. Pulcini E, McInnerney K, Gerlach R, Livinghous T, Hilmer JK, Bothner B, Flechmann P, Olerud J. E, Stewart PS.**(2012)** Phevalin (aureusimine B) production by *Staphylococcus aureus* biofilm and impacts on human keratinocyte gene expression. *Plos One*, **7**e40973.

[8] Han BH, Xu D, Choi J, Han Y, Xanthoudakis S, Roy S, Tam J, Vaillancourt J, Colucci J, Siman R, Giroux A, Robertson GS, Zamboni R, Nicholson DW, Holtzman DM. (**2002**) Selective, reversible caspase-3 inhibitor is neuroprotective and reveals distinct pathways of cell death after neonatal hypoxic-ischemic brain injury. *Journal of Biological Chemistry*, ***277***, 30128-30136.

[9] Frizler M, Lohr F, Lülsdorff M, Gütschow M. (**2011**) Facing the gem-dialkyl effect in enzyme inhibitor design: preparation of homocycloleucine-based azadipeptide nitriles. *Chemistry - A European Journal*, ***17***, 11419-11423.

[10] Gütschow M, Pietsch M, Themann A, Fahrig J, Schulze B. (**2005**) 2,4,5-Triphenylisothiazol-3(2*H*)-one 1,1-dioxides as inhibitors of human leukocyte elastase. *Journal of Enzyme Inhibition and Medicinal Chemistry*, ***20***, 341–347.

NPC Natural Product Communications

2017
Vol. 12
No. 1
111 - 114

OM-X®, a Fermented Vegetables Extract, Facilitates Muscle Endurance Capacity in Swimming Exercise Mice

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Received: July 21st, 2016; Accepted: September 23rd, 2016

The anti-fatigue effect was investigated of the probiotic supplement, OM-X®, on forced swimming capacity in mice. Mice were administered either vehicle (distilled water; DW) or OM-X® (85 mg/kg body weight) by gavage for 4 weeks. Forced swimming tests were conducted weekly using the Ishihara-modified Matsumoto swimming pool. The endurance swimming time of the final forced swimming exercise in mice fed with OM-X® group showed an approximately 2-fold increase compared with the vehicle control group. Biomedical parameters, including blood lactate, blood superoxide dismutase (SOD) activity, serum triacylglycerol (TG), hepatic total lipids (TL), TG and phospholipid (PL) were significantly lower in mice fed with OM-X® than those in the vehicle control group. Furthermore, the mRNA expression levels of carbamoyl phosphate synthetase 1 (Cps1) and arginase 1 (Arg1*)*, in the urea cycle, were increased by OM-X® feeding. Thus, our findings suggest promotion of lipid metabolism and up-regulation of the urea cycle, at least in part, for the anti-fatigue effect mediated by OM-X®.

Keywords: OM-X®, Muscle endurance capacity, Lipid metabolism, Urea cycle, Anti-fatigue.

OM-X® is a probiotic supplement that was manufactured by Dr Iichiroh Ohhira using a unique fermentation method. It is strictly hand-made and is comprised of all natural components. OM-X® is a fermented mixture of vegetables, fruits, seaweeds, and mushrooms, using 12 strains of lactic acid bacteria (LAB) and bifidobacteria. After 5 years of fermentation at room temperature, the fermented mixture contains probiotics, prebiotics such as oligosaccharides and dietary fiber, and trace amounts of vitamins, minerals, short-chain fatty acids, and amino acids. In human clinical studies, OM-X® has shown beneficial effects on bone health [1], oral ulcerations [2], and colitis [3]. Recently we further reported the inhibitory effect of OM-X® on antigen-stimulated type I allergy [4]. The inhibitory mechanisms by OM-X® was due, at least in part, to the suppression of intracellular Ca^{2+} mobilization through inhibition of the translocation of cytosolic subunits of NADPH oxidase to membrane-bound subunits and direct scavenging of ROS that are produced by NADPH oxidase.

Most people are subjected to several stressors in their modern life, resulting in the accumulation of mental and physical fatigues. In the early stage of fatigue, favorable working conditions cannot be maintained, often resulting in impaired performances. Long-term accumulation of fatigue ultimately results in death or life-threatening diseases [5, 6]. Specifically, the recovery from fatigue is crucial for athletes to maintain their high performance in competitions. In order to avoid accumulating fatigue, athletes adopt several dietary and recovery methods that are individually matched. In recent studies, traditional plants [7], green tea [8, 9], astaxanthin [10], fucoidan [11] and deep seawater [12] have demonstrated beneficial effects in the suppression of fatigue accumulation. OM-X is a naturally-fermented plant-based probiotic containing herbs, seaweed, fruits and vegetables. Therefore, the aim of this study was to evaluate the muscle endurance capacity in OM-X®-fed mice, and to prove that OM-X® is a beneficial naturally fermented extract against fatigue.

Changes in body weight, food consumption and water intake: The changes in body weight, food consumption, and water intake were similar between the vehicle control group and OM-X® group during the experimental period (Table 1).

Table 1: Body weight, food consumption, and water intakes in vehicle control mice and OM-X® fed mice for 4 weeks.

Group		water	OM-X
Body weight (g)	0 w	32.1 ± 0.8	32.2 ± 1.6
	1 w	35.1 ± 2.9	34.9 ± 2.1
	2 w	40.0 ± 2.3	39.8 ± 2.3
	3 w	42.1 ± 2.1	40.1 ± 3.0
	4 w	43.5 ± 2.4	44.2 ± 2.4
Food consumption (g/day/mouse)	0-1 w	3.0 ± 0.4	3.0 ± 0.5
	1-2 w	5.8 ± 0.3	5.8 ± 0.4
	2-3 w	5.0 ± 0.4	4.7 ± 0.7
	3-4 w	5.3 ± 0.6	5.4 ± 0.3
Water intake (mL/day/mouse)	0-1 w	5.9 ± 0.6	6.0 ± 0.4
	1-2 w	7.0 ± 0.8	7.4 ± 0.6
	2-3 w	7.0 ± 0.4	6.7 ± 0.4
	3-4 w	7.4 ± 0.5	6.5 ± 0.8

Values are given as mean ± S. E. (each group n=17).

Effect of OM-X® on endurance capacity for swimming: To assess the effect of OM-X® on endurance capacity, we employed the swimming exercise using the Ishihara-modified Matsumoto swimming pool. As shown in Figure 1, intakes of OM-X® over 2 to 4 weeks gradually recovered the endurance swimming time up to the level of 1 week. The extended endurance swimming time in mice fed with OM-X® was significantly higher than that of the control group. Specifically, the endurance swimming time of the final forced swimming exercise in mice fed with OM-X® showed approximately a 2-fold increase compared with the vehicle control group.

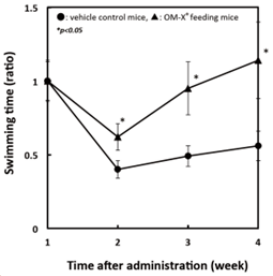

Figure 1: Effects of OM-X® on swimming exercise in mice. ●: vehicle control mice, ▲: OM-X® fed mice. Values are given as mean ± S.E. (each group n=17). $p < 0.05$ (*),

Changes in blood parameters and hepatic lipid profiles in mice fed with OM-X®: Serum components were analyzed after the last forced swimming test. Blood parameters and hepatic lipid profiles are shown in Table 2. The levels of blood lactate (p<0.01), blood SOD activity (p<0.01), serum TG (p<0.05), hepatic TL (p<0.05), TG (p<0.05) and PL (p<0.05) in mice fed with OM-X® were reduced to approximately 48, 25, 33, 26, 38, and 20%, respectively compared with those of vehicle control groups. The levels of BUN, creatinine and ketone bodies, which are the anti-fatigue and renal dysfunction indices, did not change between the vehicle control group and OM-X® group. Additionally, the activity of LDH, which is a myopathy index for over-exertion of muscle, was similar for both groups.

Table 2: Effects of OM-X® on serum biomedical parameters (blood glucose, BUN, creatinine, ketone bodies, LDH activity, lactate, SOD activity), serum lipid profiles, and hepatic lipid contents in mice.

Group	water	OM-X®
Blood glucose (mg/dL)	67.6 ± 4.7	64.0 ± 3.4
BUN (mg/100 mL)	71.9 ± 7.3	86.3 ± 6.3
Creatinine (mg/dL)	13.0 ± 0.9	12.1 ± 0.4
Ketone bodies (μmol/L)	132.6 ± 5.2	126.1 ± 8.3
LDH (Unit/L)	77.3 ± 12.9	82.6 ± 11.6
Lactate (mM)	5.3 ± 0.3	2.8 ± 0.2 **
SOD activity (%)	55.6 ± 3.4	41.5 ± 2.1 **
Serum lipid concentration		
TC (mg/100 mL)	219.3 ± 10.5	166.8 ± 9.2
TG (mg/100 mL)	366.7 ± 36.7	244.7 ± 13.2 *
PL (mg/100 mL)	299.7 ± 20.2	298.8 ± 13.5
NEFA (mEq/L)	1.4 ± 0.1	1.6 ± 0.2
Hepatic lipid profiles		
Liver weight (g)	1.8 ± 0.6	1.7 ± 0.7
TL (mg/g liver)	59.7 ± 3.8	43.9 ± 5.5 *
TC (mg/g liver)	8.8 ± 0.7	8.2 ± 0.5
TG (mg/g liver)	29.1 ± 3.1	17.9 ± 2.9 *
PL (mg/g liver)	21.0 ± 2.0	17.3 ± 1.6 *

(*p < 0.05, **p < 0.01 vs. water)

Values are given as mean±S.E. (each group n=17).

Contents of liver and muscle glycogen: Stored glycogen in liver and muscle are very important resources for energy conservation and/or maintaining blood glucose in the physiologic range. During exercise, muscle glycogen is primarily consumed, and the levels of stored glycogen in liver and muscle are low. As shown in Figure 2, there was no change in liver and gastrocnemius muscle glycogen content between mice fed with OM-X® and the vehicle control mice.

The levels of 8-OHdG in liver and kidney: To evaluate the anti-oxidative effects of OM-X®, the levels of 8-OHdG in liver and kidney, as an oxidative damage index, were measured. There were also no differences in 8-OHdG levels between mice fed with OM-X® and the vehicle control mice (Figure 3).

The mRNA expression levels of the ornithine cycle enzyme in liver: The mRNA expression of Cps1, which acts as a rate-limiting enzyme in the ornithine cycle, and Arg1, which catalyzes the hydrolysis of arginine to ornithine and urea, were increased by OM-X® feeding (Figure 4). Specifically, the levels of Cps1 in mice fed with OM-X® significantly increased by approximately 3-fold compared with the vehicle control mice. Furthermore, Arg1 levels in mice fed with OM-X® showed a trend towards an approximately 2-fold increase compared with vehicle control mice (p<0.1), but this was not statistically significant.

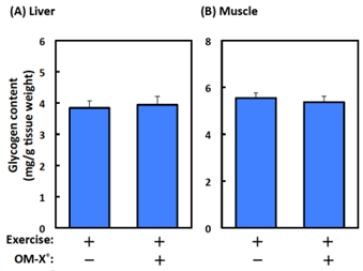

Figure 2: Effects of OM-X® on glycogen contents in exercise loaded-mice liver and muscle. (A) liver (B) muscle. Values are given as mean ± S.E. (each group n=17).

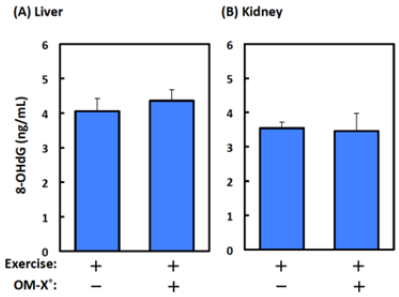

Figure 3: Effects of OM-X® on levels of 8-OHdG in exercise loaded-mice liver and kidney. (A) liver (B) kidney. Values are given as mean ± S.E. (each group n=17).

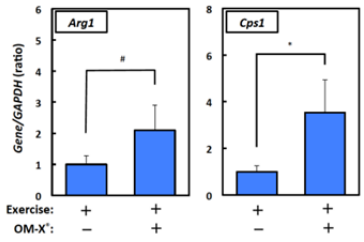

Figure 4: Effects of OM-X® on mRNA expression levels of enzymes of the urea cycle in swimming exercise loaded-mice liver. Values are given as mean ± S.E. (each group n=17). $p < 0.05$ (*), p<0.1 (#), Student's t-test.

In this study, we examined the anti-fatigue effect (improvement of muscle endurance capacity) of OM-X® using a swimming test, and showed that OM-X® markedly prolonged the swimming time in mice (Figure 1). To delineate the mechanisms underlying this anti-fatigue effect, we further measured several biomedical parameters. The levels of blood lactate, serum TG, hepatic TL, TG, and PL in mice fed with OM-X® were significantly lower than those in the vehicle control group (Table 2). Blood lactate is a glycolysis by-product of carbohydrates under anaerobic conditions [16]. Brooks *et al.* reported that blood lactate, which is produced in fast-twitch oxidative-glycolytic fibers, is translocated to the heart and/or slow-twitch oxidative fibers, and then used as an energy substrate in mitochondria [17,18]. Therefore, the decrease in blood lactate levels in mice fed with OM-X® might be due to its utilization as a mitochondrial substrate for energy production. TG in blood, adipose tissue, and muscle fiber is one of the main building blocks of FFAs. During exercise, FFAs, which are produced from TG by lipoprotein lipase, are oxidized (by β-oxidation) in the mitochondria to supply energy [19, 20]. Thus, it was thought that the reduction of serum TG level in mice fed with OM-X® was due to the production of energy through β-oxidation of FFAs. Terao *et al.* also reported that the concentrations of cholesterol, TG, and PL of very low density lipoprotein (VLDL) in long-distance runners were maintained at

low levels compared with non-athletes, suggesting that VLDL is used as an energy source during running and exercise [21]. VLDL consists of TG (approx. 55%), cholesterol ester (approx. 12%), free cholesterol (approx. 7%), PL (approx. 18%), and protein (approx. 8%; apoprotein B100, -E, C1, -C2, and -C3) and is secreted mainly by the liver. The lowering of hepatic TL, TG and PL in mice fed with OM-X® was likely involved in VLDL formation for the production of energy.

Furthermore, exercise induces free radical formation in the liver and muscles [22]. These free radicals trigger oxidative damage such as lipid peroxidation. Despite the amount of swimming exercise in the OM-X® group being higher than that in the vehicle control group, the levels of 8-OHdG in the liver and kidney were similar between the two. As the almost 2-fold increase in swimming exercise in the OM-X® group did not result in a concomitant increase in oxidative damage, elevated SOD activity was presumed to contribute to the anti-oxidative effect of OM-X®.

The accumulation of ammonia in serum leads to fatigue during exercise. Since ammonia is a by-product of the metabolism of amino acids and other nitrogen compounds, the accumulated ammonia is metabolized via the ornithine cycle (urea cycle) in the liver [23]. Therefore, the up-regulation of mRNA expression and activities of hepatic enzymes involved in ammonia metabolism contributes to the reduction of fatigue. As shown in Figure 4, the expression level of Cps1, which catalyzes the first reaction in the ornithine cycle wherein ammonia and bicarbonate combine to form carbamoyl, in mice fed with OM-X® was approximately 3-fold higher than in vehicle control mice. Although a remarkable difference was not observed in expression of Arg1, which catalyzes the hydrolysis of L-arginine into ornithine and urea in the ornithine cycle, a trend to up-regulation was observed in OM-X® fed mice. These results suggest that intake of OM-X® is capable of regulating the expression of hepatic enzymes involved in ammonia metabolism, resulting in an inhibition of the accumulation of ammonia in blood, eventually leading to lesser muscle fatigue and prolongation of exercise.

Since OM-X® also includes a sugar (55.4%), it absolutely contributes to the prolongation of exercise. Recent studies indicated that the up-regulation of glucose transporter type 4 expression through the improvement of insulin sensitivity in muscle and 5' adenosine monophosphate-activated protein kinase activation contributes to prolongation of exercise [24, 25]. We further need to study glucose metabolism in OM-X® fed mice. Based on our results, the mechanisms of anti-fatigue effect by OM-X® were due, at least in part, to (i) catabolism of glucose in OM-X®, (ii) promotion of lipid metabolism, and (iii) up-regulation of the urea cycle. It should be noted that OM-X® is a beneficial natural extract for anti-fatigue.

Experimental

OM-X® preparation: The OM-X® sample was provided by BIOBANK Co., Ltd. (Okayama, Japan). All plant ingredients used in OM-X® are nutritionally rich and safe for human consumption. The general procedure involves loading each ingredient into the manufacturing vats one by one at different stages of fermentation. When each ingredient is added, it is fermented by LAB to produce various organic acids and amino acids. In addition, the solid ingredients are broken down to facilitate absorption of their components through fermentation. Thus, various ingredients that are beneficial to human health are fermented and matured for 5 years [1-4].

Animals and diet: Four-week-old male ddY mice (Japan SLC, Inc., Hamamatsu, Japan), weighing 20-22 g were housed in plastic cages in an air-conditioned room (22 ± 2°C) with a 12-h light and dark cycle (lighting from 0700 to1900 h). All mice were fed commercial MF pellets (Oriental yeast Co., Ltd., Tokyo, Japan) and water *ad libitum* for 1 week to accustom them to the surroundings.

Swimming exercise: First, mice were subjected to a swimming exercise for 15 min to check their swimming performance. The mice that did not perform a self-motion were removed from this study. Thirty-four mice were then divided into 2 groups (n=17 per group) equally based on body weight and swimming activity. They were administrated either vehicle (distilled water; DW) or OM-X® at a dose of 85 mg/kg of body weight by gavage for 4 weeks. Forced swimming tests were then conducted weekly using the Ishihara-modified Matsumoto swimming pool [13-15]. The swimming pool (length×width×depth=90×45×45 (cm^3)) was filled with water to a depth of 38 cm (Figure 5A). A current was generated in the pool using a pump (type C-P60H; Hitachi, Tokyo, Japan), and the current strength was adjusted by a valve. The current speed at the surface was measured with a digital current meter (model SV-101-25S; Sankou, Tokyo, Japan) at the start of every swimming session (10 L/min) and was maintained at a constant speed (14 L/min). The water was maintained at a temperature of 27± 0.5°C using an electric heater. The end point of the swimming test was defined as the time at which the mice could not resist the current and failed to rise to the surface of the pool within 7 s to breathe (Figure 5B). After swimming until the end point, mice were immediately rescued and their bodies wiped with a towel. Upon completion of the test on the final day of the swimming exercise, mice were sacrificed by isoflurane anesthesia, and blood was immediately collected using a 1 mL syringe without heparin to measure blood glucose level, blood urea nitrogen (BUN), creatinine, ketone bodies, lactate, total cholesterol (TC), TG, PL, non-esterified fatty acid (NEFA), transforming growth factor (TGF)-β, lactate dehydrogenase (LDH), and SOD activity. The liver, kidney, and gastrocnemius muscle were then excised immediately, and kept at -80°C until analysis. This animal experiment was approved by the Kindai University animal use committee, and the animals were maintained according to the guidelines of Kindai University for the care of laboratory animals.

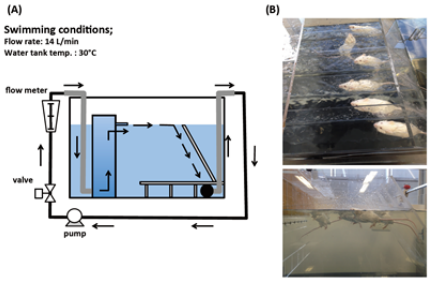

Figure 5: Equipment for forced swimming exercise. (A) Schematic diagram of the Ishihara-modified Matsumoto swimming pool, (B) appearance of swimming exercise.

Analytical procedures: The profile of serum TC, TG, PL, NEFA, blood glucose, BUN, creatinine, and ketone bodies was measured with clinical analysis kits {cholesterol CII, triglyceride G, phospholipid B, non-esterified fatty acid C test, glucose CII test, BUN-B test, creatinine test, and total ketone bodies-test, (Wako Pure Chemical Industries, Ltd., Osaka, Japan)} and lipid contents in the liver, extracted by the method of Folch *et al*., were measured in the same way. The concentration of lactate in serum was measured using a Lactate Colorimetric assay kit (BioVision Inc., CA, USA).

8-Hydroxy-2´-deoxyguanosine (8-OHdG) determination: To determine the anti-oxidative effects of OM-X® in exercised mice, we measured the 8-OHdG levels in either liver or kidney as an oxidative damage index. DNA was extracted from each tissue lysate, which was homogenized using a bead beater-type homogenizer μT-01 (TAITEC, Koshigaya, Saitama, Japan,φ5 mm SUS bead, 3000 rpm/min for 1 min) using the DNA Extractor kit (Wako). The extracted DNA from each tissue was hydrolyzed with 8-OHdG assay preparation reagent (Wako), and then measured using the 8-OHdG enzyme-linked immunosorbent assay kit (Japan Institute for the Control Aging, NIKKEN SEIL Co., Ltd, Shizuoka, Japan), and normalized to DNA concentration.

Contents of liver and muscle glycogen: The contents of liver or muscle were measured with a glycogen colorimetric assay kit (BioVision Inc.). Frozen liver or gastrocnemius muscle (10 mg) was homogenized with glycogen development buffer in a 2 mL tube using a bead beater-type homogenizer μT-01 (TAITEC,φ5 mm SUS bead, 3000 rpm/min for 1 min). The homogenized sample was centrifuged at 8,000×*g*, 4°C for 5 min, and the supernatant was assayed using the glycogen colorimetric assay kit.

Quantitative real time-PCR: Total RNA was extracted from cells using a RNeasy Mini Kit (QIAGEN Inc, Valencia, CA, USA). cDNA was synthesized from 0.25 mg of total RNA using a PrimeScript reagent kit (Takara Bio, Ohtsu, Japan). cDNA was subjected to quantitative RT-PCR using an Applied Biosystems StepOne™ Real-Time PCR system (Foster City, CA, USA). Primers for arginase 1 (Arg1), carbamoyl phosphate synthetase 1 (Cps1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Takara Bio. The expression level of each gene was determined *using the comparative Ct method* and normalized to that of GAPDH, which was used as an internal control. The PCR reaction consisted of 45 cycles (95°C for 10 s and 60°C for 40 s) after an initial denaturation step (95°C for 10 min).

***Statistical analysis*:** All data were analyzed using the Mac statistical analysis software package for Macintosh, version 2.0 (Esumi Co., Tokyo, Japan). All data are expressed as means ± standard errors (SEs). Statistical analysis was performed using the Student's t-test, and differences were considered significant when *p*-values were less than 0.05.

References

[1] Kawakami M, Ohhira I, Araki N, Inokihara K, Iwasaki H, Matsubara T. (**2003**) The influence of lactic acid bacteria (OM-X) on bone structure. *The Journal of Applied Nutrition*, ***53***, 2-8.

[2] Hashim BY, Rahman RA, Philip K. (**1999**) Lactic acid bacteria is beneficial for oral aphthous ulcerations. *Annals of Dentistry*, ***1***, 44-47.

[3] Takahata M, Fremont M, Desreumaux P, Rousseaux C, Dubuquoy C, Shimomiya Y, Nakamura Y, Miyake Y. (**2014**) Evaluation of therapeutic properties of fermented vegetable extract (OM-X®) in the model of colitis induced by *Citrobacter rodentium* in mice. *Journal of Functional Foods*, ***10***, 117-127.

[4] Itoh T, Miyake Y, Kasashima T, Shimomita Y, Nakamura Y, Ando M, Tsukamasa Y, Takahata M. (**2015**) OM-X®, fermented vegetables extract suppresses antigen-stimulated degranulation in rat basophilic leukemia RBL-2H3 cells and passive cutaneous anaphylaxis reaction in mice. *Natural Product Communications*, ***9***, 1597-601.

[5] Grandjean EP. (**1970**) Fatigue. *American Industrial Hygiene Association Journal*, ***31***, 401-411.

[6] Ream E, Richardson A. (**1996**) Fatigue: a concept analysis. *International Journal of Nursing studies*, ***33***, 519-529.

[7] Lee, JS, Kim HG, Han JM, Kim YA, Son CG. (**2015**) Anti-fatigue effect of myelophil in a chronic exercise mouse model. *European Journal of Pharmacology*, ***764***, 100-108.

[8] Murase T, Haramizu S, Shimotoyodome A, Nagasawa A, Tokimitsu I. (**2005**) Green tea extract improves endurance capacity and increases muscle lipid oxidation in mice. *The American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, ***288***, R708-R715.

[9] Bhattacharya TK, Pence BD, Ossyra JM, Gibbons TE, Perez S, McCusker RH, Kelley KW, Johnson RW, Woods JA, Rhodes JS. (**2015**) Exercise but not (-)-epigallocatechin-3-gallate or β-alanine enhances physical fitness, brain plasticity, and behavioral performance in mice. *Physiology & Behavior*, ***145***, 29-37.

[10] Ikeuchi M, Koyama T, Takahashi J, Yazawa K. (**2006**) Effects of astaxanthin supplementation on exercise-induced fatigue in mice. *Biological and Pharmaceutical Bulletin*, ***29***, 2106-2110.

[11] Chen YM, Tsai YH, Chiu YS, Wei L, Chen WC, Huang CC. (**2015**) Fucoidan supplementation improves exercise performance and exhibits anti-fatigue action in mice. *Nutrients*, ***7***, 239-252.

[12] Fan H, Tan Z, Hua Y, Huang X, Gao Y, Wu Y, Liu B, Zhou Y. (**2016**) Deep sea water improves exercise and inhibits oxidative stress in physical fatigue mouse model. *Bioscience Reports*, ***4***, 751-757.

[13] Matumoto K, Ishihara K, Tanaka K, Inoue K, Fushiki T. (**1996**) An adjustable-current swimming pool for the evaluation of endurance capacity of mice. *Journal of Applied Physiology*, ***81***, 1843-1849.

[14] Mizunoya W, Oyaizu S, Ishihara K, Fushiki T. (**2002**) Protocol for measuring the endurance capacity of mice in an adjustable-current swimming pool. *Bioscience, Biotechnology, and Biochemistry*, ***66***, 1133-1136.

[15] Ishihara K, Yamada A, Mita Y, Goto A, Ishimi T, Mabuchi H, Inoue K, Fushiki T, Yasumoto K. (**2009**) Improved swimming pool achieves higher reproducibility and sensitivity to effect of food components as ergogenic aids. *Journal of Nutritional Science and Vitaminology*, ***55***, 301-308.

[16] Schurr A. (**2008**) Lactate: a major and crucial player in normal function of both muscle and brain. *The Journal of Physiology*, ***586***, 2665-2666.

[17] Brooks GA. (**1991**) Current concepts in lactate exchange. *Medicine and Science in Sports and Exercise*, ***23***, 895-906.

[18] Brooks GA. (**1999**) Are arterial, muscle and working limb lactate exchange data obtained on men at altitude consistent with the hypothesis of an intracellular lactate shuttle? *Advances in Experimental Medicine and Biology*, ***474***, 185-204.

[19] Gorski J, Oscai LB, Palmer WK. (**1990**) Hepatic lipid metabolism in exercise and training. *Medicine and Science in Sports and Exercise*, ***22***, 213-221.

[20] Ranallo RF, Rhodes EC. (**1998**) Lipid metabolism during exercise. *Sports Medicine*, ***26***, 29-42.

[21] Terao, T, Miyoshi M, Narusawa M, Yoshioka T, Nakano S. (**1984**) Serum lipoprotein metabolisms at rest and during sub-maximal exercise in long-distance runners. *Journal of Physical Fitness Japan*, ***33***, 235-244.

[22] Witt EH, Reznick AZ, Viguie CA, Starke-Reed P, Packer L. (**1992**) Exercise, oxidative damage and effects of antioxidant manipulation. *Journal of Nutrition*, ***122***, 766-773.

[23] Wilkinson DJ, Smeeton NJ, Watt PW. (**2010**) Ammonia metabolism, the brain and fatigue; revisiting the link. *Progress in Neurobiology*, ***91***, 200-219.

[24] Holloszy JO. (**2005**) Exercise-induced increase in muscle insulin sensitivity. *Journal of Applied Physiology*, ***99***, 338-343.

[25] Richter EA, Hargreaves M. (**2013**) Exercise, GLUT4, and skeletal muscle glucose uptake. *Physiological Reviews*, ***93***, 993-1017.

NPC Natural Product Communications

2017
Vol. 12
No. 1
115 - 118

Essential Oils of *Morus alba* and *M. nigra* Leaves: Effect of Drying on the Chemical Composition

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Received: November 11th, 2016; Accepted: November 21st, 2016

Detailed GC and GC/MS analyses of essential-oil samples obtained by hydrodistillation of *Morus alba* L. and *M. nigra* L. leaves (four samples) allowed the identification of 131 constituents representing 95.1 – 96.4% of the total GC peak areas. The most abundant compounds classes were alkanes, diterpenoids, carotenoid-derived compounds and fatty acid-related constituents with *trans*-phytol (7.9 – 71.2%), (*E*,*E*)-geranyl linalool (0.2 – 8.0%), (*Z*)-bovolide (8.1%) and *n*-chain alkanes (in total, 17.5 – 52.4%) as the major constituents of the analyzed samples. In general, there were only quantitative differences noted between *M. nigra* essential oils from fresh and dry leaves. The most discernable changes included a variation in the content of the major constituents (e.g. the relative amount of *trans*-phytol and the total amount of alkanes decreased by 5.8% and ≈ 2%, respectively, while that of (*E*,*E*)-geranyl linalool increased by 7.8%). On the other hand, the composition of *M. alba* essential oil was much more significantly affected by the drying process. The highest quantitative differences were noted for *trans*-phytol, geranyl acetone, and all isomers of 4,6,8-megastigmatrien-3-one. Also, a rare plant metabolite, (*Z*)-bovolide, characteristic of leaf senescence, was only identified (8.1%) in the essential oil of *M. alba* dried leaves.

Keywords: *Morus alba*, *Morus nigra*, Metabolomics, Apocarotenoids, (*Z*)-Bovolide, Storage.

The genus *Morus* L. (Moraceae), commonly known as mulberries, comprises about 16 species of deciduous, wild-growing/cultivated trees that are spread across many temperate world regions, especially in the Northern Hemisphere [1]. Species of this genus have a long history of use in folk medicine, with a number of interesting biological/pharmacological properties already scientifically proven [2]. Specifically, *Morus alba* L. and *M. nigra* L. were shown to possess analgesic, sedative, diaphoretic, antihypertensive, antityrosinase and antidiabetic activities [2]. Only three *Morus* species (*M. alba*, *M. nigra* and *M. rubra*) are part of the Serbian flora [1]. *M. alba* (white mulberry or 'beli dud' in Serbian) is a tree native to northern China. Nowadays, white mulberry is a worldwide naturalized species cultivated to feed silkworms in the commercial production of silk [1]. A SciFinder search revealed more than 11,000 reports dealing with *M. alba*. However, only seven reports dealt with the essential-oil analysis of this particular taxon. These studies showed that the essential oil from *M. alba* leaves contained high amounts of alkanes, diterpenes and sesquiterpenes [3], while the major constituents of the essential oil isolated from dry fruits were 1,8-cineole, geraniol, linalyl acetate, α-pinene and limonene [4]. *M. nigra*, known as black mulberry or 'crni dud' in Serbian, is a deciduous fruit-bearing tree, native to Iran and Afghanistan [1]. Black mulberries are much less frequently cultivated than white mulberry trees [1]. Interestingly, based on archives from the 13th century, a black mulberry tree, planted in the courtyard of the Patriarchate of Peć, is one of the oldest plant organisms in Serbia [1]. Up to now, non-volatile secondary metabolites of *M. nigra* were the subject of several studies [5]. However, there are no previous studies on the chemical composition of the essential oil of this plant species.

The drying process of plant material has a crucial role in preventing the growth of microorganisms and, also, makes storage and transportation of plant material much easier [6]. During the drying process, evaporation of volatile phytochemicals and/or the formation of artefacts, as a result of external (e.g. oxidation by atmospheric oxygen) and internal ((non)enzymatic) degradation/transformation, can cause changes in the aroma and medicinal potential of plant material [6]. Several studies reported significant changes in the biological/pharmacological properties (e.g. antioxidant capacity), and changes in color and chemical composition of the essential oil as a consequence of plant material drying [6]. *Morus* spp. leaves can be used, for medicinal purposes, either fresh or dried [2]. However, *Morus* leaves, like other herbal teas or medicinal plants, are usually stored and consumed in dried form. For that reason, the current work is aimed to analyze the composition of the essential oil from both fresh and dried leaves of two *Morus* species (*M. alba* and *M. nigra*) cultivated in Serbia and to evaluate the influence of the drying process on the volatiles of the leaves.

Detailed GC and GC/MS analyses of four essential-oil samples obtained by hydrodistillation of both fresh and dried *M. alba* and *M. nigra* leaves, collected from mulberry trees cultivated in SE Serbia, allowed, in total, the identification of 131 constituents that represented 95.1 – 96.4% of the total GC peak areas (Table 1). The most abundant classes of compounds identified were alkanes and diterpenoids that together constituted more than 60% of sample **MA-2**, and more than 88% of the samples **MN-1**, **MN-2**, and **MA-1**. The remaining identified volatiles were carotenoid derivatives (1.4 – 24.8%) and fatty acid related constituents (tr – 8.1%). The major contributors of *M. nigra* essential oils were: *trans*-phytol (65.4 – 71.2%), (*E*,*E*)-geranyl linalool (0.2 – 8.0%) and normal chain alkanes (tricosane (2.0 – 2.8%), pentacosane (2.4 – 3.8%), heptacosane (1.9 – 2.7%), nonacosane (3.4 – 4.1%) and hentriacontane (2.4 – 3.5%)). Similarly, the bulk, by percentage, of both *M. alba* essential oils was comprised of alkanes, diterpenoids and carotenoid derivatives. *trans*-Phytol (7.9 – 61.6%) was the predominant detected constituent, followed by *cis*-bovolide (8.1%), pentacosane (8.2 – 10.9%), heptacosane (3.7 – 7.9%), nonacosane (4.2 – 12.4%) and hentriacontane (3.2 – 12.4%).

Table 1: Chemical composition of the essential oils isolated from fresh and dried leaves of *Morus alba* and *M. nigra* from Serbia.

Compound	Class[a)]	RI[b)]	*Morus nigra*[c)]		*Morus alba*[d)]		ID[e)]
			MN-1	**MN-2**	**MA-1**	**MA-2**	
			Relative content [%][i)]				
Furfural	FA	828	-	-	tr	-	f, g, h
(*E*)-3-Hexen-1-ol	FA	844	-	-	tr	-	f, g
Tiglic acid	FA	903	-	-	tr	-	f, g, h
4-Methylnonane	A	957	tr	-	-	-	f, g
Benzaldehyde	O	959	-	-	tr	-	f, g, h
Decane	A	1000	tr	-	-	-	f, g, h
Benzyl alcohol	O	1034	-	tr	tr	-	f, g, h
2-Phenylethanal	O	1051	tr	-	0.1	-	f, g
4-Methyldecane	A	1057	tr	-	-	-	f, g
2-Methyldecane	A	1068	tr	-	-	-	f, g
p-Cresol	O	1069	-	-	tr	-	f, g
Undecane	A	1100	1.0	-	-	-	f, g
Dehydrosabina ketone	M	1123	-	-	tr	-	f, g
Isophorone	C	1125	tr	-	-	-	f, g
3,7-Dimethyldecane [k)]	A	1125	tr	-	-	-	f, g
4-Ketoisophorone	C	1145	-	-	tr	-	f, g
Benzoic acid	O	1155	-	-	tr	-	f, g, h
Octanoic acid	FA	1162	-	-	tr	-	f, g, h
2-Methylundecane	A	1163	0.2	-	-	-	f, g
3-Methylundecane	A	1170	tr	-	-	-	f, g
Dodecane	A	1200	2.0	-	-	-	f, g, h
Safranal	C	1202	tr	tr	tr	tr	f, g
2,6-Dimethylundecane	A	1213	0.4	-	-	-	f, g
2-Methyldodecane	A	1264	0.3	-	-	-	f, g
3-Methyldodecane	A	1270	0.3	-	-	-	f, g
Dihydroedulan I	C	1298	-	-	tr	-	f, g
Tridecane	A	1300	1.8	tr	-	-	f, g, h
2,6-Dimethyldodecane	A	1311	tr	-	-	-	f, g
4-Vinylguaiacol	O	1311	-	tr	tr	tr	f, g
1-Methylnaphthalene	O	1315	tr	-	-	-	f, g
3,7-Dimethyldodecane	A	1317	tr	-	-	-	f, g
Eugenol	O	1353	-	-	tr	-	f, g, h
2-Methyltridecane	A	1363	tr	-	-	-	f, g
3-Methyltridecane	A	1370	tr	-	-	-	f, g
2,6,10-Trimethyldodecane [k)]	S	1375	0.2	-	-	-	f, g
(*Z*)-β-Damascenone	C	1380	-	-	tr	-	f, g
1-Undecanol	FA	1385	tr	-	-	-	f, g
1-Tetradecene	FA	1391	tr	-	-	-	f, g
Tetradecane	A	1400	0.8	-	tr	-	f, g, h
(*E*)-β-Damascenone	C	1409	-	-	tr	tr	f, g
2,6-Dimethyltridecane	A	1413	tr	-	-	-	f, g
Geranyl acetone	C	1447	-	-	-	9.8	f, g, h
Unidentified constituent [j)]		1461	tr	tr	0.1	3.2	
2-Methyltetradecane	A	1463	tr	-	-	-	f, g
3-Methyltetradecane	A	1470	tr	-	-	-	f, g
3,4-Dehydro-*β*-ionone	C	1476	0.2	-	-	tr	f, g
(*E*)-β-Ionone	C	1479	tr	-	tr	2.6	f, g
Pentadecane	A	1500	0.3	-	tr	-	f, g, h
2-Tridecanone	FA	1502	tr	-	tr	-	f, g
(*E*,*E*)-α-Farnesene	S	1503	tr	-	-	-	f, g
3,4-Dimethyl-5-pentyl-2(5*H*)-furanone (*syn*. dihydrobovolide)	FA	1512	-	-	-	tr	f, g
(*Z*)-3,4-Dimethyl-5-pentylidene-2(5*H*)-furanone (*syn*. *cis*-bovolide, (*Z*)-bovolide)[l) m)]	FA	1518	-	-	-	8.1	f, g
Caparratriene [k)]	S	1523	-	-	-	tr	f, g
(6*Z*,8*E*)-4,6,8-Megastigmatrien-3-one	C	1559	-	tr	0.1	tr	f, g
(*E*)-Nerolidol	S	1559	-	-	tr	-	f, g
2-Methylpentadecane	A	1563	tr	-	-	-	f, g
3-Methylpentadecane	A	1570	tr	-	-	-	f, g
(*Z*)-3-Hexenyl benzoate	FA	1571	-	-	tr	tr	f, g
(6*Z*,8*Z*)-4,6,8-Megastigmatrien-3-one	C	1577	0.3	1.2	0.3	4.3	f, g
2-Methyl-4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2-butenal (*syn*. boronia butenal) [k)]	C	1582	-	-	tr	-	f, g
Hexadecane	A	1600	tr	-	-	-	f, g, h
(6*E*,8*E*)-4,6,8-Megastigmatrien-3-one	C	1610	tr	tr	tr	tr	f, g
Tetradecanal	FA	1613	-	-	tr	-	f, g, h
(6*E*,8*Z*)-4,6,8-Megastigmatrien-3-one	C	1623	0.2	0.6	0.2	4.3	f, g
2-Methylhexadecane	A	1663	-	-	tr	-	f, g
1-Tetradecanol	FA	1678	-	-	tr	-	f, g, h
2-Pentadecanone	FA	1696	tr	-	tr	-	f, g
Heptadecane	A	1700	tr	tr	tr	tr	f, g, h
Pentadecanal	FA	1714	tr	-	tr	tr	f, g
Tetradecanoic acid	FA	1758	-	-	tr	-	f, g, h
Benzyl benzoate	FA	1768	-	-	tr	-	f, g
3-Methylheptadecane	A	1771	-	-	tr	-	f, g
Octyl octanoate	FA	1776	-	-	tr	-	f, g
Phenanthrene	O	1784	-	-	tr	-	f, g
1-Octadecene	FA	1793	-	-	tr	-	f, g
Octadecane	A	1800	tr	tr	tr	tr	f, g, h
Hexadecanal	FA	1816	-	-	tr	-	f, g
Neophytadiene (isomer I)	D	1835	tr	-	tr	-	f, g
Hexahydrofarnesyl acetone	C	1840	2.0	3.0	0.8	3.8	f, g
1-Hexadecanol	FA	1882	-	-	tr	-	f, g
1-Nonadecene	FA	1893	-	-	tr	-	f, g
Nonadecane	A	1900	tr	tr	0.1	tr	f, g, h
(*E*,*E*)-Farnesyl acetone	C	1909	-	-	tr	tr	f, g
Methyl palmitate	FA	1924	tr	tr	0.1	tr	f, g
Isophytol	D	1945	tr	tr	0.1	-	f, g
Hexadecanoic acid	FA	1969	-	-	2.0	-	f, g, h
Ethyl hexadecanoate	FA	1992	tr	-	-	-	f, g
1-Eicosene	FA	1994	-	-	tr	-	f, g
Eicosane	A	2000	tr	tr	0.1	tr	f, g, h
(*E*,*E*)-Geranyl linalool	D	2022	0.2	8.0	0.6	tr	f, g
Methyl (*Z*,*Z*,*Z*)-9,12,15-octadecatrienoate	FA	2096	0.4	tr	0.3	-	f, g
Heneicosane	A	2100	0.3	tr	0.7	tr	f, g, h
trans-Phytol	D	2113	71.2	65.4	61.6	7.9	f, g
Octadecanoic acid	FA	2160	-	-	0.3	-	f, g
(*Z*,*Z*,*Z*)-9,12,15-Octadecatrienoic acid	FA	2164	0.2	-	0.3	-	f, g
1-Docosene	FA	2192	-	-	0.2	-	f, g
Docosane	A	2200	0.2	tr	0.5	tr	f, g, h
Eicosanal	FA	2213	-	-	tr	-	f, g
2-Methyldocosane	A	2265	-	tr	tr	-	f, g
1-Tricosene	FA	2297	-	-	0.1	-	f, g
Tricosane	A	2300	2.0	2.8	2.8	3.0	f, g, h
2-Heneicosanone	FA	2307	-	-	tr	-	f, g
δ-Octadecalactone	FA	2347	-	tr	0.2	-	f, g
3-Methyltricosane	A	2374	-	-	tr	-	f, g
1-Tetracosene	FA	2396	tr	tr	0.7	tr	f, g
Tetracosane	A	2400	0.5	0.6	1.4	3.0	f, g, h
2-Docosanone	FA	2410	-	-	tr	-	f, g
2-Methyltetracosane	A	2465	-	-	tr	-	f, g
1-Pentacosene	FA	2497	-	-	0.8	-	f, g
Pentacosane	A	2500	2.4	3.8	8.2	10.9	f, g, h
1-Hexacosene	FA	2597	tr	tr	0.5	tr	f, g
Hexacosane	A	2600	tr	tr	0.7	2.8	f, g, h
2-Methylhexacosane	A	2665	-	-	tr	-	f, g
1-Tetracosanol	FA	2677	-	-	tr	-	f, g
1-Heptacosene	FA	2697	-	-	tr	-	f, g
Heptacosane	A	2700	1.9	2.7	3.7	7.9	f, g, h
Pentacosanal	FA	2714	-	-	tr	-	f, g
3-Methylheptacosane	A	2775	-	-	tr	-	f, g
1-Octacosene	FA	2797	tr	-	0.2	tr	f, g
Octacosane	A	2800	tr	tr	0.2	tr	f, g, h
2-Methyloctacosane	A	2864	-	-	tr	-	f, g
Nonacosane	A	2900	3.4	4.1	4.2	12.4	f, g, h
3-Methylnonacosane	A	2975	-	-	tr	-	f, g
1-Triacontene	FA	2998	-	-	0.1	tr	f, g
Triacontane	A	3000	tr	tr	0.1	tr	f, g, h
1-Hexacosyl acetate	FA	3013	-	tr	0.2	-	f, g
2-Methyltriacontane	A	3065	-	-	tr	-	f, g
Hentriacontane	A	3100	2.4	3.5	3.2	12.4	f, g, h
1-Dotriacontene	FA	3198	-	-	-	tr	f, g
Dotriacontane	A	3200	tr	-	tr	tr	f, g, h
1-Octacosyl acetate	FA	3213	-	-	tr	-	f, g
Tritriacontane	A	3300	tr	tr	0.2	tr	f, g, h
Total			**95.1**	**95.7**	**96.0**	**96.4**	
Number of constituents			71	36	96	43	
Alkanes	A		20.2	17.5	26.1	52.4	
Carotenoid derivatives	C		2.7	4.8	1.4	24.8	
Diterpenoids	D		71.4	73.4	62.3	7.9	
Fatty acids and fatty acid derivatives	FA		0.6	tr	6.0	8.1	
Monoterpenoids	M		-	-	tr	-	
Others	O		tr	tr	0.1	tr	
Sesquiterpenoids	S		0.2	-	tr	tr	

[a)] For compound class abbreviations see bottom of this Table. [b)] RI = retention indices determined relative to a homologous series of *n*-alkanes (C_8-C_{33}) on a DB-5MS column. [c)] **MN-1** = Essential-oil sample obtained by a hydrodistillation of *Morus nigra* fresh leaves, i.e. immediately after the collection of plant material, **MN-2** = Essential-oil sample obtained by a hydrodistillation of *M. nigra* leaves dried for one month at room temperature. [d)] **MA-1** = Essential-oil sample obtained by a hydrodistillation of *Morus alba* fresh leaves, i.e. immediately after the collection of plant material, **MA-2** = Essential-oil sample obtained by a hydrodistillation of *M. alba* leaves dried for one month at room temperature. [e)] ID = Identification method, f = constituent identified by a mass spectral comparison with those given in the literature [7], g = constituent identified by experimental retention index (DB-5MS column) matching with literature data [7], h = constituent identity confirmed by GC co-injection of an authentic standard. [i)] tr = trace (< 0.05%); - = not detected. [j)] MS (EI, 70 eV), *m/z* (rel. int., %): 178 (21, [M^+]), 163 (42), 151 (6), 150 (47), 136 (12), 135 (100), 122 (7), 121 (30), 109 (13), 108 (11), 107 (47), 105 (7), 95 (19), 94 (7), 93 (31), 91 (25), 85 (7), 81 (25), 80 (10), 79 (65), 78 (9), 77 (34), 71 (16), 70 (8), 69 (47), 67 (15), 66 (9), 65 (16), 57 (17), 55 (25), 53 (17), 52 (6), 51 (10), 43 (18), 42 (6), 41 (41), 40 (5), 39 (24). [k)] Relative stereochemistry was not determined. [l)] Mass spectrum and retention index were reported by G. MacLeod [8]. [m)] MS (EI, 70 eV), *m/z* (rel. int., %): 181 (4), 180 (30, [M^+]), 165 (5), 138 (15), 137 (87), 125 (29), 124 (100), 123 (8), 110 (5), 109 (12), 96 (10), 95 (6), 83 (7), 82 (14), 81 (17), 79 (6), 67 (6), 55 (59), 54 (16), 53 (10), 41 (11), 39 (12).

In general, there were only quantitative variations noted among *M. nigra* essential-oil constituents from fresh leaves (**MN-1**;

hydrodistillated immediately after the collection of plant material) and dried leaves (**MN-2**; the essential oil was isolated after a one-month drying period at room temperature). The most discernable changes included an alteration in the content of the major constituents: the content of *trans*-phytol and the total amount of alkanes decreased by 5.8 and ≈ 2%, respectively, while the relative amount of (*E*,*E*)-geranyl linalool increased by 7.8%. Contrary to this mostly quantitative compositional change in the *M. nigra* essential-oil samples during the drying of leaves, the composition of the corresponding *M. alba* essential oils (**MA-1** and **MA-2**) differed not only quantitatively, but also qualitatively. Again, *trans*-phytol was the constituent that displayed the most significant drop in its relative content; this time its relative amount was lower by *ca.* 8 times; the percentage of geranyl acetone and all isomers of 4,6,8-megastigmatrien-3-one increased after the period of drying (Table 1). Also, a γ-lactone, *cis*-bovolide, appeared in the essential oil of *M. alba* dry leaves among the major contributors (8.1%).

Carotenoid-derived compounds (apocarotenoids or norisoprenoids) reached 24.8% of the total essential oil from sample **MA-2**, while compounds of this class formed only 1.4% of the oil sample from fresh *M. alba* leaves (**MA-1**). Thus, not only the relative content of the carotenoid fraction, but also its qualitative composition, distinguished the samples of the essential oil from fresh and dried *M. alba* leaves (**MA-1** and **MA-2**; Table 1). In general, the flavor of plant material is one of the main sensory properties used in its selection, acceptance and ingestion [9]. The obtained differences, i.e. the increased amount of carotenoid derivatives, suggested that the drying process has a crucial rule for the known flavor of tea (water infusion) prepared from dried *M. alba* leaves. For example, β-ionone (violet-like, woody, floral, fruity flavor), present in both *M. alba* essential-oil samples (**MA-1** and **MA-2**; Table 1), is the main contributor to the aroma of roses despite its relatively low concentration in rose oil [10].

The increased amount and the large structural diversity of apocarotenoids found in *M. alba* dried leaves can be attributed mainly to the catalytic versatility of a family of carotenoid cleavage dioxygenases (CCDs) that catalyze the synthesis of apocarotenoid volatiles only at the leaf senescence stage, even though the CCD enzymes are present throughout the development of this plant organ [10]. A proposed biosynthetic pathway, linking most of the identified apocarotenoids from the dried *M. alba* leaves with their common precursor (phytoene), is depicted in Figure 1. Based on the structures of the biosynthesized apocarotenoids (C_{13}-norisoprenoids - β-ionone, 3,4-dihydro-β-ionone, safranal, β-damascenone and all diastereoisomers of megastigma-4,6,8-trien-3-one) it seems that the bio-oxidative cleavage reactions that involved 9,10 (9',10')-dioxygenase (CCD1, CCD7 and/or AtCCD1 enzyme, which cleave the 9,10 double bonds of their respective carotenoid substrates [10]), were the dominant ones (Figure 1).

The sample of essential oil from dried *M. alba* leaves contained a compound eluting at RI 1518 that accounted for 8.1% of the oil; this was completely absent from all other oils. A direct comparison of the mass spectrum of the compound in question (molecular ion at *m/z* 180, base peak at *m/z* 124; Figure 2) with those from the available commercial mass spectral libraries (Adams [7], Wiley 6, NIST11 and MassFinder 2.3) did not result in a positive hit. From the experience of the authors, the very same compound is known to have been the constituent of a number of other essential oils, but remained unidentified [11]. The first clues to its identity came from a consideration of another constituent present in that essential oil sample, namely, dihydrobovolide, identified based on a comparison with a mass spectrum and retention index from MassFinder 2.3, eluting (RI 1512) just before the compound of interest. Dihydrobovolide possesses a mass spectrum that displayed ions at *m/z* values that were 2 amu higher than those of the yet unidentified constituent, including its molecular ion (at *m/z* 182). This led us to consider the possibility that the compound at RI 1518 represents a dehydro derivative of dihydrobovolide. A literature search revealed that such a compound exists: (*E*/*Z*)-3,4-dimethyl-5-pentylidene-2(5*H*)-furanone, known as (*cis*/*trans*)-bovolide (Figure 2). A comparison of the reported [8] mass spectrum and RI value, for the column of the same polarity, of bovolide with those that correspond to the component from our oil corroborated the tentative identification. Thus, the component at RI 1518 was identified as one of the geometric isomers of bovolide.

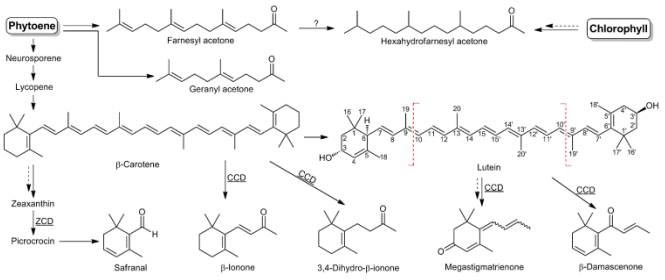

Figure 1: A proposed biosynthetic pathway for the formation of apocarotenoids in the essential oil of *M. alba* dried leaves starting from a single substrate - phytoene. ZCD is zeaxanthin cleavage dioxygenase and CCD is carotenoid cleavage dioxygenase.

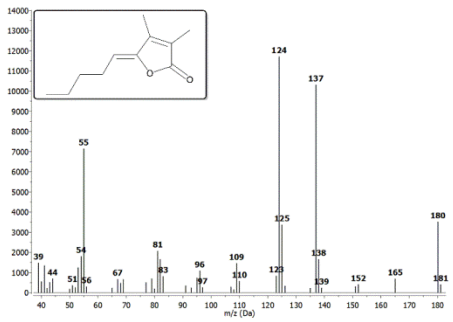

Figure 2: Mass spectrum and structure of *cis*-bovolide ((*Z*)-3,4-dimethyl-5-pentylidene-2(5*H*)-furanone).

Bovolide was first isolated and identified from butter, and was so-named because of its bovine origin [12]. Previously, Horita *et al.* confirmed that bovolide and dihydrobovolide were formed by light-induced post-harvesting biochemical transformations of native plant metabolites, usually accompanying the drying process of plant material [12c]. Although two diastereoisomers of bovolide could exist ((*Z*)- and (*E*)-3,4-dimethyl-5-pentylidene-2(5*H*)-furanone), only one is known to form as a result of such transformations of native fatty acid related plant metabolites. The dominant formation of the *Z* isomer was previously noted in the literature [12d], and that fact could be understood in terms of the apparent large difference in the minimal potential energies of the two diastereoisomers. To verify this, we calculated, using ChemBio 3D Ultra 12.0 software, at the MM2 level of theory, the ΔE mentioned between the conformations with the minimal energy of the two diastereoisomers. It amounted to ≈ 4 kcal/mol (9.4635 and 13.6868 kcal/mol for (*Z*)-3,4-dimethyl-5-pentylidene-2(5*H*)-furanone and (*E*)-3,4-dimethyl-5-pentylidene-2(5*H*)-furanone, respectively). For comparison sake, the *E* isomer of 3-hexene was only 0.21 kcal/mol more stable than the *Z* (the difference was calculated in the same manner). Such a difference in stability suggests that, in the equilibrium between the isomers, at room temperature, one would expect a domination of (*Z*)-bovolide (more than 99.99% in equilibrium). Thus, we believe that the isomer of bovolide that we detected is most probably also the thermodynamically most stable one, i.e. *Z*-isomer. It appears to

us that the trivial names *cis*-bovolide, or only bovolide (CAS No. 774-64-1), are rather confusing with regard to the stereochemistry of this compound. It is difficult to understand the meaning of *cis* in this particular case (four different groups attached to the double bond in question). We believe that the stereodesignator *Z* is much more appropriate for use, and even better to also state the nomenclature name along with the trivial one. Bovolide and dihydrobovolide, which are not connected by the regular metabolic pathway of fatty acid biosynthesis [12a,12b], were previously identified in a range of different teas [12c] and as constituents of tobacco, peppermint oil, *Lycium chinese* and *Carphephorus corymbosus* essential oils [13]. Both compounds possess a strong aroma described as celery-like and were patented for improving the flavor of tobacco [12a,12b]. Interestingly, their presence was used as a marker for exposure of plant material to light [12c].

Experimental

Plant material: Leaves of *Morus nigra* and *M. alba*, collected in July, 2015 (village Gornji Barbeš, near Gadžin Han, Serbia), were identified by Marija Marković; voucher specimens (VM0012 and VM0013) were deposited in the Herbarium of the Faculty of Science and Mathematics, University of Niš, Serbia. A portion of the leaves was subjected to hydrodistillation immediately after collection, while another portion was dried for 1 month at room temperature, without direct exposure to sunlight.

Isolation of essential oils: Fresh (3 x 300 g batches) and dried leaves (3 x 100 g batches) were subjected to hydrodistillation for 4.5 h using the original Clevenger-type apparatus [6b]. The yields of essential oils were 0.0011 and 0.0012% from the dried plant material (**MN-2** and **MA-2**, respectively) and 0.0080% (w/w) from both samples of fresh leaves (**MN-1** and **MA-1**).

GC-MS analysis: GC-MS analyses (3 repetitions) were carried out using a Hewlett-Packard 6890N gas chromatograph equipped with a fused silica capillary column DB-5MS (5% phenylmethylsiloxane, 30 m × 0.25 mm, film thickness 0.25 μm, Agilent Technologies, USA) and coupled with a 5975B mass selective detector from the same company. The injector and interface were operated at 250 and 300°C, respectively. Oven temperature was raised from 70 to 290°C at a heating rate of 5°C/min and the program ended with an isothermal period of 10 min. As a carrier gas helium at 1.0 mL/min was used. The samples, 1.0 μL of essential oil solutions in diethyl ether (10.0 mg of an essential oil sample per 1.0 mL of solvent), were injected in a pulsed split mode (the flow was 1.5 mL/min for the first 0.5 min and then set to 1.0 mL/min throughout the remainder of the analysis; split ratio 40 : 1). MS conditions were as follows: ionization voltage 70 eV, acquisition mass range *m/z* 35-650, scan time 0.32 s. Percentage composition of the essential oils was computed from the GC peak areas without any corrections. Constituents were identified by comparison of their linear retention indices (relative to C_8-C_{33} *n*-alkanes [7] on a DB-5MS column) with literature values [7] and their mass spectra with those of authentic standards, as well as those from Wiley 6, NIST11, MassFinder 2.3, and a homemade MS library with the spectra corresponding to pure substances and components of known oils, and wherever possible, by co-injection with an authentic sample.

Acknowledgments - This work was funded by the Ministry of Education, Science and Technological Development of Serbia [Project No. 172061 and TR-34012].

References

[1] Josifović M. (**1975**) *Flora of Serbia*, Vol. ***2***. Srpska akademija nauka i umetnosti, Belgrade, 50-55.

[2] (a) Bown D. (**1995**) *Encyclopedia of Herbs and Their Uses*. Dorling Kindersley, Michigan, 1-424; (b) Pianwijanpong N, Pongpan N, Suntornsuk L, Luanratana O. (**2007**) The triterpene constituents of the root bark of a hybrid between *Morus alba* L. and *M. rotundiloba* Koidz. and its antityrosinase activities. *Natural Product Communications*, ***2***, 381-384; (c) Park H-S, Shim S-M, Kim G-H. (**2013**) Inhibitory effects of ethyl acetate-soluble fraction from *Morus alba* on lipid accumulation in 3T3-L1 cells. *Natural Product Communications*, ***8***, 1579-1582; (d) Hunyadi A, Herke I, Veres K, Erdei A, Simon A, Tóth G. (**2014**) Volatile glycosides from the leaves of *Morus alba* with a potential contribution to the complex anti-diabetic activity. *Natural Product Communications*, ***9***, 145-147.

[3] Liu Z, Wang H, Liu S, Jiang N. (**2011**) Volatile components of essential oil from mulberry variety "Longsang 1" leaves. *Tianran Chanwu Yanjiu Yu Kaifa*, ***23***, 1069-1072.

[4] Kasyanov GI, Pekhov AV, Shaftan EA, Klimova ES, Shishkov GZ. (**1976**) Carbon dioxide extract of *Morus alba* L. fruit. *Rastitel'nye Resursy*, ***12***, 597-599.

[5] (a) Sanchez-Salcedo EM, Tassotti M, Del Rio D, Hernandez F, Martinez JJ, Mena P. (**2016**) (Poly)phenolic fingerprint and chemometric analysis of white (*Morus alba* L.) and black (*Morus nigra* L.) mulberry leaves by using a non-targeted UHPLC-MS approach. *Food Chemistry*, ***212***, 250-255; (b) Shukla RK, Painuly D, Shukla A, Singh J, Porval A, Vats S. (**2014**) *In vitro* biological activity and total phenolic content of *Morus nigra* seeds. *Journal of Chemical and Pharmaceutical Research*, ***6***, 200-210; (c) Zeng Q, Wang Q, Wang X, Chen H, Zhao A, Xiang Z, He N. (**2013**) Progress in study and utilization of the black mulberry, *Morus nigra* L. *Canye Kexue*, ***39***, 606-613.

[6] Blagojević PD, Radulović NS, Skropeta D. (**2015**) (Chemotaxonomic) implications of postharvest/storage-induced changes in plant volatile profiles – the case of *Artemisia absinthium* L. essential oil. *Chemistry & Biodiversity*, ***12***, 1237-1255.

[7] Adams RP. (**2007**) *Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry*, Ed. *4*. Allured Publishing Corporation, Carol Stream, 1-698.

[8] MacLeod G. (**1991**) Identification of bovolide and dihydrobovolide in beef. *Food Chemistry*, ***40***, 113-117.

[9] Preedy VR. (**2013**) *Tea in Health and Disease Prevention*. Academic Press, London, 1-1612.

[10] (a) Winterhalter P, Rouseff R. (**2002**) Carotenoid-derived aroma compounds: An introduction. *ACS Symposium Series*, ***802***, 1-17; (b) El Hadi MAM, Zhang F-J, Wu F-F, Zhou C-H, Tao J. (**2013**) Advances in fruit aroma volatile research. *Molecules*, ***18***, 8200-8229.

[11] Radulović N, Dekić M, Stojanović-Radić Z. (**2012**) Chemical composition and antimicrobial activity of the volatile oils of *Geranium sanguineum* L. and *G. robertianum* L. (Geraniaceae). *Medicinal Chemistry Research*, ***21***, 601-615.

[12] (a) Boidingh J, Taylor RJ. (**1962**) Trace constituents of butter fat. *Nature*, ***194***, 909-913; (b) Lardelli G, Dijkstra G, Harkes PD, Boldingh J. (**1966**) A new γ-1actone found in butter. *Recueil des Travaux Chimiques des Pays-Bas*, ***85***, 43-55; (c) Horita H, Hara T, Sannai A, Fujimori T. (**1985**) The light-produced volatile components of green tea. *Agricultural and Biological Chemistry*, ***49***, 3601-3603; (d) Park BR, Kim KH, Lim JW, Kim JN. (**2012**) Facile synthesis of γ-alkylidenebutenolides from Morita–Baylis–Hillman adducts. *Tetrahedron Letters*, ***53***, 36-40.

[13] (a) Demole E, Berthet D. (**1972**) A chemical study of Burley tobacco flavor (*Nicotiana tobacum* L.). Volatile to medium-volatile constituents (b.p. ≦ 84°/0.001 Torr). *Helvetica Chimica Acta*, ***55***, 1866-1882; (b) Sakata I, Hashizume T. (**1973**) Isolation and identification of 2,3-dimethyl-4-hydroxy-2-nonenoic acid lactone from shubi. *Agricultural and Biological Chemistry*, ***37***, 2441-2442; (c) Sannai A, Fujimori T, Kato K. (**1983**) Neural volatile components of 'kukoshi' (*Lycium chinese* M.). *Agricultural and Biological Chemistry*, ***47***, 2397-2399; (d) Karlsson K, Wahlberg I, Enzell CR. (**1972**) Volatile constituents of *Carphephorus corymbosus* and *Carphephorus paniculatus*. *Acta Chemica Scandinavica*, ***26***, 3839-3848.

NPC Natural Product Communications

2017
Vol. 12
No. 1
119 - 122

Vibrational and Thermal Studies of Essential Oils Derived from *Cistus ladanifer* and *Erica arborea* Shrubs

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Received: October 29th, 2016; Accepted: November 22nd, 2016

Essential oils from the two most representative shrub species from the Iberian Peninsula (namely *Cistus ladanifer* L. and *Erica arborea* L.) have been characterized by Fourier transform infrared spectroscopy (FTIR) and thermoanalytical techniques (TG/DTG and DSC). Vibrational spectra have been compared with those of components of the plants, and with those of oils, gums and resins from other species. The different content in terpenoids of *C. ladanifer* oil (mainly mono- and sesquiterpenoids) and *E. arborea* oil (mainly triterpenoids) is reflected in the ATR-FTIR by the position of the bands at around 2873 cm^{-1}, 1730 cm^{-1} and 1678 cm^{-1}. As regards their thermal behavior, *C. ladanifer*-derived oil evinced higher thermal stability than that of obtained from *E. arborea*: the pyrolysis of the former was sensitized at 210ºC, whereas for the later it occurred at 143ºC. These temperatures are high enough to state that thermolabile constituents such as terpenoids are conserved in the hydrodistillation and that this extraction process ensures the recovery of the main constituents of both essential oils.

Keywords: *Cistus ladanifer*, *Erica arborea*, Oils, TG/DTG, DSC, FTIR.

Pyrophytes are plants which have adapted to tolerate fire. “Passive pyrophytes” (e.g., *Asphodelus albus* Willd.) are able to resist the effects of fire, particularly when it passes over quickly, and hence can out-compete less resistant plants, which are damaged. “Active pyrophytes” (such as *Cistus ladanifer* L. and *Erica arborea* L.) have a similar competing advantage, but also contain volatile oils and hence encourage the incidence of fires which are beneficial to them. *C. ladanifer* (gum rockrose), native to the Western Mediterranean, is known in Spanish as ‘*Jara pringosa*’ (meaning ‘sticky shrub’) because the whole plant is covered with a sticky exudate of a fragrant resin. This resin, known as ‘labdanum’, ‘ladanum’, ‘laudanum’ or ‘ladanon’, is produced by boiling the leaves and twigs. It presents a relatively high content of ambrox (amber odor) and, consequently, it is valued in perfumery as a substitute for ambergris (from the sperm whale, which is an endangered species). *Cistus* essential oil is obtained by either hydrodistillation or steam distillation of the leaves and stems, reaching a price of over 200 €/L.

Cistus oil is relatively rich in monoterpenes (67%) and sesquiterpenes (20%) and poor in diterpenes (3%). The major constituents are α-pinene (a bronchodilator), *trans*-pinocarveol, bornyl acetate, terpinen-4-ol and camphene (monoterpenes); viridiflorol (estrogen mimic, also reported in propolis), ledol and globulol (sesquiterpene alcohols); and 15-nor-labdan-8-ol (diterpene) [1-3].

Erica arborea (tree heath) is in the heather family, *Ericaceae*. It is native to the maquis shrublands (garrigue biome) of the Mediterranean Basin, Portugal and the Canary and Madeira Islands. The leaves and flowers of *E. arborea* have been popularly used as an anti-rheumatic, a diuretic, an astringent and in the treatment of urinary infections, while its wood (briar root or *bruyère* in French) is used for making smoking pipes and jewelry.

E. arborea contains many active compounds such as flavonoids, monoterpenes, triterpenoids, phenylpropanoid glucosides and condensed tannins [4]. Pharmacological activities of the extracts from this plant have been reported to be anti-inflammatory, antioxidant and analgesic. In fact, the composition of its bark (with leaves) has been reported to include triterpenoids such as friedelin, lupeol, betulin and ursolic acid [5, 6].

According to Bessah and Benyoussef [4], the water-distilled essential oil from leaves of *E. arborea* of Algerian origin (d=0.8587; [α] +2°44′) contains 75 components, amongst which palmitic acid (33.3%), (Z,Z,Z)-9,12,15-octadecatrien-1-ol, a fatty alcohol (6.6%), and nonacosane, a straight-chain hydrocarbon (6.1%), are the major constituents [4]. Other components, present in lower proportions, are β-fenchyl alcohol, β-caryophyllene, β-bourbonene, eugenol, ionol, geranylacetone and germacrene D [4].

To the best of the authors’ knowledge, no vibrational or thermal studies have been reported in the literature for any of the two aforementioned oils. The thermal behavior of *C. ladafiner* has been studied by TG and triple shot pyrolysis [7] of the entire plant, and the studies on *E. arborea* have been aimed at the assessment of the thermal behavior of the entire plant for its integration in wildland fire spread models [8] or to measure its ignitability, combustibility and sustainability as a forest fuel [9].

In the work presented herein, the physicochemical properties of the essential oils obtained from these two pyrophytic Mediterranean shrubs have been investigated by ATR-FTIR vibrational spectroscopy and by thermogravimetric (TG) and differential scanning calorimetric (DSC) thermoanalytical techniques. These data are of interest in extraction processes for the cosmetics industry, provided that they reveal differentiation patterns of the vegetable oil fingerprints.

ATR-FTIR spectra: The wavenumbers for the main bands in the ATR-FTIR spectra of the oils of the two plants under study, together with those from leaves, roots, capsules, fine and coarse-texture components for each of the species, are summarized in Table 1. A close similarity can be observed, on the one hand, between the leaves and roots spectra and, on the other hand, between the fine and coarse components spectra. The capsules vibrational pattern for *E. arborea* exactly matched that of the fine components and is listed in the same column.

Table 1: Main bands in the ATR-FTIR vibrational spectra of *C. ladanifer* and *E. arborea* oils and various plant components. All wavenumber values are in cm^{-1}.

Cistus ladanifer					*Erica arborea*				
Oil	Leaves	Roots	Fine	Coarse	Oil	Leaves	Roots	Capsules / Fine	Coarse
3465	3412	3416	3419	3421	3360	3396	3405	3421	3412
2953	×	×	×	×	2970	×	×	×	×
2918	2919	2920	2922	2920	2931	2923	2929	2918	2919
2871	2850	×		×	2874	2852	×	2850	
×	2360	2361	2355	2361	×	2360	2361	2360	2360
×	≠	2344	2339	2343	×	≠	2344	2342	2343
1737	1733	1735	1732	1735	1721	1733	1736	1739	1740
1681	×	×	×	×	1674	×	×	×	×
×	1615	1617	1621	1617	1614	1616	1617	1616	1616
×	1515	1508	1505	1508	×	1516	1513	1508	1510
1448	1455	1452	1455	1457	1454	1455	1451	1464	1459
1375	1367	1375	1373	1375	1374	1373	1374	1375	1375
1328	≠	1320		1320	1329	≠		1331	
1245	1232	1243	1242	1243	1249	1243	1247	1246	1247
1164	1164				1152		1151		
1125									
1111					1085		1081		
×	1035	1035	1031	1035	1046	1035	1031	1050	1039
1015					1007				
952					939				
886					860				
815					821				
787					758				
635					665				
618	617	559	602	610	559		579	608	607

The oils spectra differed from those of the leaves, roots and other plant components from which they were obtained by the absence of the absorption bands at 2360 cm^{-1} (absorbed CO_2), 2344 cm^{-1} (glucose ring stretching), 1515 cm^{-1} (benzene ring stretching in lignin) and 1035 cm^{-1} (lignin), present in the latter components. Nonetheless, they all shared the presence of bands at 1455 cm^{-1} (CH_2 cellulose, lignin) and 1375 cm^{-1} (C-H cellulose, hemicellulose) [10]. Bands specific to the oils are those which appeared at 2960, 2873, 1678, 945 and 818 cm^{-1}. The bands at 2960 and 2873 cm^{-1} are assigned to asymmetrically and symmetrically stretching vibration of C–H of aliphatic CH_3 groups, due to the alkyl rest of the triglycerides present in large quantities in vegetable oils, while the band at around 1678 cm^{-1} can either be attributed to C=C stretching vibration of *cis* disubstituted olefins (RHC=CHR) or to an oxo group (α:β unsaturated) from terpenoids. The C-H out-of-plane deformation band observed between 952 and 939 cm^{-1} is highly characteristic of *trans* double bonds, and the band at around 818 cm^{-1} is related to an isopropylidine group (R_2C=CHR), usual in terpenoids [11-14].

The fact that the labdanum and tree heath oils spectra (Figure 1), as is the case for other oils, showed bands in common with resins and gums has led us to compare these spectra with those of rosehip and palm oils, myrrh and mastic resins and tragacanth gum (see Table 2). It is worth noting that, in the 2920-1160 cm^{-1} region, there is a high correspondence with the bands of *R. rubiginosa* oil, a moderate correspondence with those of other oils and resins, and low agreement with those of tragacanth gum.

In the case of *C. ladanifer* oil, whose terpene composition is favorable to low-number isoprenic units (monoterpenes and sesquiterpenes), the band at 1737 cm^{-1} (attributable to ν(CO) ester carbonyl or to terpenic oxo groups) is shared with rosehip and palm oils, whereas the composition rich in terpenes with a high number of isoprenic units (triterpenoids) exhibited by *E. arborea* oil leads to a shift of this band towards lower wavenumbers (1721 cm^{-1}). As regards the band at around 2875 cm^{-1} (attributed to symmetrical ν(C-H) from CH_3) in the *E. arborea* spectrum, it should be noted that it agrees with that of mastic resin. Another useful band in terms of differential identification of the oils is that which appears at 1681 cm^{-1} in *C. ladanifer* oil and shifted to lower wavenumbers in tree heath oil (1674 cm^{-1}) and rosehip oil (1653 cm^{-1}). This band does not appear in palm pulp oil or in tragacanth gum.

Table 2: Comparison of the vibrational spectra of *C. ladanider* and *E. arborea* oils with rosehip and palm oils, myrrh and mastic resins and tragacanth gum.

C. ladanifer		*E. arborea*		Myrrh resin	Mastic resin	Rosehip oil [15]	Palm pulp oil	Tragacanth gum	Assignments
extract	oil	extract	oil						
	3465								OH group
3362		3366	3360	3348				3335	
2969	2953	2969	2970		2949	3009	3004		asymmetric *ν*(CH) from CH_3
2915	2918	2930	2931	2925		2923	2914	2932	asymmetric *ν*(CH) from CH_2
	2871	2875	2874		2874				symmetric *ν*(CH) from CH_3
2848	2834			2854		2853	2850		
1732	1737	1735	1721	1732		1742	1736	1716	*ν*(CO) ester carbonyl of triglycerides
1712	1710				1707				
1655	1681	1674	1674	1635	1650	1653	×	×	ν(C=C) disubstituted olefins; olefinic terpenoids
1607		1614	1614	1601				1600	*ν*(C=C) aromatic ring
1462	1448	1454	1454		1459	1456	1467		*δ*(CH_2) bending deformation
				1435				1410	*δ*(C-H) from CH_2 or CH_3 groups; CH_2 cellulose/lignin
1378	1375	1374	1374	1378		1377	1389	1371	*δ*(CH_2)$_2$ bending deformation CH hemicellulose, cellulose
1341	1328	1329	1328	1335				×	*δ*(CH_3) bending deformation
			1298						
1227	1245	1249	1249	1238	1245	1238	1243	1242	*δ*(C-H), *ν*(C-O-H).
1160	1164	1152	1142		1161	1160	1170	1145	methyl ester, *ν*(CO)
1128	1125							1120	*ν*(C-C)
1106	1111				1115				
	1083	1085	1084			1098	1100		stretching vibration of C–O ester groups
	1048	1046	1045		1046				starch OH, cellulose
	1032			1023				1034	
	993	1007	1007		1008				trans double bond
950	952	939	988						*δ*(C-H) bending
876	886	860	860	841	837			892	*δ*(C-O-C)
816	815	821	838					846	R_2C=CHR isopropylidine
	787								
729		758	758	725		723	717	×	*ρ*(CH_2)$_n$
719	698		701						1,2-*cis*-disubstituted olefin
638	635	665	662	597	580				*δ*(C-H) in the furan ring
	563	559	559					595	
			527					521	

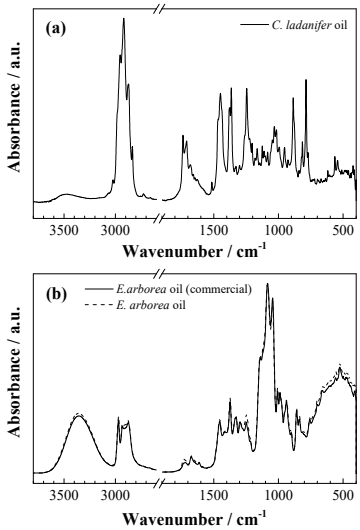

Figure 1: FTIR spectra of (a) *Cistus ladanifer* oil and (b) *Erica arborea* oil.

Thermal analysis: TG plots of gum rockrose and tree heath oils showed mass loss in the 50-240ºC and 100-220ºC temperature range, respectively, with a more abrupt pattern for *E. arborea* (Figure 2). In both cases this mass loss corresponded to pyrolysis, which was sensitized in the DTG curves by endotherms at 151ºC (*C. ladanifer*) and at 208ºC (*C. arborea*). From these temperatures, it can be seen that *E. arborea* oil presents higher thermal stability than that of *C. ladanifer*. In any case, the decomposition point of both oils is high enough to ensure the preservation of terpenoids as main constituents. The chief products of pyrolysis were straight-chain alkanes and alkenes [16]. It should be noted that heating of isopropanol extracts (not shown) resulted, after solvent evaporation, in decomposition of the oils extending up to 400 ºC.

The low-temperature DSC thermograms of the two oils under study are depicted in Figure 3. Both *C. ladanifer* and *E. arborea* oils vitrify upon cooling. Upon heating, only devitrification is observed. For *C. ladanifer* oil the glass transition is observed at T_g = (-113 ± 1) ºC and for *E. arborea* oil at T_g = (-79 ± 1) ºC.

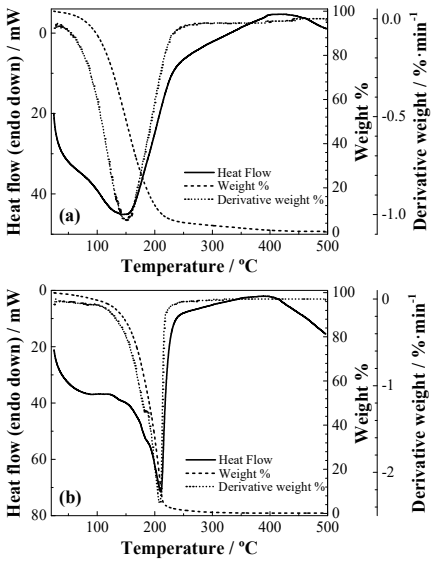

Figure 2: Thermograms for (*a*) *C. ladanifer* oil, *m* = 21.14 mg; and (*b*) *E. arborea* oil, m = 17.77 mg. The left *y* axis corresponds to the DSC curve, while the two *y* axes on the right correspond to TG/DTG curves.

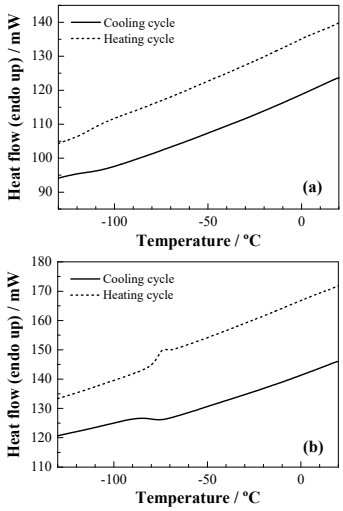

Figure 3: Low-temperature DSC thermograms for (*a*) *C. ladanifer* oil, *m* = 4.1 mg; and (*b*) *E. arborea* oil, *m* = 5.7 mg. The cooling/heating rate was $|\beta|$=50ºC·min^{-1}.

As regards the FTIR results, this technique has proven useful to identify the different components of various parts of the plants and their derived products, serving as a fingerprint technique which provides insight into the biochemical composition of the samples. As noted by Huck [17], this method is particularly suitable for the fast and simultaneous qualitative and quantitative characterization of natural products and their constituents. Thus, the data presented herein can be used for quality control in the cosmetics industry.

With regard to the thermal stability of the oils under study, it is high enough to ensure that their thermolabile constituents would be preserved during hydro-distillation, which is the method recommended by the French Pharmacopoeia for the extraction of essential oils from dried spices and the quality control of essential oils in the laboratory [18].

On another topic, provided that the two shrub species under study are active pyrophytes, the results should also be put in relation to their impact on forest fires: the flammability of the shrubs under study (and, by extension, those of their resins and oils) must be referred (apart from ignition time and moisture) to their contents of terpenoids. The high flammability of *E. arborea* can be ascribed to its high emission of terpenes throughout all the year (which can reach up to 40 $\mu g \cdot g_{DM}^{-1} \cdot h^{-1}$), in spite of the fact that its composition is relatively rich in low-volatile terpenoids, such as triterpenoids. Conversely, although *C. ladanifer* emits into the air modest amounts of terpenoids (<7 $\mu g \cdot g_{DM}^{-1} \cdot h^{-1}$), it has a very high flammability in the summer due to the potentiated emission of α-pinene, a particularly volatile monoterpene, which, as noted above, is an important component of its essential oil [19].

The essential oils from two Western Mediterranean pyrophytes, namely *C. ladanifer* and *E. arborea*, have been characterized by ATR-FTIR spectroscopy and thermal analytical (TG/DTG and DSC) techniques. Their vibrational spectra have been compared with those of other parts of the plants (leaves, roots, capsules, etc.) and with those of oils, gums and resins from other species. The specific location of the bands from unsaturated and α:β unsaturated oxogroups has been related to the different content of terpenoids of *C. ladanifer* oil (mono- and sesquiterpenoids) and *E. arborea* (triterpenoids). As regards the thermal behavior, *E. arborea* oil showed higher thermal stability than that of *C. ladanifer*, as evinced by the effects in the TG and DSC thermograms (at 210ºC and

143ºC, respectively). Thus, recognition of the TG and DSC characteristic patterns of both essential oils can also be helpful in identifying the type of oil. On the other hand, the delayed thermal decomposition of the oils under study, together with the characterization of terpenoids by ATR-FTIR, provide evidence that oil constituents that are thermolabile, such as terpenoids, are conserved in the extraction process, thus ensuring that hydrodistillation is a valuable method to recover the main constituents of these essential oils.

Experimental

Samples: *C. ladanifer* and *E. arborea* samples were collected from wild plants growing in the municipality of Ayoó de Vidriales (in the province of Zamora, Castilla y Leon, Spain) during the flowering period (Spring 2015). Oils from *Rosa rubiginosa* L. and *Elaeis guineensis* Jacq., myrrh from *Commiphora*, mastic from *Pistacia lentiscus* L. and tragacanth gum from *Astragalus* samples used for comparison purposes were of commercial origin.

Methods: Hydro-distillation of 100 g of fresh and whole leaves of *C. ladanifer* and *E. arborea* was carried out in a Clevenger-type apparatus for 3 h. The essential oils were collected, dried under anhydrous sodium sulfate and stored at 4ºC until used. To confirm their purity, essential oil composition was determined by gas chromatography coupled to mass spectrometric (GC-MS) analysis, according to the experimental conditions specified by Bessah and Benyoussef [4]. A commercial sample *E. arborea* essential oil, supplied by Radhe Shyam (Barcelona, Spain), was also tested. An alternative extraction method consisting of suspending the vegetal dry matter in amyl alcohol in a 1:2 (w/v) ratio for 30 min, under constant shaking, at room temperature, was also used to isolate high-purity oil from both plants.

Apparatus: GC/MS analysis was conducted with an Agilent Technologies 7890A apparatus (Santa Clara, CA, USA). The vibrational spectra of the materials in the 400-4000 cm^{-1} spectral range were measured using a Thermo Scientific (Waltham, MA, USA) Nicolet iS50 FT-IR spectrometer, equipped with an in-built diamond attenuated total reflection (ATR) system. Spectra of the oils were recorded at room temperature with a 1 cm^{-1} spectral resolution, and 64 scans. TG and high-temperature DSC analyses were conducted with a Perkin-Elmer (Waltham, MA, USA) STA6000 simultaneous thermal analyser by heating the samples in a slow stream of N_2 (20 $mL \cdot min^{-1}$) from room temperature up to 500ºC, with a heating rate of 20ºC·min^{-1}. Pyris v.11 software was used for data analysis. The low-temperature DSC experiments were performed in a Perkin-Elmer Pyris 1 power compensation calorimeter. A liquid nitrogen Cryofill cooling unit was used, and helium at a 20 $mL \cdot min^{-1}$ flux was employed as the purge gas. Samples were cooled to -170ºC and then heated to room temperature at a 50ºC·min^{-1} rate. The obtained data were analysed using TA Instruments Universal Analysis V4.1D software.

Acknowledgments - Financial support by the European Union LIFE+ Programme under project "CO_2 Operation: Integrated agroforestry practices and nature conservation against climate change" (ref. LIFE11 ENV/ES/000535) is gratefully acknowledged. The authors would also like to thank the support of Fundação para a Ciência e a Tecnologia (FCT) under project UID/QUI/UI0313/2013 and the COMPETE Programme.

References

[1] Gomes PB, Mata VG, Rodrigues AE. (**2005**) Characterization of the Portuguese-grown *Cistus ladanifer* essential oil. *Journal of Essential Oil Research*, ***17***, 160-165.
[2] Gülz P-G, Kobold U, Michaelis K, Vostrowsky O. (**1984**) The composition of terpene hydrocarbons in the essential oils from leaves of four *Cistus* species. *Zeitschrift für Naturforschung C*, ***39***, 699-704.
[3] Verdeguer M, Blázquez MA, Boira H. (**2012**) Chemical composition and herbicidal activity of the essential oil from a *Cistus ladanifer* L. population from Spain. *Natural Product Research*, ***26***, 1602-1609.
[4] Bessah R, Benyoussef E-H. (**2014**) Essential oil composition of *Erica arborea* L. leaves from Algeria. *Journal of Essential Oil Bearing Plants*, ***17***, 931-935.
[5] Khasan YY, Zorina AD, Matyukhina LG, Saltykova IA. (**1977**) Triterpenoids of some plants growing in Siberia. *Chemistry of Natural Compounds*, ***13***, 109-109.
[6] Hegnauer R, Hegnauer M. (**1962**) *Chemotaxonomie der Pflanzen; eine Übersicht über die Verbreitung und die systematische Bedeutung der Pflanzenstoffe*, Birkhäuser, Basel.
[7] De la Rosa JM, González-Pérez JA, González-Vázquez R, Knicker H, López-Capel E, Manning DAC, González-Vila FJ. (**2008**) Use of pyrolysis/GC–MS combined with thermal analysis to monitor C and N changes in soil organic matter from a Mediterranean fire affected forest. *Catena*, ***74***, 296-303.
[8] Cancellieri D, Leoni E, Rossi JL. (**2005**) Kinetics of the thermal degradation of *Erica arborea* by DSC: Hybrid kinetic method. *Thermochimica Acta*, ***438***, 41-50.
[9] Leroy V, Cancellieri D, Leoni E. (**2006**) Thermal degradation of ligno-cellulosic fuels: DSC and TGA studies. *Thermochimica Acta*, ***451***, 131-138.
[10] Fan M, Dai D, Huang B. (**2012**) Fourier transform infrared spectroscopy for natural fibres. In *Fourier transform–materials analysis*. Salih SM. (Ed.), InTech, Rijeka (Croatia), 45-68.
[11] Bodirlau R, Teaca CA, Spiridon I. (**2009**) Preparation and characterization of composites comprising modified hardwood and wood polymers/poly (vinyl chloride). *BioResources*, ***4***, 1285-1304.
[12] Vlachos N, Skopelitis Y, Psaroudaki M, Konstantinidou V, Chatzilazarou A, Tegou E. (**2006**) Applications of Fourier transform-infrared spectroscopy to edible oils. *Analytica Chimica Acta*, ***573***, 459-465.
[13] Moharam M, Abbas L. (**2010**) A study on the effect of microwave heating on the properties of edible oils using FTIR spectroscopy. *African Journal of Microbiology Research*, ***4***, 1921-1927.
[14] Jović O, Smolić T, Jurišić Z, Meić Z, Hrenar T. (**2013**) Chemometric analysis of Croatian extra virgin olive oils from central Dalmatia region. *Croatica Chemica Acta*, ***86***, 335-344.
[15] Martín-Ramos P, Martín-Gil J, Ramos-Sánchez MC, Navas-Gracia LM, Hernández-Navarro S, Martín-Gil FJ. (**2016**) Vibrational and thermal characterization of seeds, pulp, leaves and seed oil of *Rosa rubiginosa* L. *Boletín de la Sociedad Argentina de Botánica*, ***51***, 429-439.
[16] Alencar JW, Alves PB, Craveiro AA. (**1983**) Pyrolysis of tropical vegetable oils. *Journal of Agricultural and Food Chemistry*, ***31***, 1268-1270.
[17] Huck CW. (**2015**) Advances of infrared spectroscopy in natural product research. *Phytochemistry Letters*, ***11***, 384-393.
[18] Li Y, Fabiano-Tixier A-S, Chemat F. (**2014**) Essential oils: From conventional to green extraction. In *Essential oils as reagents in Green Chemistry*, Li Y, Fabiano-Tixier A.-S, Chemat F. (Eds.), Springer International Publishing, New York, NY, USA, 9-20.
[19] Llusia J, Peñuelas J. (**2000**) Seasonal patterns of terpene content and emission from seven Mediterranean woody species in field conditions. *American Journal of Botany*, ***87***, 133-140.

NPC Natural Product Communications

2017
Vol. 12
No. 1
123 – 126

Chemical Composition and Antibacterial Activity of the Essential Oil of *Phellodendron lavallei*

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Received: October 16th, 2015; Accepted: September 8th, 2016

The essential oils from inflorescences, fruits and leaves of *Phellodendron lavallei* Dode were analyzed by GC, GC/MS and ^{1}H NMR spectroscopy. About 100 compounds were identified. The principal components in the fruit oil were myrcene (47.7-52.0%) and limonene (38.4-40.9%), followed by germacrene D (1.2-3.2%) and (*E*)-β-caryophyllene (2.4-2.9%). The inflorescence oil contained mainly myrcene (44.8%), limonene (43.2%) and (*E*)-β-ocimene (4.1%). The main constituents of the leaf oil were limonene (26.7%) myrcene (22.1%), (*E,E*)-α-farnesene (5.6%) and α-pinene (5.0%). The antibacterial activity was examined towards both Gram-positive and Gram-negative bacteria. The fruit oil showed weak activity against the bacteria tested.

Keywords: *Phellodendron lavallei*, Essential oil composition, Myrcene, Limonene, Antibacterial activity.

Phellodendron lavallei Dode (Rutaceae) is a medium sized, deciduous tree with thick, corky bark, leathery pinnate leaves, small yellowish-green flowers and pea-sized black, aromatic fruits. The plant is native to east Asia. In Europe and North America it is cultivated in parks and along the roads because of its attractive appearance, high resistance to frost, drought, insects and pollution [1a-c]. Bark of the tree is used in Eastern medicine, due to anti-inflammatory, antibacterial, antifungal, antipyretic and hypoglycemic activities. Its leaves are effective antihepatotoxic and antiviral agent. Only few papers have described biologically active substances of this species. Alkaloids (berberine, jatrorrhizine, magnoflorine, palmatine, phellodendrine) and limonoids (limonin, obacunone) were isolated from bark [2a-d]. In leaves, flavonoids (noricariside, phellatin, phellavin) were found [3a,b]. Seeds contained 16% of lipids, including fatty acids (lauric, myristic, isopalmitic, eicosatrienoic), phospholipids and sterols (ergosterol, stigmasterol, β-sitosterol) [4].

In the literature there is no data on the essential oil of this species, and for that reason we decided to study the chemical composition of the essential oil from different parts of *P. lavallei* and the antibacterial activity of its fruit oil. The yields and chemical composition of the oils from fruits, inflorescences and leaves of the plant are presented in Table 1. The fruits were rich in essential oil (1.0-1.2%). Inflorescences and leaves contained only negligible amounts of oil. About one hundred compounds were identified, representing 98-99% of the oils. The monoterpene hydrocarbons myrcene and limonene were the principal constituents of the oils.

The fruit oil contained mainly myrcene (47.7-52.0%) and limonene (38.4-40.9%), followed by germacrene D (1.2-3.2%) and (*E*)-β-caryophyllene (2.4-2.9%). Other components occurred in amounts less than 1%. The enantiomeric ratio of limonene was: (*R*)-(+)-limonene (97.3%) : (*S*)-(-)-limonene (2.7%). The content of essential oil increased during the fruit's development, however, the oil composition varied only slightly at the same time. Drying of fruits had no influence on the yield and composition of the oil. The fruit oil of *P. lavallei* resembles that of *P. sachalinense* oil with high amounts of myrcene (50-54%) and limonene (37-40%) [5]. It differs from *P. amurense*, *P. chinense*, *P. japonicum* and *P. piriforme* oils in that the prominent component is myrcene (62-86%), whereas limonene occurs in concentration of 0.6-2% [6a-d].

The main constituents of the inflorescence oil were myrcene (44.8%), limonene (43.2%) and (*E*)-β-ocimene (4.1%). The leaf oil contained mainly limonene (26.7%), myrcene (22.1%), (*E,E*)-α-farnesene (5.6%) and α-pinene (5.0%).

P. lavallei fruit oil inhibited the growth of all tested Gram-positive and Gram-negative standard strains at concentrations between 53.0 μL/mL – 67.0 μL/mL. The lowest values of MIC for *Enterococcus faecalis* ATCC 51299 and *E. faecium* ATCC 35667 were obtained – 54.0 μL/mL and 56 μL/mL, respectively. Slightly more resistant to the oil was *Staphylococcus aureus* ATCC 433000 with a MIC of 59.0 μL/mL. A similar MIC value (59.0 μL/mL) was found for one of the Gram-negative standard strains – *Escherichia coli* ATCC 25922. The oil possessed significantly lower antibacterial activity against other Gram-negative bacteria – *Klebsiella pneumoniae* ATCC 700603 and *Acinetobacter baumannii* ATCC 19606; the MIC values were 64.0 μL/mL and 67.0 μL/mL, respectively.

The oil of *P. lavallei* contains large amounts of terpene hydrocarbons, which have rather low antimicrobial activity [7]; this is why the oil showed weak activity against the bacteria tested.

This is the first report on the essential oil of *P. lavallei*. The fruits of this species, due to their high yield of essential oil and its high content of myrcene and limonene, can be used as a source of natural myrcene and (*R*)-(+)-limonene.

Table 1: Chemical composition of the essential oils from fruits (A – fresh unripe, B – fresh ripe, C – air-dried ripe), inflorescences (D) and leaves (E) of *Phellodendron lavallei*.

Compound	RI	LRI	Percentage					Identification methods
			A	B	C	D	E	
Hex-3-en-1-ol*	844	851					0.3	RI, MS
Hex-2-en-1-ol*	857	861					0.4	RI, MS
Heptanal	882	882				tr	0.2	RI, MS
α-Pinene	933	936	0.4	0.6	0.5	2.4	5.0	RI, MS
Camphene	947	950	tr	tr	tr	0.1	0.1	RI, MS
Sabinene	969	973	0.1	0.1	0.1	0.1	0.1	RI, MS
β-Pinene	974	978	tr	tr	tr	0.1	0.1	RI, MS
Myrcene	985	987	52.0	49.0	47.7	44.8	22.1	RI, MS, ^{1}H-NMR
Hex-3-enyl acetate	999	1002					0.5	RI, MS
α-Phellandrene	1001	1002	0.3	0.3	0.2	0.1	0.3	RI, MS
α-Terpinene	1008	1013					tr	RI, MS
p-Cymene	1015	1015	tr	tr	tr	tr	tr	RI, MS
Limonene	1026	1025	38.4	40.9	40.0	43.2	26.7	RI, MS, ^{1}H-NMR
(*Z*)-β-Ocimene	1028	1029	tr	tr	tr	tr	4.2	RI, MS
(*E*)-β-Ocimene	1041	1041	0.8	0.7	0.4	4.1	3.2	RI, MS
Nonanal	1075	1076	tr	tr	tr	tr	0.4	RI, MS
Terpinolene	1084	1082	tr	tr	tr	tr	tr	RI, MS
Linalool	1087	1086	0.5	tr	tr	0.2	0.5	RI, MS
Perillene	1092	1090	tr	tr	tr	tr	1.9	RI, MS
allo-Ocimene	1116	1113	0.3	0.1	0.1	1.3	1.0	RI, MS
cis-Limonene oxide	1125	1126			0.1			RI, MS
trans-Limonene oxide	1130	1130			tr			RI, MS
Ethyl benzoate	1151	1150					0.1	RI, MS
Cryptone	1160	1160	tr	tr	tr			RI, MS
Terpinen-4-ol	1166	1164	tr	tr	0.1	tr	0.1	RI, MS
Decan-2-one	1174	1176	tr	tr	tr			RI, MS
α-Terpineol	1178	1176	tr	tr	tr	tr	tr	RI, MS
β-Cyclocitral	1201	1195	tr	tr	tr	tr	0.1	RI, MS
trans-Carveol	1203	1200	tr	tr	tr			RI, MS
cis-Carveol	1210	1210	tr	tr	tr			RI, MS
Citronellol	1212	1213	tr	tr	tr	tr	tr	RI, MS
Carvone	1216	1214	tr	tr	tr			RI, MS
(*Z*)-Hex-3-enyl 2-methylbutanoate	1218	1229[a]					0.1	RI, MS
(*Z*)-Hex-3-enyl 3-methylbutanoate	1221	1232[a]					0.2	RI, MS
Geraniol	1238	1235	tr	tr	tr	0.1	0.1	RI, MS
Methyl citronellate	1244	1245	tr	tr	tr	tr		RI, MS
Methyl nerolate	1264	1265	tr	tr	tr			RI, MS
Bornyl acetate	1270	1270	tr	tr	tr			RI, MS
Undecan-2-one	1275	1273	0.2	0.4	0.8	0.1		RI, MS, ^{1}H-NMR
Perilla alcohol	1280	1280	tr	tr	tr			RI, MS
Undecan-2-ol	1284	1284	tr	tr	0.1	tr		RI, MS
Methyl geranate	1305	1306	tr	tr	tr	tr		RI, MS
Citronellyl acetate	1334	1337	0.1	tr	0.1	0.1	0.1	RI, MS
Bicycloelemene	1337	1338	tr	tr	tr	tr	tr	RI, MS
δ-Elemene	1341	1341	0.2	0.1	0.2	tr	0.3	RI, MS, ^{1}H-NMR
Neryl acetate	1344	1342	tr	tr	tr	tr		RI, MS
α-Cubebene	1355	1355	tr	tr	tr	tr	0.1	RI, MS
Geranyl acetate	1362	1362	0.5	0.2	0.2	0.4	0.1	RI, MS, ^{1}H-NMR
N-Methyl methyl anthranilate	1372	1372				tr		RI, MS
α-Copaene	1382	1379	tr	0.1	0.1	tr	0.1	RI, MS, ^{1}H-NMR
Methyl perillate	1384	1381	tr	tr	tr			RI, MS
α-Elemene	1386	1381	tr	tr	tr	tr		RI, MS
β-Bourbonene	1388	1386	tr	tr	tr	tr		RI, MS
β-Elemene	1392	1389	0.1	0.1	0.2	tr	tr	RI, MS, ^{1}H-NMR
β-Cubebene	1394	1390	tr	tr	tr	tr	0.3	RI, MS
α-Gurjunene	1415	1413	tr	tr	tr	tr		RI, MS
β-Ylangene	1421	1420	tr	tr	tr	tr	0.3	RI, MS
β-Caryophyllene	1424	1421	2.8	2.4	2.9	0.4	1.1	RI, MS, ^{1}H-NMR
β-Copaene	1435	1430	0.2	0.2	0.3	tr	0.2	RI, MS
β-Gurjunene	1437	1437	tr	tr	tr	tr	tr	RI, MS
Isogermacrene	1447	1446	0.1	tr	0.1	tr	0.2	RI, MS
(*E*)-β-Farnesene	1450	1446	tr	tr	tr	tr	0.1	RI, MS
ζ-Muurolene	1453	1455	tr	tr	0.1	tr	0.1	RI, MS
α-Humulene	1459	1455	0.2	0.1	0.2	tr	0.2	RI, MS, ^{1}H-NMR
cis-Muurola-4(15),5-diene	1462	1462	tr	tr	0.1	tr	0.2	RI, MS
allo-Aromadendrene	1465	1463	tr	0.1	tr	tr		RI, MS
(*E*)-β-Ionone	1472	1468					0.1	RI, MS
γ-Muurolene	1476	1474	tr	tr	tr	tr	0.4	RI, MS
Tridecan-2-one	1478	1477	0.1	0.1	0.4	tr		RI, MS, ^{1}H-NMR
Germacrene D	1483	1479	1.2	2.4	3.2	0.2	3.4	RI, MS, ^{1}H-NMR
epi-Cubebol	1488	1490	tr	0.1	0.1	tr	tr	RI, MS
Bicyclogermacrene	1492	1494	tr	0.2	0.2	0.1	0.1	RI, MS, ^{1}H-NMR
α-Muurolene	1495	1496	tr	tr	0.1	tr	1.0	RI, MS
(*E*,*E*)-α-Farnesene	1499	1498	tr	tr	tr	tr	5.6	RI, MS
β-Himachalene	1503	1500					0.2	RI, MS
γ-Cadinene	1510	1507	tr	tr	tr	tr	0.9	RI, MS
Cubebol	1517	1514	tr	0.1	0.1	tr		RI, MS
δ-Cadinene	1522	1520	tr	0.2	0.4	0.2	2.6	RI, MS, ^{1}H-NMR
Cadina-1,4-diene	1527	1523	tr	tr	tr	tr	0.1	RI, MS
α-Cadinene	1534	1530	tr	tr	tr	tr	0.3	RI, MS
β-Elemol	1545	1541	0.1	tr	tr	tr	tr	RI, MS
Germacrene B	1550	1552	tr	tr	tr	tr		RI, MS
(*E*)-Nerolidol	1553	1553	0.2	tr	tr	0.1	1.2	RI, MS, ^{1}H-NMR
Germacrene D-4-ol	1573	1571	0.2	0.3	0.3	0.1	1.7	RI, MS, ^{1}H-NMR
Spathulenol	1576	1572	tr	0.1	0.1	tr	tr	RI, MS, ^{1}H-NMR

Table 1 (continued)

Compound	RI	LRI	Percentage					Identification methods
			A	B	C	D	E	
Caryophyllene oxide	1580	1578	0.1	tr	tr	0.1	0.2	RI, MS, ^{1}H-NMR
Globulol	1584	1589	tr	tr	tr	tr	tr	RI, MS
Salvial-4(14)-en-1-one	1592	1592	tr	tr	tr	tr		RI, MS
Viridiflorol	1596	1592				tr	0.1	RI, MS
1,10-di-*epi*-Cubenol	1617	1615	tr	tr	tr	tr	tr	RI, MS
1-*epi*-Cubenol	1625	1623	tr	tr	tr	tr	0.1	RI, MS
epi-α-Murolool	1633	1633	tr	0.1	0.1	0.3	1.7	RI, MS, ^{1}H-NMR
epi-α-Cadinol	1635	1633	tr	tr	tr	tr	0.1	RI, MS
α-Cadinol	1645	1643	tr	0.2	0.2	0.5	2.7	RI, MS, ^{1}H-NMR
(*E*,*E*)-Farnesol	1717	1718	0.4	0.1	0.1	0.2	0.5	RI, MS, ^{1}H-NMR
(*E*,*E*)-Farnesyl acetate	1840	1843[a]	0.1	0.1	0.1	tr	1.1	RI, MS, ^{1}H-NMR
Cembrene	1941	1938	tr	tr	tr	tr		RI, MS
Isophytol	1944	1949					0.1	RI, MS
Phytol	2108	2114					3.2	RI, MS
Aliphatic hydrocarbons C_{19}-C_{27}						tr	0.3	RI, MS
Total			99.6	99.3	99.9	99.3	98.8	
Monoterpene hydrocarbons			92.3	91.7	89.0	96.2	62.8	
Oxygenated monoterpenes			1.1	0.2	0.5	0.8	2.9	
Sesquiterpene hydrocarbons			4.8	5.9	8.1	0.9	17.8	
Oxygenated sesquiterpenes			1.1	1.0	1.0	1.3	9.4	
Aliphatic ketones			0.3	0.5	1.2	0.1		
Oil yield in fresh source (v/w, average of several replicates)			1.0	1.2	2.6	0.06	0.01	
Oil yield in dry weight (v/w)			2.7	3.0	2.9	0.2	0.04	

RI – retention indices on Rtx-1 column; LRI – retention indices of literature on DB-1 column according to MassFinder 3.1; [a] – retention indices of literature on DB-5 column [8]; tr – trace (<0.05%); * – correct isomeric form not identified.

Experimental

Plant material: *Phellodendron lavallei* Dode was collected in the Forest Experimental Station, Arboretum of Warsaw Agriculture University in Rogow, Poland. Inflorescences were harvested at the beginning of June, leaves and unripe fruits in July, and ripe fruits in October 2013. Voucher specimens (No. 2013/Pla1-2013/Pla4) were deposited in the Herbarium of the Institute of General Food Chemistry, Lodz University of Technology.

The fresh inflorescences (moisture 75.0%) and fresh leaves (moisture 72.8%) were cut into small pieces, and fresh unripe fruits (moisture 61.3%), fresh ripe fruits (moisture 62.0%) and air-dried for a month ripe fruits (moisture 10.6%) were ground. Each sample of plant material (200 g) was separately hydrodistilled in a Clevenger-type apparatus for 3 h to obtain the essential oil. After decanting and drying over anhydrous $MgSO_4$, the oils were stored at low temperature (5ºC) before analysis. The oils had a pale yellow color and intensive terpenic aroma.

Analysis of the essential oils: The chemical composition of the oils was determined by chromatographic and spectroscopic methods.

The gas chromatographic (GC) analyses were carried out on a Carlo Erba Instruments HRGC 5300 gas chromatograph operated with a split/splitless injector, a flame ionization detector using an apolar capillary column Rtx-1 (dimethylpolysiloxane), 60 m × 0.25 mm × 0.25 µm film thickness (Restek, Bellafonte, PA, USA), temperature program 50-300°C at 4°C/min, injector temperature 320°C, detector temperature 310°C. Injection volume was 0.08 µL, split ratio 1:13, carrier gas helium with a flow 4 mL/min.

The fruit oil was also analyzed by GC with a capillary column Rtx-β-Dexcst, 30 m × 0.32 mm × 0.25 µm film thickness (Restek, Bellafonte, PA, USA), temperature program 50-220°C at 4°C /min.

The gas chromatography/mass spectrometry (GC/MS) analyses were performed on a Thermo Electron Corporation Trace GC Ultra gas chromatograph equipped with a mass detector DSQ II. The MS operating parameters were: ionization voltage 70 eV, ion source temperature 200°C, mass range 33-420 m/z. Other conditions of the analyses were the same as described under GC analyses.

^{1}H NMR spectra were obtained on a Bruker DPX 250 Avance spectrophotometer using $CDCl_3$ as solvent and TMS as a standard.

Additionally, the fruit oil (15.0 g) was vacuum distilled in a Vigreux column to obtain fraction 1 (12.6 g) containing monoterpene hydrocarbons and fraction 2 (1.8 g, residue). Fraction 2 was separated by flash chromatography (FC) on Kieselgel 60, particle size 0.040-0.063 mm (Merck) with *n*-hexane and increasing amounts of diethyl ether to obtain fractions 2.1-2.6. Fraction 2.1 (835 mg), containing sesquiterpene hydrocarbons and fractions: 2.2 (310 mg), 2.3 (170 mg), 2.4 (190 mg), 2.5 (170 mg) and 2.6 (150 mg), containing oxygenated compounds, were separated on Kieselgel 60 impregnated with $AgNO_3$ to obtain fractions 2.1.1-2.1.6, 2.2.1-2.2.4, 2.3.1-2.3.4, 2.4.1-2.4.3, 2.5.1-2.5.4 and 2.6.1-2.6.4. All fractions were analyzed by GC/MS, additionally fractions: 1, 2.1.1-2.1.6, 2.2.2, 2.2.3, 2.3.2, 2.3.3, 2.4.2, 2.5.2, 2.5.3, 2.6.2 and 2.6.3 by ^{1}H NMR spectroscopy. Fraction 1 contained myrcene (57%) and limonene (42%), fraction 2.1.1 (65 mg) δ-cadinene (63%) and α-copaene (18%), fraction 2.1.2 (390 mg) (*E*)-β-caryophyllene (54%) and germacrene D (38%), fraction 2.1.3 (220 mg) germacrene D (46%) and (*E*)-β-caryophyllene (45%), fraction 2.1.4 (25 mg) β-elemene (56%), fraction 2.1.5 (58 mg) bicyclogermacrene (24%) and δ-elemene (22%), fraction 2.1.6 (33 mg) α-humulene (63%), fraction 2.2.2 (235 mg) undecan-2-one (37%), tridecan-2-one (27%) and geranyl acetate (24%), fraction 2.2.3 (35 mg) (*E*,*E*)-farnesyl acetate (36%), fraction 2.3.2 (52 mg) undecan-2-one (55%) and caryophyllene oxide (21%), fraction 2.3.3 (75 mg) germacrene D-4-ol (47%), fraction 2.4.2 (84 mg) (*E*)-nerolidol (53%), fraction 2.5.2 (102 mg) *epi*-α-muurolol (68%), fraction 2.5.3 (40 mg) spathulenol (25%), fraction 2.6.2 (70 mg) α-cadinol (62%) and fraction 2.6.3 (65 mg) (*E*,*E*)-farnesol (83%).

Identification of components of the oils and components of the fractions was based on comparison of their retention indices relative to *n*-alkanes (C_8-C_{26}) and their MS with those of commercial libraries (NIST 98.1, Wiley Registry of Mass Spectral Data 8th edn, MassFinder 3.1) and literature [8]. Major components of the oil, separated by FC, were additionally identified by comparison of their ^{1}H NMR spectra with literature data [9a-j].

A quantitative analysis (expressed as percentage of each component) was carried out by peak area normalization measurements without correction factors.

Antibacterial activity of the essential oil: The Gram-positive standard strains: *Staphylococcus aureus* ATCC 433000, *Enterococcus faecalis* ATCC 51299, *E. faecium* ATCC 35667, and Gram-negative: *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603, and *Acinetobacter baumannii* ATCC 19606 came from the collection of the Environmental Biology Department, Medical University of Lodz. The bacteria were cultivated on Columbia Agar (bioMerieux) at 37°C for 24 h under aerobic conditions. The suspensions of bacterial standard strains with an optical density of 0.5MF scale were prepared with a bioMerieux densitometer.

The antibacterial activity of the essential oil from fruits of *P. lavallei* was investigated by the micro-dilution broth method. The essential oil was diluted in ethanol and used as a stock solution. This stock solution was mixed with a 100 µL Mueller-Hinton Broth (Graso, Poland) to obtain concentrations from 52.0 µL/mL to 68.0 µL/mL. An inoculum containing $1.5 \cdot 10^8$ CFU (10 µL) per well was added to broth with various essential oil concentrations; as well as to broth with no oil added containing only ethanol at concentrations used in the dilutions of oil and were transferred to 96-well microtiter plates. The Minimal Inhibitory Concentration (MIC) was determined as the lowest concentration of oil which inhibits the visible growth of bacteria after 24 h of incubation at 37°C under aerobic conditions.

References

[1] (a) Krüssman G. (**1985**) *Manual of Cultivated Broad-Leaved Trees & Shrubs*, vol. II, Timber Press, Hong Kong, 372-373; (b) www.onlineplantguide.com/Phellodendron lavallei/Lavalle corktree (16.03.2015); (c) Meskheli M, Mchedlidze K. Gogitidze Ts. (**2010**) Anatomical and morphological description of *Phellodendron lavallei* Dode stem bark. *Georgian Medical News*, ***182***, 32-38 (SciFinder, abstract).

[2] (a) Meskheli MB, Vachnadze VYu, Vachnadze NS, Bakuridze AJ. (**2011**) Alkaloids of *Phellodendron lavallei* introduced to Western Georgia. *Chemistry of Natural Compounds*, ***47***, 154-156; (b) Meskheli MB, Vachnadze VYu, Vachnadze NS, Beraschwili DT, Bakuridze ADzh. (**2011**) Phellodendrine from *Phellodendron lavallei* introduced into Western Georgia. *Chemistry of Natural Compounds*, ***47***, 485-486; (c) Meskheli M, Vachnadze V, Beraschwili D, Bakuridze A. (**2011**) HPLC analysis of protoberberine alkaloids in *Phellodendron lavallei* D. introduced to Western Georgia. *Journal of Natural Pharmaceuticals*, ***2***, 154-157; (d) Kawaguchi J, Takanashi S, Kozuka M, Gato K, Ishida M, Kin B, Yamazaki N. (**1989**) Insecticides containing obacunone and/or limonin. *Japanese Patent*, JP 01106804 A 19890424.

[3] (a) Bodalski T, Lamer E. (**1969**) Isolation and identification of noricariside from leaves of *Phellodendron*. *Dissertationes Pharmaceuticae et Pharmacologicae*, ***21***, 181-184; (b) Glyzin VI, Bankovskii AI, Sheichenko VI, Molodozhnikov MM. (**1970**) New flavonol glycosides from *Phellodendron lavallei* and *Phellodendron amurense*. *Khimiya Prirodnykh Soedinenii*, ***6***, 762-763.

[4] Kemertelidze EP, Dalakishvili TsM, Bitadze MA, Khatiashvili NS. (**2000**) Lipids of *Phellodendron lavallei* seeds. *Chemistry of Natural Compounds*, ***36***, 272-275.

[5] Lis A, Mielczarek J. (**2006**) Chemical composition of the essential oils from fruits, leaves and flowers of *Phellodendron sachalinense* (Fr. Schmidt) Sarg. *Flavour and Fragrance Journal*, ***21***, 683-686.

[6] (a) Lis A, Boczek E, Góra J. (**2004**) Chemical composition of the essential oils from fruits, leaves and flowers of the Amur cork tree (*Phellodendron amurense* Rupr.). *Flavour and Fragrance Journal*, ***19***, 549-553; (b) Lis A, Zdrojewska A. (**2009**) Chemical composition of the essential oil from fruits of *Phellodendron chinense* C.K. Schneid. *Journal of Essential Oil Research*, ***21***, 231-234; (c) Lis A, Jóźwicka M. (**2012**) Essential oil of *Phellodendron japonicum* Maxim. *Journal of Essential Oil Research*, ***24***, 259-263; (d) Lis A, Banaszczak P. (**2011**) Compounds of the essential oil of *Phellodendron piriforme*. *Chemistry of Natural Compounds*, ***47***, 641-643.

[7] Kalemba D, Kunicka A. (**2003**) Antibacterial and antifungal properties of essential oils. *Current Medicinal Chemistry*, ***10***, 813-829.

[8] Adams RP. (**2007**) *Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry*. 4th ed. Allured Publ. Corp., Carol Stream, IL.

[9] (a) Klusner PPA, Tip L, Brandsma L. (**1991**) On the direct metalation of isoprene. *Tetrahedron, **47***, 2041-2064; (b) Joulain D, König WA. (**1998**) *The Atlas of Spectral Data of Sesquiterpene Hydrocarbons*. E.B. Verlag, Hamburg; (c) Linker U, Kersten B, Linker T. (**1995**) Potassium permanganate – mediated radical reactions: chemoselective addition of acetone to olefins. *Tetrahedron*, ***51***, 9917-9926; (d) Chandrasekhar S, Ramachandar T, Reddy MV, Takhi M. (**2000**) A single step conversion of tetrahydropyranyl ethers to acetates. *The Journal of Organic Chemistry*, ***65***, 4729-4731; (e) Kokdil G, Tanker M, Kurucu S, Topcu G. (**1997**) Essential oil analysis of *Nepeta cilicia* Boiss. *Flavour and Fragrance Journal*, ***12***, 99-101; (f) Bohlmann F, Zdero C, King RM, Robinson H. (**1984**) A hydroxygermacrene and other constituents from *Pseudobrickellia brasiliensis*. *Phytochemistry*, ***23***, 1798-1799; (g) Miyazawa M, Nankai H, Kameoka H. (**1996**) Biotransformations of acyclic terpenoids (+/-)-*trans*-nerolidol and geranylacetone by *Glomerella cingulata*. *Journal of Agricultural and Food Chemistry*, ***44***, 1543-1547; (h) Borg-Karlson AK, Norin T, Talvitie A. (**1981**) Configurations and conformations of torreyol, α-cadinol, T-muurolol and T-cadinol. *Tetrahedron*, ***37***, 425-430; (i) Ragasa CY, Ganzon J, Hofilena J, Tamboongand B, Rideout JA. (**2003**) A new furanoid diterpene from *Caesalpinia pulcherrima*. *Chemical and Pharmaceutical Bulletin*, ***51***, 1208-1210; (j) Cane DE, Iyengar R, Shiao M-S. (**1981**) Cyclonerolidol biosynthesis of the enzymatic conversion of farnesyl to nerolidyl phyrophosphate. *Journal of the American Chemical Society*, ***103***, 914-931.

NPC Natural Product Communications

2017
Vol. 12
No. 1
127 - 130

Antimicrobial Potential of *Rosmarinus officinalis* Commercial Essential Oil in the Treatment of Vaginal Infections in Pregnant Women

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Received: August 3rd, 2016; Accepted: October 1st, 2016

Treating inflammatory conditions such as vaginosis, vaginitis, and vulvovaginitis in pregnancy is a special problem due to limitations of available drugs. However, possible treatment options can be found also in naturally originated products, such as essential oils (EOs) of different plants. The aim of this study was to evaluate *in vitro* antimicrobial and toxic activities of the commercial EO of *Rosmarinus officinalis* L. (*Lamiaceae*) against five Gram-positive and Gram-negative bacterial strains and two *Candida* strains obtained from pregnant women with vaginal infection. Gas chromatography-mass spectrometry of the tested EO revealed oxygenated monoterpenes to be the major ingredients, while microdilution assay showed the highest activity on *Staphylococcus aureus* II strain at 6.2 mg/mL. After 24 hours, toxicity was determined at 19.4 mg/mL on *Artemia salina* nauplii. The obtained results show this EO to be a promising alternative therapy for vaginal infections, although further toxicity and safety research is required.

Keywords: *Rosmarinus officinalis*, Essential oil, Pregnancy, Inflammation, Antimicrobial activity, Toxicity.

Species of *Lamiaceae* are the most known sources of essential oils (EOs), among which the EO of *Rosmarinus officinalis* L. (commonly known as rosemary) is already well known for its antimicrobial activity. While carnosic and rosmarinic acid may be the main bioactive antimicrobial compounds present in rosemary extracts [1a], antibacterial efficacy of rosemary essential oil is associated with the presence of compounds such as α-pinene, camphor and (-)-bornyl acetate [1b]. Furthermore, this EO expresses additional beneficial properties, such as improving circulation and relieving pain [1c], as well as having a potential anticarcinogenic activity [1d]. There is a rising need to investigate plants used in alternative therapies to determine if their use is based on the principles of evidence-based medicine [1e]. Novel natural agents will be useful in the upcoming post-antibiotic era, with the evident need for isolation of new compounds from natural products to which antimicrobial resistance has not been detected [1a].

The genital inflammatory conditions, vaginosis, vaginitis, and vulvovaginitis, occur in more than 30% of the population of women in their reproductive years [1f]. Vaginosis is a genital inflammatory condition that occurs in women of all ages [1g] and is characterized by dysbiosis of the vaginal microbiome [1h]. The etiology of vaginosis is still not fully understood but it is most likely a result of changes in the vaginal pH, caused by substitution of the peroxide-producing *Lactobacillus* species with infective agents, both aerobic and anaerobic bacteria, and yeasts [1i]. One of the major problems with treating these conditions in pregnancy is that pregnancy itself limits the number of drugs that can be used, because of their potential teratogenic effect. The most commonly used antibiotics, such as penicillin-based drugs and metronidazole, as well as many antifungals, are increasingly less effective due to the increase of antimicrobial resistance to infectious agents [1e,1j]. However, possible treatment options can be found in various plant products, such as EOs, extracts, and tinctures. Some traditional medical practices have used such products because of their healing properties for centuries, but it is only recently that we have started to determine, isolate and understand the mechanism of action of active compounds in these products. By knowing which molecule or, even better, what specific combination of molecules, contributes the most to the antimicrobial effect of certain natural products, we can test and recommend their use in accordance with the principles of evidence-based medicine [1k]. The aim of this research was to evaluate *in vitro* possible antimicrobial and toxic activities of the commercial EO of *R. officinalis* against bacterial and *Candida* strains isolated from pregnant women with vaginal infection and to assess its possible application as an alternative treatment for inflammatory gynecological diseases.

The chemical composition of the EO determined with GC/MS is given in Table 1. Oxygenated monoterpenes were the major compounds. This finding is in agreement with the study of Hussain *et al.* [2a] in which the tested *R. officinalis* EO from Pakistan contained a high concentration of oxygenated monoterpenes (61.7%). Similarly, Teixeira *et al.* [2b] reported that the rosemary EO they analyzed contained 59.7% of oxygenated monoterpenes, which is also consistent with our findings. The major components were eucalyptol (39.0% GC/MS; 42.2% GC/FID), camphor (13.7% GC/MS; 11.6% GC/FID), α-pinene (10.3% GC/MS; 12.1% GC/FID), (1S)-(-)-β-pinene (7.2% GC/MS; 8.2% GC/FID) and endo-borneol (4.7% GC/MS; 4.3% GC/FID). Similarly, several published papers reported that the major components of *R. officinalis* EO were eucalyptol (29.2%), camphor (17.2%) and α-pinene (11.5%) [2c-2e]. The variations in the main constituent (eucalyptol and camphor) concentrations could be explained by

Table 1: Chemical composition of the rosemary essential oil (GC/MS analysis).

No of Peak	[a]Rt (mins)	[b]Chemical constituents	[c]RI	Composition (Area %; Relative %)	Formulas	MW
1	12.6	Tricyclene	933	0.8	$C_{10}H_{16}$	136.1
2	12.9	α-Pinene	944	10.3	$C_{10}H_{16}$	136.1
3	13.4	Camphene	963	4.0	$C_{10}H_{16}$	136.1
4	14.1	(1*S*)-(-)-β-Pinene	989	7.2	$C_{10}H_{16}$	136.1
5	14.2	β-Myrcene	992	1.1	$C_{10}H_{16}$	136.1
6	14.8	Pseudolimonene	1013	0.1	$C_{10}H_{16}$	136.1
7	14.9	(+)-3-Carene	1018	0.2	$C_{10}H_{16}$	136.1
8	15.3	*p*-Cymene	1035	1.9	$C_{10}H_{16}$	136.1
9	15.4	(+)-Limonene	1040	3.5	$C_{10}H_{16}$	136.1
10	15.6	Eucalyptol	1047	39.0	$C_{10}H_{18}O$	154.1
11	17.0	Linalool	1104	1.5	$C_{10}H_{18}O$	154.1
12	18.2	*trans*-Sabinol	1161	0.2	$C_{10}H_{16}O$	152.1
13	18.3	(-)-Camphor	1168	13.7	$C_{10}H_{16}O$	152.1
14	18.6	Pinocarvone	1181	0.3	$C_{10}H_{14}O$	150.1
15	18.8	endo-Borneol*	1191	4.7	$C_{10}H_{18}O$	154.1
16	18.9	Terpinen-4-ol	1195	1.8	$C_{10}H_{18}O$	154.1
17	19.2	α-Terpineol	1210	3.1	$C_{10}H_{18}O$	154.1
18	19.3	(1*R*)-(-)-Myrtenal	1215	0.6	$C_{10}H_{14}O$	150.1
19	19.6	Fenchyl acetate	1231	0.3	$C_{12}H_{20}O_2$	196.1
20	20.2	(+)-Carvone	1267	0.1	$C_{10}H_{14}O$	150.1
21	20.8	Acetic acid, 1,7,7-trimethyl-bicyclo[2.2.1]hept-2-yl ester	1298	1.6	$C_{12}H_{20}O_2$	196.1
22	23.2	Caryophyllene	1449	3.8	$C_{15}H_{24}$	204.2
23	23.9	Humulene	1486	0.3	$C_{15}H_{24}$	204.2

[a]Retention time. Compounds listed in order of elution; [b]Chemical constituents measured on the non-polar capillary column (HP-5MS, 60 m fused silica) and GC-MS detection; [c] RI (retention indices) measured relative to *n*-alkanes (C9–C18) on the non-polar column (HP-5 column) and GC-MS detection.
* endo-Borneol is (−)-Borneol

environmental and local climatic conditions, the time of harvest, the stage of plant development, or the method of extraction [2f,2g].

The chemical composition of EOs is crucial for their bioactivities [3a]. Ojeda-Sana *et al*. [3b] tested individual major components of rosemary EO against Gram-positive and Gram-negative bacteria. They noticed that 1,8-cineole (eucalyptol) and α-pinene were active against Gram-negative bacteria; camphor and borneol inhibited only Gram-positive bacteria; while myrcene had no inhibitory activity. Klančik *et al*. [3c] investigated pure rosmarinic acid against Gram-positive bacteria and found it much less effective than crude EO. Our examined rosemary EO exhibited a higher and selective antibacterial activity against the Gram-positive bacteria (Table 2), with the MIC and MBC values ranging from 6.2 to 50 mg/mL. The strongest antibacterial activity of the EO was obtained against *S. aureus* II, with MIC/MBC values of 6.2 mg/mL, while its resistance to common antibiotics was higher than that of *S. aureus* I.

In another study, Hammer *et al.* [3a] determined that rosemary EO possesses a considerable antimicrobial activity against *E. coli, P. aeruginosa,* and *S. aureus*, while our EO showed significant inhibition of growth of *S. aureus* and only a modest activity against *E. coli*. In our research, we did not detect any antimicrobial effect of rosemary EO on the *P. aeruginosa* isolate, whereas MIC/MBC activity was recorded against *P. mirabilis* (25 mg/mL). This difference compared with the latter study may be due to different origins of the isolates. Hammer *et al.* [3a] used attested isolates, while we used clinical isolates. According to some other studies, EOs exhibit a stronger activity to Gram-positive bacteria [2a,2b].

In our study the analyzed rosemary EO reached MIC/MBC values for the *S. aureus* I isolate of 25 mg/mL, while in other studies rosemary EOs achieved MIC/MBC against attested *S. aureus* at 0.3 mg/mL [2c], indicating that the clinical isolates used in our study had strong antimicrobial resistance. Furthermore, the fact that the isolated clinical strains of *E. coli* I and *E. coli* II were resistant to the commercial antibiotics used in our study but sensitive to the EO, which showed MIC and MBC/MFC values of 12.5 and 25 mg/mL, respectively, is of particular significance. This may be due to synergistic effects of the mixture of various chemical compounds, or it may be supposed that the effectiveness of EOs depends not only on their chemical composition but also on other conditions, such as pH of broth medium and strain-specific characteristics of the microorganisms [3d].

Table 2: MIC and MBC (MFC) values of the EO and common antibiotics and antimycotics (mg/mL)

	Essential oil*		Antibiotics*							
Bacterial strain	*R. officinalis*		STR		AMP		TET		CXM	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
E. coli I	25	25	nd**	nd	nd	nd	nd	nd	nd	nd
E. coli II	12.5	12.5	nd	nd	nd	nd	nd	nd	0.5	nd
S. aureus I	25	25	0.1	0.1	0.1	0.1	0.1	0.5	0.3	nd
S. aureus II	6.2	6.2	0.1	0.5	nd	nd	0.1	0.5	nd	nd
P. mirabilis	25	25	0.1	0.5	0.1	0.3	0.1	0.5	0.3	nd
P. aeruginosa	50	50	nd	nd	nd	nd	nd	nd	nd	nd
Fungal strain	*R. officinalis*						Nystatin			
	MIC		MFC		MIC		MFC			
C. albicans I	5		5		0.1		0.1			
C. albicans II	1.2		1.2		0.1		0.1			

*values are expressed in mg/mL; **nd- not detected, Tested antibiotics: STR = Streptomycin; AMP = Ampicillin; TET = Tetracycline; CXM = Cefuroxime

Our rosemary EO also showed an antifungal activity against both *Candida* isolates (1.2 – 5 mg/mL). The lowest MIC/MFC values were at 1.2 mg/mL for *C. albicans* II (Table 2). The EO antifungal activity was weaker than that of the tested antifungal drugs, which is similar to recent data showing that rosemary EO expressed antimicrobial activity on *Candida* species at 1 mg/mL [2e]. Likewise, strain-specific effects were observed for the *Candida* isolates, similarly to the research of Pintore *et al*. [2h].

LC_{50} values in the toxicity assay after 24 and 48 hours were 19.4 mg/mL and 5.4 mg/mL, respectively. Prolonged exposure to the EO (from 24 to 48 hours) resulted in increased brine shrimp mortality. There was a high linear correlation between the EO concentration and mortality after 24 (r^2 = 0.9) and 48 hours (r^2 = 0.8). These results indicate that increased EO concentrations (3.1 – 50 mg/mL) lead to an increase in EO toxicity (from 43% to 95% after 24-hour incubation and from 65% to 97% after 48-hour incubation). The brine shrimp bioassay is a quick and cheap test for establishing acute toxicity of EO. Parra *et al.* [3e] found a high correlation between *in vivo* and *in vitro* tests (r = 0.8, $p < 0.05$). Considering that EO toxicity increased with higher concentrations and a longer exposure time, it is necessary to be careful with EO dosage and application.

Our results are in agreement with the results of a previous study reporting eucalyptol and camphor to be the most prevalent constituents [3f]. However, the fact that they were the most prevalent does not necessarily mean that these compounds were responsible for the recorded antimicrobial activity. Actually, some studies have shown that carnosol, carnosic acid, and rosmarinic acid possess major antibacterial [3g], antioxidant and anti-inflammatory properties [3h 3i]. In the study of Jordan *et al*. [3j] carnosic acid was the major component credited for bacteriostatic effects. Hence, any study of EOs should take into account that each EO is specific and its composition depends on the plant it was derived from and on the type of extraction procedure applied [3a,3l].

Furthermore, the antimicrobial effect of EOs cannot be explained by a single mechanism of action or with a single active compound. It is considered that all the antiviral, antifungal and antimicrobial effects of EOs are due to synergistic effects of their numerous bioactive constituents [3d].

Promising results were seen in studies comparing the standard antimicrobial drug ciprofloxacin and rosemary oil [3e]. Moreover, some studies evaluating the synergistic effect of antibiotics and rosemary EO found that the combination of the EO with aminoglycoside antibiotics could achieve a better effect against multidrug-resistant *S. aureus* and *E. coli* [3k,3l] than antibiotics alone. Further research which would clarify the underlying mechanism of the synergy is therefore recommended.

Our results showed a significant *in vitro* antimicrobial activity of the *R. officinalis* EO on clinically isolated vaginal bacterial and *Candida* strains. We may conclude that the EO represents a potential alternative therapy for vaginal infections, when used in appropriate doses. Future research should include a wider range of bacteria and *Candida* strains, and explore the synergistic effects with common antimicrobial drugs, as well as cytotoxic activity of rosemary EO, preferably in *in vivo* studies.

Experimental

Essential oils and GC/MS analysis: The analyzed commercial EO of *R. officinalis* was purchased from MeiLab (Belgrade, Serbia, serial number ER010514) and obtained by steam distillation of the leaves and buds and stored in a refrigerator at +4°C prior to the analysis. The chemical composition was analyzed using a gas chromatography-mass spectrometry (GC/MS) after preparation by using dilution of oil with dichloromethane (1:10, w/v) and the trials were performed on an Agilent capillary gas chromatograph coupled with ta mass spectrometer (MSD) (model GC Agilent 7890A; MS 5975C). A non-polar capillary column (HP-5MS, fused silica) was used with these parameters: oven temperature was changed during a 30-min run time in the following procedure: from 50°C (for 1 min) to 100°C at 5°C/min and to 200°C at 9°C/min with the final temperature kept for 7.89 min; temperature of the injector was 250°C, with helium as carrier gas (constant flow mode, flow rate was 0.8 mL/min), split injection technique (50 : 1); ionization energy 70 eV, in the EI ionization mode; ion source temperature was 230°C; scan mass range of *m/z* 30–550 and interface line temperature 280°C. Identification of the EO components was made from their retention indexes (RI) and by comparing their mass spectra, using the Wiley Registry of Mass Spectral Data, 7th edition, NIST8 Mass Spectral Library. The other components were identified by comparison of their spectral data and RI with the literature data (our own laboratory database and mass spectra generated by the instrument software - NIST mass spectrometry and MSD ChemStation E.02.00.493), after which all identified components were confirmed by the AMDIS software program (version 2.66). The proportion of the components was obtained from the electronic integration of the MSD signals of specific ion representation.

Essential oil and GC/FID analysis: Samples of the same EO were analyzed by gas chromatography (GC) with the use of a flame ionization detector (model GC Hewlett Packard 6890; FID). Retention indices were determined with a mixture of *n*-alkanes (C9 - C18) under the same experimental conditions. The preparation of the EO for the GC/FID analysis involved dilution of dichloromethane (1:10, w/v), and the trials were performed on a Hewlett Packard capillary gas chromatograph coupled with FID non-polar capillary column (HP-5MS 60 m, 320 m ID, 0.2 µm film thickness, fused silica) used with these parameters: oven temperature was changed during the 35-min run time in the following procedure: from 50°C (for 1 min) to 100°C at 5°C/min and to 200°C at 9°C/min with the final temperature kept for 12.9 min; temperature of the injector was 250°C, with helium as the carrier gas (constant pressure mode; pressure was 13.8 psi), split injection technique (10 : 1); detector temperature 300°C, hydrogen flow 40.0 mL/min, air flow 450.0 mL/min, constant makeup flow 45.0 mL/min. Validation (and relative composition in %) of identified components was performed by GC with FID (GC/FID) and compared with experimentally acquired retention indices.

Bacterial and Candida strains: Six bacterial isolates: *Escherichia coli* I*, E. coli* II*, Staphylococcus aureus* I*, S. aureus* II*, Pseudomonas aeruginosa, Proteus mirabilis* and two *Candida* isolates, *C. albicans* I and *C. albicans* II, were obtained from the Faculty of Medicine, Clinical Centre of Vojvodina, Department of Obstetrics and Gynecology and derived from pregnant women at their regular appointments. The institutional Ethical Board of the same institution approved the study protocol.

Antimicrobial assay: The antibacterial activity, minimum inhibitory concentrations (MICs) and minimum bactericidal/fungicidal concentrations (MBCs/MFCs) of the rosemary EO were evaluated by a double-microdilution method according to the standard CLSI procedure [3m]. The tested strains were obtained from overnight cultures, grown at 37°C on nutrient agar (Müller-Hinton, MHA, Torlak, Belgrade, Serbia) for bacteria, and on malt agar (MA, Torlak, Belgrade, Serbia) for yeasts. McFarland inocula of bacteria and yeasts were prepared in sterile saline solution; reaching the final number of approximately 1.5×10^6 CFU/mL for bacteria and 1.5 x 10^5 for yeasts. Müeller Hinton broth (MHB, Torlak, Beograd, Serbia) and Sabouraud broth (SB, Torlak, Belgrade, Serbia) were used for the antimicrobial screening. A double-dilution test was performed in either MHB or SB with 1% dimethyl sulfoxide (DMSO) in a 96-well microtitre plate (Spektar, Čačak, Serbia). The final concentrations of the EO ranged from 1.2-50 mg/mL. After 24-h incubation at 37°C for the bacterial strains and 48 h for yeasts, MICs values were determined visually. MBCs/MFCs were confirmed after inoculation of MHA and MA plates with 100 µL of broth where turbidity was absent (MIC point). Nystatin (Hemofarm, Vršac, Serbia) and commercial fluconazole solution (Hemofarm, Vršac, Serbia) (2 mg/mL) were used as positive controls for yeasts (in final concentrations 0.03, 0.06, 0.13 mg/mL), while antibiotics: streptomycin, ampicillin, tetracycline, and cefuroxime (Himedia, Mumbai, India) were positive controls for bacteria (0.5 mg/mL). As a negative control, 1% DMSO was used. The test was performed in 3 replicates and the average value was taken as the final result.

Brine shrimp toxicity assay: Toxicity of the investigated rosemary EO was made by *Artemia salina* bioassay according to Meyer *et al.* [3n]. In each microwell of the plate, 10 larvae were added. The wells already contained 25 µL of specific EO dilution in 1% (DMSO) and 225 µL of artificial sea water (ASW). LC_{50} values of EO were determined by serial double dilutions in 6 repetitions (3.1 – 50 mg/mL). A mixture of 25 µL 1% DMSO (oil emulsifier) and 225 µL ASW with larvae was used as a control. For toxin control, microcystin standard MCYST-LR (SigmaAldrich, Germany) was used. Microplates were incubated under a lamp, at 30°C, without feeding the shrimps. Acute toxicity was calculated after 24 and 48 h, by counting dead nauplii. Surviving shrimps were counted under a stereomicroscope (Zeiss, Germany) and afterwards, 50 µL of methanol was added to each well. All the tests were repeated twice and performed in triplicate. Abbott's equation was applied to calculate the mortality rate: *Mortality (%) = ((n test – n control)/n control) x 100*, where n control and n test were the numbers of dead larvae in the first test and control tests. Toxicity was expressed as LC_{50} (mg/mL). LC_{50} represented the lethal concentration required to kill 50% of the population of test brine shrimps, *Artemia salina.*

Statistical analyses: Statistical analyses of the data were performed by analysis of variance (ANOVA), using Microsoft Excel XP. Duncan's multiple range test was applied for the determination of significant differences between results ($p < 0.05$) in the antimicrobial testing using Analytical Software Statistica 8.1.

Acknowledgement - This study was funded by the Ministry of Education, Science and Technological Development of the Republic of Serbia (Project No. 172058).

References

[1] (a) Moreno S, Scheyer T, Romano CS, Vojnov AA. (**2006**) Antioxidant and antimicrobial activities of rosemary extracts linked to their polyphenol composition. *Free Radical Research*, ***40***, 223-231; (b) Burt SA. (**2004**) EOs: their antibacterial properties and potential applications in foods—a review. *International Journal of Food Microbiology*, ***94***, 223-253; (c) Sagorchev P, Lukanov J, Beer AM. (**2010**) Investigations into the specific effects of rosemary oil at the receptor level. *Phytomedicine*, ***17***, 693−697; (d) Tsai CW, Lin CY, Wang YJ. (**2010**) Carnosic acid induces the NAD(P)H: quinone oxidoreductase expression in rat clone cells through the p38/nuclear factor erythroid-2 related factor pathway. *Journal of Nutrition*, ***141***, 2119−2125; (e) Nyirjesy P. (**2014**) Management of persistent vaginitis. *Obstetrics &Gynecology*, ***124***, 1135-1146; (f) Lambert JA, John S, Sobel JD, Akins RA. (**2013**) Longitudinal analysis of vaginal microbiome dynamics in women with recurrent bacterial vaginosis: Recognition of the conversion process. *PLOS ONE*, ***8***, 1-10; (g) Ravel J, Brotman RM, Gajer P, Ma B, Nandy M, Fadrosh DW, Sakamoto J, Koenig SS, Fu L, Zhou X, Hickey RJ, Schwebkeand JR, Forney LJ. (**2013**) Daily temporal dynamics of vaginal microbiota before, during and after episodes of bacterial vaginosis. *Microbiome*, ***1***, 1-29; (h) Martin DH. (**2012**) The microbiota of the vagina and its influence on women's health and disease. *The American Journal of the Medical Sciences*, ***343***, 2-9; (i) Martin DH, Zozaya M, Lillis R, Miller J, Ferris MJ. (**2012**) The microbiota of the human genitourinary tract: trying to see the forest through the trees. *Transactions of the American Clinical and Climatological Association*, ***123***, 242-256; (j) Nelson DB, Hanlon A, Nachamkin I, Haggerty CL, Mastrogiannis DS, Liu C, Fredricks DN. (**2014**) Early pregnancy changes in bacterial vaginosis-associated bacteria and preterm delivery. *Paediatric and Perinatal Epidemiology*, ***28***, 88-96; (k) Zhang AL, Xue CC, Fong H. (**2011**) Integration of herbal medicine into evidence-based clinical practice. In *Herbal Medicine: Biomolecular and Clinical Aspects*. Ch ***22***. Benzie IFF, Wachtel-Galor S. (Eds). CRC Press, Boca Raton USA. 1-16.

[2] (a) Hussain AI, Anwar F, Nigam PS, Sarker SD, Moore JE, Rao JR, Mazumdar A. (**2011**) Antibacterial activity of some Lamiaceae EOs using resazurin as an indicator of cell growth. *Food Science and Technology International*, ***44***, 1199-1206; (b) Teixeira B, Marques A, Ramos C, Neng N, Nogueira J, Saraivab JA, Nunes LM. (**2013**) Chemical composition and antibacterial and antioxidant properties of commercial EOs. *Industrial Crops and Products*, ***43***, 587-595; (c) Jiang Y, Wu N, Fu YJ, Wang W, Luo M, Zhao CJ, Zu YG, Liu XL. (**2011**) Chemical composition and antimicrobial activity of the EO of rosemary. *Environmental Toxicology and Pharmacology*, ***32***, 63-68; (d) Hussain A, Anwar F, Sherazi STH, Przybylski R. (**2008**) Chemical composition, antioxidant and antimicrobial activities of basil (*Ocimum basilicum*) EOs depends on seasonal variations. *Food Chemistry*, ***108***, 986-995; (e) Bozin B, Mimica-Dukic N, Samojlik I, Jovin E. (**2007**) Antimicrobial and antioxidant properties of rosemary and sage (*Rosmarinus officinalis* L. and *Salvia officinalis* L., Lamiaceae) EOs. *Journal of Agricultural and Food Chemistry*, ***55***, 7879–7885; (f) Janssen AM, Scheffer JJC, Baerheim-Svendsen A. (**1987**) Antimicrobial activity of EOs: a 1976-86 literature review. *Planta Medica*, ***53***, 395–398; (g) Ashour M, El-Readi M, Youns M, Mulyaningsih S, Sporere F, Efferth T, Wink M. (**2009**) Chemical composition and biological activity of the EO obtained from *Bupleurum marginatum* (Apiaceae). *Journal of Pharmacy and Pharmacology*, ***61***, 1079-1087; (h) Pintore G, Marchetti M, Chessa M, Sechi B, Scanu N, Mangano G, Tirillini B. (**2009**) *Rosmarinus officinalis* L.: Chemical modifications of the essential oil and evaluation of antioxidant and antimicrobial activity. *Natural Product Communications*, ***4***, 1685-1690.

[3] (a) Hammer KA, Carson CF, Riley TV. (**1999**) Antimicrobial activity of EOs and other plant extracts. *Journal of Applied Microbiology*, ***86***, 985-990; (b) Ojeda-Sana A, van Baren C, Elechosa M, Juárez M, Moreno S. (**2013**) New insights into antibacterial and antioxidant activities of rosemary EOs and their main components. *Food Control*, ***3***, 189-195; (c) Klančik A, Piskernik S, Jeršek B, Možina SS. (**2010**) Evaluation of diffusion and dilution methods to determine the antibacterial activity of plant extracts. *Journal of Microbiological Methods*, ***81***, 121-126; (d) Negi S. (**2012**) Plant extracts for the control of bacterial growth: Efficacy, stability and safety issues for food application. *International Journal of Food Properties*, ***156***, 7-17; (e) Lagarto Parra A, Silva Yhebra R, Guerra Sardiñas I, Iglesias Buela L. (**2001**) Comparative study of the assay of *Artemia salina* L. and the estimate of the medium lethal dose (LD_{50} value) in mice, to determine oral acute toxicity of plant extracts. *Phytomedicine*, ***8***, 395-400; (f) Lakušić D, Ristić M, Slavkovska V, Lakušić B. (**2013**) Seasonal variations in the composition of the essential oils of rosemary (*Rosmarinus officinalis*, Lamiaceae). *Natural Product Communications*, ***8***, 131-134; (g) Jordan MJ, Lax V, Rota MC, Loranand S, Sotomayor JA. (**2013**) Effect of bioclimatic area on the EO composition and antibacterial activity of *Rosmarinus officinalis* L. *Food Control*, ***30***, 463-468; (h) Garbarino JA, Troncoso N, Delpiano P, Carvajal L, Russo A. (**2006**) Antioxidant activity analysis for the selection of *Rosmarinus officinalis* L. *Natural Product Communications*, ***1***, 1123-1128; (i) Russo A, Lombardo L, Troncoso N, Garbarino J, Cardile V. (**2009**) *Rosmarinus officinalis* extract inhibits human melanoma cell growth. *Natural Product Communications*, ***4***, 1707-1710; (j) Jordán MJ, Lax V, Rota MC, Lorán S, Sotomayor JA. (**2013**) Influence of the bioclimatic area on the polyphenolic composition, and antioxidant and bacteriostatic activities of *Rosmarinus officinalis*. *Natural Product Communications*, ***8***, 817-822; (k) Barreto HM, Filho ECS, Lima E, Coutinho HDM, Morais-Braga MFB, Tavares CCA, Tintino SR, Rego JV, De Abreu APL, Lustosa MDCG, Oliveira RWG, Citó AMGL, Lopes JAD. (**2014**) Chemical composition and possible use as adjuvant of the antibiotic therapy of the EO of *Rosmarinus officinalis* L. *Industrial Crops and Products*, ***59***, 290-294; (l) Van Vuuren SF, Suliman S, Viljoen AM. (**2009**) The antimicrobial activity of four commercial EOs in combination with conventional antimicrobials. *Letters in Applied Microbiology*, ***48***, 440-446; (m) Clinical Laboratory Standards Institute. (**2008**) M02-A11 - Performance standards for antimicrobial disk susceptibility tests, Approved Standard – 11th Edition; M07-A9 - Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, Approved Standard – 9th Edition; M27-A3 and Supplement S3 Reference method for broth dilution antifungal susceptibility testing of yeasts, Approved standard – 3rd Edition. Wayne, PA, USA: Clinical Laboratory Standards Institute. 1-214; (n) Meyer BN, Ferrigni NR, Putnam JE, Jacobsen LB, Nichols DE, Mc Laughlin JL. (**1982**) Brine shrimp: a convenient general bioassay for active plant constituents. *Planta Medica*, ***45***, 31-34.

NPC Natural Product Communications

2017
Vol. 12
No. 1
131 - 134

Chemical Profile and *in vitro* Biological Activities of Essential Oils of *Nectandra puberula* and *N. cuspidata* from the Amazon

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Received: July 5th, 2016; Accepted: September 8th, 2016

Essential oils (EO) from leaves and branches of *Nectandra puberula* Schott (Nees) and from leaves of *N. cuspidata* Nees & Mart. were obtained by hydrodistillation and their chemical compositions determined by gas chromatography - mass spectrometry (GC-MS). The main compounds identified from *N. puberula* EO were apiole (22.2%), β-caryophyllene (15.1%), β-pinene (13.3%), germacrene D (8.3%), pogostol (6.6%) and bicyclogermacrene (6.4%) in the leaves; and apiole (28.1%), pogostol (19.8%) and guaiol (11.2%) in the branches. The EO of *N. cuspidata* leaves showed β-caryophyllene (26.9%), bicyclogermacrene (16.0%) and spathulenol (5.2%) as the main compounds. The EOs were subjected to antibacterial screening and displayed promising activity against *Escherichia coli* (MIC = 19.5 μg.mL^{-1}). In addition, the EOs were tested for cytotoxic activity against MCF-7 breast tumor cells and the IC_{50} values were 64.5 ± 1.6 and 117.1 ± 11.9 μg.mL^{-1} for the leaf EOs of *N. puberula* and *N. cuspidata*, respectively.

Keywords: Lauraceae, Sesquiterpenes, Phenylpropanoids, Breast cancer, *Escherichia coli*.

The Lauraceae is a pantropic family, containing about 50 genera and probably 2500-3500 species, including trees and shrubs. Southeastern Asia and tropical America are the main centers for diversity of the Lauraceae. These species frequently occur in lowland rain forests to tropical montane forests with an altitude up to ca. 4000 m [1, 2]. Many species are highlighted due to their economic importance in cooking, in carpentry and construction, papermaking, in the perfume industry, the chemical industry, and folk medicine [3]. The genus *Nectandra* belongs to the *Ocotea* complex and comprises about 114 species. It is endemic to the Neotropics with restricted distribution in tropical and subtropical Americas, with an estimated 43 species occurring in Brazil [4, 5, 6]. This genus has reported folk medicinal uses as an antifungal, anti-inflammatory, antimalarial, anticancer, analgesic, and febrifuge, as well as being used for the treatment of ulcers, among others [5].

N. puberula Schott (Nees) is commonly known in the Amazon region as "louro amarelo" and "yellow cinnamon" and is presently restricted in distribution to the north, southeast, and southern regions of Brazil [7]. It is a tree up to 20 m in height. Neolignans have been found in the extract of the trunk wood [8]. *N. cuspidata* is a shrub or tree, 5–30 m in height. The plant is not endemic to Brazil, but occurs in most states [2, 7]. A phytochemical investigation of the leaf extract resulted in the isolation and characterization of megastigmane, aporphinoid alkaloids, sesquiterpenes, polyprenols and sterols [9].

Despite the high aromatic potential, there have been few studies on the chemical composition of essential oils (EOs) of *Nectandra* species. To our knowledge, there are no previous reports on either the phytochemistry or the biological activities of the EOs of these two *Nectandra* species.

Fifty-nine volatile components were identified in the EOs from *Nectandra* species, comprising approximately 97.7% of the total composition of the oils (Table 1). The most representative class of compounds in the leaf EOs of both species was oxygenated terpenoids; about 42.3% in *N. puberula* and 76.2% in *N. cuspidata*. Comparison of the chemical profiles of the samples is shown in Figure 1. *N. puberula* oils showed a significant difference in terpene accumulation between leaves and branches. The branch oil displayed a higher concentration of oxygenated sesquiterpenoids (44.7%). The main compounds identified in the EO from *N. puberula* were apiole (22.2%), β-caryophyllene (15.1%), β-pinene (13.3%), germacrene D (8.3%), pogostol (6.6%) and bicyclogermacrene (6.4%) in the leaves, and apiole (28.1%), pogostol (19.8%) and guaiol (11.2%) in the branches. The EO of *N. cuspidata* leaves showed β-caryophyllene (26.9%), bicyclogermacrene (16.0%) and spathulenol (5.2%) as the main compounds.

The occurrence of sesquiterpene hydrocarbons and oxygenated sesquiterpenoids in *Nectandra* species has been reported. The main compounds of the EO from *N. megapotamica* leaves were α-bisabolol (62.3–69.4%) and δ-elemene (8.2–22.6%) [10]. The leaf oil of *N. salicina* growing wild in Costa Rica was dominated by atractylone (14.6%), viridiflorene (10.1%), α-pinene (9.4%), β-caryophyllene (7.2%), α-humulene (7.0%), δ-cadinene (6.1%), β-pinene (6.0%) and germacrene D (5.8%) [11]. Similarly, the leaf oil of *N. membranacea* from Costa Rica was rich in δ-cadinene (14.1%), α-copaene (12.5%), germacrene D (6.9%), α-humulene (5.6%), β-caryophyllene (5.5%), and bicyclogermacrene (4.8%), as well as the monoterpenes α-pinene (22.4%) and β-pinene (12.6%) [12]. The EO of *N. coriacea* showed valerianol (16.7%) and γ-eudesmol (12.3%) [13]. In addition, the occurrence of monoterpenoids in *Nectandra* oils was reported such as mentha-

1(7),8-diene, α-terpinolene, α-pinene, β-pinene and α-terpineol in *N. falcifolia* and *N. elaiophora* [14, 15]. However, the occurrence of phenylpropanoid derivatives such as dillapiole has not been previously reported in volatile oils from *Nectandra* species.

The antiproliferative effect of metabolites isolated from *Nectandra* species against tumor cell lines have been reported [16]. The neolignans from *N. megapotamica* displayed cytotoxic activity and induced apoptosis in leukemia cells (HL-60) [17]. The crude extract of *N. rigida* was rich in dehydrodiisoeugenol, which is reported to be a cytotoxic agent [18]. In this study, the EOs showed good activity against the MCF-7 breast tumor cell line. The IC_{50} values were 64.5 ± 1.6 and 117.1 ± 11.9 μg.mL^{-1} for EOs from leaves of *N. puberula* and *N. cuspidata*, respectively.

The most activity was observed for *N. puberula* oil, which is characterized by high contents of apiole (22.2%), a phenylpropanoid, followed by β-caryophyllene (15.1%) and β-pinene (13.3%). The cytotoxic effects against MCF-7 cell lines have been observed for phenylpropanoids such as anethole, cinnamaldehyde and eugenol [19]. Apiole was administered to mice at 1-30 mg/kg body weight through intraperitoneal injection and displayed a promising antitumor effect against colon tumors (COLO 205) in an *in vivo* xenograft model [20]. The IC_{50} values for α-pinene and β-caryophyllene have been found to be 20.6 and 19.7 μg.mL^{-1}, respectively, against MCF-7 cells [21]. In addition, β-caryophyllene potentiated the anticancer activity of paclitaxel on MCF-7 at a concentration of 10 μg.mL^{-1} [22]. The main compounds in *N. cuspidata* oil were β-caryophyllene (26.9%), bicyclogermacrene (16.0%) and spathulenol (5.2%). The EO from *N. leucantha*, rich in bicyclogermacrene (28.4%) and spathulenol (5.8%), displayed significant cytotoxic activity against murine melanoma (B16F10-Nex2), human glioblastoma (U-87) and human cervical carcinoma (HeLa) cell lines. However, the IC_{50} values against MCF-7 were 193.8 μg.mL^{-1} and 19.0 μg.mL^{-1} for the EO and bicyclogermacrene, respectively [23].

The antimicrobial property is of great importance in the applications of EO against certain human or animal pathogens [24]. In bacteria, the permeabilization of the membranes is associated with loss of ions and reduction of membrane potential, collapse of the proton pump and depletion of the ATP pool [25]. *Nectandra* leaf oils showed notable antibacterial activity against *E. coli* (MIC = 19.5 μg.mL^{-1}) (Table 2).

The antibacterial activities of the main compounds, apiole, β-caryophyllene, β-pinene, germacrene D, bicyclogermacrene and spathulenol have been reported previously [26]. The EO from *Psammogeton canescens*, rich in α-pinene (20.0%) and apiole (15.3%), exhibited strong antibacterial activity against *Escherichia coli* (MIC, 14.0 ± 0.8 μg.mL^{-1}) [27]. *Eugenia uniflora* oil, rich in apiole (11.1%) and oxygenated sesquiterpenes, was active towards two Gram-positive bacteria, *Streptococcus equi* and *Staphylococcus epidermis* [28]. The oil of the trunk bark of *Onychopetalum amazonicum*, rich in β-caryophyllene and spathulenol, exhibited good activity against *Staphylococcus epidermidis* (ATCC 12228), *Escherichia coli* (ATCC 10538) and *Kocuria rhizophila* (ATCC 9341), with MIC values of 62.5 μg.mL^{-1} [29]. In addition, β-caryophyllene exhibited strong antibacterial effect against *E. coli* (MTCC 732), with a MIC value of 9.0 ± 2.2 μM [30].

In summary, our results indicate that *Nectrandra* oils could be used as natural antimicrobial agents against *E. coli* and tested in future studies for the treatment of breast cancer.

Table 1: Chemical composition of EOs from *Nectandra* species from the Amazon.

Constituents	$RI^{calc.}$	$RI^{Lit.}$	*N. puberula* Leaves	*N. puberula* Branches	*N. cuspidata* Leaves
α-Pinene	934	932	5.0		
β-Pinene	978	974	**13.3**	1.4	
Myrcene	992	988	0.8		
Limonene	1028	1024	2.7	3.0	
Linalool	1102	1095		0.8	
δ-Elemene	1336	1335			4.4
α-Copaene	1374	1374			4.6
β-Bourbonene	1386	1387	0.5		
β-Elemene	1391	1389	2.0	2.1	0.8
β-Caryophyllene	1421	1417	**15.1**	2.8	**26.9**
β-Copaene	1427	1430			0.5
β-Gurjunene	1432	1431			0.4
α-*trans*-Bergamotene	1435	1432			3.1
γ-Elemene	1435	1434		0.9	
α-Guaiene	1440	1437	1.3	0.8	
Aromadendrene	1443	1439			0.4
α-Humulene	1452	1452	1.8	1.2	3.2
(*E*)-β-Farnesene	1458	1454			1.5
γ-Muurolene	1476	1478			0.5
Amorpha-4,7(11)-diene	1480	1479			4.5
Germacrene D	1483	1484	**8.3**	4.5	
γ-Himachalene	1484	1481			2.5
cis-Eudesma-6,11-diene	1488	1489	2.3	1.7	
unidentified	1490		1.1		
epi-Cubebol	1496	1493		1.5	
Bicyclogermacrene	1498	1500	**6.4**		**16.0**
4-*epi*-*cis*-Dihydroagarofuran	1501	1499		1.0	
α-Bulnesene	1508	1509	2.3	1.7	
β-Bisabolene	1509	1505			1.4
γ-Cadinene	1512	1513		0.8	1.2
7-*epi*-α-Selinene	1516	1520			0.3
δ-Cadinene	1523	1522	1.1	3.0	3.5
trans-Cadina-1,4-diene	1531	1533			0.5
Elemol	1551	1548		0.9	
Germacrene B	1558	1559	1.3		
Muurol-5-en-4-α-ol	1558	1559		1.2	
Maaliol	1564	1566			0.6
Caryophyllenyl alcohol	1567	1570			0.3
Spathulenol	1578	1577	1.7		**5.2**
Unidentified	1583				4.7
Caryophyllene oxide	1584	1582	2.1	1.3	
Viridiflorol	1590	1592			1.6
Guaiol	1597	1600		**11.2**	0.8
Rosifoliol	1600	1600			0.9
6-Methoxy-elemicin	1601	1595	1.7		
Rosifoliol	1610	1600		0.7	
unidentified	1621				0.6
1-*epi*-Cubenol	1626	1627			0.7
Muurola-4,10(14)-dien-1-β-ol	1630	1630		1.1	
Camphoric acid	1633	1634			0.6
allo-Aromadendrene epoxide	1637	1639			1.6
epi-α-Muurolol (=τ-Muurolol)	1640	1640		4.4	1.5
α-Muurolol (=Torreyol)	1644	1644		1.3	0.3
α-Eudesmol	1652	1652		1.3	
α-Cadinol	1653	1652			1.7
Selin-11-en-4-α-ol	1655	1658			0.4
Pogostol	1656	1651	**6.6**	**19.8**	
Unidentified	1660		0.7		
Bulnesol	1669	1670		1.4	
epi-β-Bisabolol	1670	1670			1.4
α-Bisabolol	1682	1685			1.0
Apiole	1687	1677	**22.2**	**28.1**	
Monoterpene hydrocarbons			21.7	4.4	
Oxygenated monoterpenoids				0.8	
Sesquiterpene hydrocarbons			42.3	19.5	76.2
Oxygenated sesquiterpenoids			10.3	44.7	18.0
Phenylpropanoids			23.9	28.1	
Others				0.6	
Total identified			**98.3**	**100.0**	**94.8**

$RI^{Calc.}$ = based on DB-5ms capillary column and alkane standards (C8-C32).
$RI^{Lit.}$ = based on Adams [31].

Table 2: Antimicrobial activity of *Nectandra* essential oils from the Amazon.

	Minimum inhibitory concentration (μg·mL^{-1})		
	Gentamicin (positive control)	*N. puberula*	*N. cuspidata*
P. aeruginosa	<19.5	1250	1250
E. coli	<19.5	19.5	19.5
S. epidermidis	<19.5	1250	1250
S. aureus	<19.5	625	625
B.cereus	<19.5	625	312.5

Experimental

Plant material: *Nectandra puberula* Nees was collected in Santarém (S 02° 25.0′ 10.2″ W 54° 44′ 26.9″) and a voucher (HSTM000092) was deposited in the herbarium of Universidade Federal do Oeste do Pará, Santarém, Pará State, Brazil. *N. cuspidata* was collected in Caxiuanã National Forest, Marajó Island (S 01º 44′ 18.8″ W 51º 27′ 27.4″), and a voucher (MG 104948) was deposited in the Herbarium of Museum Paraense Emílio Goeldi, Belém, Pará state, Brazil.

Leaves and branches from several mature plants were air-dried, pulverized, and subjected to hydrodistillation using a Clevenger-type apparatus (100 g, 3 h). The essential oils were dried over anhydrous sodium sulfate, and their percentage contents were calculated on the basis of the dry weight of plant material. The moisture contents of the samples were calculated after phase separation using a Dean–Stark trap (5 g, 60 min) using toluene as the solvent phase.

Gas chromatographic – mass spectral analysis: The essential oils were analyzed by GC-MS using an Agilent 6890 GC with an Agilent 5973 mass selective detector (MSD) [operated in the EI mode (electron energy = 70 eV), scan range = 40-400 amu, and scan rate = 3.99 scans/sec], and an Agilent ChemStation data system. The GC column was an HP-5ms fused silica capillary with a (5% phenyl)-polymethylsiloxane stationary phase, film thickness of 0.25 µm, length of 30 m, and internal diameter of 0.25 mm. The carrier gas was helium with a column head pressure of 48.7 kPa and a flow rate of 1.0 mL/min. Injector temperature was 200ºC and detector temperature was 280ºC. The GC oven temperature program was used as follows: 40ºC initial temperature, held for 10 min; increased at 3ºC/min to 200ºC; increased at 2ºC/min to 220°C. A 0.2%, w/v, solution of the sample in CH_2Cl_2 was prepared and 1 µL was injected using a splitless injection technique. The percentages of each component are reported as raw percentages based on total ion current without standardization. Individual components were identified by comparison of both mass spectrum and GC retention data with authentic compounds present in commercial libraries [31].

Antibacterial assay: The essential oils were screened for antimicrobial activity against *Escherichia coli* (ATCC No. 10798), *Pseudomonas aeruginosa* (ATCC No. 27853), *Bacillus cereus* (ATCC No. 14579), *Staphylococcus aureus* (ATCC No. 29213), and *Staphylococcus epidermidis* (ATCC No. 12228), using the microbroth dilution technique, as previously reported [32]. Thus, 50 µL of 1%, w/v, solution of the samples in DMSO was placed in a well of 96 well plates and 50 µL of cation-adjusted Mueller Hinton broth (CAMHB) was added. The sample solutions were then serially diluted (1:1) by transferring 50 µL of sample-CAMHB mixture to the next lane and adding 50 µL fresh CAMHB to obtain concentration from 2500 $\mu g.mL^{-1}$ to 12.5 $\mu g.mL^{-1}$. The bacteria were harvested from a fresh culture and added to each well at a concentration of approximately 1.5×10^8 colony forming units (CFU)/mL. The plates were incubated at 37°C for 24 h and the final minimum inhibitory concentration (MIC) was determined as the lowest concentration with no turbidity. Gentamicin was used as positive antibiotic control and DMSO was used as negative control.

Cytotoxic assay: MCF-7 human breast adenocarcinoma cells (ATCC No. HTB-22) were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 30 mM HEPES, $NaHCO_3$, and penicillin streptomycin. *In vitro* cytotoxic activity of essential oils on MCF-7 cells was performed using the 96-well MTT assay, as previously reported [33]. Cells were plated into 96-well cell culture plates at a concentration of 1.2×10^4 cells/well and a volume of 100 µL in each well. The plate was then labeled and incubated at 37°C and 5% CO_2 for 48 h. By then, the cells had reached 70-80% confluent growth. The supernatant fluid was gently aspirated (without touching the bottom of the well to avoid removing cells) and replaced with 100 µL growth medium containing 1.0 µL and 0.5 µL of essential oil (1% in DMSO), giving a final concentration of 100 and 50 $\mu g.mL^{-1}$. The plate was then incubated at 37°C and 5% CO_2 for 48 h. Then, the liquid was gently aspirated from each well. In a tube, 10 mL feeding medium was mixed with 2 mL of MTT stock solution (and was protected from light). Into each well, 100 µL of the MTT solution was added and the pre-read absorbance was immediately measured spectrophotometrically at 570 nm (using a Molecular Devices SpectraMax Plus 384 microplate reader. Formazan crystals were formed over the course of 4 h at 37°C and 5% CO_2. After 4h, DMSO was used to dissolve the purple crystals. The amount of MTT-formazan produced was determined spectrophotometrically at 570 nm. Growing medium, DMSO, and tingenone (100 $\mu g.mL^{-1}$) served as negative, compound, and positive controls, respectively. Solutions were added to wells in 8 replicates. Average absorbances, standard deviations, and percent kill ratios (% $kill_{oil}$ / % $kill_{control}$) were calculated. Median inhibitory concentrations (IC_{50}) were determined using the Reed-Muench method [34].

Acknowledgments – The authors are grateful to CNPq - Programa "Ciências sem fronteiras" for financial support.

References

[1] Shi G-L, Xie Z-M, Li H-M. (**2014**). High diversity of Lauraceae from the Oligocene of Ningming, South China. *Palaeoworld*, ***23***, 336-356.
[2] Rohwer JG. Lauraceae. K Kubitzki, Rohwer JG., Bittrich V. (Eds.) (**1993**) *The families and genera of vascular plants II Flowering plants, dicotyledons, Magnoliid, Hamamelid and Caryophyllid families*. Springer-Verlag, Berlin, Heidelberg, New York, pp. 366-391.
[3] Marques CA. (**2001**) Importância econômica da família Lauraceae Lindl. Floresta e Meio Ambiente, ***8***,195-206.
[4] Chanderbali AS, Werff H, Renner SS. (**2001**) Phylogeny and historical biogeography of Lauraceae: evidence from the chloroplast and nuclear genomes. *Annals of the Missouri Botanical Garden*, ***88***,104-134.
[5] Macías-Villamizar VE, Cuca-Suárez LE, Coy-Barrera ED. (**2015**) Genus *Nectandra*: Phytochemistry and biological activity. *Boletín Latinoamericano y del Caribe de Plantas Medicinales y Aromáticas*, ***14***, 317-342.
[6] Baitello JB, Lorea-Hernández FG, Moraes PLR, Esteves R, Marcovino JR. (**2003**) Lauraceae. In *Flora Fanerogâmica do Estado de São Paulo*. Vol. 3. Wanderley MGL, Shepherd GJ, Giulietti AM, Melhem T. (Eds.). Fapesp-Rilla, São Paulo, 149-223
[7] Zanon MMF, Goldenberg R, De Moraes PLR. (**2009**) O gênero *Nectandra* Rol. ex Rottb. (Lauraceae) no Estado do Paraná, Brasil. *Acta Botanica Brasilica*, ***23***, 22-35.
[8] Moro JC, Fernandes JB, Vieira PC, Yoshida M, Gottlieb OR, Gottlieb HE. (**1986**) Neolignans from *Nectandra puberula*. *Phytochemistry*, ***26,*** 269-272.
[9] Batista AL, Yoshida NC, Garcez FR, Garcez WS. (**2015**) Chemical constituents from *Nectandra cuspidata* Nees with Lauraceae. *Biochemical Systematics and Ecology*, ***61,*** 229-231.
[10] Romoff P, Ferreira MJP, Padilla R, Toyama DO, Fávero OA, Lago JHG. (**2010**) Chemical composition of volatile oils from leaves of *Nectandra megapotamica* Spreng. (Lauraceae). *Química Nova*, ***33,*** 1119-1121.

[11] Cicció JF, Chaverri C, Díaz C. (**2009**) Volatile compounds of *Nectandra salicina* (Lauraceae) from Costa Rica and their cytotoxic activity on cell lines. *Química Nova*, ***32***, 417-420.
[12] Wu X, Vogler B, Haber WA, Setzer WN. (**2006**) A phytochemical investigation of *Nectandra membranacea* from Monteverde, Costa Rica. *Natural Products Communications*, ***1***, 465-468.
[13] Pino JA, Marbot R, Paya A, Chao D, Herrera D. (**2005**) Aromatic plants from western Cuba. III. Composition of the leaf oils of *Lippia strigulosa* (Mart. et Gal.) Mold. and *Nectandra coriacea* (Sw.) Griseb. *Journal of Essential Oil Research*, ***17,*** 648-650.
[14] Torres AM, Ricciardi GAL, Agrelo de Nassif AE, Ricciardi AIA, Dellacassa E. (**2005**) Examen del aceite esencial de *Nectandra angustifolia* (Schrad.) Nees & Mart. ex Nees. Comunicaciones Científicas y Tecnológicas. Universidad Nacional del Nordeste, Argentina, Resumen E-013, http://www.unne.edu.ar/unnevieja/Web/cyt/com2005/index.htm, accessed on April 2016.
[15] Naves YR. (**1951**) Etudes sur les matieres vegetales volatiles-CXVIII-Sur l'huile essentielle du Louro inhamuy (*Nectandra elaiphora* Barb Rodr) de l'Amazonie Bresilienne. *Bulletin de la Société Chimique de France*, ***18***, 987-991.
[16] Cuca LE, Coy ED, Alarcón MA, Fernández A, Aristizábal FA. (**2011**) Cytotoxic effect of some natural compounds isolated from Lauraceae plants and synthetic derivatives. *Biomedica*, ***31***, 335-343.
[17] Ponci V, Figueiredo CR, Massaoka MH, de Farias CF, Matsuo AL, Sartorelli P, Lago JH. (**2015**) Neolignans from *Nectandra megapotamica* (Lauraceae) display *in vitro* cytotoxic activity and induce apoptosis in leukemia cells. *Molecules*, ***20***, 12757-12768.
[18] Le Quesne PW, Larrahondo JE, Raffauf RF. (**1980**) Antitumor plants. X. Constituents of *Nectandra rigida*. *Journal of Natural Products*, ***43***, 353-359.
[19] (a) Nakagawa Y, Suzuki T. (**2003**) Cytotoxic and xenoestrogenic effects via biotransformation of *trans*-anethole on isolated rat hepatocytes and cultured MCF-7 human breast cancer cells. *Biochemical Pharmacology*, ***66***, 63-73; (b) Liao J-F, Huang S-Y, Jan Y-M, Yu L-L, Chen C-F. (**1998**) Central inhibitory effects of water extract of *Acori graminei* rhizoma in mice. *Journal of Ethnopharmacology*, ***61***, 185-193; (c) Jaganathan SK, Supriyanto E. (**2012**) Antiproliferative and molecular mechanism of eugenol-induced apoptosis in cancer cells. *Molecules*, ***17***, 6290-6304; (d) Carvalho AA, Andrade LN, Sousa EBV, De Sousa, DP. (**2015**) Antitumor phenylpropanoids found in essential oils. *BioMed Research International* 2015:392674.
[20] Wei PL, Tu SH, Lien HM, Chen LC, Chen CS, Wu CH, Huang CS, Chang HW, Chang CH, Tseng H, Ho YS. (**2012**) The *in vivo* antitumor effects on human COLO 205 cancer cells of the 4,7-dimethoxy-5-(2-propen-1-yl)-1,3-benzodioxole (apiole) derivative of 5-substituted 4,7-dimethoxy-5-methyl-l,3-benzodioxole (SY-1) isolated from the fruiting body of *Antrodia camphorata. Journal of Cancer Research and Therapeutics,* ***8***, 532-536.
[21] Cole RA, Bansal A, Moriarity DM, Haber WA, Setzer WN. (**2007**) Chemical composition and cytotoxic activity of the leaf essential oil of *Eugenia zuchowskiae* from Monteverde, Costa Rica. *Journal of Natural Medicines*, ***61***, 414-417.
[22] Legault J, Pichette A. (**2007**) Potentiating effect of beta-caryophyllene on anticancer activity of alpha-humulene, isocaryophyllene and paclitaxel. *Journal of Pharmacy and Pharmacology*, ***59***, 643-647.
[23] Grecco SS, Martins EG, Girola N, de Figueiredo CR, Matsuo AL, Soares MG, Bertoldo Bde C, Sartorelli P, Lago JH. (**2015**) Chemical composition and *in vitro* cytotoxic effects of the essential oil from *Nectandra leucantha* leaves. *Pharmaceutical Biology*, ***53***, 133-137.
[24] Bakkali F, Averbeck S, Averbeck D, Idaomar M. (**2008**) Biological effects of essential oils - A review. *Food and Chemical Toxicology*, ***46***, 446-475.
[25] Knobloch K, Pauli A, Iberl B, Weigand H, Weis N. (**1989**) Antibacterial and antifungal properties of essential oil components. *Journal of Essential Oil Research*, ***1***, 119-128.
[26] Schmidt JM, Noletto JA, Vogler V, Setzer WN. (**2007**) Abaco bush medicine: Chemical composition of essential oils of four aromatic medicinal plants from Abaco Island, Bahamas. *Journal of Herbs, Spices & Medicinal Plants*, ***12***, 43-65.
[27] Kazemi M, Rostami H. (**2015**) Chemical composition, antimicrobial and antioxidant activities of the essential oil of *Psammogeton canescens*. *Natural Product Research*, ***29***, 277-280.
[28] Lago JH, Souza ED, Mariane B, Pascon R, Vallim MA, Martins RC, Baroli AA, Carvalho BA, Soares MG, dos Santos RT, Sartorelli P. (**2011**) Chemical and biological evaluation of essential oils from two species of Myrtaceae - *Eugenia uniflora* L. and *Plinia trunciflora* (O. Berg) Kausel. *Molecules*, ***16***, 9827-9837.
[29] de Lima BR, da Silva FM, Soares ER, de Almeida RA, da Silva Filho FA, Pereira Junior RC, Hernandez Tasco ÁJ, Salvador MJ, Koolen HH, de Souza AD, Pinheiro ML. (**2016**) Chemical composition and antimicrobial activity of the essential oils of *Onychopetalum amazonicum* R.E.Fr. *Natural Product Research*, ***31***, 1-4.
[30] Dahham SS, Tabana YM, Iqbal MA, Ahamed MB, Ezzat MO, Majid AS, Majid AM. (**2015**) The anticancer, antioxidant and antimicrobial properties of the sesquiterpene β-caryophyllene from the essential oil of *Aquilaria crassna*. *Molecules*, ***20***, 11808-11829.
[31] Adams RP. (**2007**) *Identification of essential oil components by gas chromatography / mass spectrometry*, 4th Ed. Allured Publishing, Carol Stream, Illinois, USA.
[32] Setzer MC, Moriarity DM, Lawton RO, Setzer WN, Gentry GA, Haber WA. (**2003**) Phytomedicinal potential of tropical cloudforest plants from Monteverde, Costa Rica. *Revista de Biología Tropical*, ***51***, 647-674.
[33] Palazzo MC, Wright HL, Agius BR, Wright BS, Moriarity DM, Haber WA, Setzer WN. (**2009**) Chemical compositions and biological activities of leaf essential oils of six species of Annonaceae from Monteverde, Costa Rica. *Records of Natural Products*, ***3***, 153-160.
[34] Reed LJ, Muench H.. (**1938**) A simple method of estimating fifty per cent endpoints. *American Journal of Hygiene*, ***27***, 493-497.

NPC Natural Product Communications

2017
Vol. 12
No. 1
135 - 138

Chemical Composition and Acaricidal Activity of *Thymus algeriensis* Essential Oil against *Varroa destructor*

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Received: May 16th, 2016; Accepted: November 30th, 2016

The aim of the present study was to determine the chemical composition and evaluate the acaricidal activity of *Thymus algeriensis* essential oil (TAEO) against *Varroa destructor.* This ectoparasitic mite is a pest of the honey bee *Apis mellifera*. The essential oil from the aerial parts of *T. algeriensis*, obtained by hydrodistillation, was obtained in a yield of 2.8± 0.2%, w/w. The TAEO was analyzed by GC and GC/MS. Thirty-four compounds were identified, representing 99.3% of the oil. The main constituents were carvacrol (48.4%), γ-terpinene (14.9%), *p*-cymene (14.7%), and thymol (5.6%). Four lots were constituted at the level of an apiary in order to study the dynamics of the *Varroa destructor* and its host, *Apis mellifera*. After diagnosis by the biological method "install of diapers", the lots were treated at different doses of TAEO (0.1, 0.3 and 0.5%). TAEO was sprayed on top of the hives. The results show that TAEO at 0.5% resulted in a decrease in the rate of infestation of *Varroa destructor*, causing a mortality rate of 32.6% without negative effect on the nesting of the queen. The essential oil of *T. algeriensis* could be used as a bioacaricidal agent.

Keywords: *Thymus algeriensis* L., *Varroa destructor*, *Apis mellifera*, Essential oils, Acaricidal activity.

The honey bee, *Apis mellifera* L., is critical for crop pollination and honey production. The ectoparasitic mite *Varroa destructor* is a pest of the honey bee. This mite, which feeds on haemolymph of brood and adult bees, causes colony disorder, weakness, decrease in brood and deformation of bees [1a]. It also reduces the ability of the colony to pollinate plants [1b]. The parasite destroys the mechanical protective barriers of the integument and impairs the immune system of the bees. The disease caused by the *Varroa* mites is called varroasis and is one of the main causes of economic damage to the beekeeping industry. Varroasis was reported for the first time in Umm Teboul (East of Algeria) in June 1981, in an apiary of a beekeeping cooperative. Currently, this parasite has spread rapidly throughout the country. Natural products having components with various modes of action might provide an effective solution to the problem of varroaosis [1c]. Essential oils might be an option to control *V. destructor*. Several authors report that these oils might be useful in reducing both mite infestation rates and hive contamination [1d-1e].

The genus *Thymus* L. (Lamiaceae) comprises more than 250 species growing wild throughout the world. This genus is represented by eleven species in the flora of Algeria, one of which is *T. algeriensis* Boiss. et Reut. *T. algeriensis* is the most endemic widespread North African species and well known as "*Zaatar*". The chemical composition of *T. algeriensis* essential oil has been extensively studied in different Maghreb countries [2a-2o], and the existence of several chemotypes has been revealed (Table 1). Therefore, the aims of this study were to analyze further the composition of the essential oil of wild *T. algeriensis* from Ain-Defla (Northern Algeria), and to determine its acaricidal activity against *V. destructor* and its host, *A. mellifera*. To the best of our knowledge, this study represents the first report on acaricidal activity of *T. algeriensis* essential oil.

Extraction afforded a yellow-red oil with a very strong and persistent odor of *Thymus*. The essential oil yield was 2.8 ± 0.2%, w/w, (n= 03). This plant can be assigned to one of the oil-rich species of the Lamiaceae. The percentages and retention indices of the identified oil components are listed in Table 2 in the order of their elution on the HP-5MS column. Thirty-four components were identified, accounting for 99.3% of the total oil. This was found to be rich in monoterpene phenols (carvacrol: 48.4% and thymol: 5.6%) and their corresponding monoterpene hydrocarbon precursors: *p*-cymene (14.7%) and γ-terpinene (14.9 %). Oxygenated monoterpenes were the predominent chemical group (58.4%), followed by monoterpenes (38.2%), whereas the sesquiterpenoid content was very low (2.7%). Chemical profiling of the TAEO sample revealed that it could belong to the carvacrol chemotype, as is quite common for Algerian samples of this species. The compositions of samples from North African countries showed that carvacrol and thymol were individually or jointly the predominent components. Thus, thymol (36.8%) and myrcene (20.2%) were reported as the major components for *T. algeriensis* from Libya [2c], while other authors cited thymol (14.4–65.1%) and carvacrol (22.8–70.3%) as the major compounds for Moroccan samples [2b].

According to the main compounds, several chemotypes were identified in wild plants growing in Tunisia [2h]. In Algeria, two chemotypes were found: thymol/ *p*-cymene/γ-terpinene (i) and

Table 1: Major compounds of *T. algeriensis* essential oils (%) from various countries.

Country	Ref [a]	Major compounds [b] (%)								
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Libya	[2c]		36.8	20.2						
	[2a]	4.7	38.5	8.9	7.2					
	[2i]		38.5	8.9	7.1					
	[2b]	22.8-70.3	14.4-65.1							
	[2m]	8.1	37.8	11.7	15.1					
Morocco	[2k]	80.4	3.4	5.0	2.0	1.8				
		49.3	0.8	2.6	0.9	0.8				
	[2j]								20.5	27.7
	[2h]						17.7		15.5	8.2
Algeria	[2g]		29.5	13	6.9					
				9.9			6.5	10.6	6.5	10.1
	[2e]		29.2	6.8		43.3				
	[2l]	4	71		3	0.5				
	[2d]		62.7			78.8				

[a] Ref: References; [b] Compound: **1**. Carvacrol; **2**. Thymol; **3.** *p*-Cymene; **4**. γ-Terpinene, **5**. Linalool; **6**. 1,8 –Cineole; **7**. 4-Terpineol; **8**. α-Pinene; 9. Camphor.

Table 2: Chemical composition of *Thymus algeriensis* essential oil from Northern Algeria (mean of triplicates).

Compound	RI[a]	RI[b]	Area (%)
α-Thujene	930	923	1.4±0.6
α-Pinene	939	930	1.1±0.4
Camphene	944	945	tr
Sabinene	975	970	tr
β-Pinene	979	973	tr
1-Octen-3 ol	977	977	0.2±0.1
β-Myrcene	991	988	2.7±0.9
α- Phellandrene	1003	1002	0.3±0.1
α-Terpinene	1017	1015	2.2±0.9
***p*-Cymene**	1025	**1023**	**14.7± 2.6**
Limonene	1029	1028	0.8±0.3
trans β-Ocimene	1050	1048	tr
γ-Terpinene	1060	**1059**	**14.9± 2.8**
cis-Sabinene hydrate	1070	1066	0.1±0.1
α-Terpinolene	1089	1088	0.1±0.1
Linalool	1097	1098	1.2±0.6
1-Octen-3- yl-acetate	1116	1113	0.4±0.1
Camphor	1146	1145	tr
Borneol	1175	1167	0.1±0.1
Terpinen -4-ol	1177	1177	1.5±0.7
trans-Dihydrocarvone	1201	1197	0.1±0.1
Carvacrol methyl ether	1245	1244	1±0.6
Thymol	1290	**1293**	**5.6±1.8**
Carvacrol	1299	**1306**	**48.4±4.2**
Terpinyl acetate	1347	1340	0.1±0.1
α-Gurjunene	1411	1413	0.7±0.2
β-Caryophyllene	1419	1423	0.1±0.1
Aromadendrene	1441	1443	0.2±0.1
Alloaromadendrene	1460	1465	0.1±0.1
Bicyclosesquiphellandrene	1473	1479	0.1±0.1
Ledene	1493	1499	0.2±0.1
γ-Cadinene	1506	1517	0.1±0.1
δ-Cadinene	1523	1526	1±0.4
Caryophyllene oxide	1583	1582	0.2±0.1

tr: traces (<0.1); RI[a], retention indices [4a]; RI[b], retention indices relative to C7–C30 on HP-5MS capillary column. Monoterpene hydrocarbons: 38.2%. Oxygen-containing monoterpenes: 58.4%; Sesquiterpene hydrocarbons: 2.5%; Oxygen-containing sesquiterpenes: 0.2%; Total identified: 99.3%

terpinyl acetate / nerolidol / α-pinene / borneol / bornyl acetate (ii) [2g]. The oil composition of two *T. algeriensis* samples collected from the same location was different with one thymol-rich and the other linalool-rich [2d]**.** TAEO showed a large variability and displayed different chemical profiles. This great variability and diversity observed in the chemical composition of the essential oils of *Thymus* can be attributed to many factors, including climatic and soil variations, stage of the vegetative cycle, seasonal variation, and the method of preservation and extraction.

Diagnosis of the presence of *Varroa destructor* mite in an apiary revealed the existence of the mite in all the hives. All colonies of the hive were parasitized by the *Varroa* mite and presented a degree of infestation variable from 1.4 to 34%. There is a correlation between the number of *Varroa* collected daily and the total population within the colony. The mite population can be estimated throughout the year by multiplying the daily mite drop by: 250 - 500 or 20 - 40 when the brood is either absent or present, respectively [3a].

In order to ensure the survival of the colony the following year, anti-*Varroa* treatment is imperative, and some authors propose critical periods of intervention [3b] to the apiculture industry. To reduce the infestation threshold to 50 mites, it is necessary that the acaricidal treatment is conducted in August before the breeding of the winter bee brood [3c].

In order to determine the effect of treatment doses on the nesting of the queen, calculation of the brood area is necessary. The essential oils were diluted with analytical grade ethanol to the following concentrations: 0.1, 0.3, and 0.5%, v/v. The results of the treatments are represented in Figure 1. Lot D presents a very high brood area (1147 cm^2), followed by lot B (913 cm^2), A (903 cm^2), and C (447.5 cm^2), respectively. The hives treated by TAEO (0.5%) represent the best brood area with an evolution from 925 - 1147 cm^2 corresponding to an increase of 225 cm^2. This means that this treatment had no adverse effect on the activity of the bee colony and the laying of the queen. As for the duration of application of treatment, the difference is not significant (p = 0.4123). Finally, the treatment did not disrupt the population of the hive; the brood remained compact and hatching continued normally, indicating the safety of the essential oils to the colony bees. The treatment with TAEO had no influence on the activity of the colony bees and the laying of the queen. Application of TAEO, in winter (from14/01 to 16/03) reduced the infestation of various lots infected by *V. destructor*. The results of the treatments at different concentrations (0.1, 0.3, and 0.5%) are represented in Figure 2. Mortality rates obtained were: A (4.1%), B (24.0%), C (32.4%) and D (32.6%), an average number of dead mites: A (173), B (435) C (1274) and D (1366). These results are consistent with those reported in the literature [3d]. Concerning the factor treatment dose, there is a clear significant difference (p<0.05) between the four lots. Statistical analysis shows that treatment of lots A and B had no effect on the mortality of *Varroa*; then, it is very significant for lots C and D, which shows the effectiveness of treatment by the TAEO concentration of 0.5%. There is a strong correlation between the number of mites killed and the concentrations of the oils tested. One possible explanation for this result is the presence of carvacrol and thymol as major components and the synergic effect with other monoterpenes, such as *p*-cymene, and γ-terpinene. Indeed, previous studies have found that carvacrol was acaricidal against several species of ticks, while carvacrol and γ-terpinene acted as miticides [3e]. Thymol and structurally related compounds like *p*-cymene are effective as acaricides; it has an acaricidal activity against *Varroa*, but while decreasing the laying of the queen [3f]. γ-Terpinene, another active substance of *Thymus* oil, has a very good varroacidal effect. However, the use of *Thymus* oil containing γ-terpinene at a concentration of 250 µg/L of air has proved to be very toxic for both *V. destructor* and bees [1c]. On the other hand, the third application of TAEO (0.5%) presented the most important rate of mortality (340 mites) during the treatment. The statistical analysis also reveals that this application (p= 0.012) corresponds to the stadium phoretic of the *Varroa* mite (Figure 2). During the period of development of the laying and nesting of the queen, the natural

mortality of *Varroa* decreases. This result is consistent with the literature [3g]. Also, it is slightly higher than that obtained by spraying oxalic acid at 0.56 g per colony on adult worker bees, where a mite mortality rate in the phoretic stage of 25.9% was observed [3h]. So, it is much higher than that obtained by applying, for 12 days, an extract of *Lantana camara*, when the infestation rate was reduced to 0.20% [3i]. Treatment with *Citrus aurantium* and *Cymbopagon flexuosus* reduced the average percentage of *Varroa* infestation by 100% after the fourth week [3j]. However, the spray of thyme oils resulted in the death of 65.9% of *Varroa* [3k]. In late autumn, the *Varroa* mite is very sensitive to essential oils due to the formation of a cluster and the absence of brood [3l]. When the external temperature range is from 15-20°C, some authors recommend the application of products containing thymol, because, beyond this range, outside temperature affects negatively the efficiency of the thymol and bee activity [1c]. These conditions are for our study and in the climatic conditions of Algeria in late winter with a temperature of 12-19° C. It follows that the application, in winter, of *T. algeriensis* essential oil by spraying inside the hives is very effective against infestation by *V. destructor* and for maintaining the activity of colony bees and the laying of the queen.

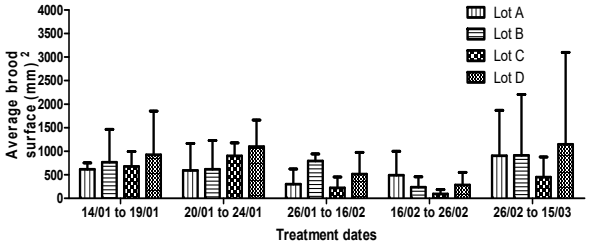

Figure 1: Average brood area during the spraying period of *T. algeriensis* essential oils (TAEO) at different doses. Lot A: control (untreated colonies), Lot B: TAEO (0.1%), Lot C: TAEO (0.3%), Lot D: TAEO (0.5%).

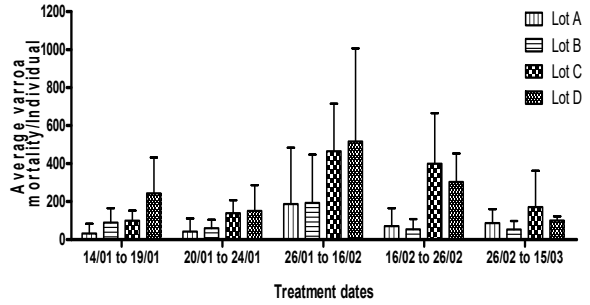

Figure 2: Average mortality of *Varroa* during the spraying period of *T. algeriensis* essential oils (TAEO) at different doses. Lot A: control (untreated colonies), Lot B: TAEO (0.1%), Lot C: TAEO (0.3%), Lot D: TAEO (0.5%).

The use of natural products as acaricides may represent an important alternative for the control of *V. destructor* since they are rich sources of bioactive compounds that are biodegradable. The present findings demonstrate that *T. algeriensis* essential oil may be used as a natural acaricide. Carvacrol, thymol, *p*-cymene, and γ-terpinene are the major compounds in *T. algeriensis* essential oil and may act synergistically to produce the observed acaricidal action against *V. destructor*. Treatment with TAEO (concentration 0.5%) applied in the climatic conditions of Algeria in late winter, is effective against the mite *V. destructor* and has no influence on the activity of the colony bees and the laying of the queen.

Experimental

Biological material: This study has been carried out in an apiary installed at Ain Defla (Algeria). Twenty hives were randomly distributed in 4 blocs (A, B, C, and D), each block containing 5 hives. Block A (hives witnesses) was left without applications. The other blocs were treated with different doses (0.1, 0.3, and 0.5%) of *T. algeriensis* essential oil in 96% ethanol.

Plant material and essential oil hydrodistillation: Aerial parts of wild *T. algeriensis* were collected in the first week of June 2013 in Mekhatria within the region of Ain-Defla located in northern Algeria (at 140 Km Northwest of Algiers - latitude: 36°25' N; longitude: 2° 21'7" E; Altitude: 365m). A voucher specimen was deposited in the Herbarium of the Agronomic Departement, Djilali Bounaama University of Khemis Miliana. Air-dried plants (50 g) with 600 mL distilled water (1:12, w/v) were separately subjected to hydrodistillation for 2 h using a Clevenger-type apparatus. All experiments were conducted in triplicate and results were expressed on the basis of dry weight.

Essential oil analysis: Ten mg of essential oil was dissolved in 5 mL of diethyl ether. The essential oils were analyzed by gas chromatography coupled to a flame ionization detector (GC-FID) and by gas chromatography coupled to a mass spectrometer (GC-MS).

GC-FID analysis: The analysis of the oil was carried out by means of an Agilent technology HP GC 6890 system with a flame ionization detector (FID), using a capillary column coated with 5 % phenyl-methyl siloxane (30 m x 0.25 mm x 0.25 µm film thickness Agilent Technologies, Hewlett-Packard, CA, USA). The temperature program was as follows: 40°C during1 min, then raised in a first ramp to 200°C at 6°C/min, followed by a second ramp to 280°C at 30°C/min, and finally kept at 280°C during 2 min. Injection was realized in splitless mode at 280°C; the volume injected was 1 µL of diluted oil (10 mg of oil/5 mL diethyl ether). Detector temperature was fixed at 300°C; Carrier gas was helium at 1 mL/min.

GC-MS analysis: GC/MS was performed with an Agilent HP 6890 GC system coupled with an Agilent HP 5973 Network Mass Selective Detector operated by HP Enhanced ChemStation software. Analytical conditions were fixed as follows: Agilent HP-5MS capillary column (30 m × 0.25 mm, df = 0.25 µm), a split-splitless injector at 250°C (splitless mode), temperature program: from 40°-250°C at 6°C/min, mobile phase: carrier gas helium at 1 mL/min. The mass spectra were recorded in EI mode (70 eV), scanned mass range: from 35 to 500 amu. Source and quadrupole temperatures were fixed at 230°C and 150°C, respectively. The identification of the components was performed on the basis of chromatographic retention indices and by comparison of the recorded spectra with computed spectral library (Wiley 275. L) [4a]. For sesquiterpene hydrocarbons, further confirmations were obtained by comparing the mass spectra with data from the literature [4a-4b]. Retention indices (RI) were calculated by means of a mixture of homologue *n*-alkanes (C_7-C_{30}) analyzed under the same chromatographic conditions used for the analysis of essential oils [4a].

Diagnosis of the presence of the* Varroa *mite before treatment by spraying: A diagnosis using the biological method "Installing the swaddling clothes" was performed before the treatment with *T. algeriensis* essential oil during the summer period. It allows the detection of the presence of the parasite and then to confirm and to assess the degree of infestation. Also, the diagnosis allowed us to establish a procedure to follow in order to preserve the bees in the best possible conditions [4c]. Among the biological methods for the diagnosis, the utilization of diapers or cover-bottoms was chosen. These swaddling clothes were smeared with fat (Vaseline)

supported by a grid on the floor of the hive. The swaddling clothes were then removed and examined carefully using a magnifying glass to detect dead *Varroa* mites. This method lasted for 30 days during which the swaddling clothes were replaced in the morning once every 3 days.

Treatment of the* Varroa *mites by spraying: The technique used was the spraying of TAEO at different doses (0.1, 0.3, and 0.5% in 96% ethanol). It was applied to the top of the frames of the hive using sprayers to ensure contact of the treatment with the *Varroa* mites. The treatments were applied all 6 days during the winter period from 14/01/2012 to 26/02/2012. Then, dead *Varroa* mites were counted on the dashboard greased all 3 days with the aid of a magnifying glass. The surface of the brood was calculated using the equation of an ellipse.

References

[1] (a) Anderson DL, Trueman JWH. (**2000**) *Varroa jacobsoni* (Acari: Varroidae) is more than one species. *Experimental and Applied Acarology*, ***24***, 165-189; (b) De Jong D, Morse RA, Eickwort GC. (**1982**) Mite pest of honey bees. *Annual Review of Entomology*, ***27***, 229-252; (c) Imdorf A, Bogdanov SO, Choa RI, Calderone NW. (**1999**) Use of essential oils for the control of *V. jacobsoni* Oud. in honey bee colonies. *Apidology*, ***30***, 209-228; (d) Al-Abbadi A, Nazer IK**. (2003)** Control of *Varroa* mite (*Varroa destructor*) on honeybees by aromatic oils and plant materials. *Agricultural and Marine Sciences*, ***8***, 15-20; (e) González-Gómez R, Otero-Colina G, Villanueva-Jiménez JA, Peña-Valdivia CB, Santizo-Rincón JA. (**2012**) Repellency of the oily extract of neem seeds (*Azadirachta indica*) against *Varroa destructor* (Acari: Varroidae). *Experimental and Applied Acarology*, ***56***, 261-70.

[2] (a) Giweli AA, Džamić AM, Soković MD, Ristić MS, Marin PD. (**2013**) Chemical composition, antioxidant and antimicrobial activities of essential oil of *Thymus algeriensis* wild-growing in Libya. *Central European Journal of Biology*, 504–511; (b) Benjilali B, Hammouni M, M'Hamedi A, Richard H. **(1987)** Essential oil composition of different Moroccan thyme varieties: principal component analysis. *Sciences des Aliments*, ***7*,** 275-299; (c) Aboutabl EA, El-dahmy SI. (**1995**) Chemical composition and antimicrobial activity of essential oil of *Thymus algeriensis* Boiss. *Bulletin of Faculty of Pharmacy*, *Cairo University*, ***33***, 87–90; (d) Houmani Z, Azzoudj S, Naxakis G, Skoula M. (**2002**) The essential oil composition of Algerian Zaâtar: *Origanum* ssp. and *Thymus* ssp. *Journal of Herbs, Spices and Medicinal Plants*, ***9***, 275–280; (e) Dob T, Dahmane D, Benabdelkader T, Chelghoum C. (**2006**) Studies on the essential oil composition and antimicrobial activity of *Thymus algeriensis* Boiss. et Reut. *International Journal of Aromatherapy*, ***16***, 95–100; (f) Hazzit M, Baaliouamer A. (**2007**) Composition of essential oils of *Thymus algeriensis* Boiss. et Reut. and *Thymus numidicus* Poiret from Algeria. *Rivista Italiana* EPPOS, ***43***, 11–18; (g) Hazzit M, Baaliouamer A, Verissimo AR, Faleiro ML, Miguel MG. (**2009**) Chemical composition and biological activities of Algerian *Thymus* oils. *Food Chemistry*, ***116***, 714-721; (h) Ben El Hadj Ali I, Zaouali Y, Bejaoui A, Boussaid M. (**2010**) Variation of the chemical composition of essential oils in Tunisian populations of *Thymus algeriensis* Boiss. et Reut. (Lamiaceae) and implication for conservation. *Chemistry & Biodiversity*, ***7***, 1276-1289; (i) Nikolić M, Glamočlija J, Ferreira ICFR, CRC, Fernandes Â, Marković T, Marković D, Giweli A, Soković M. (**2014**) Chemical composition, antimicrobial, antioxidant and antitumor activity of *Thymus serpyllum* L., *Thymus algeriensis* Boiss.et Reut and *Thymus vulgaris* L. essential oils. *Industrial Crops and Products*, ***52***, 183-190; (j) Amarti F, Satrani B, Ghanmi M, Aafi A, Farah A, Aarab L, El Ajjouri M, Guedira A, Chaouch A. (**2011**) Activité antioxydante et composition chimique des huiles essentielles de quatre espèces de thym du Maroc. *Acta Botanica Gallica*, ***158***, 513-523; (k) Jaafari A, Ait Mouse H, Rakib EM, Ait M'barek L, Tilaoui M, Benbakhta C, Boulli A, Abbad A, Zyad A. (**2007**) Chemical composition and antitumor activity of different wild varieties of Moroccan thyme. *Revista Brasileira de Farmacognosia*, ***17***, 477-491; (l) Chemat S, Cherfouha R, Meklati BY, Belanteur K. (**2012**) Composition and microbial activity of thyme (*Thymus algeriensis* genuinus) essential oil. *Journal of Essential Oil Research*, ***24***, 5-11; (m) Zayyad N, Farah A, Bahhou J. (**2014**) Chemical analysis and antibacterial activity of essential oils from three species of Thymus*: Thymus zygis*, *T. algeriensis,* and *T. bleicherianus*. *Bulletin de la Société Royale des Sciences de Liège*, ***83***, 2014, 118-132; (n) Ben Jabeur M, Hamada W. (**2015**) Antifungal activity of chemically different essential oils from wild Tunisian *Thymus* spp. *Natural Product Research*, ***29***, 869-873; (o) Ben El Hadj Ali I, Chaouach M, Bahri R, Chaieb I, Fethia M. (**2015**) Chemical composition and antioxidant, antibacterial, allelopathic and insecticidal activities of essential oil of *Thymus algeriensis* Boiss. et Reut. *Industrial Crops and Products*, ***77***, 631-639.

[3] (a) Martin S. (**1998**) A population model for the ectoparasitic mite *Varroa jacobsoni* in honey bee (*Apis mellifera*) colonies. *Ecological Modelling*, ***109***, 267-281; (b) Kozac P, Eccles L, Tam J, Kemper M, Rawn D, Guzman E, Kelly P. (**2012**) *Varroa-mite sampling and monitoring infestation levels*. OMAFRA Infosheet Ontario, Ministry of Agriculture, Food and Rural Affairs; (c) Noireterre P. (**2011**) Biologie et pathogénie du *Varroa destructor*, *Bulletin des GTV*, ***62***, 101-106; (d) Otten C. (**1991**) Vergleichende Untersuchungen zum Populations wachstum von *Varroa jacobsoni* Oud. in Völkernvon *Apis mellifera* L unterschiedlicher geographischer Herkunft. Ph D Thesis, JW Goethe Universität, Frankfurt, Germany; (e) Iori A, Grazioli D, Gentile E, Marano G, Salvatore G. (**2005**) Acaricidal properties of the essential oil of *Melaleuca alternifolia* Cheel (tea tree oil) against nymphs of *Ixodes ricinus*. *Veterinary Parasitology*, ***129***, 173-176; (f) Burley LM, Fell RD, Saacke RG. (**2008**) Survival of honey bee (*Hymenoptera*: *Apidae*) spermatozoa incubated at room temperature from drones exposed to miticides. *Journal of Economic Entomology*, ***101,*** 1081-1087; (g) Bruneau E. (**2005**) Dépérissement des ruchers en Région wallonne : Etat des lieux. *Abeilles & Cie*, ***104***, 8-11; (h) Al Toufailia H, Scandian L, Ratnieks FLW. (**2015**) Towards integrated control of *Varroa*: 2. Comparing application methods and doses of oxalic acid on the mortality of phoretic *Varroa destructor* mites and their honey bee hosts. *Journal of Apicultural Research*, ***54***, 108-120; (i) Zaitoun AA, Madkour MH. (**2012**) Acaricidal activity of some plant extracts on parasitic bee mite, *Varroa destructor. Journal of Food, Agriculture & Environment,* ***10***, 1153-1155; (j) Abd El-Wahab TE, Ebadah IMA, Zidan EW. (**2012**) Control of *Varroa* mite by essential oils and formic acid with their effects on grooming behaviour of honey bee colonies. *Journal of Basic and Applied Scientific Research*, ***2***, 7674-7680; (k) Hamaad RFM, Eldoksch HA, Abdel Samed AM, Abdel Moein NM. (**2008**) Effect of essential oils and a thymol formulation for controlling *Varroa destructor* in honeybee (*Apis mellifera*) colonies. *Egypt Journal Agricultural Research*, ***86***, 951-961; (l) Noel R, Amrine JW. (**1996**) Formic acid fumigator for controlling honey bee mites in bee hive. Biology research in the UK. *American Bee Journal*, ***137***, 382-385.

[4] (a) Adams RP. (**2001**) *Identification of Essential Oils Components by Gas Chromatography / Mass Spectrometry*. Allured Publ., Carol Stream, Illinois; (b) Joulain D, König WA. (**1998**) *The Atlas of Spectral Data of Sesquiterpene Hydrocarbons*. E.B. – Verlag Hambourg; (c) Robaux P. (**1988**) *Varroa jacobsoni*: Problems with diagnosis and control in Europe. In *Africanizad Honey Bee and Bee Mites*. Nedham G, Page R, Delfinado - Baker M, Bowman CE. (Eds). Ellis Horwood, Chichester, U.K, 370-372.

NPC Natural Product Communications

2017
Vol. 12
No. 1
139 - 146

Cereal Lignans, Natural Compounds of Interest for Human Health?

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Received: August 11th, 2016; Accepted: September 27th, 2016

Cereals are suggested to be the most important sources of lignan in the diets of western populations. Recent epidemiological studies show that European subpopulations in which the major source of lignans are cereals, display lower disease frequency regarding metabolic and cardiovascular diseases. The biological mechanisms of lignan are several. Beyond their antioxidant and anti-inflammatory actions at nutritional doses some lignans regulate the activity of specific nuclear receptors (NRs), such as the estrogen receptors (ERs), and also NRs that are central switches in glucose and fatty acid metabolism such as PPARα, PPARγ and LXRs, highlighting them as selective nuclear receptor modulators (SNRMs). These include enterodiol (END) and enterolactone (ENL), the metabolites produced by the gut microbiota from food lignans. The available knowledge suggests that given some additional research it should be possible to make 'function' claims for a regular intake of lignans-rich foods related to maintaining a healthy metabolism.

Keywords: Lignan, Cereal bran, Obesity, Metabolic syndrome, Health claims.

Introduction: Lignans are a group of compounds found in fiber-rich foods such as cereals, oilseed, nuts, vegetables (brassica), and fruits (berries) typically regarded as components of healthy diets [1-3]. They are expected to have antioxidant, anti-inflammatory and hormone-mimicking effects in humans both as native compounds and after fermentative conversion in the colon [4-7].

Intakes of lignan-rich foods have been shown to be correlated with a lower incidence and progression of diseases / health conditions related to the metabolic syndrome, type 2 diabetes, cardiometabolic risk factors, specific types of cancers and overall mortality [8-12]. The most studied food sources rich in lignans, such as oilseeds (flaxseed and sesame seeds), cereals, and in particular cereal fibers, may contain sufficient concentrations of specific lignans to exert functional effects in humans. Amongst cereals, wheat and rye contain the highest concentration of lignans, mostly in their bran fraction. The most abundant lignans here are 7-hydroxymatairesinol and syringaresinol [14, 15].

Recent studies have highlighted some of the possible mechanisms of action of lignans. Almost all are endowed with antioxidant and anti-inflammatory activities. However, some of these compounds have also shown the ability to bind and activate/inhibit nuclear receptors (NRs) such as the estrogen receptors (ERs) [7], the peroxisome proliferator activate receptor gamma (PPARγ) [16], and the liver X receptor alpha (LXRα) [17]. The regulation of gene expression through these receptors may require the metabolization of most plant lignans to the mammalian lignans enterodiol (END) and enterolactone (ENL) [18], while some lignans like 7-hydroxymatairesinol are shown to engage NRs also in their unmetabolized form [19]. Metabolization of lignans requires gut microbial processes to release them from the food matrix and undergo deglycosylation in the intestine and conversion in the liver to active metabolites. Several of the lignans that have been studied so far have shown interesting roles in promoting human health. Some of them were identified in plants to be of pharmaceutical and cosmetic interest although the diet remains the major route of intake of these chemicals [5, 13, 20, 21]. In particular cereal lignans are present in high amounts in the daily diet of western populations and are likely to be the dietary plants that provide most of the beneficial bioactive lignans in Europe, the US and Canada [5, 13, 20, 21]. Here we review the recent and ongoing research on the characterization of the health effects of cereal lignans and their mechanisms.

Methods: A comprehensive computer literature search of the Pub-Med/MEDLINE, Embase and Scopus databases was conducted to find relevant published articles about the role of lignans in health. No beginning date limit was used; the search was updated until January 2016. Only articles in the English language were selected. To expand our search, references of the retrieved articles were also screened for additional studies.

Lignan chemistry and biochemistry: The chemistry of lignans has been studied for the past 65 years. They possess very different structural composition and can be found in nature in vegetables, mostly in exudates and resins, although they can be present in different parts of the plant. Lignans are polyphenolic substances derived from phenylalanine via dimerization of substituted cinnamic alcohols, known as monolignols, to a dibenzylbutane skeleton and they are found mostly in plants as aglycones, esters or glycosides. These dimeric phenylpropanoids show two C6-C3 units attached to the central carbon C8 while, when two phenylpropanoid units are found coupled in a different manner, like C5-C5', the new term "neolignan" has been adopted (Figure 1) [22].

The lignans can be classified into eight chemical groups and several subgroups based upon the way in which oxygen is incorporated into the skeleton and on the cyclization pattern [22]. Lignan subgroups arise via the shikimic acid pathway from the reduction of cinnamic acid derivatives produced from aromatic aminoacids (L-phenylalanine and L-tyrosine) and subsequently reduced via coenzyme A to alcohols that are the main precursors of all lignans (*p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol) in plants. The enantioselective dimerization of two coniferyl alcohol monomers via intermolecular 8,8' oxidative coupling, generate pinoresinol, a major lignan in food seeds and grains [23].

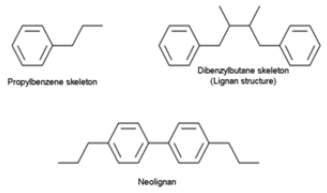

Figure 1: Basic structure of lignans. a and b) The propylbenzene skeleton and the dibenzylbutane skeleton of lignan attached C6-C3 to the central carbon C8. c) Two phenylpropanoid units of a neolignan coupled C5-C5'.

Subsequent metabolization steps generate lariciresinol and secoisolariciresinol through pinoresinol/lariciresinol reductase and secoisolariciresinol dehydrogenase respectively. Secoisolariciresinol is the common precursor of all dibenzylbutyrolactol lignans including matairesinol, yatein and aryltetralin lignans (Figure 2). These subclasses of lignans includes some important bioactive chemicals from which several structural analogues have been derived, studied and employed as pharmaceuticals [24].

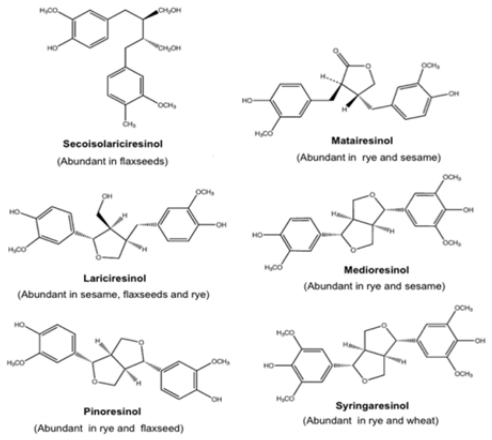

Figure 2: Major lignans in oilseeds and cereals. Secoisolariciresinol is the common precursor of all dibenzylbutyrolactol lignans. These subclasses of lignans include some important bioactive chemicals from which several structural analogues have been derived, studied and employed as pharmaceuticals [24].

Lignans can be converted by intestinal microbiota, under strictly anaerobic condition, to the mammalian lignans, enterodiol (END) and enterolactone (ENL). These compounds, also called enterolignan, following metabolization steps in the gut acquire specific bioactivities and healthy effects, some of which have been shown to occur through the interaction with nuclear receptors [18]. The biotransformation of plant lignans to enterolignan has been extensively studied with secoisolariciresinol (SDG) through the pathway consisting of glycoside hydrolysis, demethylation and dehydroxylation and intermediates [25]. The metabolization steps will be further discussed in the last paragraph of this review in a more detailed manner.

Lignans in nutrition, availability and food sources: Several of the lignans under study are present in plants of a very different kind throughout the whole plant kingdom. Some of them are highly concentrated in plants of medical interest, i.e. *Podophyllum* species containing podophyllotoxin, which is particularly interesting for its anticancer properties [24, 26]. High concentrations of lignans are also found in plants of nutritional interest. The richest are by far oilseeds such as flaxseed and sesame, with concentrations up to 1% of the seed weight. These seeds are consumed in functional amounts in defined geographical regions of the world, in particular the Middle East, North Africa and Asia.

Cereals, nuts, broccoli (*Brassica* species), fruits, berries and beverages (tea, coffee, beer wine) are suggested to be the most important sources of lignans in the diets of Europeans. According to a Dutch lignan database, the average daily intake of lignans in the Netherlands was 1000 to 2000 μg/day and in the Finnish population 1081 μg/day, ranging from 1136 μg/day for men to 1036 μg/day for women) [13]**.** In another study in the Finnish population the average lignan intake based on urinary excretion of lignan was 1224 μg/day [27]**.** Sweden registered the highest intake in Europe with 1773 μg/day total lignans for women and 1947 μg/day for men. Intakes between 1062 and 1188 μg/day have been reported in south Europe (Italy) [13] and 600 μg/day in the USA (Breast Cancer and Environment Research Project Puberty Study) [28]**.** Higher levels of intake (up to 13.5 mg/day) have been reported in previous studies in the USA and Canada [21]**.** In the east, a Chinese study reported intakes measured through the urinary excretion of mammalian lignans giving values in the range of 0.30 and 1.18 nmol/mg creatinine of enterodiol and enterolactone, respectively. The levels of enterodiol were two-fold higher in the Chinese population compared with US women in a similar age range, while enterolactone was observed at similar levels in both populations [29]**.**

Cereal lignans: Up to date, the most investigated lignans have been extracted from food sources such as flaxseed and sesame seeds, the first being known as the richest food source of the lignan secoisolariciresinol and the latter being the most consumed lignan-rich food in the world with a unique lignan composition. However, in the western countries cereals are the major source of lignans (because of their regular daily consumption) with an impact that may influence human health (Table 1).

Wheat: Wheat is the most consumed cereal in the diet in Europe, the USA, and Canada. It is well accepted and has an attractive low price as food, but also as a lignan source. By wheat milling the grain is split into two major parts, the flour (about 70-80%), and the bran fraction, which is approximately 15%. This last fraction contains most of the grain lignans (70-85%), which are mostly situated within the aleurone cells, the single cell layer at the inner site of the bran.

Novel milling and dry-fractionation techniques and new extraction and analytical methods have recently allowed full-scale separation of aleurone cells from the other layers of wheat bran, yielding a fiber rich concentrate which contains many nutrients and well-known bioactive lignans, as well as several new ones with potent bioactivity (Figure 3) [31, 32].

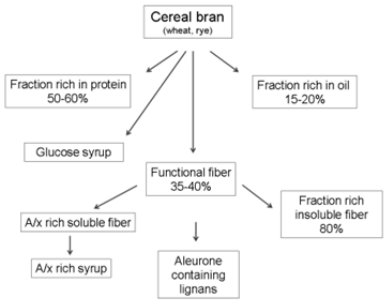

Figure 3: A schematic representation of the fractionation of cereal bran (approximately 15% of the grain). Most of the grain lignan (70-85%) is situated within the aleurone, the single cell layer at the inner site of the bran. Separation of the aleurone cells from the other layers of the cereal bran yields a fiber rich concentrate which, beyond well-known and new lignans, contains many other nutrients.

Table 1: Concentration of main lignan in different food sources.

	Lignan (μg/100 g)						
Food sources	Pinoresinol	Syringaresinol	Lariciresinol	Secoisolariciresinol	Matairesinol	7-Hydroxymatairesinol	Sesamine
Flaxseeds	871	48	1780	165759	529	35	ND
Sesame	47136	205	13060	240	1137	7209	62724
Rye bran	1547	3540	1503	462	729	1017	ND
Wheat bran	138	882	672	868	410	2787	ND
Oat bran	567	297	766	90	440	712	ND
Barley bran	71	140	133	42	42	541	ND

Altogether, wheat grains may contain up to 1 mg lignans/100 g and the large majority of these reside in the bran layer containing up to 20 mg lignans /100 g bran. 7-Hydroxymatairesinol and syringaresinol are the dominant lignans in wheat although secoisolariciresinol, lariciresinol, matairesinol and other less characterized lignans, such as 7-oxomatairesinol are also present in variable concentrations depending on the variety [33, 34] (Table 2).

Table 2: Concentration of total lignans in wheat bran and rye bran.

Wheat bran	Amounts of total lignan 5-20 mg/100 g *(depending on the variety)*
Total lignan	7-hydroxymatairesinol, syringaresinol, secoisolariciresinol, lariciresinol, matairesinol
New lignan	Iso-hydroxymatairesinol, 7-oxomatairesinol
Rye bran	Amounts of total lignan 10-20 mg/100 g *(depending on the variety)*
Total lignan	syringaresinol, pinoresinol, lariciresinol, isolariciresinol, 7-hydroxymatairesinol, matairesinol, secoisolariciresinol.

Different varieties of wheat have been profiled for their content of lignans. In a work by Dinelli *et al*. [35], old and modern Italian wheat varieties cropped in the same location and growing season have been analyzed. The study shows that both free and bound phenols are very variable in content and, on the average, the bound fraction contributed 72.0% to the total phenolic content. The obtained metabolomic fingerprints showed the presence of several classes of compounds which included flavonoids, coumarins, stilbenes, proanthocyanidins and lignans, some of which may have health promoting effects. The HPLC-ESI-TOF-MS analysis identified 104 compounds and also highlighted remarkable differences in the phytochemical fingerprints of old and modern wheat varieties. Six ancient wheat genotypes (Bianco Nostrale, Frassineto, Gentil Rosso, Gentil Rosso Mutico, Marzuolo d'Aqui, Verna) showed phenolic profiles with a number of total compounds and isomer forms including the lignan syringaresinol, with much higher contents than in the modern cultivars, indicating that they might be better sources of phenolic compounds with added value in terms of potential health-promoting components [35]**.**

Rye: Among cereal grains, rye has the highest concentration of lignans, mostly as glycosides which, like in wheat, are concentrated in the outer layers of the kernel [36]**.** The most abundant are pinoresinol, syringaresinol, 7-hydroxymatairesinol, lariciresinol and isolariciresinol. When considering the whole rye grain, 7-hydroxymatairesinol is the dominant lignin, followed by syringaresinol (Table 2).

In studies aimed at identifying novel urinary biomarkers of wholegrain rye bread intake versus refined wheat bread, the profiling of several metabolites, including END and ENL, allowed the identification of wholegrain rye as a food source providing a higher concentration of urinary phenols [37]. In their study, Bondia-Pons *et al*. experimentally found higher levels of five lignans after intake of wholegrain rye bread than after intake of refined white bread. This was associated with a lower fasting plasma insulin level and lower 24-h urinary C-peptide excretion [37]**.** The highest concentration of plasma and urinary levels of entrolignans have been measured in subjects traditionally consuming wholegrain rye at the highest amounts in North and East Europe, Russia and China, where the production of rye is mostly concentrated (1 to 4 million tons/year).

Cereal consumption and bioprinciples of accessibility: Regarding the nutritional aspect of cereal grains and bran, studies suggest that whole grains have superior nutritional values compared with foods enriched with fibers. This is because the bioactive components are more abundant in the whole grain and particularly concentrated in the aleurone layer. Moreover, beyond lignans, the bran fraction of cereals contains most of the micronutrients and complex carbohydrates of the grains, in particular beta-glucans and arabinoxylans, which are promising nutraceuticals. Nonetheless, as recently evidenced by an EU study consortia on European cohorts, the consumption of whole seeds or grains supposed to provide health benefits, often does not fulfil the expectations (www.healthgrain.eu). This might be due to the fact that although cereals are good sources of lignans, their bioabsorption from the consumption of the whole grains is limited. This is likely due to their macro- and microstructure and consequently texture of the raw flour which influences their functionality in the gastrointestinal tract, specifically component absorption, bioavailability, and metabolization to enterolignans [38]**.** The studies carried out by two groups in the Healthgrain consortium working on wholegrain products, including wheat, showed in fact that subjects consuming whole grains did not achieve significant changes in traditional biomarkers of MetS (www.healthgrain.eu**).** It is now known that this is partially due to the inaccessibility of the bioactive principles contained in the natural matrices because of their insufficient breakdown and digestion but also absorption. The major problem identified by bioavailability studies was the poor bioaccessibility of the active principles. Two studies from Anson *et al.* [39, 40], evidenced that release and conversion of phenolic acids to their microbial metabolites was enhanced by bioprocessing of wheat bran to more bioavailable formulations. In the bioprocessed bread, the bran was processed by yeast fermentation combined with enzyme treatment, consisting of cell-wall-degrading enzymes, mainly xylanase, cellulase, glucanase and feruloyl-esterase [40]**.**

Nutritional impact on health of lignans. The metabolic syndrome (MetS): The high prevalence of MetS and excessive adipose tissue accumulation (in particular, abdominal obesity) is a major threat to public health, being associated with a substantial decrease in health-related quality of life and an increase in economic costs due to disease treatment, as well as lower social productivity.

MetS is a multiplex and life-based risk factor that consists of several correlated risks of metabolic origin. In addition to dyslipidemia, hypertension and hyperglycemia, the syndrome carries a proinflammatory state [41]**.** Subjects affected by MetS are at increased risk of cardiovascular diseases (2-fold), type-2 diabetes (5-fold), gallbladder disease, certain types of cancer and psychosocial problems (www.lipgene.tcd.ie,

www.nutrition.org.uk/lipgene, www.oecd.org.) [42]. More than 130 million Europeans are estimated to have MetS and 56 million type-2 diabetes [43] and the numbers are estimated to increase for the next decades. Thus, MetS is 'the' major health challenge in Europe and the western countries today but further, the same tendency is also seen in many less developed countries, e.g. India. New strategies and very extensive research and initiatives related to the long-term prevention and reduction of incidence and severity of cardiovascular diseases, type-2 diabetes and obesity have been undertaken by the EU Commission and the WHO for both prevention and cure, to halt the problem [44-46]. Amongst the identification of fields of intervention, the better characterization of the bioactive compounds within many foods seems to be a promising area. In fact, although poor diets and physical inactivity are the driving forces behind MetS, appropriate dietary strategies may positively impact on disease predisposition and evolution.

For the assessment of the nutritional effects of lignan consumption on human health, different European networks such as the EPIC-Potsdam Study have been particularly suited to serve as epidemiological reference cohorts for results comparison. They have been recently used to investigate the role of lignans on disease occurrence using medically verified data. The analyzed endpoints of chronic diseases following lignan intake were: a) incidence of the metabolic syndrome (NHANES-National Health and Nutrition Examination Survey [47]; b) type-2 diabetes (EPIC-InterAct Study) [9]; c) cardiometabolic risk factors (NHANES) [11]; d) specific types of cancers (DietCompLyf Study) [12]; (EPIC Study) [10, 48-51], (EPIC-Norfolk) [52-54]; and e) overall mortality (EPIC-Spain) [10]. From these studies it is apparent that European subpopulations in which the major source of lignans is cereals [9], display considerable lower disease frequency and beneficial effects in the control of metabolic diseases like dyslipidemia and type-2 diabetes. Further, other recent observations in different populations report that lignan intake is associated with lower levels of triglycerides, lower fasting glucose, and fasting insulin serum levels in males aged 20-60 years. These data are associated with higher HDL-cholesterol levels [2, 11, 29, 55-58]. Some studies have also been conducted in animal models in which partially purified lignan fractions have been shown to improve blood lipids and glycemic control [59, 60]. The results from all these studies underline the need to understand better the mechanisms of action of these compounds in maintaining a healthy metabolism.

Mechanisms of lignans on health: Although impressive advances have been seen in the last years as regards the mechanisms of action for the most thoroughly investigated dietary polyphenols (lignans, stilbenes, isoflavones, etc.), most of their health-related effects have been attributed to their activity as antioxidants [61, 62]. However, a certain amount of data is now becoming available on the activity of these compounds at doses lower than those required to exert biological antioxidant activities, but sufficient to activate fundamental cell pathways, several of which are regulated by the nuclear receptors (NRs). The availability of data regarding the regulation of different NRs at relatively low doses, stimulated the characterization of the effects of lignans in tissues and cells at doses that are consistent with the doses reached in the blood through the diet (nutritional doses) [7, 18]. Among others, it has been shown that, at nutritional doses, some lignans can regulate the activity of specific NRs such as the estrogen receptors (ERs) with an efficiency that in some cases approximate to that of the endogenous ligand 17ß-estradiol [18]. This highlighted the importance of their actions as hormone mimics suggesting that they are effective on several physiological functions. Based on these observations, molecular and cellular studies are now starting to support the data provided by epidemiological studies and dietary interventions. Significant effects have in fact been shown for regulatory mechanisms related to lipid metabolism, glucose homeostasis, cholesterol biosynthesis and insulin biosynthesis and secretion, [18, 60, 61, 63-65].

Several data on lignans as estrogenic compounds regulating ER-dependent pathways have been published [19, 66]. However, recent progress in the area indicate that the term "phytoestrogen" may be poorly representative of the complex activities of lignans when considering as functional targets metabolic functions regulated through the ERs in the liver or adipose tissues, and not representative at all when considering lignan activity through metabolic sensors like the peroxisome proliferator-activated receptors. It is in fact becoming frequent to observe that the same molecules that regulate the ERs, also regulate other NRs that play fundamental actions in metabolic tissues. The engagement and regulation of PPARα, PPARγ, and LXRs that are central regulators of glucose metabolism, fatty acid metabolism and insulin action, evidence the lignans as real SNRMs (Selective Nuclear Receptors Modulators). Known examples are the flaxseed lignan secoisolariciresinol diglucoside (SDG), which can regulate the ERs [6], PPARs [67] and LXRs [17], and unpublished observations. A lignan isolated from medicinal plants, honokiol [68], is a non-adipogenic PPARγ agonist [69] and a ligand of the adipogenic RXRß receptor [70]. Meso-dihydroguaiaretic acid (MDGA), a major component of *Myristica fragrans*, a traditionally used food spice in several countries [71], competes with the LXRα agonist T0901317 for binding to the LXRα domain [17]. The sesame lignans sesamin and episesamin, can stimulate fatty acid synthesis and oxidation in the liver *via* activation of PPARα, can attenuate nutritional fibrosing steatohepatitis through up-regulation of PPARγ [72], down-regulate PPARγ antiinflammatory effects in 3T3-L1 preadipocytes [16], and can suppress macrophage-derived chemokine expression in human monocytes *via* epigenetic ER- and PPARα–mediated regulation [73]. The mammalian enterolignans, enterodiol (ENL) and enterolactone (END) generated through the metabolization of dietary lignans by the intestinal microbiota bind both the ERs [18] and likely the LXRs, as evidenced by unpublished *in silico* studies (personal communication from Ingemar Pongratz). Activity has also been reported for (+)-pinoresinol through the negative regulation of the human thyroid hormone receptor ß [74], likely interfering with adipose cell differentiation [75]. Negative regulation has also been shown by ENL through the androgen receptor (AR) and by sesamin through the pregnane X receptor (PXR), while secoisolariciresinol and ENL are able to activate PXR in reporter assays suggesting an effect in the metabolism of CYP3A substrates [76]. A few cereal lignans have also been tested *in vitro* and found to be effective inhibitors of adipocyte differentiation. Pinoresinol, cycloisolariciresinol, lariciresinol, seicoisolariciresinol in the concentration range of 1-1000 nM, are effective in inhibiting 3T3-L1 cell differentiation [77] and although their fine mechanism of action has still to be clarified, it is likely that it involves the ability to regulate nuclear receptors such as PPARs, ERs and LXRs [65, 78, 79].

Microbial metabolization of lignans: Lignans occur in food in the form of esters, glycoconjugates, and polymers. These compounds are not directly bioavailable and require metabolic steps which involve both mammalian (glucuronidation and to a lesser degree, sulfation) and gut microbial processes [80]. When lignan glycosides are consumed with the diet, they are released from the matrix after mastication, are deglycosylated and are converted in the liver to glucuronidated [81], methylated and sulfated derivatives [82] that can be identified in human urine [83], but also to enterodiol and enterolactone by the intestinal microbiota [20] (Figure 4).

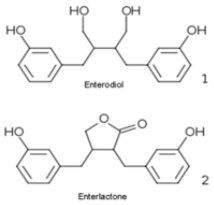

Figure 4: Mammalian lignans. The enterolignans (or mammalian lignan) enterodiol (END) and enterolactone (ENL) are lignan metabolites that are produced in the intestine from plant lignans by the gut microbiota.

Probiotic lactobacilli and bifidobacteria, which have evolved within the colonic ecosystem where indigestible oligo- and polysaccharides are their sole carbon sources, bear several glycosyl-hydrolases and can contribute to the release of the aglycones from glycoconjugated phytochemicals [80]**.** β-Glucosidase-positive probiotic bacteria were proved to release the aglycones of lignans *in vitro* [84]**.** Despite the structural diversity of food lignans and their very different plant origins (i.e. oily matrices versus cereal bran), lignans are converted to enterolignans by the gut microbiota [20]**.** Following metabolization, enterodiol and enterolactone may reach a level high enough for signal pathway regulation in the liver, lung, kidney, heart and brain tissues [83] and pharmacokinetic studies show that these microbial metabolites have high systemic clearance and short half-lives in the blood circulation, before being excreted as hepatic conjugates with the urine [85]. Dietary consumption of probiotics and limited digestible or indigestible food constituents such as oligosaccharides (prebiotics) and polyphenols or both (synbiotics), regulate the numbers and types of microbes which can influence energy utilization from the diet and regulate energy expenditure and storage. However, the genera of the microbiota that contribute to the metabolism of lignans are not yet sufficiently characterized, although investigations are underway and some gut microbiota strands have been identified as major lignan metabolizers in humans [86] and in animals [4, 87] and may thus represent possible beneficial factors for health. Associations are starting to be reported between the composition of the intestinal microbiota, lignan metabolites and the risk of diseases. Recent results suggest that urinary levels of ENL and/or END are associated with a lower risk of type 2-diabetes in US women [5]**,** inflammation [88] and obesity and metabolic alterations in US men [55]**,** and to the inhibition of inflammation and pro-carcinogenic microenvironments in animal tissues [66]**.**

Conclusions: Today, more than 20 different lignans have been identified in seeds and cereals consumed as food worldwide and many others are found in cosmetic and medicinal plants [89]**.** Many of these have not been studied before and may possess potent biological activity, but also, amongst the most studied ones, data are not sufficient to draw definitive considerations for the present. However, the health effects of the most abundant food lignans in ameliorating different chronic diseases and related co-morbidities are being extensively characterized regarding absorption, bioavailability and activity following intake of whole foods, enriched foods, and fractions and extracts. Although so far most of these studies deal mainly with oilseeds lignans, an increasing number of observations indicate that regular intakes of wholegrain cereals and their products may exert a level of protection not observed with the ingestion of refined cereals, and recent research suggests that part of these effects may well be contributed by the lignan components [90]**.**

To conclude, the reviewed body of research suggests that given additional efforts in clinical trials, it may be possible to make 'function' claims for lignans related to maintaining a healthy metabolism and the prevention/amelioration of the metabolic syndrome and related pathologies. Importantly, this effect may be elicited through mechanisms that involve their direct agonistic/antagonistic action with different types of nuclear receptors.

Acknowledgements - We are grateful to Francesco Sainato and Alessandro Bulla for their helpful English writing and secretarial assistance.

Conflict of interest: No conflicts of interest needs to be disclosed by any of the authors.

References

[1] Nooyens AC, Milder IE, van Gelder BM, Bueno-de-Mesquita HB, van Boxtel MP, VerschurenWM. (**2015**) Diet and cognitive decline at middle age: the role of antioxidants. *British Journal of Nutrition*, ***113***, 1410-1417.

[2] Khalesi S, Irwin C, Schubert M. (**2015**) Flaxseed consumption may reduce blood pressure: a systematic review and meta-analysis of controlled trials. *Journal of Nutrition*, ***145***, 758-765

[3] Tresserra-Rimbau A, Rimm EB, Medina-Remón A, Martínez-González MA, de la Torre R, Corella D, Salas-Salvadó J, Gómez-Gracia E, Lapetra J, Arós F, Fiol M, Ros E, Serra-Majem L, Pintó X, Saez GT, Basora J, Sorlí JV, Martínez JA, Vinyoles E, Ruiz-Gutiérrez V, Estruch R, Lamuela-Raventós RM. (**2014**) Investigators PS. Inverse association between habitual polyphenol intake and incidence of cardiovascular events in the PREDIMED study. *Nutrition Metabolism and Cardiovascular Disease*, ***24***, 639-647.

[4] Schogor AL, Huws SA, Santos GT, Scollan ND, Hauck BD, Winters AL, Kim EJ, Petit HV. (**2014**) Ruminal *Prevotella* spp. may play an important role in the conversion of plant lignans into human health beneficial antioxidants. *PLoS One* ***9***, e87949.

[5] Sun Q, Wedick NM, Pan A, Townsend MK, Cassidy A, Franke AA, Rimm EB, Hu FB, van Dam RM. (**2014**) Gut microbiota metabolites of dietary lignans and risk of type 2 diabetes: a prospective investigation in two cohorts of U.S. women. *Diabetes Care*, ***37***, 1287-1295.

[6] Saggar JK, Chen J, Corey P, Thompson LU. (**2010**) Dietary flaxseed lignan or oil combined with tamoxifen treatment affects MCF-7 tumor growth through estrogen receptor- and growth factor-signaling pathways. *Molecular Nutrition and Food Research*, ***54***, 415-425.

[7] Damdimopoulou P, Nurmi T, Salminen A, Damdimopoulos AE, Kotka M, van der Saag P, Strauss L, Poutanen M, Pongratz I, Mäkelä S. (**2011**) A single dose of enterolactone activates estrogen signaling and regulates expression of circadian clock genes in mice. *Journal of Nutrition, **141***, 1583-1589.

[8] Zamora-Ros R, Agudo A, Luján-Barroso L, Romieu I, Ferrari P, Knaze V, Bueno-de-Mesquita HB, Leenders M, Travis RC, Navarro C, Sánchez-Cantalejo E, Slimani N, Scalbert A, Fedirko V, Hjartåker A, Engeset D, Skeie G, Boeing H, Förster J, Li K, Teucher B, Agnoli C, Tumino R, Mattiello A, Saieva C, Johansson I, Stenling R, Redondo ML, Wallström P, Ericson U, Khaw KT, Mulligan AA, Trichopoulou A, Dilis V, Katsoulis M, Peeters PH, Igali L, Tjønneland A, Halkjær J, Touillaud M, Perquier F, Fagherazzi G, Amiano P, Ardanaz E, Bredsdorff L, Overvad K, Ricceri F, Riboli E, González CA. (**2012**) Dietary flavonoid and lignan intake and gastric adenocarcinoma risk in the European Prospective Investigation into Cancer and Nutrition (EPIC) study. *American Journal of Clinical Nutrition*, ***96***, 1398-1408.

[9] Zamora-Ros R, Forouhi NG, Sharp SJ, González CA, Buijsse B, Guevara M, van der Schouw YT, Amiano P, Boeing H, Bredsdorff L, Clavel-Chapelon F, Fagherazzi G, Feskens EJ, Franks PW, Grioni S, Katzke V, Key TJ, Khaw KT, Kühn T, Masala G, Mattiello A, Molina-Montes E, Nilsson PM, Overvad K, Perquier F, Quirós JR, Romieu I, Sacerdote C, Scalbert A, Schulze M, Slimani N, Spijkerman AM, Tjonneland A, Tormo

MJ, Tumino R, van der A DL, Langenberg C, Riboli E, Wareham NJ. (**2013**) The association between dietary flavonoid and lignan intakes and incident type 2 diabetes in European populations: the EPIC-InterAct study. *Diabetes Care*, ***36***, 3961-3970.

[10] Zamora-Ros R, Jiménez C, Cleries R, Agudo A, Sánchez MJ, Sánchez-Cantalejo E, Molina-Montes E, Navarro C, Chirlaque MD, María Huerta J, Amiano P, Redondo ML, Barricarte A, González CA. (**2013**) Dietary flavonoid and lignan intake and mortality in a Spanish cohort. *Epidemiology*, ***24***, 726-733.

[11] Frankenfeld CL. (**2014**) Cardiometabolic risk factors are associated with high urinary enterolactone concentration, independent of urinary enterodiol concentration and dietary fiber intake in adults. *Journal of Nutrition*, ***144***, 1445-1453.

[12] Swann R, Perkins KA, Velentzis LS, Ciria C, Dutton SJ, Mulligan AA, Woodside JV, Cantwell MM, Leathem AJ, Robertson CE, Dwek MV. (**2013**) The DietCompLyf study: a prospective cohort study of breast cancer survival and phytoestrogen consumption. *Maturitas*, ***75***, 232-240.

[13] Tetens I, Turrini A, Tapanainen H, Christensen T, Lampe JW, Fagt S, Håkansson N, Lundquist A, Hallund J, Valsta LM, group PWw. (**2013**) Dietary intake and main sources of plant lignans in five European countries. *Food and Nutrition Research*, ***57***.

[14] Smeds AI, Eklund PC, Sjöholm RE, Willför SM, Nishibe S, Deyama T, Holmbom BR. (**2007**) Quantification of a broad spectrum of lignans in cereals, oilseeds, and nuts. *Journal of Agricultural and Food Chemistry*, ***55***, 1337-1346.

[15] Smeds AI, Jauhiainen L, Tuomola E, Peltonen-Sainio P. (**2009**) Characterization of variation in the lignan content and composition of winter rye, spring wheat, and spring oat. *Journal of Agricultural and Food Chemistry*, ***57***, 5837-5842.

[16] Freise C, Trowitzsch-Kienast W, Erben U, Seehofer D, Kim KY, Zeitz M, Ruehl M, Somasundaram R. (**2013**) (+)-Episesamin inhibits adipogenesis and exerts anti-inflammatory effects in 3T3-L1 (pre)adipocytes by sustained Wnt signaling, down-regulation of PPARγ and induction of iNOS. *Journal of Nutritional Biochemestry, **24***, 550-555.

[17] Sim WC, Park S, Lee KY, Je YT, Yin HQ, Choi YJ, Sung SH, Park SJ, Park HJ, Shin KJ, Lee BH. (**2014**) LXR-α antagonist meso-dihydroguaiaretic acid attenuates high-fat diet-induced nonalcoholic fatty liver. *Biochemical Pharmacology*, ***90***, 414-424.

[18] Penttinen P, Jaehrling J, Damdimopoulos AE, Inzunza J, Lemmen JG, van der Saag P, Pettersson K, Gauglitz G, Mäkelä S, Pongratz I. (**2007**) Diet-derived polyphenol metabolite enterolactone is a tissue-specific estrogen receptor activator. *Endocrinology*, ***148***, 4875-4886.

[19] Cosentino M, Marino F, Ferrari M, Rasini E, Bombelli R, Luini A, Legnaro M, Delle Canne MG, Luzzani M, Crema F, Paracchini S, Lecchini S. (**2007**) Estrogenic activity of 7-hydroxymatairesinol potassium acetate (HMR/lignan) from Norway spruce (*Picea abies*) knots and of its active metabolite enterolactone in MCF-7 cells. *Pharmacological Research*, ***56***, 140-147.

[20 Peñalvo JL, López-Romero P. (**2012**) Urinary enterolignan concentrations are positively associated with serum HDL cholesterol and negatively associated with serum triglycerides in U.S. adults. *Journal of Nutrition*, ***142***, 751-756.

[21] Boucher BA, Cotterchio M, Curca IA, Kreiger N, Harris SA, Kirsh VA, Goodwin PJ. (**2012**) Intake of phytoestrogen foods and supplements among women recently diagnosed with breast cancer in Ontario, Canada. *Nutrition and Cancer*, ***64***, 695-703.

[22] Cunha WR, Andrade ML, Cassio Sala Veneziani R, Ambrosio RS, Bastos JK. (**2012**) Lignans: Chemical and biological properties. In *Phytochemicals - A global perspective of their role in nutrition and health*. Venketeshwer Rao (Ed), InTech, ISBN 978-953-51-0296-0

[23] Suzuki S, Yamamura M, Hattori T, Nakatsubo T, Umezawa T. (**2007**) The subunit composition of hinokiresinol synthase controls geometrical selectivity in norlignan formation. *Proceedings of the National Academy of Sciences USA*, ***104***, 21008-21013.

[24] Yousefzadi M, Sharifi M, Chashmi NA, Behmanesh M, Ghasempour A. (**2010**) Optimization of podophyllotoxin extraction method from *Linum album* cell cultures. *Pharmaceutical Biology*, ***48***, 1421-1425.

[25] Clavel T, Henderson G, Alpert CA, Philippe C, Rigottier-Gois L, Doré J, Blaut M. (**2005**) Intestinal bacterial communities that produce active estrogen-like compounds enterodiol and enterolactone in humans. *Applied Environemental Microbiology*, ***71***, 6077-6085.

[26] You Y. (**2005**) Podophyllotoxin derivatives: current synthetic approaches for new anticancer agents. *Current Pharmaceutical Design*, ***11***, 1695-1717.

[27] Nurmi T, Mursu J, Peñalvo JL, Poulsen HE, Voutilainen S. (**2010**) Dietary intake and urinary excretion of lignans in Finnish men. *British Journal of Nutrition*, ***103***, 677-685.

[28] Mervish NA, Gardiner EW, Galvez MP, Kushi LH, Windham GC, Biro FM, Pinney SM, Rybak ME, Teitelbaum SL, Wolff MS. (**2013**) BCERP. Dietary flavonol intake is associated with age of puberty in a longitudinal cohort of girls. *Nutrition Research*, ***33***, 534-542.

[29] Wu H, Pan A, Yu Z, Qi Q, Lu L, Zhang G, Yu D, Zong G, Zhou Y, Chen X, Tang L, Feng Y, Zhou H, Li H, Demark-Wahnefried W, Hu FB, Lin X. (**2010**) Lifestyle counseling and supplementation with flaxseed or walnuts influence the management of metabolic syndrome. *Journal of Nutrition*, ***140***, 1937-1942.

[30] Smeds AI, Willför SM, Pietarinen SP, Peltonen-Sainio P, Reunanen MH. (**2007**) Occurrence of "mammalian" lignans in plant and water sources. *Planta*, ***226***, 639-646.

[31] Brouns F, Hemery Y, Price R, Anson NM. (**2012**) Wheat aleurone: separation, composition, health aspects, and potential food use. *Critical Review in Food Science and Nutrition*, ***52***, 553-568.

[32] van der Kamp JW, Poutanen K, Seal CJ, Richardson DP. (**2014**) The HEALTHGRAIN definition of 'whole grain'. *Food and Nutrition Research*, ***58***, 22100.

[33] Cukelj N, Jakasa I, Sarajlija H, Novotni D, Curić D. (**2011**) Identification and quantification of lignans in wheat bran by gas chromatography-electron capture detection. *Talanta*, ***84***, 127-132.

[34] Smeds AI, Saarinen NM, Eklund PC, Sjöholm RE, Mäkelä SI. (**2005**) New lignan metabolites in rat urine. *Journal of Chromatography B Analytical Technology in Biomedical and Life Science*, ***816***, 87-97.

[35] Dinelli G, Segura-Carretero A, Di Silvestro R, Marotti I, Arráez-Román D, Benedettelli S, Ghiselli L, Fernadez-Gutierrez A. (**2011**) Profiles of phenolic compounds in modern and old common wheat varieties determined by liquid chromatography coupled with time-of-flight mass spectrometry. *Journal of Chromatography A*, ***1218***, 7670-7681.

[36] Mazur W. (**1998**) Phytoestrogen content in foods. Baillieres *Clinical Endocrinology and Metabolism*, ***12***, 729-742.

[37] Bondia -Pons I, Barri T, Hanhineva K, Juntunen K, Dragsted LO, Mykkänen H, Poutanen K. (**2013**) UPLC-QTOF/MS metabolic profiling unveils urinary changes in humans after a whole grain rye versus refined wheat bread intervention. *Molecular Nutrition and Food Research*, ***57***, 412-422.

[38] Niemi P, Tamminen T, Smeds A, Viljanen K, Ohra-aho T, Holopainen-Mantila U, Faulds CB, Poutanen K, Buchert J. (**2012**) Characterization of lipids and lignans in brewer's spent grain and its enzymatically extracted fraction. *Journal of Agricultural and Food Chemistry*, ***60***, 9910-9917.

[39] Anson NM, Selinheimo E, Havenaar R, Aura AM, Mattila I, Lehtinen P, Bast A, Poutanen K, Haenen GR. (**2009**) Bioprocessing of wheat bran improves *in vitro* bioaccessibility and colonic metabolism of phenolic compounds. *Journal of Agricultural and Food Chemistry*, ***57***, 6148-6155.

[40] Anson NM, Havenaar R, Vaes W, Coulier L, Venema K, Selinheimo E, Bast A, Haenen GR. (**2011**) Effect of bioprocessing of wheat bran in wholemeal wheat breads on the colonic SCFA production *in vitro* and postprandial plasma concentrations in men. *Food Chemistry*, ***128***, 404-409.

[41] Wang Y, Yu Q, Chen Y, Cao F. (**2013**) Pathophysiology and therapeutics of cardiovascular disease in metabolic syndrome. *Current Pharmaceutical Design*, ***19***, 4799-4805.

[42] Ehrhardt MJ, Mulrooney DA. (**2015**) Metabolic syndrome in adult survivors of childhood cancer: the intersection of oncology, endocrinology, and cardiology. *Lancet Diabetes and Endocrinolology*, ***3***, 494-496.

[43] Tamayo T, Rosenbauer J, Wild SH, Spijkerman AM, Baan C, Forouhi NG, Herder C, Rathmann W. (**2014**) Diabetes in Europe: an update. *Diabetes Research and Clinical Practice*, ***103***, 206-217.

[44] 2003 RoaJWFEC (**2003**) Diet, nutrition and the prevention of chronic disease. In Serie WTR (Ed) Geneva.

[45] Communities. GPoCotE (**2005**) Promoting healthy diets and physical activity: a European dimension for the prevention of the obesity and chronic disease. In Protection DGHaC, (Ed) Brussels.

[46] Level. WpopcaC. (**2007**) A strategy of Europe on nutrition, overweight and obesity related health issues. Brussels.

[47] Struja T, Richard A, Linseisen J, Eichholzer M, Rohrmann S. (**2014**) The association between urinary phytoestrogen excretion and components of the metabolic syndrome in NHANES. *European Journal of Nutrition*, ***53***, 1371-1381.

[48] Zamora-Ros R, Knaze V, Luján-Barroso L, Kuhnle GG, Mulligan AA, Touillaud M, Slimani N, Romieu I, Powell N, Tumino R, Peeters PH, de Magistris MS, Ricceri F, Sonestedt E, Drake I, Hjartåker A, Skie G, Mouw T, Wark PA, Romaguera D, Bueno-de-Mesquita HB, Ros M, Molina E, Sieri S, Quirós JR, Huerta JM, Tjønneland A, Halkjær J, Masala G, Teucher B, Kaas R, Travis RC, Dilis V, Benetou V, Trichopoulou A, Amiano P, Ardanaz E, Boeing H, Förster J, Clavel-Chapelon F, Fagherazzi G, Perquier F, Johansson G, Johansson I, Cassidy A, Overvad K, González CA. (**2012**) Dietary intakes and food sources of phytoestrogens in the European Prospective Investigation into Cancer and Nutrition (EPIC) 24-hour dietary recall cohort. *European Journal of Clinical Nutrition*, ***66***, 932-941.

[49] Zamora-Ros R, Fedirko V, Trichopoulou A, González CA, Bamia C, Trepo E, Nöthlings U, Duarte-Salles T, Serafini M, Bredsdorff L, Overvad K, Tjønneland A, Halkjaer J, Fagherazzi G, Perquier F, Boutron-Ruault MC, Katzke V, Lukanova A, Floegel A, Boeing H, Lagiou P, Trichopoulos D, Saieva C, Agnoli C, Mattiello A, Tumino R, Sacerdote C, Bueno-de-Mesquita HB, Peeters PH, Weiderpass E, Engeset D, Skeie G, Argüelles MV, Molina-Montes E, Dorronsoro M, Tormo MJ, Ardanaz E, Ericson U, Sonestedt E, Sund M, Landberg R, Khaw KT, Wareham NJ, Crowe FL, Riboli E, Jenab M. (**2013**) Dietary flavonoid, lignan and antioxidant capacity and risk of hepatocellular carcinoma in the European prospective investigation into cancer and nutrition study. *International Journal of Cancer*, ***133***, 2429-2443.

[50] Zamora-Ros R, Ferrari P, González CA, Tjønneland A, Olsen A, Bredsdorff L, Overvad K, Touillaud M, Perquier F, Fagherazzi G, Lukanova A, Tikk K, Aleksandrova K, Boeing H, Trichopoulou A, Trichopoulos D, Dilis V, Masala G, Sieri S, Mattiello A, Tumino R, Ricceri F, Bueno-de-Mesquita HB, Peeters PH, Weiderpass E, Skeie G, Engeset D, Menéndez V, Travier N, Molina-Montes E, Amiano P, Chirlaque MD, Barricarte A, Wallström P, Sonestedt E, Sund M, Landberg R, Khaw KT, Wareham NJ, Travis RC, Scalbert A, Ward HA, Riboli E, Romieu I. (**2013**) Dietary flavonoid and lignan intake and breast cancer risk according to menopause and hormone receptor status in the European Prospective Investigation into Cancer and Nutrition (EPIC) Study. *Breast Cancer Research and Treatment*, ***139***, 163-176.

[51] Zamora-Ros R, Sacerdote C, Ricceri F, Weiderpass E, Roswall N, Buckland G, St-Jules DE, Overvad K, Kyrø C, Fagherazzi G, Kvaskoff M, Severi G, Chang-Claude J, Kaaks R, Nöthlings U, Trichopoulou A, Naska A, Trichopoulos D, Palli D, Grioni S, Mattiello A, Tumino R, Gram IT, Engeset D, Huerta JM, Molina-Montes E, Argüelles M, Amiano P, Ardanaz E, Ericson U, Lindkvist B, Nilsson LM, Kiemeney LA, Ros M, Bueno-de-Mesquita HB, Peeters PH, Khaw KT, Wareham NJ, Knaze V, Romieu I, Scalbert A, Brennan P, Wark P, Vineis P, Riboli E, González CA. (**2014**) Flavonoid and lignan intake in relation to bladder cancer risk in the European Prospective Investigation into Cancer and Nutrition (EPIC) study. *British Journal of Cancer*, ***111***, 1870-1880.

[52] Ward HA, Kuhnle GG. (**2010**) Phytoestrogen consumption and association with breast, prostate and colorectal cancer in EPIC Norfolk. *Archives of Biochemistry and Biophysics*, ***501***, 170-175.

[53] Ward HA, Kuhnle GG, Mulligan AA, Lentjes MA, Luben RN, Khaw KT. (**2010**) Breast, colorectal, and prostate cancer risk in the European Prospective Investigation into Cancer and Nutrition-Norfolk in relation to phytoestrogen intake derived from an improved database. *American Journal of Clinical Nutrition*, ***91***, 440-448.

[54] Mulligan AA, Kuhnle GG, Lentjes MA, van Scheltinga V, Powell NA, McTaggart A, Bhaniani A, Khaw KT. (**2013**) Intakes and sources of isoflavones, lignans, enterolignans, coumestrol and soya-containing foods in the Norfolk arm of the European Prospective Investigation into Cancer and Nutrition (EPIC-Norfolk), from 7 d food diaries, using a newly updated database. *Public Health Nutrition*, ***16***, 1454-1462.

[55] Xu C, Liu Q, Zhang Q, Jiang ZY, Gu A. (**2015**) Urinary enterolactone associated with liver enzyme levels in US adults: National Health and Nutrition Examination Survey (NHANES). *British Journal of Nutrition*, ***114***, 91-97.

[56] Fukumitsu S, Aida K, Shimizu H, Toyoda K. (**2010**) Flaxseed lignan lowers blood cholesterol and decreases liver disease risk factors in moderately hypercholesterolemic men. *Nutrition Research*, ***30***, 441-446.

[57] Narasimhulu CA, Selvarajan K, Litvinov D, Parthasarathy S. (**2015**) Anti-atherosclerotic and anti-inflammatory actions of sesame oil. *Journal of Medicinal Food*, ***18***, 11-20.

[58] Zhang W, Wang X, Liu Y, Tian H, Flickinger B, Empie MW, Sun SZ. (**2008**) Dietary flaxseed lignan extract lowers plasma cholesterol and glucose concentrations in hypercholesterolaemic subjects. *British Journal of Nutrition*, ***99***, 1301-1309.

[59] Hong L, Yi W, Liangliang C, Juncheng H, Qin W, Xiaoxiang Z. (**2013**) Hypoglycaemic and hypolipidaemic activities of sesamin from sesame meal and its ability to ameliorate insulin resistance in KK-Ay mice. *Journal of the Science of Food and Agriculture*, ***93***, 1833-1838.

[60] Kim MH, Choi YY, Han JM, Lee HS, Hong SB, Lee SG, Yang WM. (**2014**) Ameliorative effects of *Schizandra chinensis* on osteoporosis via activation of estrogen receptor (ER)-α/-β. *Food and Function*, ***5***, 1594-1601.

[61] Wan Y, Li H, Fu G, Chen X, Chen F, Xie M. (**2015**) The relationship of antioxidant components and antioxidant activity of sesame seed oil. *Journal of the Science of Food and Agriculture*, ***95***, 2571-2578.

[62] Soory M. (**2012**) Nutritional antioxidants and their applications in cardiometabolic diseases. *Infectious Disorders - Drug Targets*, ***12***, 388-401.

[63] Zhang X, Xu X, Xu T, Qin S. (**2014**) β-Ecdysterone suppresses interleukin-1β-induced apoptosis and inflammation in rat chondrocytes via inhibition of NF-κB signaling pathway. *Drug Development Research*, ***75***, 195-201.

[64] Spilioti E, Holmbom B, Papavassiliou AG, Moutsatsou P. (**2014**) Lignans 7-hydroxymatairesinol and 7-hydroxymatairesinol 2 exhibit anti-inflammatory activity in human aortic endothelial cells. *Molecular Nutrition and Food Research*, ***58***, 749-759.

[65] Freise C, Querfeld U. (**2015**)The lignan (+)-episesamin interferes with TNF-α-induced activation of VSMC via diminished activation of NF-κB, ERK1/2 and AKT and decreased activity of gelatinases. *Acta Physiologica (Oxford)*, ***213***, 642-652.

[66] Dikshit A, Gomes Filho MA, Eilati E, McGee S, Small C, Gao C, Klug T, Hales DB. (**2015**) Flaxseed reduces the pro-carcinogenic micro-environment in the ovaries of normal hens by altering the PG and oestrogen pathways in a dose-dependent manner. *British Journal of Nutrition*, ***113***, 1384-1395.

[67] Fukumitsu S, Aida K, Ueno N, Ozawa S, Takahashi Y, Kobori M. (**2008**) Flaxseed lignan attenuates high-fat diet-induced fat accumulation and induces adiponectin expression in mice. *British Journal of Nutrition*, ***100***, 669-676.

[68] Yang S, Yang D, Hu K, Zhou H, Guo Y, Du G, Lu Y. (**2015**) Certification of a new certified reference material of honokiol. *Analytical Bioanalytical Chemistry*, ***407***, 5849-5855.

[69] Atanasov AG, Wang JN, Gu SP, Bu J, Kramer MP, Baumgartner L, Fakhrudin N, Ladurner A, Malainer C, Vuorinen A, Noha SM, Schwaiger S, Rollinger JM, Schuster D, Stuppner H, Dirsch VM, Heiss EH. (**2013**) Honokiol: a non-adipogenic PPARγ agonist from nature. *Biochimica et Biophysica Acta*, ***1830***, 4813-4819.

[70] Jung CG, Horike H, Cha BY, Uhm KO, Yamauchi R, Yamaguchi T, Hosono T, Iida K, Woo JT, Michikawa M. (**2010**) Honokiol increases ABCA1 expression level by activating retinoid X receptor beta. *Biological and Pharmaceutical Bulletin*, ***33***, 1105-1111.

[71] Jeon JS, Oh SJ, Lee JY, Ryu CS, Kim YM, Lee BH, Kim SK. (**2015**) Metabolic characterization of meso-dihydroguaiaretic acid in liver microsomes and in mice. *Food Chemical and Toxicology*, ***76***, 94-102.
[72] Periasamy S, Hsu DZ, Chang PC, Liu MY. (**2014**) Sesame oil attenuates nutritional fibrosing steatohepatitis by modulating matrix metalloproteinases-2, 9 and PPAR-γ. The *Journal of Nutritional Biochemestry*, ***25***, 337-344.
[73] Hsieh CC, Kuo CH, Kuo HF, Chen YS, Wang SL, Chao D, Lee MS, Hung CH. (**2014**) Sesamin suppresses macrophage-derived chemokine expression in human monocytes via epigenetic regulation. *Food and Function*, ***5***, 2494-2500.
[74] Ogungbe IV, Crouch RA, Demeritte T. (**2014**) (-) Arctigenin and (+) pinoresinol are antagonists of the human thyroid hormone receptor β. *Journal of Chemical Information and Modeling*, ***54***, 3051-3055.
[75]] Jiang D, Ma S, Jing F, Xu C, Yan F, Wang A, Zhao J. (**2015**) Thyroid-stimulating hormone inhibits adipose triglyceride lipase in 3T3-L1 adipocytes through the PKA pathway. *PLoS One **10***, e0116439.
[76] Jacobs MN, Nolan GT, Hood SR. (**2005**) Lignans, bacteriocides and organochlorine compounds activate the human pregnane X receptor (PXR). *Toxicology and Applied Pharmacology*, ***209***, 123-133.
[77] Biasiotto G, Penza M, Zanella I, Cadei M, Caimi L, Rossini C, Smeds AI, Di Lorenzo D. (**2014**) Oilseeds ameliorate metabolic parameters in male mice, while contained lignans inhibit 3T3-L1 adipocyte differentiation *in vitro*. *European Journal of Nutrition*, ***53***, 1685-1697.
[78] Jennewein C, Kuhn AM, Schmidt MV, Meilladec-Jullig V, von Knethen A, Gonzalez FJ, Brüne B. (**2008**) Sumoylation of peroxisome proliferator-activated receptor gamma by apoptotic cells prevents lipopolysaccharide-induced NCoR removal from kappaB binding sites mediating transrepression of proinflammatory cytokines. *Journal of Immunology*, ***181***, 5646-5652.
[79] Lin CH, Shen ML, Zhou N, Lee CC, Kao ST, Wu DC. (**2014**) Protective effects of the polyphenol sesamin on allergen-induced T(H)2 responses and airway inflammation in mice. *PLoS One*, ***9***, e96091.
[80] Rossi M, Amaretti A, Leonardi A, Raimondi S, Simone M, Quartieri A. (**2013**) Potential impact of probiotic consumption on the bioactivity of dietary phytochemicals. *Journal of Agricultural and Food Chemestry*, ***61***, 9551-9558.
[81] Zhu L, Ge G, Zhang H, Liu H, He G, Liang S, Zhang Y, Fang Z, Dong P, Finel M, Yang L. (**2012**) Characterization of hepatic and intestinal glucuronidation of magnolol: application of the relative activity factor approach to decipher the contributions of multiple UDP-glucuronosyltransferase isoforms. *Drug Metabolism and Disposition*, ***40***, 529-538.
[82] Jin JS, Hattori M. (**2010**) Human intestinal bacterium, strain END-2 is responsible for demethylation as well as lactonization during plant lignan metabolism. *Biological and Pharmaceutical Bulletin*, ***33***, 1443-1447.
[83] Jan KC, Chang YW, Hwang LS, Ho CT. (**2012**) Tissue distribution and cytochrome P450 inhibition of sesaminol and its tetrahydrofuranoid metabolites. *Journal of Agricultural and Food Chemestry*, ***60***, 8616-8623.
[84] Davis CD, Milner JA. (**2009**) Gastrointestinal microflora, food components and colon cancer prevention. *Journal of Nutritional Biochemistry*, ***20***, 743-752.
[85] Mukker JK, Singh RS, Muir AD, Krol ES, Alcorn J. (**2015**) Comparative pharmacokinetics of purified flaxseed and associated mammalian lignans in male Wistar rats. *British Journal of Nutrition*, ***113***, 749-757.
[86] Hullar MA, Lancaster SM, Li F, Tseng E, Beer K, Atkinson C, Wähälä K, Copeland WK, Randolph TW, Newton KM, Lampe JW. (**2015**) Enterolignan-producing phenotypes are associated with increased gut microbial diversity and altered composition in premenopausal women in the United States. *Cancer Epidemiological, Biomarkers and Prevention*, ***24***, 546-554.
[87] Woting A, Clavel T, Loh G, Blaut M. (**2010**) Bacterial transformation of dietary lignans in gnotobiotic rats. *FEMS Microbiology Ecology*, ***72***, 507-514.
[88] Eichholzer M, Richard A, Nicastro HL, Platz EA, Linseisen J, Rohrmann S. (**2014**) Urinary lignans and inflammatory markers in the US National Health and Nutrition Examination Survey (NHANES) 1999-2004 and 2005-2008. *Cancer Causes and Control*, ***25***, 395-403.
[89] Zhang J, Chen J, Liang Z, Zhao C. (**2014**) New lignans and their biological activities. *Chemistry and Biodiversity*, ***11***, 1-54.
[90] Gil A, Ortega RM, Maldonado J. (**2011**) Wholegrain cereals and bread: a duet of the Mediterranean diet for the prevention of chronic diseases. *Public Health Nutrition*, ***14***, 2316-2322.

NPC Natural Product Communications

2017
Vol. 12
No. 1
147 - 150

The Rare Amino Acid Building Block 3-(3-furyl)-Alanine in the Formation of Non-ribosomal Peptides

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Received: July 13th, 2016; Accepted: September 13th, 2016

Microorganisms have made considerable contributions to the production of peptide secondary metabolites, many of them with therapeutic potential, *e.g.*, the fungus-derived immunosuppressant cyclosporine A and the antibiotic daptomycin originating from *Streptomyces*. Most of the medically used peptides are the product of non-ribosomal peptide synthetases (NRPS), incorporating apart from proteinogenic also unique, non-proteinogenic amino acids into the peptides. An extremely rare such amino acid is 3-(3-furyl)-alanine. So far, only few peptides have been found that contain this residue, including the rhizonins, bingchamide B and endolides. The producer of the rhizonins was proven to be the bacterial endosymbiont *Burkholderia endofungorum* inside the fungus *Rhizopus microsporus*. The microbial origin, chemistry and bioactivity of the 3-(3-furyl)-alanine containing peptides are the focus of this review.

Keywords: Peptides, NRPS, Furyl-alanine, Endolide.

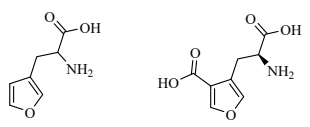
Among the huge class of secondary metabolites, peptides are a restricted group of structurally diverse compounds produced by prokaryotes like bacteria, and eukaryotes, *e.g.* fungi [1]. They are often essential for the survival and growth of the microbial producers in the rivalry of their natural habitat, acting as siderophores, toxins or signaling molecules [2]. Many of these peptides have important medical applications as antibiotics, *e.g.*, daptomycin, and immunosuppressants, *e.g.*, cyclosporine A [3]. In terms of biosynthesis, some of these peptides originate from ribosome-derived precursor molecules and gain their final structure by post-translational modifications, *e.g.*, microcins, microviridins and polytheonamides [4]. A second group of these peptides, including most of the medically used ones, are produced by non-ribosomal peptide synthetases (NRPS), which function as multi-modular enzymes connecting small building blocks, predominantly amino acids, but in some cases also carboxy acids [5]. The latter group often contains unique, non-proteinogenic amino acids, such as hydroxyphenylglycines in vancomycin and (4*R*)-4-[(*E*)-2-butenyl]-4-methyl-L-threonine in cyclosporine A [2]. Over 500 different amino acids are known to date, either found as partial structures in peptides, as free low molecular weight natural products, or are metabolic intermediates [6]. 3-(3-Furyl)-alanine (**1**) is such a non-proteinogenic, heterocyclic amino acid incorporated in non-ribosomal peptides, but to date not encountered free in nature (Figure 1). In contrast, the 3-(3-furyl)-alanine carboxy derivative, L-3-(3-carboxyfuran-4-yl)-alanine (**2**) was isolated from the fruiting bodies of the gilled non-edible mushrooms *Phyllotopsis nidulans* and *Tricholomopsis rutilans* (Figure 1) [7,8]. For this component no biological activity has been described to date. Chemically synthesized L-3-(3-furyl)- and L-3-(2-furyl)-alanine, however, exhibit growth inhibitory activity towards both bacteria and fungi [9], making them potentially useful agrochemical fungicides, industrial microbicides and wood preservatives [10].

3-(3-Furyl)-alanine as a building block in bioactive natural products: Even though 3-(3-furyl)-alanine is so far not known as an unbound amino acid, fungi and bacteria incorporate this residue into peptide structures with diverse bioactivities (Figure 2, Table 1).

Table 1: Peptides incorporating 3-(3-furyl)-alanine.

Source	Peptide	Activity
Burkholderia endofungorum (bacterial)	Rhizonin A, B (**3**,**4**)	Hepatotoxic effects in rats [15] and plant growth promoting activity [17]
Streptomyces bingchenggensis (bacterial)	Bingchamide B (**5**)	*In vitro* cytotoxic activity towards human colon cancer cell line HCT-116 (IC_{50}= 18 µg mL^{-1}) [18]
Stachylidium sp. (fungal)	Endolide A (**6**)	Affinity to the vasopressin receptor 1A (K_i = 7.04 µM) [19]
	Endolide B (**7**)	Affinity to the serotonin receptor subtype $5HT_{2b}$ (K_i = 0.77 µM) [19]

The first described naturally occurring peptides containing 3-(3-furyl)-alanine were rhizonin A (**3**) and B (**4**), at that time isolated from *Rhizopus microsporus*, a fungus used to prepare fermented food [11]. Interestingly, the two 3-(3-furyl)-alanine units in rhizonin A possess opposite absolute configurations as determined from X-ray crystallography. The rhizonins were regarded as the first mycotoxins isolated from fungi (Zygomycota), with most known mycotoxin producing strains belong to the fungal phyla Ascomycota or Basidiomycota [12]. It was later found that the true producer of rhizonins is in fact the bacterial endosymbiont *Burkholderia endofungorum,* which resides inside the fungal cytosol [13]. This was also true for the polyketide-derived antimitotic macrolide rhizoxin, initially believed to be a product of *R. microsporus* but later discovered to be produced by *Burkholderia rhizoxinica* [14].



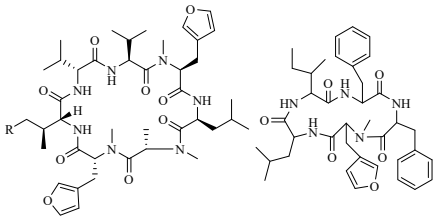
3-(3-furyl)-alanine (**1**) L-3-(3-carboxyfuran-4-yl)-alanine (**2**)

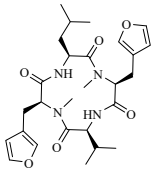
Figure 1: 3-(3-Furyl)-alanine and its carboxy-derivative.

Rhizonin A exhibited potent hepatotoxic effects in rats [15], and cytotoxicity, which was mainly ascribed to its 3-(3-furyl)-alanine partial structure. Synthetic replacement of this moiety with phenylalanine gave analogues **8** and **9** with reduced cytotoxicity and

improved *in vitro* fat-accumulation inhibitory activity in 3T3-L1 murine adipocytes [16]. Controversially, rhizonin A showed plant growth promoting activity in lettuce seed germination, and thus, the rhizobacterial strain *Burkholderia* sp. KCTC11096 is to be explored for supporting sustainable agricultural production [17].

The second report of a 3-(3-furyl)-alanine building block is related to the cyclic pentapeptide, bingchamide B (**5**), isolated from a soil-dwelling strain of *Streptomyces bingchenggensis* [18]. The absolute configuration of the amino acid building blocks of bingchamide B, including 3-(3-furyl)-alanine, was not determined. Bingchamides exhibited *in vitro* cytotoxic activity towards human colon cancer cell line HCT-116 with an IC_{50} value of 18 μg mL^{-1} for bingchamide B and an IC_{50} value of 14.1 μg mL^{-1} for its natural analogue bingchamide A (**10**), featuring a phenylalanine residue in place of the 3-(3-furyl)-alanine [18].





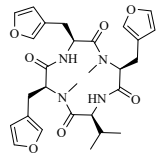
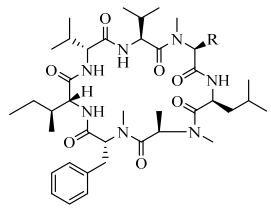
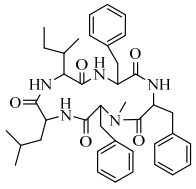


Figure 2: Peptides featuring a 3-(3-furyl)-alanine amino acid residue.





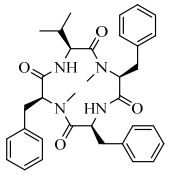


Figure 3: Bioactive peptide analogues featuring phenylalanine amino acid residues.

Recently, the novel tetrapeptides endolide A (**6**) and B (**7**) were isolated from a solid culture of the marine sponge-derived fungus *Stachylidium* sp. [19]. Here, the two 3-(3-furyl)-alanine units in endolide A were assigned the L-configuration as determined from X-ray crystallography and Marfey's analysis. Hitherto the endolides were regarded as the first fungal peptides containing 3-(3-furyl)-alanine. Whether the endolides are presumably produced by an endofungal bacterium, as in the case of rhizonins, could not be unequivocally proven, as it was not yet possible to isolate and culture axenic bacteria from the *Stachylidium* sp. fungus [19]. Unlike the other 3-(3-furyl)-alanine containing peptides rhizonin A and bingchamide B, the endolides did not exhibit any *in vitro* cytotoxicity. Interestingly, they have affinity to G protein-coupled receptors (GPCRs) in radioligand binding assays. Endolide A showed affinity to the vasopressin receptor 1A with a K_i of 7.04 μM, while endolide B had selective affinity to the serotonin receptor subtype $5HT_{2b}$ with a K_i of 0.77 μM [19].

A structural analogue of the endolides, hirsutide (**11**), was isolated from the entomopathogenic fungus *Hirsutella* sp. Hirsutide features phenylalanine residues instead of the 3-(3-furyl)-alanine residues in endolide B [20]. Initially no bioactivities were reported for hirsutide. However, its recent chemical synthesis and detailed pharmacological screening revealed potent antibacterial activity against the Gram-negative bacteria *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, with additional anthelmintic activity and minor cytotoxic effects against Dalton's lymphoma ascites and Ehrlich's ascites carcinoma cell lines with IC_{50} values of 14 and 22 μM, respectively [21].

Putative biosynthesis of peptides and 3-(3-furyl)-alanine: Peptide secondary metabolites are assembled either through the ribosomal machinery, *i.e.*, through direct gene translation [4], or by multi-modular non-ribosomal peptide synthetases (NRPS) [2]. In the translation system, decoding of the trinucleotide sequence (codon) information stored in the messenger RNA determines the peptide amino acid sequence. Here each codon assigns a single amino acid from the known pool of 20 L-configured proteinogenic amino acids [22].

On the other hand, NRPS assembly lines are divided into modules termed initiation and elongation modules, where each module is responsible for integrating a dedicated substrate into the growing peptide chain. This is done with the help of catalytic domains, a sub-division of each module. Fundamental domains are indispensable for the selection and activation of the building blocks (adenylation, A-domain), the tethering of the product to the enzyme (peptidyl carrier protein, PCP-domain) and peptide bond formation (condensation, C-domain). Structural diversity arises due to the presence of auxiliary domains imparting unique structural features to the non-ribosomal peptides. These include epimerization (E) domains for incorporating D-amino acids, methylation (Mt) domains for introducing methyl groups and oxidation (Ox) and reduction (R) domains for the formation of heterocyclic rings. Finally, a terminal thioesterase (TE) domain catalyzes peptide release and at times cyclization [23].

A closer look into the structural architecture of the rhizonins, bingchamides and endolides proposes a non-ribosomal biosynthetic origin. This is implied due to the incorporated amino acid residues, *i.e.*, *N*-methylated, sometimes D-configured, and structurally unusual non-proteinogenic in nature, *i.e.*, 3-(3-furyl)-alanine. For the rhizonins, total DNA isolated from the cultured *B. endofungorum* afforded NRPS gene fragments. However, the exact NRPS genes involved in the biosynthesis of rhizonins were not yet revealed and further studies are underway [13]. Analysis of the *S. bingchenggensis* genome yielded gene clusters postulated to encode NRPSs. These include modules with adenylation domain substrate specificities, correlating with the structures of bingchamides [24]. However, no proof for a responsible biosynthetic gene cluster is currently available.

In NRP biosynthesis, not only the multi-modular NRPS assembly line is of importance, but also the formation of the building blocks. Often the respective enzymes are encoded as part of or adjacent to

the biosynthetic gene cluster [25,26]. So far, no biosynthetic studies were conducted to reveal the biosynthetic origin of 3-(3-furyl)-alanine, apart from it being considered a potential substrate for the NRPS biosynthetic machinery. Taking known biosynthetic pathways for similar furans into consideration, several pathways for 3-(3-furyl)-alanine may be proposed here.

A furan ring as such is encountered in many secondary metabolites. In terpenoids, furan ring biosynthesis utilizes active isoprene units, *i.e.*, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), as seen in the diterpenoids divinatorins (**12**) and bibifuran (**13**) [27,28]. The signaling molecules methylenomycin furans (MMFs) (**14**) and the furanylcarbonyl containing acyl rhamnosides (**15**) are formed by a mixed acetate-glycerol pathway [29-32]. The latter involves the condensation of a dihydroxyacetone-derived three-carbon unit originating from glycerol and a β-ketothioester intermediate derived from a polyketide synthase or fatty acid metabolism (Figure 5). In contrast, the hydroxyfuranone (**16**) and methoxyfuranone (**17**), volatile strawberry aroma compounds, are obtained from carbohydrate metabolism, *i.e.*, from D-fructose [33].

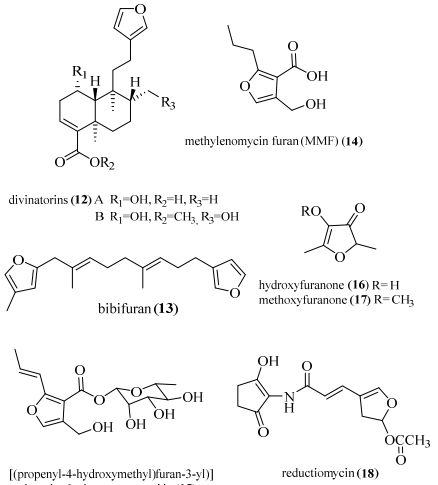


Figure 4: Selected metabolites featuring a furan moiety.

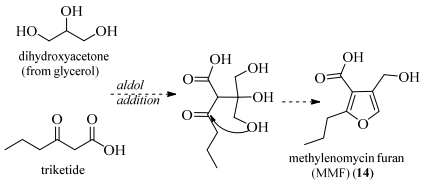


Figure 5: Biosynthesis of methylenomycin furan through a mixed acetate-glycerol pathway, adopted from literature [27,30].

However, the association of the furan skeleton with an amino acid, *i.e.*, alanine, implies other precursor molecules to be involved in its biosynthesis. 3-(3-Furyl)-alanine is an analog of the shikimate-derived aromatic amino acid phenylalanine [34]. Thus, the structural relationship between the 3-(3-furyl)-alanine and the phenylalanine skeleton, may point to the possibility of furyl-alanine being formed by ring cleavage of a phenylalanine intermediate. Such a ring cleavage is known for several metabolites such as the mycotoxin patulin [35], the betalain plant pigments [36], and pyrrolobenzodiazepine anti-tumor antibiotics [37]. Feeding of isotopically labeled phenylalanine to the respective microbial cultures could clarify this presumption.

On the other side however, the unique dihydrofuranacrylic acid moiety of the antibiotic reductiomycin (**18**), a *Streptomyces* metabolite, also shares the basic carbon skeleton with 3-(3-furyl)-alanine [38]. In this case, labeling studies showed that the dihydrofuranacrylic acid moiety is derived from a symmetrical product of the shikimate pathway, *i.e.*, *p*-hydroxybenzoic acid, by aromatic ring cleavage (Figure 6) [39].

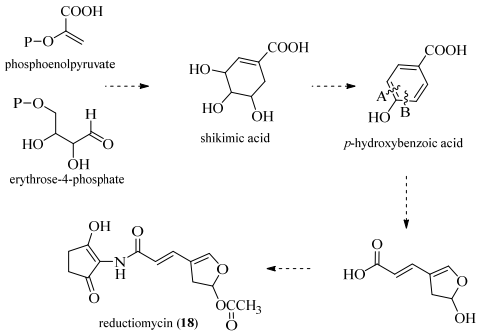


Figure 6: Biosynthesis of the dihydrofuranacrylic acid moiety of reductiomycin via the shikimate pathway, adopted from literature [38,39]. Cleavage occurs either at site A, or alternatively at B, resulting in different isotope labeling patterns (not shown here).

Which metabolic pathway underlies the biosynthesis of the unique 3-(3-furyl)-alanine moiety in the peptides, and whether all the peptides incorporating such a moiety have a similar biosynthetic origin is yet to be revealed.

Conclusion: Microorganisms have made considerable contributions to the production of peptide secondary metabolites, many of them with therapeutic potential, *e.g.*, the immunosuppressant cyclosporine A [40] and the antibiotic daptomycin [41]. Indeed, peptides that occur in nature as secondary metabolites share structural features like a cyclic architecture, *N*-methylation of amide bonds, and incorporation of atypical building blocks, such as D-amino acids, carboxy acids and non-proteinogenic amino acids. Such features bestow several attractive attributes in terms of receptor affinity and selectivity and improved proteolytic stability, making them interesting drug scaffolds [3]. With the ongoing interest in peptide-based drugs [42], 3-(3-furyl)-alanine containing peptides add an interesting structural variation to this group of natural products. However, a deeper understanding of their biosynthesis and bioactivities should be sought, in order to be implemented in the drug-design process.

Acknowledgments - Financial support came from the Ministry of Higher Education of the Government of Egypt.

References

[1] Uhlig T, Kyprianou T, Martinelli FG, Oppici CA, Heiligers D, Hills D, Calvo XR, Verhaert P. (**2014**) The emergence of peptides in the pharmaceutical business: From exploration to exploitation. *EuPA Open Proteomics*, ***4***, 58-69.

[2] Schwarzer D, Finking R, Marahiel MA. (**2003**) Nonribosomal peptides: from genes to products. *Natural Product Reports*, ***20***, 275-287.

[3] Passioura T, Katoh T, Goto Y, Suga H. (**2014**) Selection-based discovery of drug-like macrocyclic peptides. *Annual Review of Biochemistry*, ***83***, 727-752.

[4] Arnison PG, Bibb MJ, Bierbaum G, Bowers AA, Bugni TS, Bulaj G, Camarero JA, Campopiano DJ, Challis GL, Clardy J, Cotter PD, Craik DJ, Dawson M, Dittmann E, Donadio S, Dorrestein PC, Entian KD, Fischbach MA, Garavelli JS, Göransson U, Gruber CW, Haft DH, Hemscheidt TK, Hertweck C, Hill C, Horswill AR, Jaspars M, Kelly WL, Klinman JP, Kuipers OP, Link AJ, Liu W, Marahiel MA, Mitchell DA, Moll GN, Moore

BS, Müller R, Nair SK, Nes IF, Norris GE, Olivera BM, Onaka H, Patchett ML, Piel J, Reaney MJ, Rebuffat S, Ross RP, Sahl HG, Schmidt EW, Selsted ME, Severinov K, Shen B, Sivonen K, Smith L, Stein T, Süssmuth RD, Tagg JR, Tang GL, Truman AW, Vederas JC, Walsh CT, Walton JD, Wenzel SC, Willey JM, van der Donk WA.(**2013**) Ribosomally synthesized and post-translationally modified peptide natural products: overview and recommendations for a universal nomenclature. *Natural Product Reports*, ***30***, 108-160.

[5] Hur GH, Vickery CR, Burkart MD. (**2012**) Explorations of catalytic domains in non-ribosomal peptide synthetase enzymology. *Natural Product Reports*, ***29***, 1074-1098.

[6] Wagner I, Musso H. (**1983**) New naturally occurring amino acids. *Angewandte Chemie International Edition English*, ***22***, 816-828.

[7] Doyle RR, Levenberg B. (**1974**) L-3-(3-carboxyfuran-4-yl)alanine, a new amino acid from the mushroom *Phyllotopsis nidulans*. *Phytochemistry*, ***13***, 2813-2814.

[8] Hatanaka S, Niimura Y. (**1975**) L-3-(3-carboxy-4-furyl)alanine from *Tricholomopsis rutilans*. *Scientific Papers of the College of General Education, University of Tokyo*, ***25***, 35-37.

[9] Lewis DE, Dunn FW. (**1963**) β-3-Furylalanine, a new analogue of phenylalanine. *Archives of Biochemistry and Biophysics*, ***107***, 363-366.

[10] Aleu J, Bustillo AJ, Hernández-Galán R, Collado IG. (**2006**) Biocatalysis applied to the synthesis of agrochemicals. *Current Organic Chemistry*, ***10***, 2037-2054.

[11] Steyn PS, Tuinman AA, van Heerden FR, van Rooyen PH, Wessels PL, Rabie CJ. (**1983**) The isolation, structure, and absolute configuration of the mycotoxin, rhizonin A, a novel cyclic heptapeptide containing *N*-methyl-3-(3-furyl)alanine, produced by *Rhizopus microsporus*. *Journal of the Chemical Society, Chemical Communications*, 47-49.

[12] Hanson JR. The chemistry of fungi ©2008, RSC Publishing. PDF eISBN: 978-1-84755-832-9. DOI:10.1039/9781847558329.

[13] Partida-Martinez LP, de Looss CF, Ishida K, Ishida M, Roth M, Buder K, Hertweck C. (**2007**) Rhizonin, the first mycotoxin isolated from the Zygomycota, is not a fungal metabolite but is produced by bacterial endosymbionts. *Applied and Environmental Microbiology*, ***73***, 793–797.

[14] Partida-Martinez LP, Hertweck C. (**2007**) A gene cluster encoding rhizoxin biosynthesis in "*Burkholderia rhizoxinica*", the bacterial endosymbiont of the fungus *Rhizopus microsporus*. *ChemBioChem*, ***8***, 41-45.

[15] Wilson T, Rabie CS, Fincham, JE, Steyn PS, Schipper MA. (**1984**) Toxicity of rhizonin A, isolated from *Rhizopus microsporus*, in laboratory animals. *Food and Chemical Toxicology*, ***4***, 275-281.

[16] Shimokawa K, Yamada K, Uemura D. (**2009**) Structure-based hybridization of the bioactive natural product rhizonin A and ternatin leading to a selective fat-accumulation inhibitor against 3T3-L1 adipocytes. *Bioorganic and Medicinal Chemistry Letters*, ***19***, 867-869.

[17] Kang SM, Khan AL, Hussain J, Ali L, Kamran M, Waqas M, Lee I-J. (**2012**) Rhizonin A from *Burkholderia* sp. KCTC11096 and its growth promoting role in lettuce seed germination. *Molecules*, ***17***, 7980-7988.

[18] Xiang WS, Wang JD, Wang XJ, Zhang J. (**2009**) Bingchamides A and B, two novel cyclic pentapeptides from the *Streptomyces bingchenggensis*: Fermentation, isolation, structural elucidation and biological properties. *Journal of Antibiotics (Tokyo)*, ***62***, 501- 505.

[19] Almeida C, El Maddah F, Kehraus S, Schnakenburg G, König GM. (**2016**) Endolide A and B, vasopressin and serotonin-receptor interacting *N*-methylated peptides from the sponge-derived fungus *Stachylidium* sp. *Organic Letters*, ***18***, 528-531.

[20] Lang G, Blunt JW, Cummings NJ, Cole AL, Munro MH. (**2005**) Hirsutide, a cyclic tetrapeptide from a spider-derived entomopathogenic fungus, *Hirsutella* sp. *Journal of Natural Products*, ***68***, 1303-1305.

[21] Dahiya R, Maheshwari M, Kumar A. (**2009**) Towards the synthesis and biological evaluation of hirsutide. *Monatshefte Chemie*, ***140***, 121-127.

[22] Katoh T, Goto Y, Shamim Reza Md, Suga H. (**2011**) Ribosomal synthesis of backbone macrocyclic peptides. *Chemical Communications*, ***47***, 9946-9958.

[23] Marahiel MA. (**2009**) Working outside the protein-synthesis rules: Insights into non-ribosomal peptide synthesis. *Journal of Peptide Science*, ***15***, 799–807.

[24] Wang XJ, Yan YJ, Zhang B, An J, Wang JJ, Tian J, Jiang L, Chen YH, Huang SX, Yin M, Zhang J, Gao AL, Liu CX, Zhu ZX, Xiang WS. (**2010**) Genome sequence of the milbemycin-producing bacterium *Streptomyces bingchenggensis*. *Journal of Bacteriology*, 4526-4527.

[25] Al Toma RS, Brieke C, Cryle MJ, Süssmuth RD. (**2015**) Structural aspects of phenylglycines, their biosynthesis and occurrence in peptide natural products. *Natural Product Reports*, ***32***, 1207-1235.

[26] Du L, Sánchez C, Shen B. (**2001**) Hybrid peptide-polyketide natural products: biosynthesis and prospects toward engineering novel molecules. *Metabolic Engineering*, ***3***, 78-95.

[27] Kutrzeba L, Dayan FE, Howell J, Feng J, Giner JL, Zjawiony JK. (**2007**) Biosynthesis of salvinorin A proceeds via the deoxyxylulose phosphate pathway. *Phytochemistry*, ***68***, 1872-1881.

[28] Göthel Q, Muñoz J, Köck M. (**2012**) Formyleleganolone and bibifuran, two metabolites from the brown alga *Bifurcaria bifurcata*. *Phytochemistry Letters*, ***5***, 693-695.

[29] Corre C, Haynes SW, Malet N, Song L, Challis GL. (**2010**) A butenolide intermediate in methylenomycin furan biosynthesis is implied by incorporation of stereospecifically ^{13}C-labelled glycerols. *Chemical Communications*, ***46***, 4079-4081.

[30] Corre C, Song L, O'Rourke S, Chater KF, Challis GL. (**2008**) 2-Alkyl-4-hydroxymethylfuran-3-carboxylic acids, antibiotic production inducers discovered by *Streptomyces coelicolor* genome mining. *Proceedings of the National Academy of Sciences*, ***105***, 17510-17515.

[31] Grond S, Langer HJ, Henne P, Sattler I, Thiericke R, Grabley S, Zähner H, Zeeck A. (**2000**) Secondary metabolites by chemical screening, 39 acyl α-L-rhamnopyranosides, a novel family of secondary metabolites from *Streptomyces* sp.: isolation and biosynthesis. *European Journal of Organic Chemistry*, 929-937.

[32] Hoffmann L, Grond S. (**2004**) Mixed acetate-glycerol biosynthesis and formation of benzoate directly from shikimate in *Streptomyces* sp. *European Journal of Organic Chemistry*, 4771-4777.

[33] Wein M, Lewinsohn E, Schwab W. (**2001**) Metabolic fate of isotopes during the biological transformation of carbohydrates to 2,5-dimethyl-4-hydroxy-3(2H)-furanone in strawberry fruits. *Journal of Agricultural and Food Chemistry*, ***49***, 2427-2432.

[34] Lewis DE, Dunn FW. (**1964**) β-3-Furylalanine, a new analog of phenylalanine. *Archives of Biochemistry and Biophysics*, ***107***, 363-366.

[35] Puel O, Galtier P, Oswald IP. (**2010**) Biosynthesis and toxicological effects of patulin. *Toxins*, ***2***, 613-631.

[36] Gandía-Herrero F, García-Carmona F. (**2013**) Biosynthesis of betalains: yellow and violet plant pigments. *Trends in Plant Science*, ***18***, 334-343.

[37] Gerratana B. (**2012**) Biosynthesis, synthesis, and biological activities of pyrrolobenzodiazepines. *Medicinal Research Reviews*, ***32***, 254-293.

[38] Beale JM, Lee JP, Nakagawa A, Omura S, Floss HG. (**1986**) Biosynthesis of the antibiotic reductiomycin. *Journal of the American Chemical Society*, ***108***, 331-332.

[39] Cho H, Beale JM, Cynthia G, Mocek U, Nakagawa A, Omura S, Floss HG (**1993**) Studies on the biosynthesis of the antibiotic reductiomycin in *Streptomyces xanthochromogenus*. *Journal of the American Chemical Society*, ***115***, 12296-12304.

[40] Thell K, Hellinger R, Schabbauer G, Gruber CW. (**2014**) Immunosuppressive peptides and their therapeutic applications. *Drug Discovery Today*, ***19***, 645-653.

[41] Steenbergen JN, Alder J, Thorne GM, Tally FP. (**2005**) Daptomycin: a lipopeptide antibiotic for the treatment of serious Gram-positive infection. *Journal of Antimicrobial Chemotherapy,* ***55***, 283-288.

[42] Fosgerau K, Hoffmann T. (**2015**) Peptide therapeutics: current status and future directions. *Drug Discovery Today*, ***20***, 122-128.

Natural Product Communications

Manuscripts in Press
Volume 12, Number 1 (2017)

Accounts/Reviews

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2017
Volume 12, Number 1

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