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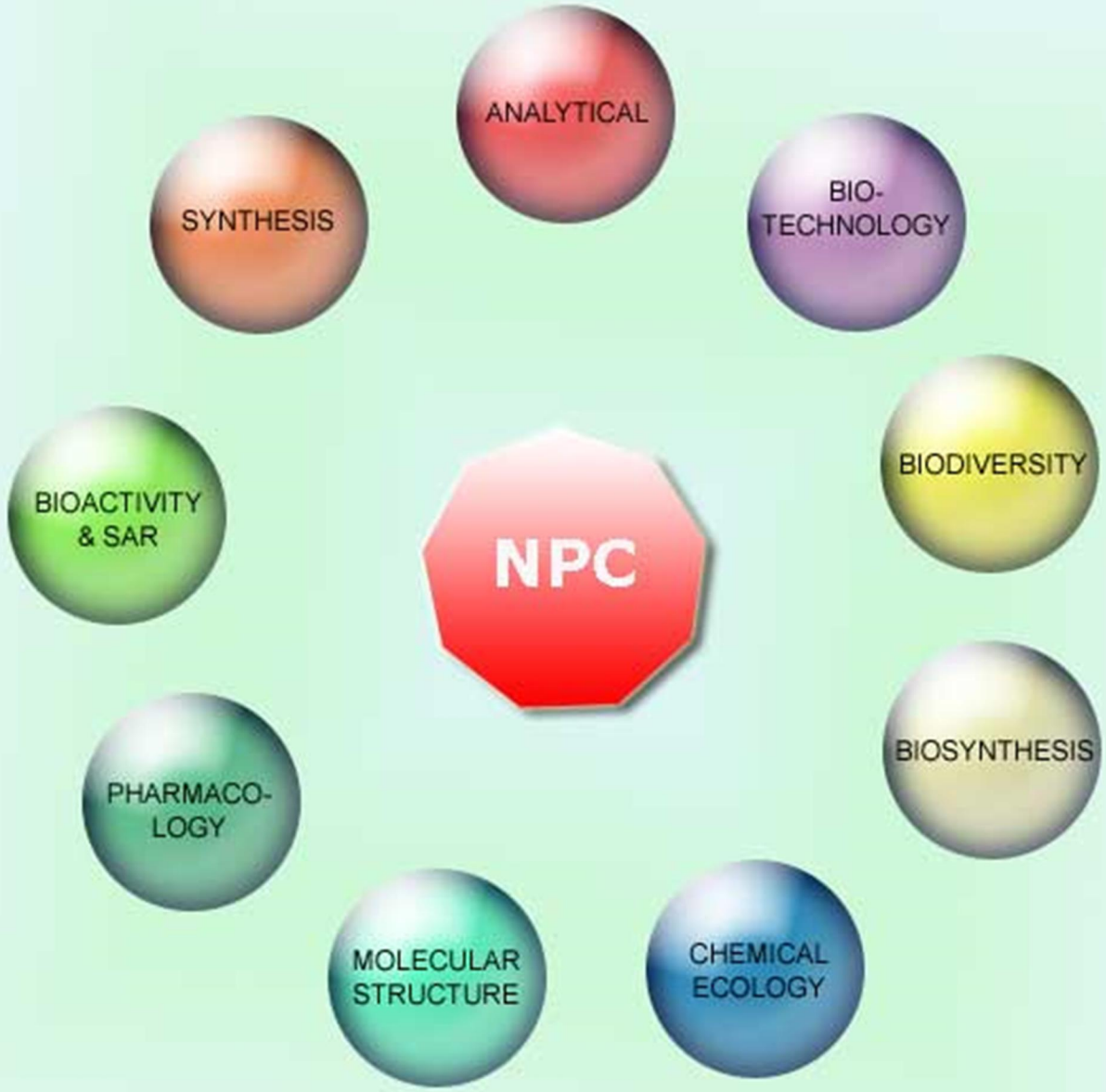

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New Humulenes from *Hyptis incana* (Labiatae)

Mitsuru Satoh*[,a], Yoshio Satoh[b], Yasuhiro Anzai[a], Daisuke Ajisawa[a], Keiichi Matsuzaki[c], Mitsuko Makino[c] and Yasuo Fujimoto[d]

[a]*Laboratory of Drug Design and Medicinal Chemistry, Showa Pharmaceutical University, 3-3165 Higashitamagawagakuen, Machida, Tokyo 194-8543 Japan*

[b]*Department of Clinical and Biomedical Sciences, Showa Pharmaceutical University, 3-3165 Higashitamagawagakuen, Machida, Tokyo 194-8543 Japan*

[c]*School of Pharmacy, Nihon University, 7-7-1 Narashinodai, Funabashi, Chiba 274-8555, Japan*

[d]*Department of General Studies, College of Humanities and Sciences, Nihon University, 3-25-40 Sakurajosui, Setagaya-ku, Tokyo 156-8550, Japan*

ms@ac.shoyaku.ac.jp

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Two new humulene-type sesquiterpenes, named hyptishumulene I (**1**) and II (**2**), have been isolated, together with eight known compounds, a humulene-type sesquiterpene (**3**), a monoterpene (**4**) and six abietane-type diterpenoids (**5-10**) from the aerial parts of *Hyptis incana* (Labiatae). The cytotoxic activity of the isolated compounds against mouse leukemia cells (L1210) was examined. The abietane-type diterpenoids (**5-10**) showed rather potent growth inhibitory activity (IC_{50}<15 μM), while the new humulene-type compounds (**1** and **2**) exhibited moderate activity (IC_{50}>50 μM).

Keywords: *Hyptis incana*, Labiatae, Humulene-type sesquiterpene, Cytotoxicity, Leukemia cell.

Hyptis incana Willd. ex Steudel (Labiatae, local name in Brazil: Salva do Marajo) is distributed widely throughout the Amazon area in South America. The plant has been used by native people as a tonic, to control perspiration, and to treat acne [1]. During the course of our studies on the isolation of biologically active substances from South American medicinal plants [2,3], we investigated the cytotoxic activity of alcohol extracts of this plant and found that the extracts exhibited potent growth inhibitory activity against leukemia cells (L1210). In this paper, we describe the isolation from *H. incana*, structure elucidation and growth inhibitory activity of two new humulene-type sesquiterpenes, named hyptishumulene I (**1**) and II (**2**), along with eight known compounds, a humulene-type sesquiterpene (**3**), which was isolated from *Torilis japonica* [4] and the red alga *Laurencia lobata* [5], a monoterpene (**4**) [6], six abietane-type diterpenes {7-methoxy-rosmanol (**5**) [7], 7-ethoxyrosmanol (**6**) [7], rosmanol (**7**) [7,8], epirosmanol (**8**) [8], isorosmanol (**9**)[8] and safficinolide (**10**) [9]}.

The dried aerial parts of *H. incana* were extracted with 80% EtOH. The crude extract was fractionated on a Diaion HP-20 column eluted successively with H_2O, 40%MeOH, 70%MeOH, MeOH, and acetone. The growth inhibitory activity of each fraction against L1210 cells was tested, and the 70%MeOH and MeOH fractions (>90% inhibition at the concentration of 50 μg/mL) were found to exhibit potent activities. Compounds (**1-10**) were isolated from these fractions by purification using silica gel chromatography and HPLC (Figure 1).

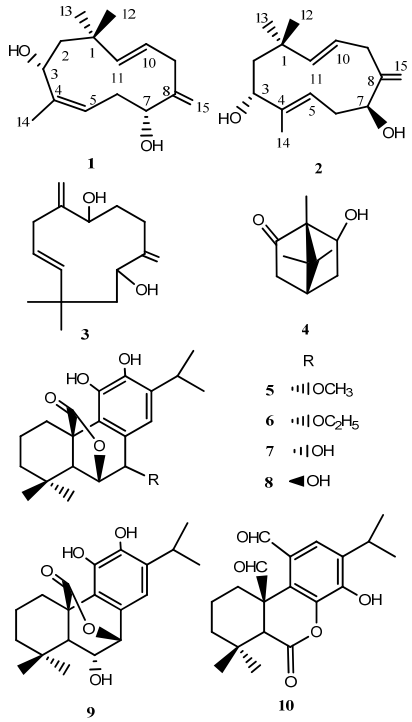

Figure 1: Structures of Compounds **1-10**.

Hyptishumulene I (**1**) was obtained as a colorless amorphous powder, $[\alpha]_D^{20}$ –24.6 (*c* = 0.1, $CHCl_3$). Its high-resolution HR-EI-MS showed the $[M]^+$ ion peak at *m/z* 236.1791, corresponding to the molecular formula $C_{15}H_{24}O_2$ (calcd. for 236.1777). The ^{1}H-, ^{13}C- , ^{1}H-^{1}H correlation spectroscopy (COSY) and heteronuclear single quantum coherence (HSQC) spectra of **1** showed signals due to two tertiary methyls (δ_H 0.98-δ_C 30.8, δ_H 1.16-δ_C 24.3), a vinyl methyl (δ_H 1.73-δ_C 17.0), three methylenes, (δ_H 1.45, 1.85-δ_C 49.7, δ_H 2.03, 2.74-δ_C 35.3, δ_H 2.66, 2.98-δ_C 35.0), an exomethylene (δ_H 4.87, 4.99-δ_C 113.2), two *sec*-alcohols (δ_H 4.41-δ_C 67.3, δ_H 4.06-δ_C 77.2), a *trans*-disubstituted double bond [δ_H 5.34 (d, *J* = 16.0 Hz) - δ_C 141.5, δ_H 5.39 (ddd, *J* = 6.0, 6.2, 16.0 Hz)- δ_C 124.0], a vinyl proton (δ_H 5.11-δ_C 122.8), an sp^3 quaternary carbon (δ_C 36.0), and two sp^2 quaternary carbons (δ_C 138.3, 149.6).

Table 1: ^{1}H- and ^{13}C NMR data for hyptishumulene I (**1**) and II (**2**).

	^{1}H-NMR (600 MHz, δ, $CDCl_3$)		^{13}C-NMR (150 MHz, δ, $CDCl_3$)	
position	**1**	**2**	**1**	**2**
1			36.0	35.1
2α	1.85 dd (6.3, 14.1)	1.76 dd (11.6, 12.2)	49.7	46.0
2β	1.45 dd (2.1, 14.1)	1.40 dd (2.2, 12.2),		
3	4.41 dd (2.1, 6.3)	4.13 dd (2.2, 11.6)	67.3	75.8
4			138.3	139.9
5	5.11 ddd (1.8, 1.8, 12.0)	5.09 ddd (0.8, 7.7, 7.9)	122.8	125.0
6α	2.03 ddd (1.8, 6.0, 14.0)	2.64 ddd (4.1, 7.9, 12.0)	35.3	37.0
6β	2.74 ddd (7.2, 12.0, 14.0)	2.14 ddd (7.7, 10.6, 12.0)		
7	4.06 dd (6.0, 7.2)	3.92 dd (4.1, 10.6)	77.2	72.9
8			149.6	153.3
9α	2.98 dd (6.0, 14.0)	2.93 dd (5.3, 12.2)	35.0	41.3
9β	2.66 dd (6.2, 14.0)	2.52 dd (10.3, 12.2)		
10	5.39 ddd (6.0, 6.2, 16.0)	4.85 ddd (5.3, 10.3, 15.5)	124.0	124.3
11	5.34 d (16.0)	5.05 d (15.5)	141.5	138.8
12	1.16 s	1.14 s	24.3	32.5
13	0.98s	0.98 s	30.8	23.8
14	1.73 t (1.8)	1.59 d (0.8)	17.0	10.1
15	4.87 d (1.8)	5.01 s	113.2	112.0
	4.99 d (1.8)	5.21 s		

The numbers in parentheses are *J* values in Hz

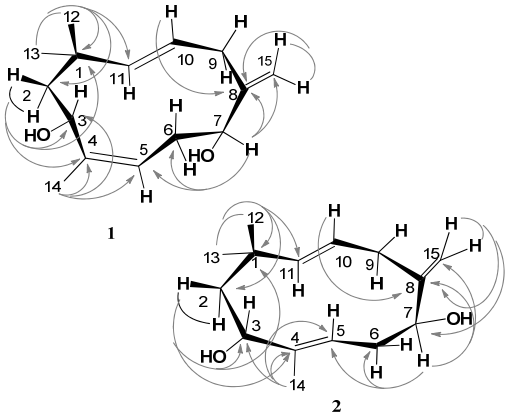

Figure 2: HMBC correlations for **1** and **2**.

The presence of the *sec*-alcohol groups was further confirmed by acetylation. Treatment of **1** with acetic anhydride-pyridine gave a diacetate whose oxymethine proton signals showed acetylation shifts (δ_H 4.06→ δ_H 4.77, δ_H 4.41→ δ_H 5.44). Connectivity of these functional groups was investigated by measuring the heteronuclear multiple bond coherence (HMBC) spectrum.

As shown in Figure 2, the proton signals at δ1.16 and δ0.98 assignable to a *geminal* dimethyl group (C-12 and -13) showed correlations with carbon signals at δ 141.5 (C-11), δ 36.0 (C-1) and δ 49.7 (C-2), suggesting the connectivity from C-10 to C-2 through C-1. The vinyl methyl proton signal at δ_H 1.73 (H-14) exhibited long-range correlations with the carbon signals at δ_C 138.3 (C-4), 122.8 (C-5) and δ_C 67.3 (C-3), which showed the cross peaks with the proton signals at δ_H 1.45 and 1.85 (H-2). These results indicated the presence of the C-3-C-5 sequence, including the vinyl methyl group at C-14 and the connectivity of C-3 and C-2. In addition, the HMBC spectrum of **1** showed correlations between the proton signal at δ_H 4.06 (H-7) due to the other *sec*-alcohol group and the carbon signals at δ_C 122.8 (C-5), 35.3 (C-6), δ_C 113.2 (C-15) and δ_C 149.6 (C-8), which, in turn, correlated with the proton signals at δ_H 2.66 and 2.98 (H-9) and at δ_H 5.39 (H-10). Thus, the structure of **1** was deduced to be a humulene-type sesquiterpene (Table 1).

The stereostructure of **1** was investigated by analysis of its nuclear Overhauser effect enhancement (NOE) difference spectra. As shown in Figure 3, the proton signal at δ 1.73 (H-14), due to a vinyl methyl group, showed a cross peak with the vinyl proton signal at δ 5.11 (H-5), which had correlation with the proton signal at δ 4.06 (H-7), thereby suggesting the *Z*-configuration of the C-$_{(4)}$-C-$_{(5)}$ double bond and α-configuration of the hydroxy group at C-7 [10].

The coupling constants of H-7 (*J* = 6.0, 7.2 Hz) are also supportive of the β-configuration for H-7 by taking into account the Karplus' rule, because the dihedral angles between H-7 and H-6α, and H-6β in hyptishumulene are approximately 170° and 20°, respectively, on inspection using a molecular model. The stereochemistry at C-3 was also deduced by difference NOE experiments on **1**. Irradiation at δ 4.41 (H-3) produced significant enhancement of the proton signal at δ 1.16 (H-12) and δ 2.74 (H-6β), indicating an α-configuration of the hydroxy group at C-3. Furthermore, NOEs were observed between the following proton signals: H-7 and H-5, H-15; H-9α and H-11; H-10 and H-9β, H-12; H-11 and H-9α, H-13; H-2α and H-13, H-14; and H-2β and H-12. These spectral data are explained satisfactorily by the stereostructure shown in Figure 3. The structure of **1** was also supported by it being the most stable (Figure 3', minimized energy: 13.2963 kcal/mol) by molecular mechanics calculation (MM2) [11].

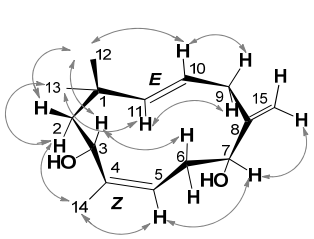

Figure 3: NOE correlations for **1**.

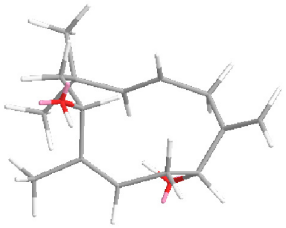

Figure 3'

Hyptishumulene II (**2**) was obtained as a colorless amorphous powder $[\alpha]_D^{20}$ −30.4° (*c* = 0.1, $CHCl_3$). Its HR-EI-MS showed the $[M]^+$ ion peak at *m/z* 236.1786, corresponding to the molecular formula $C_{15}H_{24}O_2$ (calcd. for 236.1777), which is the same as that of compound **1**.

The ^{1}H- and ^{13}C-NMR spectra of **2** were very similar to those of **1**, except for a few differences (Tables 1). The spectral data indicated that **2** should be a stereoisomer of **1**. Comparison of the NOESY spectra of **1** and **2**, showed that the H-5 signal (δ 5.09) of **2** had cross peaks with H-3 (δ 4.13) and H-6β (δ 2.14), while the corresponding signal (δ 5.11) of **1** exhibited correlations with H-7 (δ 4.06) and H-14 (δ 1.73). These results indicated that the C-$_{(4)}$-C-$_{(5)}$ double bond of **2** is the *E*-form. In addition, the β-orientation of the hydroxy group at C-7 was confirmed by observation of NOE correlations between H-7 and H6α, H-9α, as well as the NOEs shown in Figure 4. Thus, the structure of **2** was represented as shown in Fig. 1.

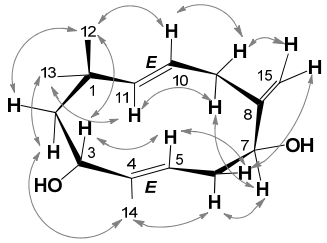

Figure 4: NOE Correlations for **2**.

The structures of compounds **3-10** were identified by comparison of their spectral data with those described in the literature.

Growth inhibitory activity: The effects of the isolated compounds (**1-10**) on the growth of leukemia cells (L1210) were examined. The abietane-type diterpenoids (**5-10**) showed rather potent growth inhibitory activity (IC_{50}<15 μM), while the new humulene-type

compounds (**1** and **2**) exhibited moderate activity (IC_{50}>50 μM) (Table 2). Compounds **5-9** induced apoptosis of neuroblastoma cells [12].

Table 2: Growth inhibitory effects of the compounds isolated from *H. incana* against leukemia cells (L1210) (n=5).

Compounds	IC_{50} (μM)
1	> 50
2	> 50
3	> 50
4	> 50
5	13
6	13
7	12
8	12
9	12
10	15

Experimental

General: ^{1}H and ^{13}C NMR spectra were measured on Bruker AV300 and AV600 spectrometers in $CDCl_3$, with tetramethylsilane (TMS) as an internal standard. MS were recorded on a JEOL JMS-600 spectrometer. Optical rotations were recorded using a JASCO DIP-360 digital polarimeter. CC was performed on either Diaion HP-20 resin (Mitsubishi Kasei) or silica gel (Wakogel C-200). HPLC was conducted with JASCO PU-980 or Senshu SSC 3220 pumps equipped with an ERMA ERC-7522 (RI) detector. TLC was performed using silica gel 60 F_{254} (Merck) precoated plates and detection was carried out by spraying with 10% sodium phosphomolybdate in methanol, followed by heating.

Plant material: *Hyptis incana* was purchased in 1999, and identified by Pharmacist Gilbert Rubens Biancalana (Laboratorio Farmaervas Ltda. in Sao Paulo, Brazil). The voucher specimen was deposited in the herbarium of Showa Pharmaceutical University.

Extraction and isolation: The dried aerial parts of *H. incana* (2858 g) were extracted with 80% EtOH (20 l × 3) with ultrasonication. The extract was concentrated *in vacuo* to give a brown solid (750 g). This (550 g) was chromatographed on a Diaion HP-20 column eluted successively with stepwise gradients of 10 L each of H_2O, 40% MeOH, 70% MeOH, MeOH, and acetone, and then each eluate was concentrated *in vacuo* to afford 5 fractions [H_2O (168.0 g), 40% MeOH (57.7 g), 70% MeOH (74.8 g), MeOH (143.6 g), and acetone (21.1 g)]. The cytotoxicity of each fraction against L1210 cells was tested, and the MeOH and 70% MeOH fractions were found to be the most active.

The 70% MeOH fraction (50.0 g) was chromatographed on a silica gel column eluted successively with solvents of increasing polarity [*n*-hexane: EtOAc = 1: 1 (5 L, Fr. 1-4), 1: 5 (5 L, Fr. 5-9), and EtOAc (5 L, Fr. 10)] to afford 10 fractions. Fraction 3 (3.0 g) was further fractionated by HPLC [Senshu Pak, PEGASIL-Silica 60-5, 10 × 250 mm (column A), *n*-hexane: EtOAc = 1: 1, flow rate: 3.0 mL/min] to afford 15 fractions (Fr. 3-1 to 3-15). Fraction 3-11 (retention time (Rt): 18.0–19.5 min) was further purified by HPLC [PEGASIL-ODS, 10 × 250 mm (column B), MeOH: H_2O = 8: 2, flow rate: 3.0 mL/min] to give **1** (13 mg, Rt: 8.0 min).

Hyptishumulene I (1)
Colorless amorphous powder.
$[\alpha]_D^{20}$: –24.6 (*c* 0.1, $CHCl_3$).
HR-EI-MS *m*/*z*: 236.1791 (calcd. for $C_{15}H_{24}O_2$ 236.1777),
^{1}H NMR ($CDCl_3$, 600 MHz) and ^{13}C NMR ($CDCl_3$, 150 MHz): Table 1.

Fraction 3-12 (Rt: 20.5–22.0 min) was purified using HPLC (column B, MeOH: H_2O= 8: 2, flow rate: 3.0 mL/min) to give **2** (6 mg, Rt: 7.0 min).

Hyptishumulene II (**2**)
Colorless amorphous powder.
$[\alpha]_D^{20}$: –30.4 (*c* 0.1, $CHCl_3$).
HR-EI-MS *m*/*z*: 236.1786 (calcd. for $C_{15}H_{24}O_2$ 236.1777),
^{1}H NMR ($CDCl_3$, 600 MHz) and ^{13}C NMR ($CDCl_3$, 150 MHz): Table 1.

Fraction 3-10 (retention time (Rt): 16.0–17.5 min) was further purified by HPLC [PEGASIL-ODS, 10 × 250 mm (column B), MeOH: H_2O = 8: 2, flow rate: 3.0 mL/min] to give **3** (10 mg, Rt: 6.0 min).

Fraction 3-2 (retention time (Rt): 8.0–8.8 min) was further purified by HPLC [column A, *n*-hexane: EtOAc = 1: 1, flow rate: 3.0 mL/min] to give **4** (12 mg, Rt: 8.5 min).Purification of a part (100 mg) of fraction 1 by HPLC (column A, *n*-hexane: EtOAc = 3: 1, flow rate: 5.0 mL/min) gave **5** (methoxyrosmanol, 15 mg, Rt: 6.0 min) and **6** (ethoxyrosmanol, 33 mg, Rt: 7.5 min). Separation of a part (100 mg) of fraction 2 by HPLC (column A, *n*-hexane: EtOAc = 5: 2, flow rate: 5.0 mL/min) afforded **7** (rosmanol, 20.5 mg, Rt: 6.5 min), **8** (epirosmanol, 9.8 mg, Rt: 8.0 min) and **9** (isorosmanol, 7.3 mg, Rt: 13.0 min). Purification of part (50 mg) of fraction 2 by HPLC [PEGASIL-ODS, 20 × 150 mm, MeOH, flow rate: 8.0 mL/min] gave **10** (safficinolide, 8.5 mg, Rt: 4.5 min)

Acetylation of hyptishumulene I (1): A mixture of **1** (1 mg) and five drops (large excess) of acetic anhydride in pyridine (0.5 mL) was allowed to stand for 12 h at room temperature. The mixture was poured into water and then extracted with AcOEt (10 mL). The AcOEt solution was washed with brine (10 mL x 2), dried over Na_2SO_4 and concentrated *in vacuo* to give an acetate (1 mg).

^{1}H NMR δ: 0.94 (3H, s), 0.99 (3H, s), 1.18 (3H, s), 1.57 (1H, m), 1.64 (1H, m), 1,92 (3H, s, -Ac) 1.99 (3H, s, -Ac), 2.00 (1H, m), 2.02 (1H, m), 2.76 (1H, m), 2.83 (1H, m), 4.77 (1H, m), 4.82 (1H, s), 4.89 (1H, s), 5.11 (1H, m), 5.36 (1H, m), 5.38 (1H, m), 5.44 (1H, m).
MS: *m*/*z* 320 $[M]^+$.

Cytotoxicity assay: The effects of isolated compounds on the growth of leukemia cells (L1210) were investigated as follows. Cells were suspended in RPMI 1640 medium (NISSUI) containing 1% kanamycin sulfate, 10% fetal bovine serum, and supplemented with L-glutamine. Cells were plated in a 96-well microplate (100 μL/well) at a density of 3.0×10^4 cells/well. After incubating the plate for 24 h at 37°C in a humidified 5% CO_2 atmosphere, compounds **1-10** in MeOH (2 μL/well) at various concentrations (n = 5) were added and the plate was incubated for an additional 48 h under the same conditions. Control wells (n = 5) containing the same volume of MeOH (2 μL/well) were incubated in each assay. Ten μL of Cell Counting Kit-8 [13, 14] was added to each well, then, the microplate was incubated for 3 h in a 5% CO_2 atmosphere at 37°C. The absorbance (A) of each well was measured at 450 nm using a Microplate reader (MTP-450, Corona Electric). The percent inhibition was calculated by comparing $A_{control}$ and $A_{treated}$.

$$\text{inhibition (\%)} = (1 - A_{\text{treated-blank}} / A_{\text{control-blank}}) \times 100$$

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Inhibitory Effects against Pasture Weeds in Brazilian Amazonia of Natural Products from the Marine Brown Alga *Dictyota menstrualis*

Rainiomar Raimundo Fonseca[a], Antonio Pedro Silva Souza Filho[b], Roberto Campos Villaça[c] and Valéria Laneuville Teixeira[c,*]

[a]*Programa de Pós-Graduação em Química, Instituto de Química, Universidade Federal Fluminense, Niterói, RJ, 24020-150, Brazil*

[b]*Lab. Agroindústria, EMBRAPA Amazônia Oriental/ CPATU, Belém, Pará, 66095-100, Brazil*

[c]*Departamento de Biologia Marinha, Instituto de Biologia, P.O. Box 100.644, Niterói, RJ, 24001-970, Brazil*

valerialaneuville@gmail.com

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Fractions of the acetone extract and a mixture of two diterpenes from the marine brown alga *Dictyota menstrualis* were prepared with the aim of identifying potential effects on the germination of seeds and on elongation of the radicle and hypocotyl of the weeds *Mimosa pudica* and *Senna obtusifolia*. The bioassay on seed germination was performed in controlled conditions of 25ºC temperature and a 12 hour photoperiod, while the one concerning radicle and hypocotyl elongation was performed at the same temperature, though adopting a photoperiod of 24 hours. The results varied according to the receptor species, the fraction utilized, and its concentration. TLC analysis of the fractions and comparison with isolated products indicated that the diterpenes pachydictyol A and isopachydictyol A were the most abundant compounds in fraction HE, whereas the diterpene 6-hydroxy-dichotomano-2, 13-diene-16, 17-dial (**3**) was the most abundant compound in fractions DC and EA. Analysis of less polar fractions (in *n*-hexane, dichloromethane and ethyl acetate) revealed values of less than 30% inhibition. On the other hand, the ethanol/water fraction was the most active in all instances. The biological activity of these fractions must be due to the presence of known diterpenes and/or sulfated polysaccharides isolated in earlier studies.

Keywords: *Dictyota menstrualis*, Marine natural product, *Mimosa pudica*, *Senna obtusifolia*, Inhibitory effects.

In the Amazon region, the occurrence of weeds is regarded as the most serious biological problem faced by the ranchers, and weed control represents one of the highest costs of production. Therefore, the definition of new strategies that enable reduction of infestation in pastures to an acceptable level may guarantee higher productivity in the long term by assuring the longevity of crops. In addition to increasing the economic profit, these strategies may be useful to soften the social and environmental dissatisfaction that this type of activity has awakened nationally and internationally due to the use of aggressive bio defensives [1-3]. Our group has been studying the natural products of marine brown algae for over 25 years. During this period, many biological activities were recognized for the natural products of benthic algae. In the previous study, we have shown the inhibitory effects of the crude extract from the marine red alga *Plocamium brasiliense* (Greville) M. A. Howe & W. R. Taylor [4]. The positive results obtained stimulated new tests with other species of seaweed.

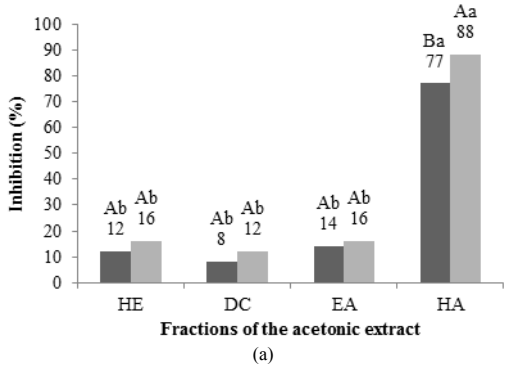
The marine brown alga *Dictyota menstrualis* (Hoyt) Schnetter, Hörning & Weber-Peukert is very common along the Brazilian coast and known as a prolific producer of bioactive diterpenes [5-9]. The diterpenes are the main components of the hexane, dichloromethane and acetone extracts of these algae. On the other hand, the sulfated polysaccharides (heterofucans) are the major components of the more polar extracts from the Brazilian population [10].

Fifteen diterpenes are known for *D. menstrualis*, isolated from five populations in several regions of the world: one from North Carolina (USA) [11,12], one from the area around Sydney (Australia) [13], two from the coast of the State of Rio de Janeiro (Brazil) and two from the São Pedro and São Paulo Archipelago (Brazil) [8,9]. Besides, studies using diterpenes isolated from *D. menstrualis* from the Brazilian littoral indicate that the secondary metabolites are used as a defense mechanism against amphipod herbivores [14].

Indeed, investigation of allelopathy has traditionally been conducted utilizing phytochemicals and metabolites from terrestrial plants [e.g. 15]. Allelopathy is considered to be a phenomenon in which a plant (including microorganisms) directly or indirectly causes either positive or negative effects on the development of other plants by liberating chemical substances in the environment. Nevertheless, a study on allelopathy may well be applied to a seaweed species such as *D. menstrualis* since it produces various bioactive diterpenes [8]. Allelopathy may represent an excellent alternative strategy for the management of pastures, allowing the control of infestations of undesired plants as weeds and, as a consequence, avoid great losses, especially regarding livestock. Moreover, this phenomenon has ecological importance, notably when it comes to providing alternative sources of chemical structures that can be used to produce agricultural biodefensives [16, 17].

In the present study, the inhibitory effects of fractions of different polarities, and a mixture of pachydictyol A and isopachydictyol A obtained from the acetone extract of *D. menstrualis* was evaluated in bioassays concerning germination and development of radicle and hypocotyl using as receptor species the weeds *Mimosa pudica* L. and *Senna obtusifolia* (L.) Irwin & Barney.

The inhibitory effects were evaluated of different fractions {*n*-hexane (HE), dichloromethane (DC), ethyl acetate (EA), and ethanol/water (HA)} of an acetone extract of *D. menstrualis*. The aim of the first assay was to analyze the effects of these fractions on the germination of seeds (Figure 1a-c). The results presented in Figure 1 revealed that the ethanol/water fraction(HA) had a



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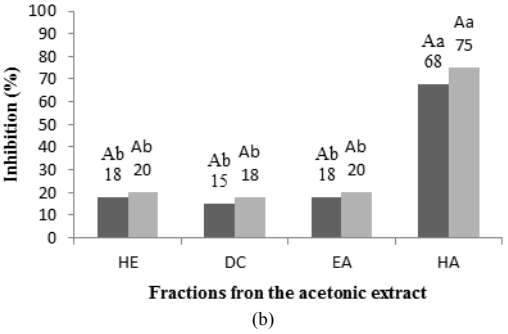

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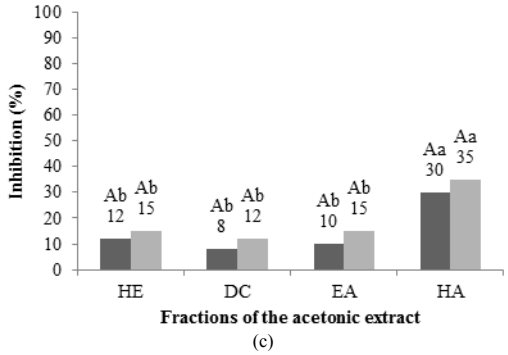


(c)

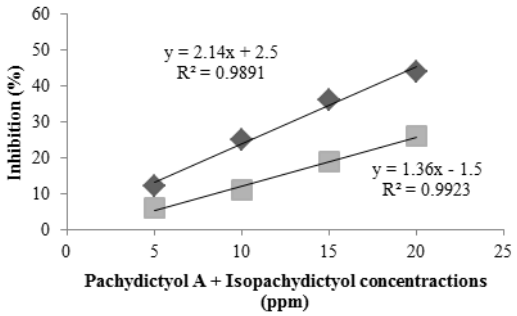
Mimosa pudica *Senna obtusifolia*

Figure 1: Effects of fractions HE, DC, EA and HA on (a) the germination of seeds, (b) on radicle elongation, and (c) on hypocotyl elongation of two weeds (*Mimosa pudica* and *Senna obtusifolia*). The data are expressed as percentage of inhibition in relation to control treatment (distilled water). HE = *n*-Hexane fraction, DC = Dichloromethane fraction, EA = Ethyl acetate fraction, and HA = Ethanol/water (7:3) fraction. Extracts with the same solvent from *M. pudica* and *S. obtusifolia* do not differ by Tukey (5%).

potent inhibitory effect on the germination of seeds, ranging from 88% to 77% inhibition for *M. pudica* and *S. obtusifolia*, respectively. On the other hand, the apolar (HE) and intermediate polarity fractions (DC and EA) presented values ranging from 8 to 20% inhibition.

These results revealed that the fractions from *D. menstrualis* were more active than the crude extract of the red alga *P. brasiliense* [4], since the dichloromethane extract of the latter contains active components capable of inhibiting the germination of seeds of *M. pudica* and *S.obtusifolia* by merely 25 and 14%, respectively.

The effect of each fraction on radicle elongation is presented in Figure 1b. The results show that the ethanol/water fraction of *D. menstrualis* (HA) contains active components that inhibit by 75 and 68% the radicle elongation of *M. pudica* and *S. obtusifolia*, respectively.



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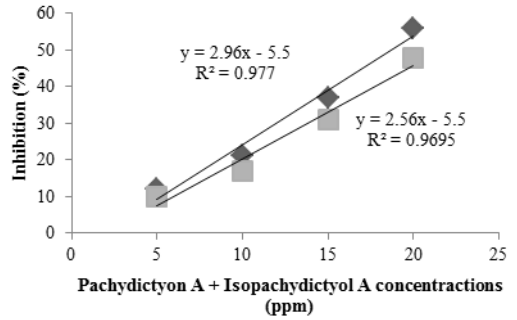

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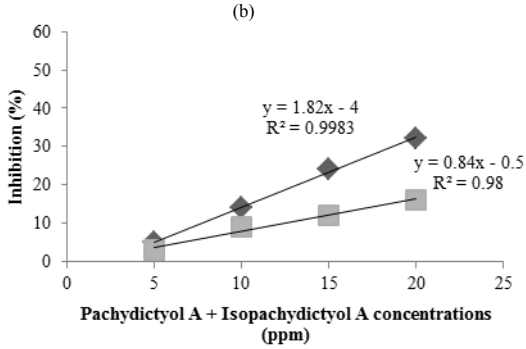


(c)

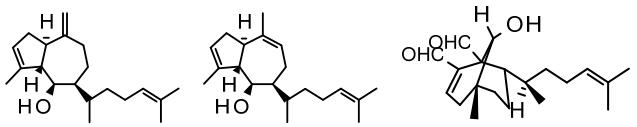
Mimosa pudica *Senna obtusifolia*

Figure 2: Effects of pachydictyol A+isopachydictyol A (in ppm) (a) on seed germination, (b) on radicle elongation, and (c) on hypocotyl elongation of two weeds. The data are expressed as percentage inhibition in relation to control (distilled water).

Finally, the effect of each fraction on hypocotyl development of the weeds is shown in Figure 1c. Again, the ethanol/water fraction was the most effective (35% for *M. pudica* and 30% for *S. obtusifolia*). In fact, it is important to note that the *D. menstrualis* fractions tested were more efficient against *M. pudica* in all experiments.

The inhibition values observed (Figure 1a, b and c) demonstrate that the ethanol/water fraction has a far more intense inhibitory effect on seed germination (88 and 77%) and radicle elongation (75 and 68%) than on hypocotyl elongation (35 and 30%, respectively).

Considering that the results reflect the inhibitory potential of chemical substances according to their polarities, it was possible to verify that the compounds present in HA are strongly active, whereas those in HE, DC and EA presented low inhibition rates. Thus, the results obtained qualify HA as a potential alternative source of chemical substances that might inspire more advanced studies regarding the production of agricultural biodefenses.



1 **2** **3**

The chemical composition of *D. menstrualis* is characterized by cyclic diterpenes known as prenylated guaianes, α, β-unsaturated aldehydes [5-9, 11-13], and sulfated polysaccharides [10]. TLC analysis of the fractions and comparison with isolated products indicated that the diterpenes pachydictyol A (1) and isopachydictyol A (2) were the most abundant compounds in fraction HE. In the fractions DC and EA, the most abundant compound was the diterpene 6-hydroxy-dichotomano-2, 13-diene-16, 17-dial (3).

The effects of the unseparated diterpenes pachydictyol A and isopachydictyol A are presented in Figure 2. The mixture had a satisfactory inhibitory effect in dose-dependent concentrations (5-20 ppm) on the germination of seeds, ranging from 14-48% inhibition of *M. pudica* and from 6-28% of *S. obtusifolia*).

The results obtained indicate an effective inhibitory potential for the ethanol/water fraction of the alga. This inhibitory effect must be due to polar diterpenes and/or sulfated polysaccharides [18,19]. The other fractions (HE, DC and AC), however, presented low inhibitory potential compared with HA. Therefore, we may conclude that the ethanol/water fraction of *D. menstrualis* can become a promising alternative for the management of pastures.

Experimental

Biological material: *Dictyota menstrualis* (Dictyotaceae, Phaeophyceae) was collected at Atol das Rocas reef, Rio Grande do Norte State (lat. 03º51'03"S, long. 33º40' 29"W), Brazil, in February 2005. Atol das Rocas reef is a marine biological reserve in the northeast of Brazil, and is the only atoll in the South Atlantic. The collection was performed with federal authorization for *collection,* SISBIO/IBAMA number 17532, by scuba diving at a depth of 2-5 m. The algae were triaged in the place of collection and air-dried. They were then placed in thermic boxes to be transported to ALGAMAR laboratory where the identification was performed by one of us (RCV). Voucher specimens were deposited in the Herbarium of Universidade do Estado do Rio de Janeiro (HRJ).The receptor plants used in bioassays were two of the main weeds that occur in areas of cultivated pastures in the Amazon region, known as sensitive plant (*Mimosa pudica*) and mata-pasto (*Senna obtusifolia*). Seeds of these 2 plants were collected in the Municipality of Castanhal, Pará State, in the Brazilian Amazon region. The seeds were cleaned and purged in seed conservation chambers. Seed dormancy was overcome as established by earlier studies [2,3].

Extraction: The dried algae were powdered in an industrial blender, yielding 50 g of powder, which was extracted with acetone (100%) at room temperature for 10 days. The acetone extracts were obtained by exhaustive extraction (using a decanting funnel as a container). The solvent was evaporated under reduced pressure, yielding a brownish residue (6 g). This was mixed with 40 g of silica gel. First, the mixture was stirred and filtered through filter paper with 800 mL *n*-hexane (HE, 92 mg). The second extraction was performed with 800 mL of dichloromethane (CH_2Cl_2), yielding 62 mg of extract (DC). The third extraction was made with 800 mL of ethyl acetate (EtOAc), yielding 130 mg after evaporation of the solvent (EA). Finally, the ethanol/water extract (HA) was prepared using 800 mL of ethyl alcohol/water (7:3). The ethanol was evaporated in a rotary evaporator; this was followed by lyophilization of the residue, yielding 240 mg (HA). All fractions were prepared at a 1% concentration for biological tests. The *n*-hexane fraction (HE) was submitted to silica gel chromatography (*n*-hexane/EtOAc), eluting with 30% EtOAc in *n*-hexane to afford 20 mg of unseparated diterpenes pachydictyol A and isopachydictyol A, in relative proportions 3:2. The NMR and IR spectra of the hydroalcoholic extracts presented characteristic signals of sulfated polysaccharides.

Bioassays: The bioassays on germination were developed in controlled conditions: constant temperature (25ºC) and photoperiod of 12 h. Germination was monitored for 15 days, involving daily counting and elimination of germinated seeds, considered as those presenting radicle extension equal to or superior to 2 mm [2-3]. Each transparent Petri dish measured 9.0 cm in diameter and received 20 seeds. Regarding radicle and hypocotyl elongation, bioassays were performed under controlled conditions of constant temperature (25°C) and a photoperiod of 24 h in a germination chamber with cool white fluorescent lamps and a luminous flux of 10 $\mu mol.m^{-2}.s^{-1}$. Six pre-germinated seeds were placed in a transparent Petri dish for approximately 2-3 days. At the end of 10 days of growth, extension of the radicle and hypocotyl were measured. Each Petri dish was covered with a qualitative filter paper and received 3.0 mL of fraction, diterpene or an equal volume of distilled water (control). In the evaluation of fractions, a concentration was used of 1%, w/v, of each fraction and the volume of water replaced was 3.0 mL. In the evaluation of diterpenes, 4 concentrations were used, 5, 10, 15 and 20 ppm. Only after the beginning of each bioassay was either the fraction or diterpenes added. From this moment on, only distilled water was added when necessary.

Statistical analysis: The experimental design for all bioassays was entirely randomized and 3 repetitions were performed. The data were submitted to analysis of variance by the F test and the averages were compared by Tukey test ($P < 5\%$). All the analyses were performed in SAS program [20].

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Isolation of the Plant Hormone (+)-Abscisic acid as an Antimycobacterial Constituent of the Medicinal Plant Endophyte *Nigrospora* sp.

Trevor N. Clark[a], Katelyn Ellsworth[a], Haoxin Li[a], John A. Johnson[a] and Christopher A. Gray[a,b]*.

[a]*Department of Biology, University of New Brunswick, 100 Tucker Park Rd, Saint John, NB, Canada, E2L 4L5.*
[b]*Department of Chemistry, University of New Brunswick, 100 Tucker Park Rd, Saint John, NB, Canada, E2L 4L5.*

cgray@unb.ca

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An extract of the endophytic fungus *Nigropsora* sp. (isolate TC2-054) from the Canadian medicinal plant *Fragaria virginiana* exhibited significant antimycobacterial activity against *Mycobacterium tuberculosis* H37Ra. Bioassay guided fractionation revealed that linoleic acid derivatives and the plant hormone (+)-abscisic acid (ABA) were responsible for the observed antimycobacterial activity. This activity of ABA has not been previously reported.

Keywords: Abscisic acid, Antimycobacterial activity, Endophyte, *Fragaria virginiana*, Canadian medicinal plant, *Nigropsora* sp.

Endophytic fungi are increasingly being recognized as an important source of bioactive natural products [1] and the biosynthetic potential of endophytes isolated from medicinal plants has recently been highlighted [1f]. As part of our research on Canadian medicinal plants, we have so far isolated 81 endophytic fungi from a selection of twelve plants that have been used therapeutically by the First Nations peoples of the Canadian Maritimes [2]. Antimicrobial screening of this endophyte library indicated that an extract of the spent fermentation broth of *Nigropsora* sp. isolate TC2-054 obtained from the medicinal plant *Fragaria virginiana* exhibited significant antimycobacterial activity and, therefore, warranted further investigation.

The EtOAc extract of a bench-scale (2 L), two-week fermentation of TC2-054 was subjected to a modified Kupchan partition protocol with the *n*-hexane and CH_2Cl_2 fractions showing significant activity towards *M. tuberculosis* H37Ra. NMR analysis of the *n*-hexane fraction showed it to contain significant amounts of linoleic acid derivatives that are known to exhibit antimycobacterial activity [3] and was not further fractionated. However, normal phase HPLC of the CH_2Cl_2 fraction led to the isolation of (+)–abscisic acid [4] (ABA; 6.5 mg/L; Figure 1) as the only antimycobacterial constituent [*M. tuberculosis* H37Ra; MIC: 200 μg/mL; IC_{50} (± SD): 40.8 ± 3.7 μg/mL). LC-MS screening did not detect ABA in a *F. virginiana* extract prepared from leaves collected at the same time as those used for the isolation of the *Nigrospora* sp.

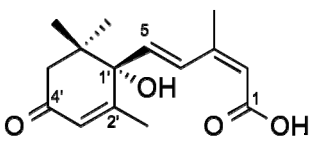

Figure 1: (+)–Abscisic acid (ABA).

The role of ABA as a plant stress hormone has been well documented since its isolation in 1961 [4a]. More recently, it has been shown to be involved in a wide range of developmental physiological processes in higher plants [5a] and found also to be produced by lower plants, lichens and fungi [5b], including a species of *Nigrospora* isolated from a Thai sea-fan [5c]. Many of these fungi are recognized endophytes and phytopathogens [5b, d] and the production of ABA by these fungi may be a factor in the development and maintenance of plant-fungus relationships [5b]. ABA has recently been shown to be an important factor in fruit ripening in the strawberry, *Fragaria ananassa* [5e], posing intriguing questions relating to the role of endophyte derived ABA in *F. virginiana*.

The antimycobacterial activity of ABA has not been previously described, and although it exhibited only moderate activity against *M. tuberculosis* H37Ra, it did not show any observable toxicity to mammalian cells; no difference in viability was observed between HEK293 cells treated with abscisic acid (at concentrations up to 250 μg/mL) and the corresponding vehicle controls [analysis of 250 μg/mL data: t-test, F = 0.052, *P* = 0.83, 4 d.f. and Mann-Whittney, U = 6.0, *P* = 0.70; mean cell viability (± SD) 93 ± 10% compared with negative control]. Given its low toxicity to human cells, further work should focus on defining the mode of action of ABA against mycobacteria as it may reveal a selective cellular target that could be exploited in future tuberculosis drug development.

Experimental

Isolation and identification of Nigrospora sp. (TC2-054): Endophytes were isolated from the leaves of *Fragaria virginiana* Duchesne collected from the wood-lot on the UNB campus in Saint John, New Brunswick, Canada (N 45° 18.375' W 66° 05.616') in August 2010. Plants were identified by Dr Stephen Clayden of the New Brunswick Museum and a voucher specimen has been deposited in the New Brunswick Museum Herbarium (Number: NBM VP-37478). Leaf surfaces were sterilized by immersion in 5.25% aqueous sodium hypochlorite for 5 sec, sterile distilled water for 10 sec and 70% EtOH for 15 sec. The sterile tissue was rinsed with sterile distilled water, blotted dry on an autoclaved paper towel, and aseptically cut into pieces that were placed onto 2% malt extract agar and incubated at ambient room temperature. Endophytic fungi were subcultured on 2% malt extract agar until pure cultures were obtained. Isolate TC2-054 was identified taxonomically as *Nigrospora* sp. through examination of colony and

spore morphology, with the taxonomic classification being confirmed by comparison of the internal transcribed spacer and 5.8S rRNA gene (ITS) DNA regions with corresponding sequences available in the GenBank database (NCBI, US Government), as previously described. The sequence data derived from isolate TC2-054 has been submitted to GenBank and assigned accession number KC916673.

Biological assays: Anti-mycobacterial activity against *M. tuberculosis* strain H37Ra (ATCC 25177) and cytotoxicity against HEK 293 was evaluated as previously described [6].

Fermentation and extraction: TC2-054 was fermented in 2% malt extract broth at room temperature for 2 weeks (2 L; 20 ×100 mL batches in 250 mL Erlenmeyer flasks). The fungal material was separated from the broth using vacuum filtration, and the broth was extracted with EtOAc (3×300 mL). The organic fraction was concentrated *in vacuo* to give a crude extract (79 mg).

Bioassay guided fractionation: The crude extract (79 mg) was dissolved in 9:1 MeOH/H_2O (50 mL) and extracted with hexanes (3 × 25 mL) before being diluted with H_2O (25 mL) and extracted with CH_2Cl_2 (3 × 25 mL). The aqueous fraction was concentrated *in vacuo,* dissolved in H_2O (50 mL) and extracted with EtOAc (3 × 25 mL) and *n*-BuOH (3 × 25 mL). The 5 partition fractions were concentrated *in vacuo* with the CH_2Cl_2 fraction (46 mg) showing strong bioactivity. The CH_2Cl_2 fraction (46 mg) was subjected to normal phase HPLC (1:1, hexanes/EtOAc) to give **1** (13 mg)

Extraction and LC-MS screening of Fragaria virginiana: Freeze-dried ground plant material (20.5 g) was extracted using a Soxhlet apparatus for 11.5 h with MeOH (200 mL). The crude extract was then concentrated *in vacuo* to give a green oil (6.54 g). The crude extract (0.5 mg/mL in MeOH, 20 μL injections) was subjected to LC/MS using a Phenomenex Kinetex C18 column (50 x 4.60 mm, 2.6 μm) eluted with a 0.1% formic acid H_2O/MeOH gradient (1 min at 95:5 H_2O/MeOH, 7 min linear transition to 1:99 H_2O:MeOH for a further 7 min) at 500 μL/min on an Agilent 1100 LC system coupled to an AB Sciex API 2000 triple quadrupole MS (TurboIonSpray source run in positive mode). The presence of ABA was analyzed by selected reaction monitoring of the pseudo-molecular ion (*m*/*z* 265) to dehydrated product ion (*m*/*z* 247) transition. Under these conditions, an ABA standard eluted with a retention time of 6.6 min and was detectable in MeOH solutions at a concentration of ≥ 25 ng/mL. No peak corresponding to ABA was observed in the *F. virginiana* crude extract. Subsequent co-injections of the crude extract and ABA standard confirmed that ABA would be detectable at ≥ 100 ng/g in the crude extract.

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New Cembranoid Diterpene from the South China Sea Soft Coral *Sarcophyton* sp.

Fei Cao, Jing Zhou, Kai-Xia Xu, Meng-Qi Zhang and Chang-Yun Wang*

Key Laboratory of Marine Drugs, *Ministry of Education*, *School of Medicine and Pharmacy*, *Ocean University of China*, *Qingdao 266003*, *China*

changyun@ouc.edu.cn

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One new oxygenated cembranoid diterpene, named sarcophytol W (**1**), along with six known analogues (**2**–**7**) were isolated from a soft coral *Sarcophyton* sp. collected from the South China Sea. Their structures were elucidated by spectroscopic analyses and by comparison with previously reported data. The absolute configuration of **1** was assigned on the basis of the absolute configuration of the related congener **2**, which was determined by application of the modified Mosher's method. All the compounds were evaluated for their antibacterial and antifouling activities.

Keywords: Soft coral, *Sarcophyton* sp., Cembrane diterpenoid, Absolute configuration, Bioactivity.

Diterpenoids from marine organisms possess a wide array of unusual structures. Among them, cembrane diterpenoids with the 14-carbon cyclic nucleus have been frequently found in soft corals [1]. These cembrane diterpenoids possess various biological activities such as ichthyotoxic [2], antifouling [3], antitumor [4], neuroprotective [5], antiinflammatory [6] and cytotoxic activities [7].

Recently, in the course of our investigation of new bioactive substances from soft corals collected from the South China Sea [8–13], a soft coral *Sarcophyton* sp. attracted our attention because the EtOAc portion showed lethal activity toward brine shrimps, *Artemia salina*. Chemical investigation of the active extract led to the isolation of seven cembrene diterpenoids, named sarcophytol W (**1**), (2*E*,7*E*)-4,11-dihydroxy-1,12-oxidocembra-2,7-dien (**2**) [14], (–)-marasol (**3**) [15], (+)-11,12-epoxy-11,12-dihydrocembrene-C (**4**) [16], (+)-11,12-epoxysarcophytol A (**5**) [17], sarcophytol B (**6**) [18], and sarcolactone A (**7**) [3]. Their structures were elucidated by NMR spectroscopic methods and comparison with previously reported data in the literature. Among these isolated compounds, **1** is a new cembrane diterpenoid possessing 3,14-epoxy and 11,12-epoxy groups (Figure 1).

Sarcophytol W (**1**) was obtained as a colorless oil. Its molecular formula was determined as $C_{20}H_{32}O_3$ on the basis of positive HRESIMS (*m/z* 321.2422 $[M + H]^+$; calc. 321.2430), with five degrees of unsaturation, demonstrating the presence of an additional oxygen with respect to (–)-marasol (**3**) [15]. Careful comparison of the ^1H and ^{13}C NMR data of **1** with those of **3** showed a close structural relationship between them. The main differences were the presence of the typical signals for a trisubstituted epoxy functionality (δ_H 2.57, 1H, dd, J = 2.4, 6.6 Hz; δ_C 66.6, CH and 60.8, C) in **1**, instead of a double bond (δ_H 5.11, 1H, br d, J = 9.2 Hz, H-11; δ_C 131.2, CH, C-11 and 129.8, C, C-12) in **3**, suggesting **1** is an 11,12-epoxy derivative of **3**. Analysis of 2D NMR experiments and in particular of HMBC correlations from H_3-20 to C-11, C-12, and C-13, and ^1H-^1H COSY correlations (Figure 1) confirmed the proposed structure and allowed us to assign all carbon and proton resonances (Table 1).

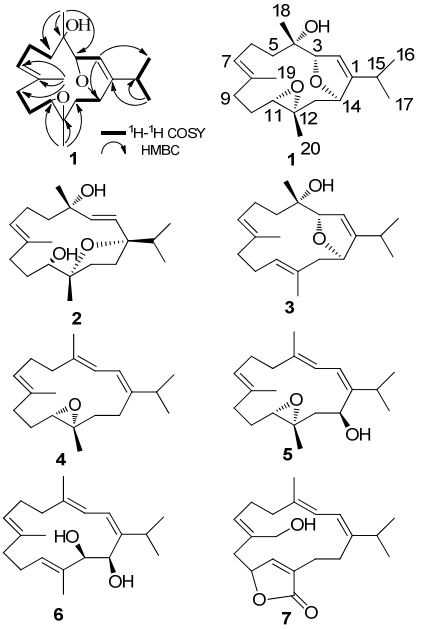

Figure 1: ^1H-^1H COSY and key HMBC correlations for compound **1** and structures of compounds **1**–**7**.

The relative configuration of **1** was assigned on the basis of 1D NOE measurements and 2D NOESY experiments. In the NOESY spectrum, correlations between H-3/H-14, H-3/H-11, H-14/H_3-20 and H-11/H_3-18 revealed the β orientation of H-3, H-11, H-14, H_3-18, and H_3-20. Also, in the selective NOE experiments, the result of irradiation of H-3, H-11, H-14, H_3-18 and H_3-20 was compatible with the 2D NOESY experiments (Figure 2).

The absolute configuration of **1** was assigned on the basis of a shared biogenesis with **2**. The relative and absolute configurations of **2** were determined by 1D NOE experiments and the modified Mosher's method. In the selective 1D NOE experiments with **2**, irradiation of H_3-17 resulted in the enhancement of H_3-18 and H_3-20, and irradiation of H-11 resulted in the enhancement of H_3-20, indicating that they should be on the same face of the

Table 1: ^{1}H and ^{13}C NMR spectroscopic data ($CDCl_3$) of **1**.

position	δ_C	δ_H *J* in Hz
1	151.8	–
2	118.1	5.43 (lH, d, 1.8)
3	87.5	4.54 (lH, m)
4	74.6	–
5	40.6	1.93 (Ha, ddd, 14.4, 10.8, 1.8), 1.60 (Hb, ddd, 14.4, 7.8, 1.8)
6	21.7	2.18 (Ha, m), 2.13 (Hb, m)
7	126.0	5.35 (1H, t, 6.0)
8	132.6	–
9	36.5	2.20 (2H, m)
10	25.0	1.76 (2H, m)
11	66.4	2.58 (1H, dd, 6.6, 2.4)
12	60.6	–
13	45.5	2.18 (Ha, dd, 13.6, 4.2), 1.01(Hb, d, 13.6)
14	82.8	4.80 (1H, m)
15	26.2	2.17 (1H, m)
16	22.8	1.03 (3H, d, 6.6)
17	21.2	1.12 (3H, d, 6.6)
18	22.8	1.00 (3H, s)
19	16.6	1.59 (3H, s)
20	16.5	1.34 (3H, s)

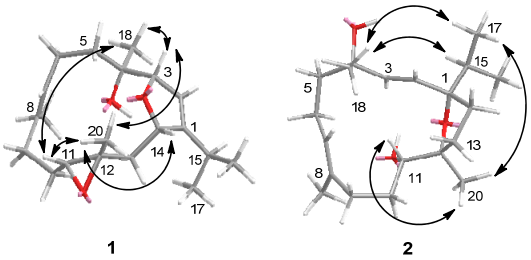

Figure 2: Selected NOE correlations for compounds **1** and **2**.

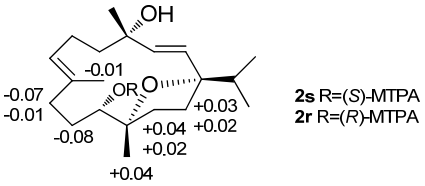

Figure 3: Values of $\Delta\delta_{H(S-R)}$ (measured in $CDCl_3$) of the MTPA esters of compound **2**.

molecule (Figure 2). Thus, the relative configurations of all asymmetric carbons of **2** were assigned as *β*-faces of H_3-18, H-11, H_3-20 and the isopropyl group. The absolute configuration of **2** was established by the modified Mosher's method [19]. Treatment of **2** with (*R*)-(+)-*α*- and (*S*)-(–)-*α*-methoxy-*α*-(trifluoromethyl)phenyl-acetyl chloride (MTPA-Cl) gave the corresponding (*S*)- and (*R*)-MTPA esters, **2s** and **2r**, respectively. The ^{1}H NMR signals of the two MTPA esters were assigned on the basis of their ^{1}H–^{1}H COSY spectra. The $\Delta\delta_{H(S-R)}$ values were then calculated (Figure 3). Following the literature [19], the results indicated that the absolute configuration of C-11 was *S*. Therefore, the absolute configuration of **2** was assigned as 1*S*,4*S*,11*S*,12*S*. Correspondingly, the absolute configuration of **1** could also be inferred according to a shared biogenesis as 3*S*,4*S*,11*S*,12*S*,14*R*. Compound **1** was thus identified as (1*Z*,3*S*,4*S*,7*E*,11*S*,12*S*,14*R*)-3,14-epoxy-11,12-epoxy-4-hydroxycembra-1,7-diene.

In this study, six known cembraniod diterpenoids (**2**–**7**) were isolated from the *Sarcophyton* sp.; these had been obtained from soft corals of the family Alcyoniidae in previous reports [16,18,20]. Interestingly, (–)-marasol (**3**) was isolated from *S. infundibuliforme* and *S. glaucum* [13, 20], whereas the enantiomer of **3**, (+)-marasol, was found in the gorgonian *Plexaura flexuosa* of the Plexauridae family [21]. More interestingly, their biosynthetic precursor, sarcophytol A, was found explicitly in both families. These results could partly support the conclusion that the families Alcyoniidae and Plexauridae are closely related taxonomically, which was inferred from our previous chemical investigations [13].

The antibacterial activity of **1**–**7** was evaluated against a panel of pathogenic bacteria *in vitro*. Compound **6** showed antibacterial activity against *Bacillus cereus*, *Staphylococcus albus* and *Vibrio parahaemolyticus* with MIC values of 3.13, 1.56, and 0.50 *μ*M, respectively. The antifouling activity of all the isolated compounds was also evaluated, but only **3** exhibited an antifouling effect on larval settlement of the barnacle *Balanus amphitrite* at a concentration of 10.0 *μ*g/mL.

In conclusion, seven oxygenated cembranoid diterpenes (**1**–**7**) were obtained from the soft coral *Sarcophyton* sp., of which **1** is a new compound possessing 3,14-epoxy and 11,12-epoxy groups. The absolute configuration of **1** was assigned on the basis of the absolute configuration of the related congener **2**, which was determined by application of the modified Mosher's method. In addition, the results reflected part of the chemotaxonomic significance among Alcyoniidae and Plexauridae.

Experimental

General: Optical rotations were measured on a JASCO P-1020 digital polarimeter at room temperature. UV spectra were recorded on an UV-2501PC spectrophotometer, and IR spectra on a Nicolet Nexus 470 spectrometer. ^{1}H and ^{13}C NMR spectra were obtained using a JEOL Eclips-600 (600 and 150 MHz) spectrometer. ESI-MS and HR-ESI-MS were recorded on a Q-TOF Ultima Global GAA076 LC mass spectrometer. HPLC separation was performed with a Waters 1525 semi-preparative HPLC system coupled with a Waters 2996 photodiode array detector. A Kromasil C_{18} preparative HPLC column (250×10 mm, 5 *μ*m) was used. All solvents were of analytical grade (Shanghai Chemical Plant, Shanghai, People's Republic of China). Silica gel (200–300 mesh; Qingdao Marine Chemical Group Co., Qingdao, People's Republic of China), octadecylsilyl (ODS) silica gel (45–60 mm; Merck KGaA, Darmstadt, Germany), and Sephadex LH-20 (Amersham Biosciences Inc., Piscataway, NJ, USA) were used for column chromatography. Precoated silica gel GF254 plates (Yantai Zifu Chemical Group Co., Yantai, People's Republic of China) were used for analytical TLC analyses.

Biological material: The sample of *Sarcophyton* sp. was collected by scuba diving in Xuwen Coral Reef Area, Guangdong Province, China, and was subsequently identified by Prof. Hui Huang of South China Sea Institute of Oceanology, Chinese Academy of Sciences. A voucher specimen (XW-18) was deposited at the Key Laboratory of Marine Drugs, Ministry of Education, Ocean University of China, Qingdao, China.

Extraction and isolation: *Sarcophyton* sp. (950.0 g, wet weight) was homogenized, followed by extraction with ethanol (95%, v/v) and MeOH/$CHCl_3$ (1:1), successively. The organic layer was filtered and concentrated under reduced pressure to give a residue (50.6 g), which was partitioned between EtOAc and H_2O (EtOAc:H_2O = 2:1), 6 times. The EtOAc extract (8.4 g) was first applied to silica gel CC eluting with light petroleum containing increasing amounts of EtOAc to yield 6 fractions (Fr.1 – Fr.6). Fr.2 was separated by silica gel CC (light petroleum: EtOAc = 98:2), followed by Sephadex LH-20 CC (light petroleum: $CHCl_3$:MeOH = 2:1:1) to obtain **3** (38.6 mg) and **4** (1.20 g). Fr.3 was separated by silica gel CC (light petroleum: EtOAc = 15:1) to offer Fr.3-1 and Fr.3-2. Fr.3-1 was further separated by Sephadex LH-20 CC (light petroleum: $CHCl_3$: MeOH = 2:1:1) to yield **1** (40.0 mg). Fr.3-2 was purified by semi-preparative HPLC (MeOH: H_2O = 85:15) to obtain **5** (38.1 mg). Fr.4 was subjected to repeated chromatography over Sephadex LH-20 to give **6** (25.2 mg). Fr.5 was separated by silica gel CC (light petroleum: EtOAc = 4:1), followed by Sephadex

LH-20 CC (light petroleum: $CHCl_3$: MeOH = 2:1:1), and further purified by semi-preparative HPLC (MeOH: H_2O = 80:20) to yield **7** (93.5 mg). Fr.6 was first subjected to silica gel CC, then purified by semi-preparative HPLC to give **2** (16.4 mg).

Sarcophytol W (**1**)
Colorless oil.
$[\alpha]_D^{20}$: +69 (*c* 0.41, $CHCl_3$).
UV λ_{max} (MeOH) nm: 206 (3.01).
IR (KBr): 3046, 2933, 1453, 1366, 1029, 971, 943 cm^{-1}.
^1H NMR and ^{13}C NMR: Table 1.
HRESIMS: *m/z* $[M + H]^+$, calcd for $C_{20}H_{33}O_3$: 321.2430; found: 321.2422.

Preparation of the (S)- and (R)-MTPA ester derivatives of 2: To a stirred solution of **2** (1.5 mg) in pyridine (500 μL) was added 4-(dimethylamino)pyridine (2 mg) and (*R*)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (MTPA-Cl, 10 μL). The mixture was stirred at room temperature for 12 h. The reaction mixture was then passed through a disposable pipet packed with silica gel and eluted with light petroleum and EtOAc (3:1) to give the (*R*)-Mosher ester **2s**. Treatment of **2** (1.5 mg) with (*S*)-MTPA-Cl (10 μL) as described above yielded the corresponding (*R*)-Mosher ester **2r**.

2-(*S*)-MTPA ester (**2s**)
^1H NMR (600 MHz $CDCl_3$): 5.89 (1H, d, *J* = 15.6 Hz, H-3), 5.58 (1H, d, *J* = 15.6 Hz, H-2), 5.47 (1H, d, *J* = 2.4, 10.2 Hz, H-11), 5.22 (1H, d, *J* = 9.0 Hz, H-7), 3.51 (3H, s, OCH_3-MTPA), 2.17 (1H, m, H-9a), 2.14 (1H, m, H-13a), 2.05 (1H, m, H-14a), 1.83 (1H, m, H-14b), 1.80 (1H, m, H-13b), 1.76 (1H, m, H-9b), 1.67 (3H, s, H-19), 1.66 (2H, m, H-10), 1.65 (1H, m, H-15), 1.31 (3H, s, H-18), 1.06 (3H, s, H-20), 0.84 (3H, d, *J* = 6.6 Hz, H-17), 0.81 (3H, d, *J* = 6.6 Hz, H-16).
ESIMS: *m/z* 561 $[M + Na]^+$.

2-(*R*)-MTPA ester (**2r**)
^1H NMR (600 MHz, $CDCl_3$): 5.89 (1H, d, *J* = 15.6 Hz, H-3), 5.58 (1H, d, *J* = 15.6 Hz, H-2), 5.48 (1H, d, *J* = 2.4, 10.2 Hz, H-11), 5.23 (1H, d, *J* = 9.0 Hz, H-7), 3.53 (3H, s, OCH_3-MTPA), 2.18 (1H, m, H-9a), 2.10 (1H, m, H-13a), 2.02 (1H, m, H-14a), 1.83 (1H, m, H-9b), 1.81 (1H, m, H-14b), 1.78 (1H, m, H-13b), 1.74 (2H, m, H-10), 1.68 (3H, s, H-19), 1.63 (1H, m, H-15), 1.30 (3H, s, H-18), 1.02 (3H, s, H-20), 0.83 (3H, d, *J* = 6.6 Hz, H-17), 0.80 (3H, d, *J* = 6.6 Hz, H-16).
ESIMS: *m/z* 561 $[M + Na]^+$.

Antibacterial activity assay: Antibacterial activity was evaluated by the conventional broth dilution assay [22]. Eight bacterial strains, *Bacillus cereus*, *Tetragenococcus halophilus*, *Staphylococcus albus*, *S. aureus*, *Escherichia coli*, *Pseudomonas putida*, *Nocardia brasiliensis*, and *Vibrio parahaemolyticus* were used, and ciprofloxacin was employed as a positive control.

Antifouling bioassay: Antifouling activity of the compounds on the larval settlement of the barnacle *Balanus amphitrite* was tested according to literature procedures [23].

Supplementary data: ^1H, ^{13}C, ^1H-^1H COSY, HMQC, HMBC, 1D NOE ,2D NOESY and mass spectra of **1**; 1D NOE spectra of **2**; ^1H and mass spectra of the (*R*)- and (*S*)-MTPA esters of **2** ; ^1H and ^{13}C date of **3** are also available.

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Crotofolane Diterpenoids from *Croton caracasanus*

Katiuska Chávez[a], Reinaldo S. Compagnone[b], Ricarda Riina[c], Alexander Briceño[d], Teresa González[d], Emilio Squitieri[b], Carlos Landaeta[b], Humberto Soscún[d] and Alírica I. Suárez[a,*]

[a]*Facultad de Farmacia*, *Universidad Central de Venezuela*, *Caracas*, *Venezuela*
[b]*Escuela de Química*, *Facultad de Ciencias*, *Universidad Central de Venezuela*, *Caracas*, *Venezuela*
[c]*Real Jardín Botánico*, *RJB-CSIC*, *Plaza Murillo 2*, *Madrid 28014*, *Spain*
[d]*Centro de Química*, *Instituto Venezolano de Investigaciones Científicas (IVIC)*, *Caracas*, *Venezuela*

alirica.suarez@ciens.ucv.ve

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Four new crotofolane-type diterpenoids, crotocarasin (A-D) (**1-4**), together with the known crotofolin E, were isolated from a dichloromethane extract of the stems of *Croton caracasanus* Pittier. The structures of the new compounds were determined by spectroscopic methods, and the structure of **3** was further confirmed by single-crystal X-ray data analyses.

Keywords: Diterpenoids, Crotofolanes, *Croton caracasanus* Pittier, Euphorbiaceae.

The genus *Croton* (Euphorbiaceae), one of the largest genera of flowering plants [1], is a rich source of terpenoids. A great variety of structurally diverse diterpenoids such as abietanes [2], neo-clerodanes [3] labdanes [4], and *ent*-kauranes [5] are frequently found in *Croton* species. The presence of cembranoids [6] and nor-crotofolane-type diterpenoids [7] has also been reported. The unusual structure of one crotofolane diterpenoid was reported for the first time from *C. corylifolius* [8]; the compound was named crotofolin A (**6**). Furthermore, a series of compounds, crotofolins B, C, D and E, were identified from the same species [9] and from *C. dichogamus* [10]. More recently, new crotofolanes were reported from *C. cascarilloides* [11]. As part of our continuing interest in the chemistry of *Croton* species occurring in Venezuela [12-15], we now report the isolation and structural elucidation of four new diterpenoids with a crotofolane skeleton, isolated from stems of *C. caracasanus* Pittier, a species closely related to *C. corylifolius.* The *in vitro* cytotoxicity of the isolated metabolites against PC-3, HeLa, and MCF-7 tumor cell lines was also assessed. From a chemotaxonomic point of view, it is of interest to note that these crotofolane diterpenoids have been isolated only from related species of *Croton.*

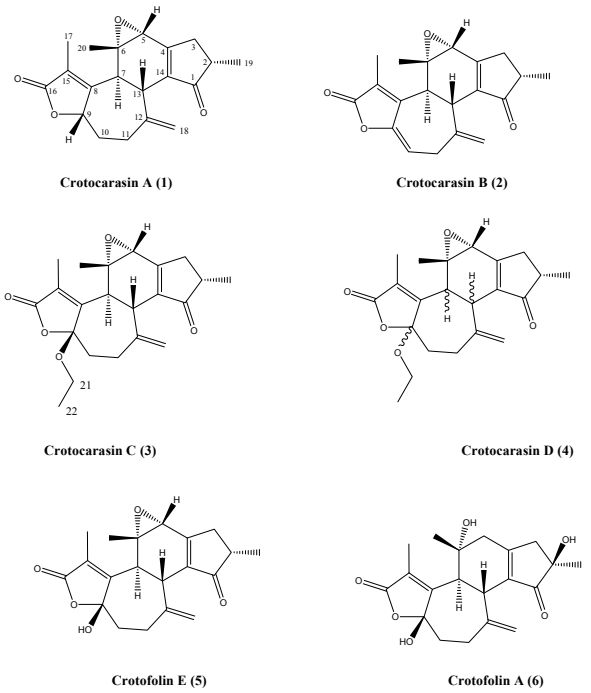

Figure 1: Structures of compounds **1**-**6**.

Crotocarasin A (**1**) was isolated as a colorless sticky solid, $[\alpha]_D^{25}$ -33.3 (*c* 1.00, $CHCl_3$). Its molecular formula, $C_{20}H_{22}O_4$, was established by HREIMS as 326.1500, and its ^{13}C NMR spectrum indicated a structure with 10 indices of hydrogen deficiency. The IR spectrum of **1** revealed absorption bands for an α,β-unsaturated γ-lactone (1753 cm^{-1}), α,β-unsaturated carbonyl (1663 and 1630 cm^{-1}) and terminal olefin (901 cm^{-1}) functionalities. The ^1H and ^{13}C NMR spectra (Tables 1 and 2) showed signals due to three different methyl groups, at δ_H 1.18 (d, *J*= 7.7 Hz, H-19), at δ_H 1.20 (s, H-20) and, a vinylic one at δ_H 1.93 (s, H-17). A terminal olefinic methylene group at δ_H 4.46 and 4.94 (s, H-18), and one allylic proton at δ_H 3.14 (s, H-5) were also evident. ^{13}C NMR and DEPT data (Table 2) indicated the presence of three methyl, four methylene and five methine carbons, one of them oxygenated, at δ_C 82.3 ppm (C-9). The crotofolane skeleton was proposed on the basis of the mentioned features and the COSY, HMQC, and HMBC (Figure 2) correlations, and also by comparison of spectroscopic data of compound **1** with those of crotofolin E (**5**), isolated many years ago from *C. corylifolius* and also isolated in this work (Figure 1).

The differences between the NMR spectra of **1** and **5** could be explained by the absence of the hydroxyl group. Instead, the presence of a broad singlet integrating for one proton at δ_H 5.00 (brs, H-9) identified another oxo-allylic proton on C-**9**, which was corroborated by the difference in the chemical shifts of the carbon in this position (δ_C 82.3 vs 108.7) (Figure 1). NOESY NMR experiments were performed for crotocarasin A (**1**); NOE correlations between the methyl group (H-20) and the oxo-allylic proton (H-5) were observed. The relationship between H-13 and

Table 1: ^{13}C NMR spectral data at 67.5 MHz [$CDCl_3$ (**1**- **4**) and CD_3OD (**5**)].

	1	2	3	4	5
Position			**δ_C**		
1	207.6	207.3	208.0	208.8	209.6
2	40.9	41.1	40.8	40.6	40.9
3	36.4	36.2	36.5	37.4	36.0
4	165.8	166.2	165.7	166.2	167.9
5	54.6	55.0	55.0	56.4	54.5
6	59.4	59.8	60.0	61.2	59.8
7	52.7	53.3	52.8	41.3	53.0
8	161.9	141.6	159.5	162.1	160.2
9	82.3	148.8	110.0	108.7	108.7
10	38.3	113.4	36.9	36.3	41.5
11	35.9	39.0	34.7	28.5	34.5
12	145.8	146.3	146.2	137.7	147.0
13	40.8	40.6	40.2	44.6	39.9
14	144.9	147.1	145.2	145.7	144.4
15	128.5	130.2	129.7	123.4	129.4
16	173.1	169.4	170.9	171.4	171.6
17	10.2	11.3	10.0	11.0	8.6
18	112.8	113.0	112.9	110.1	111.6
19	17.3	17.4	17.4	17.5	16.2
20	19.7	19.4	20.1	22.0	18.9
21	----	----	60.0	59.0	----
22	----	----	15.5	15.3	----

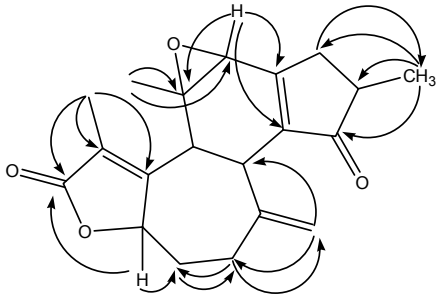

Figure 2: HMBC correlations for crotocarasin A (**1**).

H-9 was evident, indicating that both of them are on the same face of the structure. Due to this, the stereochemistry of the previous isolated crotofolanes was established on the basis of X-ray studies and similarities of the NMR data; here we propose that crotocarasin A (**1**) has a similar relative configuration to crotofolin E (**5**).

Crotocarasin B (**2**) was isolated as a yellow sticky solid with $[\alpha]_D^{25}$ –56.6 (*c* 1.00, $CHCl_3$).The ^{13}C NMR and mass spectrometric data of **2** indicated that it has the molecular formula $C_{20}H_{20}O_4$, thus accounting for eleven elements of unsaturation. The IR spectrum showed bands at 1735, 1710 and 1458 cm^{-1}. In the ^{13}C NMR spectrum of **4** (Table 2), a trisubstituted olefin was deduced from the signals at δ_C 148.8 (C-9) and δ_C 113.4 (C-10), additional to the terminal olefin also found in the previously described compounds. The relationship of each proton and carbon was established from HMQC and HMBC spectra. Close examination of the NMR data (Tables 1 and 2) shows that **2** has the same framework as **1** and **5**, and differs only at C-9 and C-10 (Figure 1)**.** An olefin between these two carbons, confirmed by the strong correlations observed in the HMBC experiment [δ_H 5.97 (H-10) with δ_C 148.8 (C-9), δ_C 141.6 (C-8) and δ_C 39.0 (C-11)] agrees with the proposed structure of **2**, which could be obtained biogenetically by dehydration of crotofolin E (**5**), and is named by us as crotocarasin B.

Crotocarasin C (**3**) was isolated as white crystals, mp 236-238°C, $[\alpha]_D^{25}$ -78.4 (*c* 1.00, $CHCl_3$), and gave a molecular formula of $C_{22}H_{26}O_5$, as determined by HREIMS. Comparison of the ^1H and ^{13}C NMR (Tables **1** and **2**) with those of **5**, suggested a similar structure, but with additional signals. The presence of a multiplet at δ_H 3.80 (m, H-21), integrating for two protons, and a new methyl signal at δ_H 1.19 (t, *J* = 7.2 Hz, H-22), showed the presence of an ethoxy group in this compound. ^{13}C NMR and DEPT spectra showed 22 carbon atoms with 26 attached protons. The signals that identify the principal subunits found in **1** and **5** were also visualized. Close examination of 2D NMR, HMQC, and HMBC data indicated that the crotofolane skeleton was the same as in **5**, and that the ethoxy group found in **3** was attached to C-9. In the NOESY experiment on crotocarasin C (**3**), the NOE correlations more evident were between methyl H-20 and the oxo-allylic H-5. None was observed for protons H-7 and H-13, and with the same criteria used above for crotocarasin A, the relative stereochemistry of the chiral centers was proposed for crotocarasin C (**3**). In order to verify the stereochemical assignment made by NMR experiments, the structure of **3** was determined by single crystal X-ray analysis (Figure 3). The molecular structure of **3** is shown in Figure 1 with the respective labels.

Table 2: ^1H NMR spectral data at 270 MHz [$CDCl_3$ (**1**-**4**) and CD_3OD (**5**)].

	1	2	3	4	5
Position		**δ_H (mult, *J* in Hz)**			
1	---	---	---	---	---
2	2.47 (m)	2.48 (m)	2.48 (m)	2.48 (m)	2.48 (m)
3	2.44 (m), 2.88 (m)	2.43 (m)	2.43 (m), 2.93 (2dd, 6.4; 3.7)	2.54 (m), 2.99 (m)	2.41 (m), 3.04 (m)
4	---	---	---	---	---
5	3.14 (s)	3.16 (s)	3.14 (s)	3.40 (s)	3.30 (s)
6	---	---	---	---	---
7	2.83 (brs)	2.80 (d, 11.6)	2.77 (brs)	3.18 (brs)	2.74 (d, 11.6)
8	---	---	---	---	---
9	5.00 (brs)	---	---	---	---
10	1.23 (m), 2.61 (d, 3.7)	5.97 (dd, 6.4; 3.5)	1.34 (dt, 14.0; 4.9), 2.81(brt, 2.7)	2.04 (m), 2.34 (m)	1.48 (dt, 12.9; 5.7), 2.58 (m)
11	2.23 (dt, 12.6; 3.7), 2.64 (m)	3.18 (m)	2.20 (dt, 13.4; 4.2), 2.42 (m)	2.39 (m), 2.72 (m)	2.53 (m)
12	---	---	---	---	---
13	3.01 (m)	3.21 (m)	3.48 (td, 11.6; 3.5)	3.15 (s)	3.59 (td, 11.6; 3.5)
14	---	---	---	---	---
15	---	---	---	---	---
16	---	---	---	---	---
17	1.93 (s)	2.08 (s)	1.89 (s)	1.83 (s)	1.85 (s)
18	4.46 (s), 4.94 (s)	4.53 (s), 5.04 (s)	4.44 (s), 4.92 (s)	4.30 (s), 4.76 (s)	4.44 (s), 4.90 (s)
19	1.18 (d, 7.7)	1.20 (d, 7.4)	1.17 (d, 7.4)	1.22 (d, 7.3)	1.17 (d, 7.7)
20	1.20 (s)	1.23 (s)	1.25 (s)	1.46 (s)	1.29 (s)
21	---	---	3.80 (m)	3.55 (m)	---
22	---	---	1.19 (t, 7.2)	1.22 (t, 6.9)	---

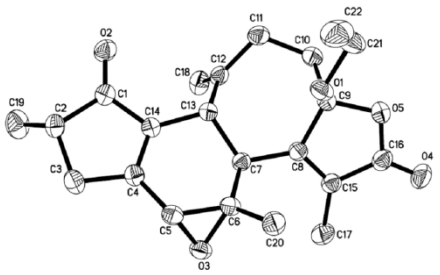

Figure 3: ORTEP drawing of compound **3**. No absolute configuration was determined (Hydrogen atoms were omitted for clarity).

Crotocarasin D (**4**) was isolated as a colorless oil, $[\alpha]_D^{25}$ -48.8 (*c* 1.00, $CHCl_3$), and gave the same molecular formula $C_{22}H_{26}O_5$ as **3**, deduced from HREIMS and ^{13}C NMR. The ^1H NMR spectrum resembled compound **3**, but with the relevant difference of one methyl signal integrating for 6 protons, which was identified at δ_H 1.22 (d, *J* = 7.3 Hz, H-19 and t, *J* = 6.9 Hz, H-22); the correlations between carbons and protons at this chemical shift indicated that two methyl groups are involved with this signal; the carbon at δ_C 17.5 (C-19) correlates with the doublet for H-19, and the signal at δ_C 15.3 (C-22) is correlated with the triplet for H-22, respectively. The other two methyl groups present in **3** were found in **4**, also as two singlets at δ_H 1.83 (s, H-17) and δ_H 1.46 (s, H-20). The differences in ^1H and ^{13}C NMR spectra between **3** and **4** (see Tables 1 and 2) involved mainly the seven membered ring and the gamma lactone. These differences could be explained by possible conformational changes in these rings. The NOESY NMR experiment of crotocarasin D (**4**) did not show any correlation between H-7/H-13. GIAO/CAM-B3LYP/6-31+G(d,p) calculations were performed on the three possible diastereomers to compare theoretical and experimental ^1H and ^{13}C NMR spectra, and thus predict the configuration of this new crotofolane, but were not conclusive (results not shown). Different experiments are necessary to propose the relative stereochemistry in the diastereomer of **3** named as crotocarasin D (**4**). An attempt to obtain a crystal for this compound was unsuccessful.

Crotocarasins A-D (**1**-**4**) were tested for cytotoxic activity against human tumor cell lines PC-3, HeLa, and MCF-7, using the MTT method [16]. Adriamycin was used as a positive control. IC_{50} values > 50 μM were obtained, which indicate that these crotofolanes were not cytotoxic.

Experimental

General experimental procedures: Melting points were measured on a Kofler hot-stage melting point instrument and are uncorrected. Specific rotations were acquired with an ATAGO Polax-2L polarimeter. IR spectra were recorded on a Shidmazu 470 spectrophotometer, EIMS on a Varian Saturn 2000, and HREIMS with a Finnigan Trace mass spectrometer. NMR spectra were measured on a JEOL 270 MHz, and Bruker AMX-500. Chemical shifts are given in ppm referenced to the residual non-deuterated solvent signal ($CHCl_3$ 7.24 and 77.0 ppm). CC was performed using Si gel (70-230 mesh) from Scharlau. TLC analysis was carried out using plastic precoated plates (Merck, Si gel plates $GF_{254,}$ 0.2 mm) and the compounds were visualized using either a UV lamp λ = 254 nm or by spraying with *p*-anisaldehyde. All solvents used were of analytical grade.

Plant material: Aerial parts of *C. caracasanus* Pittier were collected around Caracas, Venezuela, in August 2009 and identified by Dr Ricarda Riina. A voucher specimen (VEN 382364) has been deposited at the Herbario Nacional de Venezuela.

Extraction and isolation: The air-dried and powdered stems (213 g) of *C. caracasanus* were extracted by maceration with MeOH (2 x 2L) at room temperature for 15 days, and the combined extracts were concentrated *in vacuo*. The residue was dissolved in a mixture of MeOH: H_2O (1:1), and then partitioned in turn with *n*-hexane, dichloromethane, and EtOAc, to afford *n*-hexane (1.38 g), CH_2Cl_2 (0.99 g), EtOAc (0.70 g) and a residual hydro-methanolic fraction (2.75 g). CC of the dichloromethane fraction over Si gel, using a CH_2Cl_2: EtOAc (90:10) mixture with increasing polarity afforded fractions A-M. Fraction D furnished crotocarasin B (**2**) (55.0 mg); fraction E was subjected to chromatography with Si-gel eluting with *n*-hexane:EtOAc:MeOH (65:30:5) to yield crotocarasin C (**3**) (29.3 mg). Crotocarasin D (**4**) (23.1 mg) was obtained pure after purification of fraction J by CC on Si gel using a step gradient of CH_2Cl_2:*n*-hexane (0-5%) as eluent. In the same manner, fraction G gave crotofolin E (**5**) (51.5 mg). Preparative TLC ($CHCl_3$: EtOAc, 88:12) of fraction I afforded crotocarasin A (**1**) (19.2 mg).

Crotocarasin A (**1**)
Colorless sticky solid.
$[\alpha]_D^{25}$: –33.3 (*c* 1.00, $CHCl_3$).
IR ν_{max} film: 3473, 1753, 1704, 1663, 1630, 1447, 901 cm^{-1}
^1H NMR and ^{13}C NMR: Table **2** and **1**.
HREIMS: *m/z* 326.1510 $[M]^+$; calcd for $C_{20}H_{22}O_4$: 326.1512.

Crotocarasin B (**2**)
Yellow sticky solid.
$[\alpha]_D^{25}$: –56.6 (*c* 1.00, $CHCl_3$)
IR ν_{max} film: 3233, 1735, 1710, 1651, 1632, 1458, 902 cm^{-1}.
^1H NMR and ^{13}C NMR: Table **2** and **1**.
HREIMS: *m/z* 324.1360 $[M]^+$; calcd for $C_{20}H_{20}O_4$: 324.1356.

Crotocarasin C (**3**)
White crystals.
MP: 236-238°C.
$[\alpha]_D^{25}$: –78.4 (*c* 1.00, $CHCl_3$).
IR ν_{max} film: 3491, 1758, 1707, 1670, 1650, 1630, 1441, 940 cm^{-1}.
^1H NMR and ^{13}C NMR: Table **2** and **1**.
HREIMS: *m/z* 370.1775 $[M]^+$; calcd for $C_{22}H_{26}O_5$: 370.1773.

Crotocarasin D (**4**)
Colorless oil.
$[\alpha]_D^{25}$: –48.8 (*c* 1.00, $CHCl_3$).
IR ν_{max} film: 3464, 1763, 1705, 1597, 1433, 1120, 938, 910 cm^{-1}.
^1H NMR and ^{13}C NMR: Table **2** and **1**.
HREIMS *m/z*: 370.1771 $[M]^+$; calcd for $C_{22}H_{26}O_5$: 370.1773.

Crotofolin E (**5**)
White crystals.
MP: 207-209°C.
$[\alpha]_D^{25}$: –76.4 (*c* 1.00, $CHCl_3$).
IR ν_{max} film: 3465, 1737, 1695, 1651, 1628, 1456, 952, 902 cm^{-1}.
^1H NMR and ^{13}C NMR: Table **2** and **1**.
HREIMS: *m/z* 342.1459 $[M]^+$; calcd for $C_{20}H_{22}O_5$: 342.1461.
Note: several NMR assignments were different from those reported in the reference and here are reassigned according to 2D NMR data, which was not available in the original publication [9].

Crystal data of crotocarasin C (3): Compound **3** was crystallized from $CHCl_3$ solution by slow solvent evaporation. A colorless block crystal was selected for crystallographic measurements. Intensity data were recorded at room temperature on a Rigaku AFC-7S diffractometer equipped with a Mercury CCD detector using monochromatic Mo(Kα) radiation (λ = 0.71070 Å). Experimental details on unit cell and intensity measurements can be found in the

CIF file deposited at the Cambridge Crystallographic Data Centre with CCDC number 867457. The structure was solved by Direct Methods and refined by full-matrix least-squares on F^2. The H-atoms on C were placed in calculated positions using a riding atom model with fixed C-H distances [0.93 Å for C(sp^2), 0.96 Å for C(sp^3, CH_3), and 0.97 Å for C(sp^3, CH_2)]. All the H atoms were refined with isotropic displacement parameters set to 1.2 × *U*eq for C(sp^2) and 1.5 for C(sp3) of the attached atom. All the refinement calculations were made using SHELXTL-NT [16].Molecular formula = $C_{22}H_{26}O_5$,molecular mass = 370.4393, crystal system = orthorhombic, space group = $P2_12_12_1$, unit cell dimensions: a = 10.478 (3) Å, b = 12.250 (3) Å, c = 15.590 (3) Å; V = 2001.0 (9) $Å^3$, 4; ρ = 1.230 Mg/m^3; μ = 0.086 mm^{-1}, F(000) =792, λ(Mo Kα).Theta range for data collection = 4.20° to 55.40°; limiting indices = -12≤ *h*≤ *12,-11* ≤ *k* ≤ *14, -18,* ≤ *l* ≤ *18*; collected reflections 23828; independent reflections 3272 [R(int) = 0.0360]; Data/Restraints/Parameters = 3948/0/245; Goodness-of-fit on F^2 = 1.096; Final R indices [$I>2\alpha$ (I)]; R indices (all data) R_1= 0.0546, wR_2= 0.1006; Largest diff. Peak and hole0.137 and -0.139 $eÅ^{-3}$.

Supplementary data: Spectral data, theoretical calculations and cytotoxicity values are available.

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Development and Validation of a Modified Ultrasound-Assisted Extraction Method and a HPLC Method for the Quantitative Determination of Two Triterpenic Acids in *Hedyotis diffusa*

Yu-Chiao Yang[a], Ming-Chi Wei[b,*], Hui-Fen Chiu[a] and Ting-Chia Huang[c]

[a]*Department and Graduate Institute of Pharmacology*, *Kaohsiung Medical University*, *Kaohsiung 80708*, *Taiwan*
[b]*Department of Environmental Engineering & Science*, *Chia Nan University of Pharmacy and Science*, *Tainan 71710*, *Taiwan*
[c]*Department of Chemical Engineering*, *National Cheng Kung University*, *Tainan 70101*, *Taiwan*

s120702@mail.chna.edu.tw; *ycyang@kmu.edu.tw*

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In the present study, the oleanolic acid (OA) and ursolic acid (UA) contents of *Hedyotis diffusa* and *H. corymbosa* were determined by a rapid, selective and accurate method combining modified ultrasound-assisted extraction (MUAE) and HPLC. Compared with traditional extraction methods, MUAE reduced the extraction time, the extraction temperature and the solvent consumption and maximized the extraction yields of OA and UA. Furthermore, the combined MUAE–HPLC method was applied to quantitate OA and UA in plant samples and exhibited good repeatability, reproducibility and stability. The mean recovery studies (one extraction cycle) for OA and UA were between 91.3 and 91.7% with RSD values less than 4.5%. The pioneer method was further applied to quantitate OA and UA in six samples of *H. diffusa* and five samples of *H. corymbosa*. The results showed that the OA and UA content in the samples from different sources were significantly different. This report is valuable for the application of *H. diffusa* and *H. corymbosa* obtained from different regions in clinical research and pharmacology.

Keywords: *Hedyotis diffusa*, *Hedyotis corymbosa*, Oleanolic acid, Ursolic acid, Modified ultrasound-assisted extraction.

The dried whole plants of *Hedyotis diffusa* Willd. (synonym *Oldenlandia diffusa* Willd., family Rubiaceae) are called "Peh-Hue-Juwa-Chi-Cao" in Chinese. The extracts of *H. diffusa* possess various pharmacological properties, such as anti-oxidative, anti-angiogenic, antimutagenic, neuroprotective, anti-inflammatory, hepatoprotective and anticancer activities [1,2]. Up to now, four major classes of compounds, triterpenoids, flavonoids, anthraquinones and steroids, have been reported as bioactive compounds from this herb [1,3]. Among these components, triterpenic acids such as oleanolic acid (OA) and ursolic acid (UA) have been extensively studied for their pharmacological properties due to their high abundance in medicinal herbs. Both have minimal toxicity and have been shown to display numerous biological properties with therapeutic potential [4]. In recent years, studies have shown that UA and OA induce apoptosis in a wide variety of cancer cells, including hepatocellular carcinoma [5], prostate carcinoma [6], colorectal cancer [7], acute myelogenous leukemia [8], skin tumorigenesis [9], cervical carcinoma [10] and lung carcinoma [11].

Several publications have reported the analysis of OA and UA in other raw materials using HPLC methods [12-14]. However, the simultaneous determination of OA and UA in plant extracts is a difficult task due to their structural similarity [15]. In this paper, a simple, rapid and accurate HPLC method coupled with a photo diode array (PDA) detector is described for the analysis of OA and UA from *H. diffusa*. The optimal conditions for the analytical method were also investigated for the best resolution and highest sensitivity of detection. In addition, *H. corymbosa* (L.) Lam has also been used in Taiwan for treating the same conditions as *H. diffusa*. To compare the efficacy of the two herbs, the OA and UA contents of *H. diffusa* and *H. corymbosa* were also analyzed.

Recently, traditional solvent [16], ultrasonic-assisted [12,13,17,18] and supercritical carbon dioxide [13] extraction techniques have been employed in the extraction of OA and UA from different materials. In the case of ultrasonic-assisted extraction (UAE), the principal advantage is that it is the simplest and most economical technique and is easy to scale up to industrial level. Several probable mechanisms for the ultrasonic enhancement of extraction, such as cell disruption, improved penetration and enhanced swelling and hydration processes, have been cited as leading to enhanced extraction [19-20].

In this study, we focussed primarily on the development of an efficient technique for the extraction and determination of OA and UA contents in *H. diffusa* and *H. corymbosa.* The novel method consists of heat-reflux extraction (HRE) (5–10 min), followed by UAE, which is the first time that this novel method has been investigated. The effects of processing parameters were further evaluated. The optimized conditions giving the highest OA and UA yields were recorded and used to compare the results obtained with those found using conventional UAE and HRE. Moreover, a simple, rapid and accurate HPLC method was developed and optimized for the analysis of OA and UA in extracts of *H. diffusa* and *H. corymbosa*.

Because various parameters potentially affect the UAE process, the optimization of the experimental conditions represents a critical step in the development of a UAE method. In this study, UAE parameters such as the type of solvent, solvent consumption, extraction time, extraction temperature, duty cycle, liquid/solid ratio, material particle size and ethanol-water mixture, were optimized. However, the extraction yields of OA and UA (shown in Table 1) still did not match those of the extracts prepared by HRE

(one extraction cycle). Therefore, MUAE in the presence of two stages of extraction was employed in order to enhance the extraction efficiency. Moreover, the conventional HRE of OA and UA from *H. diffusa* was also optimized and compared with MUAE and UAE. The optimized conditions and extraction yields of OA and UA obtained by MUAE, UAE and HRE are listed in Table 1 (one extraction cycle). The application of MUAE significantly reduced the extraction time, extraction temperature and solvent consumption, and achieved superior OA and UA yields. This was attributed to the cavitational effects of ultrasound disruption of plant cell walls and facilitating the release of the target constituents. This release enhanced the mass transport of the solvent from the continuous phase into the plant cells. These results clearly demonstrated that the developed MUAE method provided a good alternative for the extraction of OA and UA from *H. diffusa*.

Table 1: Comparison of optimum extraction parameters for HRE, UAE and MUAE methods.

Extraction parameters	Extraction method		
	HRE	UAE	MUAE
Herbal sample	HD1	HD1	HD1
Particle size (mm)	0.2–0.5	0.2–0.5	0.2–0.5
Plant weight (g)	5	5	5
Frequency (kHz)	–	40	40
Duty cycle (%)	–	79	79
Stirring rate (rpm)	200	–	200
Time (min)	60	40	40
Temperature (°C)	Boiling point	55	55
Liquid/solid ratio (mL/g)	14	12	12
Extraction cycle	1	1	1
OA			
Yield (mg/g)[a]	0.59 ± 0.03	0.56 ± 0.03	0.60 ± 0.02
Ethanol (%)[b]	70	60	60
RSD (%)[c]	4.42	5.35	3.82
UA			
Yield (mg/g)[a]	3.28 ± 0.14	3.06 ± 0.14	3.37 ± 0.13
Ethanol (%)[b]	90	80	80
RSD (%)[c]	4.20	4.57	3.94

[a]Values are written as the mean (mg/g) ± standard deviation (SD) of six replications. The amounts are expressed as mg of the target compound per g of the plant on a dry weight basis. [b]Ethanol concentration in water (v/v). [c]RSD (%) = (SD/mean) × 100.

The optimization of the HPLC conditions for the analysis of triterpenic acids in *H. diffusa* was achieved by varying the mobile phase composition, flow rate and column temperature. The chromatograms of standard and ultrasonically extracted *H. diffusa* are compared. OA and UA were satisfactorily separated under the optimum conditions described above, with retention times of 50.44 ± 0.06 and 56.62 ± 0.08 min, respectively. No interference peaks from the endogenous constituents of the extract of *H. diffusa* were found in the region of the investigated compounds. Furthermore, the two compounds of interest were unequivocally confirmed by the retention times and UV spectra of the authentic standards. Therefore, this HPLC system was simple, easy to use, and effective for the identification and quantification of OA and UA in *H. diffusa*.

The calibration curve was constructed using a linear regression of the theoretical concentration of an analyte versus the corrected peak area. The mean regression equations and their correlation coefficients were calculated to be $Y = 1.8083 \times 10^6 X + 7.8839 \times 10^4$ and $R^2 = 0.997$ for OA, and $Y = 1.5469 \times 10^6 X - 1.6539 \times 10^4$ and $R^2 = 0.998$ for UA. The analytical procedure was also sensitive with respect to the limits of detection (LOD) and limits of quantification (LOQ) for OA and UA (0.056 and 0.094 μg/mL, respectively, for the LOD and 0.18 and 0.31 μg/mL, respectively, for the LOQ).

The precision and accuracy of the developed method was evaluated by measuring intra- and inter-day variability in terms of the relative standard deviation (RSD) and the relative error (RE). The standard solutions, at four different concentration levels, were analyzed at least six times within the same day, and the RSD and RE values obtained were less than 5.0 and 4.0% for OA and less than 4.5 and 4.0% for UA. Similarly, to measure the inter-day variability, the same concentration of the two standards was run over at least six consecutive days, and the values were 6.5 and 5.7% for OA and 6.3 and 5.9% for UA. Therefore, the criteria for the precision and accuracy for analyzing the OA and UA samples were fulfilled in the developed analytical method.

Table 2: Repeatability and reproducibility of the developed MUAE–HPLC method and stability of the sample.

Analytes	Repeatability[a]		Reproducibility[b]		Stability (RE (%))[c]	
	Mean (mg/g)	RSD (%)[d]	Mean (mg/g)	RSD (%)[d]	2 4 h	48 h
OA	0.60	3.82	0.60	5.98	–1.07	1.46
UA	3.37	3.94	3.21	6.63	–0.98	–1.12

[a]Six samples of HD1 from the same source were extracted and analyzed under the optimum conditions during one day. [b]The extraction yields obtained from six independent extractions (HD1) performed on five consecutive days. [c]RE (%) = [(mean observed concentration after 24 h (or 48 h) − initial mean concentration)/initial mean concentration] × 100. [d]RSD (%) = (SD/mean)×100.

To study the repeatability of the developed MUAE−HPLC method, six herbal samples (HD1) from the same source were extracted under the optimized conditions and analyzed using the established method. Table 2 (one extraction cycle) reveals that the RSD of the OA and UA content in six replicate herbal samples was 3.82 and 3.94%, respectively, indicating that the developed method had good repeatability. Furthermore, the reproducibility was evaluated by calculating the extraction yields obtained from six independent extractions performed on five consecutive days. The obtained RSD for OA and UA were less than 6.7%, which were within the acceptance criteria and indicated that the combined MUAE−HPLC method was accurate, reliable, and reproducible.

The same sample solution was stored at 25°C and analyzed every 12 h over 48 h to evaluate the stability of the samples. The results showed that the relative error (RE) of OA and UA were 1.07 and 0.98%, respectively, after 24 h and 1.46 and 1.12%, respectively, after two days. The results listed in Table 2 indicate that OA and UA were relatively stable in 60% ethanol solution for at least two days.

Table 3: Extraction recoveries of spiked analytes.

Analytes[a]	Content (mg)	Spiked (mg)	Found[b] (mg)	Recovery[c] (%)	Mean[d] (%)	RSD[d] (%)	Mean[e] (%)	RSD[e] (%)
OA	2.67	1.5	4.06 ± 0.19	92.67 ± 4.34	91.72	4.5	100.84	5.67
		3.0	5.47 ± 0.26	93.33 ± 4.45				
		4.5	6.79 ± 0.28	90.16 ± 3.81				
UA	14.95	8.0	22.20 ± 0.89	90.63 ± 3.71	91.29	4.3	100.39	5.41
		15.0	28.78 ± 1.21	92.20 ± 3.87				
		25.0	37.71 ± 1.75	91.04 ± 4.26				

[a]Three different quantities of the authentic standards were added to the sample solution (HD1). The mixtures were extracted (1 extraction cycle) and analyzed using the optimized MUAE–HPLC method. [b]The experiments were repeated six times at each level. Values are expressed as the mean ± standard deviation (SD). [c]Recovery (%) = [(found value – content)/spiked value] × 100. [d]One extraction cycle. [e]Three extraction cycles.

Table 3 shows the recoveries of OA and UA after applying the combined MUAE−HPLC method. As listed in Table 3, the mean recoveries of OA and UA were 91.72%, with an average RSD of 4.56% (n = 6), and 91.29%, with an average RSD of 4.32% (n = 6), respectively (1 extraction cycle). The mean extraction recoveries of OA and UA reach 100% after three extraction cycles using MUAE in the present work. The recovery results revealed that the MUAE method was adequate and appropriate for the investigated analysis. The recovery tests also confirmed that the HPLC method was reliable and accurate for OA and UA.

Using optimized experimental conditions, the developed MUAE−HPLC method was applied for the simultaneous quantitation of OA and UA in the extracts of six samples of *H. diffusa* (HD1 to HD6) and five of *H. corymbosa* (HC7 to HC11) collected from different areas. The calculated content of the two components are summarized in Table 4 (one extraction cycle). There were significant variations in the OA and UA contents in the different samples of *H. diffusa* and *H. corymbosa*. The OA and UA contents in the six batches of *H. diffusa* ranged from 0.32 to 0.80 mg/g and from 1.75 to 3.42 mg/g, respectively. The RSD values calculated for the average content (HD1 to HD6) were 21.7% for OA and 28.2% for UA. For *H. corymbosa*, the OA and UA contents in the five batches ranged from 1.53 to 2.15 mg/g and from 6.36 to 8.54 mg/g, respectively. The RSD values calculated for the average content (HC7 to HC11) were 12.4% for OA and 11.4% for UA. These results were not surprising, as previous studies have revealed that the content of bioactive components in plants could vary greatly between cultivars [21]. Notwithstanding, further studies on the influence of genetic and environmental factors on bioactive content should be performed. Table 4 also reveals that the amount of OA in all the plant samples was markedly lower than the amount of UA. These results are valuable for the application of *H. diffusa* and *H. corymbosa* to clinical research and pharmacology.

Table 4: The mean contents of OA and UA present in 11 samples of *H. diffusa* and *H. corymbosa.*

Sample[a]	OA		UA	
	Content[b] (mg/g)	RSD[c] (%)	Content[b] (mg/g)	RSD[c] (%)
HD1	0.60± 0.02	3.17	3.37 ± 0.13	3.94
HD2	0.32 ± 0.01	3.84	1.75 ± 0.07	4.05
HD3	0.80 ± 0.02	2.97	3.42 ± 0.13	3.76
HD4	0.59 ± 0.02	4.25	2.16 ± 0.07	3.27
HD5	0.76 ± 0.03	3.87	2.94 ± 1.01	3.09
HD6	0.50 ± 0.01	3.06	1.97 ± 0.08	4.23
HC7	1.79 ± 0.08	4.22	7.04 ± 0.25	3.51
HC8	1.53 ± 0.05	3.43	6.36± 0.19	2.96
HC9	2.15 ± 0.08	3.58	8.54 ± 0.33	3.85
HC10	1.94 ± 0.06	2.94	7.85 ± 0.31	4.01
HC11	1.80 ± 0.07	4.03	7.99 ± 0.30	3.74

[a]Six different *H. diffusa* and five different *H. corymbosa* samples were extracted using the optimized MUAE process (1 extraction cycle), and the extracts were analyzed by the developed HPLC method. [b]Values are expressed as the mean ± standard deviation (SD) of six replications and are calculated based on the dry weight of the plant. [c]RSD (%) = (SD/mean) × 100.

Experimental

Plant material: Six batches of dried, whole *H. diffusa* plants (Sample HD1 to HD6) and 5 samples of *H. corymbosa* (Sample HC7 to HC11) were purchased from different local Chinese medicinal shops (Taiwan). The air-dried whole plants were pulverized in a knife mill, and parts were sieved to produce samples with sizes of 0.6–0.85, 0.5–0.6, 0.21–0.5 and <0.21 mm. The moisture content (% dry weight basis) was determined by drying at 105°C to a constant mass and was 11.32 to 12.82% for samples HD1 to HC11. All the yields and compositions were calculated based on a moisture-free basis and represent the mean values of at least 6 experiments.

Chemicals: Oleanolic acid (97%) and ursolic acid (95%) were purchased as HPLC reference standards from the Sigma Chemical Co. (St. Louis, MO, USA). Methanol (99.9%), ethanol (99.9%), acetone (99.7%), acetonitrile (99.9%), ethyl acetate (99.9%), *n*-hexane (95%) and 85% phosphoric acid were bought from Merck Co. (Darmstadt, Germany), while cyclohexane and sulfuric acid were supplied by Tedia (Fairfield, OH, USA) and Acros Organics (Morris Plains, NJ, USA), respectively. Deionized water was prepared using a Milli-Q reverse osmosis unit from Millipore (Bedford, MA, USA).

Preparation of standard solutions: Standard stock solutions of OA and UA were prepared daily. The standard mixture solutions containing OA and UA at low (40 μg/mL), medium (200 μg/mL) and high (500 μg/mL) concentrations were prepared in a similar manner to yield the quality-control (QC) samples.

Modified ultrasound-assisted extraction (MUAE): MUAE was carried out with a working frequency of 40 kHz and 185W (Branson B-33510E-DTH, USA) of power; this extraction was operated in a pulse mode that provided 95 s of pulse on, followed by 25 s of pulse off for every 120 s (79% on/off time, work with the hands). There were 2 stages of extraction: HRE and UAE. An HRE (200 rpm) was used to obtain good solvent-to-plant material contact (10 min). For all experiments, 10 min HRE was followed by UAE. The MUAE conditions were then utilized at various ratios of solvent to raw material (6–22 mL/g), ultrasound amplitude (0–100%), ethanol concentrations (0–100 %), particle sizes (0.85–1.0, 0.6–0.85, 0.5–0.6, 0.21–0.5 and <0.21 mm), extraction times (5–60 min), extraction temperatures (30–80°C) and extraction cycles (1–4). At least 6 replicates were performed for each extraction condition tested, and all analyses were repeated 6 times.

Heat-reflux extraction (HRE): HRE was performed by mixing 5 g of dried, powdered plant with an appropriate amount of extraction solvent (100 mL) in a 250 mL conical flask that was immersed in a temperature-controlled water-bath, with stirring (200 rpm). The follow-up extractions were performed using the method detailed above for MUAE. The post-treatment of the extracts was also the same as that mentioned for MUAE.

High-performance liquid chromatography (HPLC): The HPLC analysis of OA and UA was performed on a Jasco HPLC system (Jasco, Tokyo, Japan) with a LiChrospher® C-18 analytical column (250 mm × 4 mm i.d., 5-μm particle size) (Merck, Darmstadt, Germany). The mobile phase was composed of acetonitrile (A) and 0.1% aqueous H_3PO_4 (B). The gradient was as follows: 0–25 min, 22–23% (solvent A), flow rate of 1 mL/min; 25–40 min, 23–23% (solvent A), flow rate of 1.0–1.5 mL/min; and 40–60 min, 23–90% (solvent A), and flow rate of 1.5–1.0 mL/min. The temperature of the column was maintained at 40°C, and the effluent was monitored at 210 nm. Detailed descriptions are available elsewhere [12].

Calibration curves: Eleven-point calibration curves for OA and UA were constructed. Working solutions at concentrations of 3, 6, 8, 10, 40, 90, 200, 400, 600, 800 and 1000 μg/mL were prepared for OA, and 10, 20, 40, 70, 100, 120, 200, 400, 600, 800 and 1000 μg/mL for UA. This operation was repeated on 6 consecutive days with freshly prepared calibration standards to select the most appropriate regression model.

Limits of detection and quantification: The LOD and LOQ of the analysis method were determined as the analyte concentrations giving rise to signal-to-noise ratios of 3 and 10, respectively. For this purpose, the baseline noise was also evaluated by the injection of 20 μL of the mobile phase B (blank) in 6 replications.

Precision and accuracy, analysis repeatability and stability: The precision and accuracy of the HPLC analysis method were established across the analytical range for OA and UA at 3 concentrations of OA and UA (40, 200 and 500 μg/mL). The intra-day precision was established as the repeatability of the assay and determined by calculating the RSD (%) for the repeated measurements, while the inter-day precision was evaluated as the intermediate precision of the assay. To determine the intra-day precision and accuracy for each standard, the same mixed standard

solutions were examined 6 times within one day. The inter-day precision and accuracy were established by analyzing each sample on 6 consecutive days; each sample was injected 5 times on each day. The assay precision was expressed by the RSD (%) between the replicate measurements, while accuracy was defined as the RE (%), which was calculated using the formula RE (%) = (mean of observed concentration – spiked concentration)/spiked concentration × 100. The injection repeatability was analyzed by injecting the same standard mixture solution 6 times sequentially, while analysis repeatability was examined by injecting 6 different samples obtained through the same sample preparation procedure. For the stability test, the same real sample used for analysis repeatability was analyzed after 48 h at room temperature.

Extraction recovery: To assess the extraction recoveries of OA and UA from *H. diffusa*, 3 different quantities (low, medium and high) of the authentic standards were added to the sample solution before extraction. The follow-up extractions and HPLC analyses were performed in the same manner as described above.

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New Triterpenoid Saponins from the Roots of *Saponaria officinalis*

Barbara Moniuszko-Szajwaj[a,*], Łukasz Pecio[a], Mariusz Kowalczyk[a], Ana M. Simonet[b], Francisco A. Macias[b], Małgorzata Szumacher-Strabel[c], Adam Cieślak[c], Wiesław Oleszek[a] and Anna Stochmal[a]

[a]*Department of Biochemistry*, *Institute of Soil Science and Plant Cultivation*, *State Research Institute*, *Czartoryskich 8*, *24-100 Puławy*, *Poland*

[b]*Grupo de Alelopatia*, *Departamento de Quimica Organica*, *Facultad de Ciencias*, *Universidad deCadiz*, *C/Republica Saharaui*, *s/n*, *11510 Puerto Real*, *Cadiz*, *Spain*

[c]*Department of Animal Nutrition and Feed Management*, *RUMEN PULS*, *Poznan University of Life Sciences*, *Wołyńska 33*, *60-637 Poznań*, *Poland*

bszajwaj@iung.pulawy.pl

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Three new triterpenoid saponins (**1-3**), along with nine known saponins, were isolated from the roots of *Saponaria officinalis* L. Two of them: vaccaroside D (**4**) and dianchinenoside B (**5**) are known, but not previously reported for *S. officinalis*, and seven others: saponarioside C (**6**), D (**7**), F (**8**), G (**9**), I (**10**), K (**11**), and L (**12**) have been previously isolated from this plant. The structures of the new saponins were established as 3-*O*-*β*-D-xylopyranosyl-16*α*-hydroxygypsogenic acid-28-*O*-[*β*-D-glucopyranosyl-(1→6)]-*β*-D-glucopyranoside (**1**), 3-*O*-*β*-D-xylopyranosyl-16*α*-hydroxygypsogenic acid-28-*O*-[*β*-D-glucopyranosyl-(1→3)]-[*α*-D-galactopyranosyl-(1→6)-*α*-D-galactopyranosyl-(1→6)-*β*-D-glucopyranosyl-(1→6)]-*β*-D-glucopyranoside (**2**) and 3-*O*-*β*-D-xylopyranosyl-gypsogenic acid-28-*O*-[*β*-D-glucopyranosyl-(1→3)]-[6-*O*-(3-hydroxy-3-methylglutaryl)-*β*-D-glucopyranosyl-(1→6)]-*β*-D-glucopyranoside (**3**). Their structures were elucidated by extensive spectroscopic methods, including 1D- (^{1}H, ^{13}C) and 2D-NMR (D QF-COSY, TOCSY, ROESY, HSQC and HMBC) experiments, as well as high-resolution electrospray ionization mass spectrometry (HR-ESI-MS), ESI-MS/MS and acid hydrolysis.

Keywords: *Saponaria officinalis* L., Caryophyllaceae, Triterpenoid saponins, NMR.

Saponaria officinalis L., commonly named soapwort or bouncing-bet, is a perennial plant belonging to the family Caryophyllaceae. Its native range extends throughout Europe, northern Africa and west to central Asia, but currently is also cultivated in many countries across the world. It contains a high level of saponins and, like other such plants, it is used as an expectorant to remove catarrh from the upper respiratory tract. In ancient times, when soap was unknown, *S. officinalis* was used for cleaning and washing because of its detergent properties [1-3]. The saponin fraction of *S. officinalis* has shown an anti-inflammatory activity *in vitro* against carrageenan-induced rat-paw edema and inhibited prostaglandin synthetase. Purified saponins have indicated hypocholesterolemic effects *in vitro*, which is believed to be due to the ability of saponin to form an insoluble complex with cholesterol, preventing its absorption from the small intestine. Saponins also indicate spermicidal activity, which may result from their hemolytic property [4]. Unfortunately, apart from its beneficial effects, this plant is toxic for ruminants. It leads to photosensitization followed by liver and kidney degeneration and gut problems [5a]. Beside the pharmacological properties of saponins from *S. officinalis*, like anticancer, immunomodulatory and cytotoxity activities [5b], Czaban and co-workers reported antifungal activities of the saponin fraction against *Gaeumannomyces graminis* var. *tritici* and *Fusarium culmorum*, which are pathogens of cereals [5c].

S. officinalis is a rich source of triterpenoid glycosides [6] with gypsogenic acid, hydroxygypsogenic acid and quillaic acid aglycones, mostly occurring as bisdesmosides. The highest yield of saponins in Caryophyllaceae species is usually located in roots or seeds [5b], therefore the study of root material led to the isolation of three novel saponins and two not described previously in this plant. The structural elucidation of these triterpenoid saponins was based on chemical methods and spectroscopic techniques.

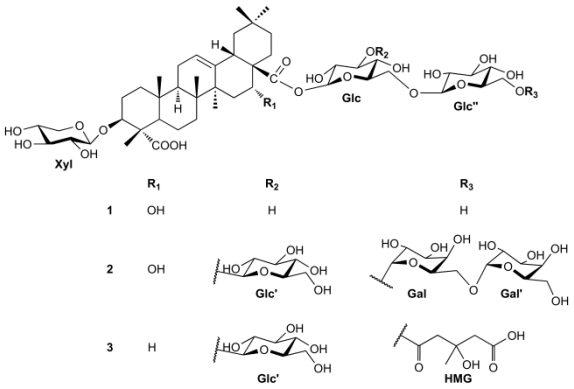

Figure 1: Structure of compounds **1**-**3**.

The 70% MeOH extract of the roots of *S. officinalis* was subjected to successive chromatographic steps to provide three new triterpene glycosides (**1**-**3**), along with nine known saponins.

Compound **1** exhibited in the HR-ESI-MS, the $[M-H]^-$ peak at *m/z* 957.4684 consistent with the molecular formula $C_{47}H_{73}O_{20}$. Its positive-ion ESI-MS displayed a quasimolecular ion at *m/z* 981 $[M+Na]^+$ and fragmentation ion peaks were observed at *m/z* 849 $[M+Na-132]^+$, 831 $[M+Na-H_2O-132]^+$, 819 $[M+Na-162]^+$, 657 $[M+Na-162-162]^+$, 525 $[M+Na-162-162-132]^+$, 365 $[Na+162+162+H_2O]^+$, and 347 $[Na+162+162]^+$. Its spectral features and physiochemical properties suggested **1** to be a triterpenoid saponin. From the 47 carbons, 30 were assigned to the aglycone part and 17 to the oligosaccharide moiety (Tables 1 and 2). The six sp^3 hybrid carbon signals at δ_C 13.2, 16.6, 17.9, 25.0, 27.6, and 33.5, and the two sp^2 hybrid carbon signals at δ_C 122.9 and 144.9 together with the information from ^{1}H NMR analysis [six methyl

Table 1: ^{13}C and ^{1}H-NMR data for the aglycone moieties of compounds **1-3** in pyridine-d_5 (δ in ppm, J in Hz).[a,b]

	1		2		3	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	39.4	1.64[b], 1.20[b]	39.4	1.61[b], 1.16[b]	39.1	1.57[b], 1.11[b]
2	26.8	2.20[b], 1.97[b]	26.8	2.20[b], 1.95[b]	26.6	2.20[b], 1.94[b]
3	85.8	4.65 dd (11.9, 3.9)	85.2	4.62[b]	85.4	4.62 dd (12.0, 3.1)
4	53.8	-	53.8	-	53.7	-
5	52.5	2.04[b]	52.6	1.96[b]	52.4	1.90[b]
6	21.8	1.55[b], 1.48[b]	21.8	1.60[b], 1.47[b]	21.6	1.58[b], 1.46[b]
7	33.5	1.76[b], 1.35[b]	33.5	1.68[b], 1.23[b]	32.7	1.82[b], 1.68[b]
8	40.9	-	40.8	-	40.5	-
9	47.8	1.96[b]	47.9	1.91[b]	48.6	1.76[b]
10	37.1	-	37.2	-	37.0	-
11	24.2	2.02[b]	24.3	1.98[b], 1.92[b]	24.1	1.95[b], 1.87[b]
12	122.9	5.61 t (3.1)	122.9	5.58 t (3.4)	123.0	5.40 t (3.3)
13	144.9	-	144.8	-	144.4	-
14	42.4	-	42.5	-	42.4	-
15	36.5	2.51 d (14.0), 1.69[b]	36.5	2.36[b], 1.62[b]	28.5	2.17[b], 1.04[b]
16	74.7	5.26 br s	74.6	5.20 br s	23.6	1.98[b], 1.85[b]
17	49.5	-	49.5	-	47.3	-
18	41.6	3.53 dd (14.2, 4.3)	41.6	3.44 dd (14.2, 3.8)	41.9	3.12[b]
19	47.5	2.77 t (13.6) 1.36 dd (12.5, 4.1)	47.6	2.70 t (13.6) 1.29[b]	46.5	1.68[b], 1.19[b]
20	31.2	-	31.2	-	31.0	-
21	36.3	2.40[b], 1.27[b]	36.3	2.35[b], 1.25[b]	34.2	1.55[b], 1.23[b]
22	32.6	2.40[b], 2.17[b]	32.5	2.31[b], 2.06[b]	33.4	1.27[b], 1.05[b]
23	181.7	-	181.6	-	180.8	-
24	13.2	1.58 s	13.2	1.58 s	13.0	1.55 s
25	16.6	1.01 s	16.6	1.00 s	16.4	0.92 s
26	17.9	1.15 s	17.9	1.10 s	17.7	1.08 s
27	27.6	1.79 s	27.6	1.77 s	26.3	1.19 s
28	176.4	-	176.1	-	176.5	-
29	33.5	0.97 s	33.5	0.95 s	33.1	0.86 s
30	25.0	1.05 s	25.1	1.01 s	24.0	0.83 s

[a]The assignments were based upon 2D-COSY, 2D-TOCSY, HSQC and HMBC spectra. [b]Overlapped signals.

Table 2: ^{13}C and ^{1}H-NMR data for the sugar moieties of compounds **1-3** in pyridine-d_5 (δ in ppm, J in Hz).[a,b,c]

	1		2		3	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
Xyl-1	106.6	5.03 d (7.8)	106.6	5.02 d (7.3)	106.5	5.01 d (7.4)
2	75.7	3.96 t (8.1)	75.8	3.98 t (8.0)	75.6	3.98[b]
3	78.4	4.11[b]	78.3	4.10[b]	78.0	4.09[b]
4	71.5	4.19[b]	71.5	4.20[b]	71.4	4.22[b]
5ax.	67.5	3.70 t (10.8)	67.5	3.71 t (11.0)	67.4	3.70 t (10.7)
5eq.		4.32 dd (10.5, 5.0)		4.36 dd (11.0, 5.0)		4.36 dd (11.2, 5.0)
Glc-1	96.2	6.26 d (8.2)	95.5	6.21 d (8.3)	95.1	6.22 d (8.1)
2	74.3	4.06 t (8.6)	72.3	4.06 t (8.0)	73.3	4.18 t (8.3)
3	79.1	4.20 t (8.9)	88.7	4.22[b]	88.8	4.27[b]
4	71.3	4.29 t (9.3)	68.6	4.22[b]	69.3	4.24[b]
5	78.4		77.9	4.05[b]	78.0	4.14[b]
6a	69.8	4.71 dd (11.1, 1.5)	69.5	4.61 dd (11.3, 3.7)	69.9	4.80 d (9.6)
6b		4.35 dd (11.2, 3.5)		4.20		4.31 dd (11.3, 4.0)
Glc'-1			106.0	5.24 d (8.0)	106.1	5.30 d (7.8)
2			75.0	4.00 t (8.2)	75.8	4.04 t (8.6)
3			78.1	4.16 t (8.7)	78.5	4.16 t (9.0)
4			71.3	4.12 t (8.7)	71.9	4.09 t (9.0)
5			78.9	4.00[b]	78.7	3.98[b]
6a			62.9	4.51 d (10.0)	62.5	4.53 dd (11.9, 1.6)
6b				4.24 dd (11.1, 3.8)		4.23 dd (12.0, 4.0)
Glc''-1	105.7	5.03 d (7.8)	105.8	4.88 d (7.8)	105.5	4.98 d (7.8)
2	75.5	4.01 t (8.0)	75.2	3.93 t (8.3)	75.3	3.98 t (8.3)
3	78.9	4.22[b]	78.4	4.07 t (9.5)	78.4	4.18 t (8.7)
4	71.9	4.22[b]	72.1	4.11 t (8.5)	72.1	3.99[b]
5	78.7	3.90 ddd (7.8, 4.9, 2.4)	76.5	3.87 ddd (9.0, 5.0, 2.4)	78.4	4.03[b]
6a	63.0	4.49 dd (11.8, 2.3)	68.1	4.51 dd (11.5, 3.5)	65.2	5.11[b]
6b		4.37 dd (11.7, 5.0)		4.14 dd (10.5, 4.7)		4.64[b]
Gal-1			100.8	5.42 d (3.7)		
2			70.5	4.62 dd (10.0, 3.4)		
3			71.8	4.49 dd (11.0, 3.5)		
4			71.5	4.57[b]		
5			70.5	4.73 t (6.2)		
6a			68.6	4.47 dd (10.1, 6.5)		
6b				4.24 dd (10.3, 5.8)		
Gal'-1			101.2	5.55 d (3.7)		
2			70.6	4.64 dd (10.0, 2.3)		
3			72.1	4.55 dd (9.7, 3.3)		
4			72.0	4.63 d (3.8)		
5			73.1	4.55 dd (5.9, 5.9)		
6			63.0	4.40 2H, d (6.2)		
HMG -1					172.2	-
2					47.3	3.10[b]
						2.96 d (14.0)
3					70.4	-
4					47.3	3.10[b]
						3.05 d (13.8)
5					172.2	-
6					28.8	1.69 s

[a]The assignments were based upon 1D-TOCSY, 2D-COSY, 2D-TOCSY, HSQC, HSQC-TOCSY and HMBC spectra. [b]Overlapped signals. [c]^{13}C chemical shifts of substituted residues are underlined.

proton singlets at δ_H 0.97, 1.01, 1.05, 1.15, 1.58, and 1.79 and a broad triplet vinyl proton at δ_H 5.61 (t, J = 3.1 Hz)] indicated that the aglycone possesses an olean-12-ene skeleton. The appearance of H-16 as a broad singlet at δ_H 5.26 indicated that 16-OH was *α*-oriented. This was supported by the high-frequency shifts of C-15 (δ_C 36.5) and C-16 (δ_C 74.7) in the ^{13}C NMR spectrum of **1**. Two high-frequency shifts at δ_C 181.7 (indicating an unsubstituted carboxylic group) and 176.4 were assigned to C-23 and C-28 on the basis of 1D ^{13}C and HMBC experiment, respectively. Detailed NMR analysis identified the aglycone as 16*α*-hydroxygypsogenic acid [7a]. The high-frequency shift of the carbinol function at δ_C 85.8 (C-3) and low-frequency shift of the carboxylic function at δ_C 176.4 (C-28) showed the bisdesmosidic structure of **1**. The observation of two overlapping anomeric signals at δ_H 5.03 (d, J = 7.8 Hz) and a signal at δ_H 6.26 (d, J = 8.2 Hz) correlating with signals at δ_C 105.7, 106.6 and at δ_C 96.2 in the *g*-HSQC spectrum, respectively, suggested that this compound possesses three sugar moieties. The individual sugar units were identified on the basis of proton resonance analysis of each sugar unit. These determinations were carried out by a combination of DQF-COSY and 1D-TOCSY NMR experiments. 1D TOCSY subspectra were obtained from selective excitation of the anomeric protons. The selective TOCSY experiment for the signal at δ 6.26 revealed spin system characteristics of a *β*-glucose unit. The anomeric signals at δ_H 5.03 were too close to obtain independent subspectra in the 1D TOCSY experiment, but a different ratio of the pair of signals could be observed, indicating the presence of *β*-glucose and *β*-xylose units. These observations were supported by the 1D ROESY correlation between H-1 and H-3/H-5 protons of the sugar units. Analysis of the *g*-HSQC experiments allowed the assignments of signals of the ^{13}C NMR spectrum corresponding to the three sugar units. The sequence of the three sugar units was deduced from the 1D ROESY and HMBC spectra, in which long range correlations were observed from H-$1_{Glc''}$ (δ 5.03) to H-$6b_{Glc}$ (δ 4.35) and C-6_{Glc} (δ 69.8), H-1_{Glc} (δ 6.26) to C-28 of the aglycone (δ 176.4), H-1_{Xyl} (δ 5.03) to H-3 of the aglycone (δ 4.65) and C-3 of the aglycone (δ 85.8). Thus the structure of **1** was established as 3-*O*-*β*-D-xylopyranosyl-16*α*-hydroxygypsogenic acid-28-*O*-[*β*-D-glucopyranosyl-(1→6)]-*β*-D-glucopyranoside, a new triterpenoid saponin. A similar compound was reported in *Dianthus superbus* (another member of Caryophyllaceae family), but containing an *α*-L-arabinopyranosyl unit attached to C-3 of the aglycone [7a].

Compound **2**, $C_{65}H_{103}O_{35}$ (HR-ESI-MS $[M-H]^-$ at *m/z* 1443.6276), exhibited a fragment-ion peak at *m/z* 1263 $[M-H-H_2O-162]^-$, corresponding with the loss of a hexosyl moiety. Other fragmentation ion peaks were observed at *m/z* 1119 $[M-H-162-162]^-$, 939 $[M-H-162-162-H_2O-162]^-$, 809 $[M-H-162-162-162-H_2O-132]^-$, 633 $[M-H-162-162-162-162-162]^-$, and 485 $[M-H-162-162-162-162-162-H_2O-132]^-$. Comparison of ^{1}H and ^{13}C NMR spectra (Tables 1 and 2) of **2** with those of **1** indicated that **2** had the same aglycone as **1**. The difference was observable in the saccharide moieties. Thus the aglycone of **2** was also determined to be 16*α*-hydroxygypsogenic acid. Structure elucidation of the sugar portions was achieved by 1D TOCSY, DQF-COSY, *g*-HSQC, *g*-HSQSC-TOCSY and *g*-HMBC experiments. The 1D TOCSY subspectra obtained by irradiating the six anomeric proton signals, allowed the determination of the nature of the sugar units. The signals at δ 6.21 (d, J = 8.3 Hz), 5.24 (d, J = 8.0 Hz) and 4.88 (d,

J = 7.8 Hz) showed the typical spin system of *β*-glucose units, whereas the subspectra obtained by irradiating the signal at δ 5.02 (d, *J* = 7.3 Hz) were established as belonging to a *β*-xylose unit. The characteristic spin system for the two *α*-galactoses was obtained by irradiation of signals at δ 5.55 (d, *J* = 3.7 Hz) and 5.42 (d, *J* = 3.7 Hz). The doublet at δ 5.55 generated a 1D TOCSY subspectrum with only two signals at δ 4.64 (dd, *J* = 10.0, 2.3 Hz) and 4.55 (dd, *J* = 9.7, 3.3 Hz). Similarly, the doublet at δ 5.42 generated a 1D TOCSY subspectrum with two signals at δ 4.62 (dd, *J* = 10.0, 3.4 Hz) and 4.49 (dd, *J* = 11.0, 3.5 Hz). Additional 1D TOCSY experiments conducted for isolated signals at δ 4.73 (t, *J* = 6.2 Hz) and 4.40 (d, 6.2) led to the identification of the H-6/H-4 sequences. These observations were supported by the 1D ROESY correlation between H-3/H-5 protons of the sugar units. Analysis of the *g*-HSQC and *g*-HSQC-TOCSY experiments allowed the assignments of signals of the ^{13}C NMR spectrum corresponding to the six sugar units. The sequence of the six sugar units was deduced from the 1D ROESY and HMBC spectra, in which long range correlations were observed from H-$1_{Glc'}$ (δ 5.24) to H-3_{Glc} (δ 4.22) and C-3_{Glc} (δ 88.7), H-$1_{Gal'}$ (δ 5.55) to H-$6b_{Gal}$ (δ 4.24) and C-6_{Gal} (δ 68.6), H-1_{Gal} (δ 5.42) to H-$6b_{Glc''}$ (δ 4.14) and C-$6_{Glc''}$ (δ 68.1), H-$1_{Glc''}$ (*δ* 4.88) to H-$6b_{Glc}$ (δ 4.20) and C-6_{Glc} (δ 69.5), H-1_{Glc} (δ 6.21) to C-28 of the aglycone (δ 176.1), H-1_{Xyl} (δ 5.02) to H-3 of the aglycone (δ 4.62) and C-3 of the aglycone (δ 85.2). Thus the structure of **2** was established as 3-*O*-*β*-D-xylopyranosyl-16*α*-hydroxygypsogenic acid-28-*O*-[*β*-D-glucopyranosyl-(1→3)]-[*α*-D-galactopyranosyl-(1→6)-*α*-D-galactopyranosyl-(1→6)-*β*-D-glucopyranosyl-(1→6)]-*β*-D-glucopyranoside. A triterpenoid saponin with *α*-D-galactopyranose as a component sugar is rare in nature, but has been reported from *Saponaria officinalis* and *Silene rubicunda*. Compound **2** is the first triterpenoid saponin containing two *α*-D-galactose units [2a,7b].

Compound **3** exhibited in its HR-ESI-MS, the $[M-H]^-$ peak at *m/z* 1247.5732 consistent with the molecular formula $C_{59}H_{91}O_{28}$. Fragment-ion peaks were observed at *m/z* 1103 $[M-H-144]^-$ and 923 $[M-H-144-H_2O-162]^-$, corresponding with the successive elimination of the 3-hydroxy-3-methylglutaryl (HMG) moiety and hexosyl moiety, respectively. Other fragmentation ion peaks were observed at *m/z* 1066 $[M-H-H_2O-162]^-$, 617 [M-H-144-162-162-162]$^-$, and 485 [M-H-144-162-162-162-132]. The aglycone of **3** was similar to **1** and **2** except for the lack of a hydroxyl group at C-16. This was supported by the low-frequency shifts of C-15 (δ_C 28.5) and C-16 (δ_C 23.6) in the ^{13}C NMR spectrum of **3** (Tables 1 and 2). Thus the aglycone of **3** was determined to be gypsogenic acid. Structure elucidation of the sugar portions was achieved by 1D TOCSY, DQF-COSY, *g*-HSQC, *g*-HSQSC-TOCSY and *g*-HMBC experiments. The 1D TOCSY subspectra obtained by irradiating the four anomeric proton signals, allowed the determination of the nature of the sugar units. Signals at δ 6.22 (d, *J* = 8.1 Hz), 5.30 (d, *J* = 7.8 Hz), and 4.98 (d, *J* = 7.8 Hz) showed the typical spin system of *β*-glucose units, whereas the subspectra obtained by irradiating the signal at δ 5.01 (d, *J* = 7.4 Hz) were established as belonging to a *β*-xylose unit. These observations were supported by the 1D ROESY correlation between H-1 and H-3/H-5 protons of the sugar units. Analysis of the *g*-HSQC and *g*-HSQC-TOCSY experiments allowed the assignment of signals of the ^{13}C NMR spectrum corresponding to the four sugar units. The sequence of the four sugar units was deduced from the 1D ROESY and HMBC spectra, in which long range correlations were observed from H-$1_{Glc'}$ (δ 5.30) to H-3_{Glc} (δ 4.27) and C-3_{Glc} (δ 88.8), H-$1_{Glc''}$ (δ 4.98) to H-$6b_{Glc}$ (δ 4.31) and C-6_{Glc} (δ 69.9), H-1_{Glc} (δ 6.22) to C-28 of the aglycone (δ 176.5), H-1_{Xyl} (δ 5.01) to H-3 of the aglycone (δ 4.62) and C-3 of the aglycone (δ 85.4). Additional signals, not belonging to the sugar and aglycone parts, were observed in the ^{1}H and ^{13}C spectra of **3**, suggesting the presence of a 3-hydroxyl-3-methylglutaryl (HMG) moiety: -CH_3 group at δ_H 1.69 (s) and δ_C 28.8, two methylenes [δ_H 3.10 and 2.96 (d, *J* = 14.0 Hz)], δ_C 47.3 and [δ_H 3.10 and 3.05 (d, *J* = 13.8 Hz)], δ_C 47.3 and two overlapping carboxylic groups at δ_C 172.2, and one quaternary carbinol carbon at δ_C 70.4. The ester bond between HMG-1 and C-$6_{Glc''}$ was suggested by high-frequency shifts for the H-$6_{Glc''}$ (δ_H 5.11 and 4.64) and for the C-$6_{Glc''}$ (δ_C 65.2), as the HMBC connectivity was not observed. Thus the structure of **3** was established as 3-*O*-*β*-D-xylopyranosyl-gypsogenic acid-28-*O*-[*β*-D-glucopyranosyl-(1→3)]-[6-*O*-(3-hydroxy-3-methylglutaryl)-*β*-D-glucopyranosyl-(1→6)]-*β*-D-glucopyranoside. A similar compound was reported in *Dianthus superbus*, but containing a *β*-D-glucopyranosyl unit attached to C-3 of the aglycone [7a].

Moreover, nine known compounds were identified as vaccaroside D (**4**) [7c], dianchinenoside B (**5**) [8a], saponarioside C (**6**), D (**7**), F (**8**) and G (**9**) [2a] and saponarioside I (**10**), K (**11**) and L (**12**) [2b], by comparing their NMR data with those in the literature. Compounds **4** and **5** were isolated from this genus for the first time.

Usually, several oleane-type saponins are accompanied in one species but *Saponaria officinalis* like *Vaccaria segetalis* contains saponins that are based on more than three different sapogenins [5b]. Both new and known saponins have gypsogenic acid, hydrogypsogenic acids and their derivatives [2b,c] as an aglycone component. Existing research suggests that *S. officinalis* is lacking gypsogenin, in contrast to the rest of the Caryophyllaceae family, in which the occurrence of gypsogenin is more frequent than that of gypsogenic acid [5b].

Almost all isolated saponins had a bisdesmosidic linkage, with the exception of saponarioside K and vaccaroside B, which are monodesmosides. The linking points for sugar moieties to the triterpenoid skeletons in *S. officinalis* are at C-3 and C-28. For the first time in this plant we proved the presence of 3-hydroxyl-3-methylglutaryl in a sugar chain – compound **3.**

In spite of the fact that *Saponaria officinalis* is widespread over different climes we still know little about its phytochemical composition, especially about the presence of saponins and their structures. Since this plant is used in folk medicine there is a need for confirmation of its composition by modern methods and proper standardization of plant material used in experiments.

Experimental

General experimental procedure: 1D and 2D NMR, Varian INOVA-600 spectrometer; ESI-MS/MS, Thermo LCQ Advantage Max mass spectrometer; HR-ESI-MS, Maldi SYNAPT G2-S HDMS Qq-TOF spectrometer; CC, Sephadex LH-20; VLC, LiChroprep C18 (Merck). HPLC, was performed on a Kromasil C18 column (10 x 250 mm, 5 μm) using a Gilson semi-preparative HPLC equipped with a PrepELS II (Evaporative Light Scattering) detector (the drift tube temperature was maintained at 65°C and the pressure of the nebulizer gas – nitrogen – was 47 psi).

Plant material: Dry, ground roots of *Saponaria officinalis* were purchased from a commercial source (Herbapol in Cracow, Poland). A voucher sample has been deposited at the Department of Biochemistry and Crop Quality, Institute of Soil Science and Plant Cultivation, State Research Institute, Pulawy.

Extraction and isolation: The isolation of the saponin fraction was performed according to previously developed procedures [8b]. Powdered (500 g) and defatted (497 g) roots of *S. officinalis* were

extracted 3 times with 80% MeOH at room temperature for 24 h. After each extraction the crude extract was filtered. The combined extracts were concentrated under reduced pressure and lyophilized (233 g). The extract was suspended in H_2O and applied to a vacuum liquid chromatograph (100 x 60 mm, LiChroprep C18, 40-63 µm) preconditioned with water. Sugars and phenolics were removed with water and 30% MeOH. Saponins were then eluted from the column with 90% MeOH. The solvent was evaporated *in vacuo* and the residue lyophilized, obtaining 49.9 g of crude saponin mixture, which was suspended in H_2O and extracted with EtOAc saturated with water. This extraction separated nonpolar compounds, which went into the EtOAc layer, such as catechins and yielded 45.41 g of saponins. The purified saponin fraction was suspended in H_2O and then partitioned successively between H_2O and *n*-BuOH saturated with water. The *n*-BuOH soluble fraction was fractionated by CC (Sephadex LH-20, 44 x 450 mm, 25-100 µm, Sigma-Aldrich) preconditioned with 40% MeOH containing 0.1% HCOOH, and eluted with 40% MeOH containing 0.1% HCOOH. This separation gave 5 subfractions. Individual saponins were separated from the subfractions using a semi-preparative high-pressure chromatograph (Gilson) equipped with a PrepELS II (Evaporative Light Scattering) detector and a Kromasil C18 column (10 x 250 mm, 5µm; Eka Chemicals AB). The flow rate was 5 mL min^{-1}. 0.1% HCOOH was used as mobile phase A and acetonitrile containing 0.1% HCOOH as mobile phase B. The drift tube temperature was maintained at 65°C and the pressure of the nebulizer gas – nitrogen – was 47 psi. In this way all compounds were isolated.

3-*O*-*β*-D-Xylopyranosyl-16*α*-hydroxygypsogenic acid-28-*O*-[*β*-D-glucopyranosyl (1→6)]-*β*-D-glucopyranoside (**1**)
^{1}H and ^{13}C NMR: Tables 1 and 2.
ESI-MS/MS: *m/z* 957 $[M-H]^-$ and 981 $[M+Na]^+$, 849, 831, 819, 809, 657, 525, 365, 347.
HR-ESI-MS: *m/z* 957.4684 $[M-H]^-$, calcd. for $C_{47}H_{73}O_{20}$: 957.4695.

3-*O*-*β*-D-Xylopyranosyl-16*α*-hydroxygypsogenic acid-28-*O*-[*β*-D-glucopyranosyl-(1→3)]-[*α*-D-galactopyranosyl-(1→6)-*α*-D-galactopyranosyl-(1→6)-*β*-D-glucopyranosyl-(1→6)]-*β*-D-glucopyranoside (**2**)
^{1}H and ^{13}C NMR: Tables 1 and 2.
ESI-MS/MS: *m/z* 1443 $[M-H]^-$, 1263, 1119, 939, 633, 485.
HR-ESI-MS: *m/z* 1443.6276 $[M-H]^-$; calcd. for $C_{65}H_{103}O_{35}$: 1443.6280.

3-*O*-*β*-D-Xylopyranosyl-gypsogenic acid-28-*O*-[*β*-D-glucopyranosyl-(1→3)]-[6-*O*-(3-hydroxy-3-methylglutaryl)-*β*-D-glucopyranosyl-(1→6)]-*β*-D-glucopyranoside (**3**)
^{1}H and ^{13}C NMR: Tables 1 and 2.
ESI-MS/MS: *m/z* 1247.5 [M-H], 1103, 1066, 923, 617, 485.
HR-ESI-MS: *m/z* 1247.5707 $[M-H]^-$; calcd. for $C_{59}H_{91}O_{28}$: 1247.5732.

Acid hydrolysis of saponins and determination of absolute configuration of monosaccharides: Each compound (2.5 mg) was hydrolysed in 1.5 mL solution of 3 M HCl (dioxane-H_2O, 1:2) at 90ºC for 5 h. After dioxane was removed from the hydrolysis reaction mixture under a stream of nitrogen, the aglycones were extracted with EtOAc (3×1 mL) and dried. The aqueous layer containing sugars was neutralized using an Amberlite IRA-400 ion exchange resin in its hydroxide form and concentrated under a stream of nitrogen at 60˚C and then used for sugar determination. To determine the absolute configuration of the monosaccharide ingredients of isolated compounds, the method of Tanaka *et al.* [8c] was used, with slight modification. Sugars of each sample were dissolved in pyridine (0.5 mL) to which 1 mg of L-cysteine methyl ester hydrochloride was added. The mixture was kept at 60˚C for 1 h, and then 0.2 µL of *o*-tolyl isothiocyanate was added to the mixture, which was heated at 60˚C for 1 h. Each mixture was directly analysed by reversed-phase HPLC using a 616-Pump, 966-Photodiode Array detector 717-plus Autosampler (Waters) and an Eurospher-100 C18 (250 mm x 4 mm; 5 µm Knauer) column. MeCN-H_2O (25:75, v/v) containing 50 mM H_3PO_4 was used as mobile phase at a flow rate 0.8 mL min^{-1} and a column temperature of 35ºC. Detection was performed by UV at 250 nm. The absolute configuration was determined by comparing the retention times of sugars with derivatives prepared in a similar way from standard monosaccharides (Sigma-Aldrich). The following sugars were detected for **1** and **3**: D-glucose and D-xylose, and for **2**: D-glucose, D-xylose and D-galactose.

Supplementary data: NMR spectra (^{1}H and ^{13}C NMR, HSQC, HMBC, TOCSY, ROESY and DQF-COSY), HR-ESI-MS and ESI-MS/MS spectra for the new compounds (**1**-**3**).

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Minor Triterpene Saponins from Underground Parts of *Lysimachia thyrsiflora*: Structure elucidation, LC-ESI-MS/MS Quantification, and Biological Activity

Irma Podolak[a,*], Paweł Żmudzki[c], Paulina Koczurkiewicz[a,b], Marta Michalik[b], Paweł Zajdel[c] and Agnieszka Galanty[a]

[a]*Department of Pharmacognosy*, *Pharmaceutical Faculty*, *Medical College*, *Jagiellonian University*, *Medyczna 9*, *30-688 Cracow*, *Poland*

[b]*Department of Cell Biology*, *Faculty of Biochemistry*, *Biophysics and Biotechnology*, *Jagiellonian University*, *Gronostajowa 7*, *30-387 Cracow*, *Poland*

[c]*Department of Medicinal Chemistry*, *Pharmaceutical Faculty*, *Medical College*, *Jagiellonian University*, *Medyczna 9*, *30-688 Cracow*, *Poland*

mfpodola@cyf-kr.edu.pl

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Two minor triterpene saponins, one previously not reported, were isolated from the underground parts of *Lysimachia thyrsiflora* L. The structures were determined based on a combination of one- and two-dimensional NMR techniques, and mass spectrometry, as 30-*O*-β-D-glucopyranosyl-3β,16α,30-trihydroxy-olean-12-en-28-yl acetate 3-*O*-β-D-glucopyranosyl-(1→4)-*O*-[β-D-glucopyranosyl-(1→2)-]-*O*-α-L-arabinopyranoside (denoted as thyrsiloside A), and davuricoside L. Quantitative determination of these two compounds, along with other saponins present in *L. thyrsiflora* underground parts, was performed by a developed UPLC-ESI-MS/MS method, validated according to the ICH guidelines. The relative total content of triterpene saponins amounted to 1.19 μg/mg dw. Both **4** and **5** were present in minor quantities, 0.031 and 0.077 μg/mg dw, respectively. Assays performed on a panel of human cancer cell lines showed antiproliferative activity against prostate carcinoma PC-3 (GI_{50} after 48 h = 20 and 24 μg/mL, respectively).

Keywords: *Lysimachia thyrsiflora*, Olean-12-ene saponins, UPLC-ESI-MS/MS quantification, Cytotoxicity, Antifungal activity.

Lysimachia L. is a genus of over hundred species, both wild and cultivated in Europe and Asia. Many of them have been used in traditional folk medicine for their anticholecystitic, anthelminthic, and antihypertensive properties. Pentacyclic triterpene glycosides are the most characteristic constituents within this genus, and generally have oleanane-derived sapogenols of two structural types: **I**. 13β,28-epoxy and **II**. Δ^{12}-17-CH_2OH. Compounds of type **I**, with a completely saturated pentacyclic skeleton, are fairly uncommon and are found almost exclusively in the families Myrsinaceae and Primulaceae [1]. Although the genus *Lysimachia* is traditionally classified in Primulaceae, some recent phylogenetic data suggest its relocation to the family Myrsinaceae [2]. *L. thyrsiflora* L. (tufted loosestrife) is among five representatives of this genus reported in Poland. In addition to flavonoids, ecdysteroids and benzoquinones, the plant contains triterpene saponins. While tri- and tetrasaccharides of priverogenin A were reported in the aerial parts [3,4], in our own previous investigations of *L. thyrsiflora* we isolated cyclaminorin (**1**), ardisicrispin A (**2**), and ardisicrenoside B (**3**) from the underground parts [5,6]. These compounds exhibited interesting biological activities, such as the uterocontractile activity of **1** and **2** [7,8], cytotoxicity of **2** towards human cancer cell lines [9,10], and antifungal activity of **1** against *Candida* yeasts [7].

As part of our continuing phytochemical investigations of the *Lysimachia* genus, in this paper we report the isolation and structure elucidation of two additional triterpene saponins, 30-*O*-β-D-glucopyranosyl-3β,16α,30-trihydroxy-olean-12-en-28-yl acetate 3-*O*-β-D-glucopyranosyl-(1→4)-*O*-[β-D-glucopyranosyl-(1→2)-]-*O*-α-L-arabinopyranoside (**4**) and davuricoside L (**5**) from the underground parts of *L. thyrsiflora*. Of these, **4** is a new compound and both were isolated for the first time from this species.

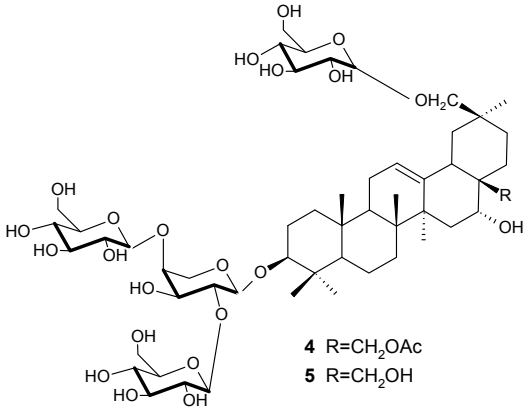

Figure 1: Structures of compounds **4** and **5**.

Furthermore, an UPLC-ESI-MS/MS method was developed and validated for the quantitative determination of **4** and **5**, along with other triterpene glycosides (**1**-**3**) in this plant species. In the context of biological activity of *Lysimachia* saponins, our present work has also focused on the screening of isolated compounds against human tumor and normal cell lines, as well as fungal strains, to establish potential structure-activity correlations.

A combination of repetitive open column and preparative silica gel chromatography of the MeOH extract of the underground parts of *L. thyrsiflora* yielded compounds **4** and **5** (Figure 1).

Compound **4** showed a $[M + Na]^+$quasimolecular ion peak at *m/z* = 1157. 57 in the positive HR-ESI-TOF-MS ($C_{55}H_{90}O_{24}Na$)

indicating a molecular mass of 1134 Da. The negative-ion FAB-MS showed the major ion peak at *m/z* 1133.4, which was assigned to [M – H]$^-$, a peak corresponding to the loss of one hexose unit at *m/z* 971.4 [M – H – 162]$^-$, and two hexose units at *m/z* 809.3 [M – H – 162 – 162]$^-$. The IR spectrum, apart from absorption bands due to the presence of hydroxyl groups (3333 cm^{-1}), showed a significant absorption peak at 1737 cm^{-1}(ester). The ^{13}C NMR spectrum displayed 55 carbons, of which 30 were assigned to the aglycone part, 23 to the oligosaccharide moiety, and the remaining 2 to an acetyl group (Tables 1 and 2). The ^{1}H NMR spectrum showed the presence of six tertiary methyl groups [δ_H 0.86, 0.95, 0.96, 0.98, 1.06, 1.37] and one olefinic proton [δ_H 5.36 (t, J = 3.6 Hz)]. The corresponding carbons were identified by a HSQC experiment [δ_C 16.9, 17.7, 28.1, 16.2, 28.5, 27.6, respectively]. The chemical shifts of the two olefinic carbons [δ_C 124.1 and 144.2], and comparison of the remaining chemical shifts (analyzed with DEPT and HSQC) with those established for pentacyclic triterpenes [11], revealed the characteristic carbon pattern of an olean-12-ene skeleton. Moreover, characteristic signals due to an acetyl group were observed [δ_H 2.03, δ_C 20.9, 173.2]. The structural assignment was initiated from the long-range coupling networks observed between the methyl protons and the adjacent carbons in the HMBC experiment (Figure 2). Two methyl groups (C-23 and C-24) and a methyl and CH_2OR group (C-29 and C-30, respectively) were shown to be geminal pairs, and the six methyls exhibited the following key connectivities: H-23 (δ_H 1.06) and H-24 (δ_H 0.86) to C-3 (δ_C 91.4), C-4 (δ_C 40.4), C-5 (δ_C 57.0), and carbon resonances of each other (δ_C 28.5; 16.9); H-25 (δ_H 0.98) to C-5 (δ_C 57.0), C-9 (δ_C 48.2), C-10 (δ_C 37.8); H-26 (δ_H 0.95) to C-7 (δ_C 34.0), C-9 (δ_C 48.2), and C-14 (δ_C 42.7); H-27 (δ_H 1.37) to C-8 (δ_C 41.0), C-14 (δ_C 42.7), and C-15 (δ_C 35.2); H-29 (δ_H 0.96) to C-19 (δ_C 43.4), C-20 (δ_C 35.6), and C-30 (δ_C 76.0).

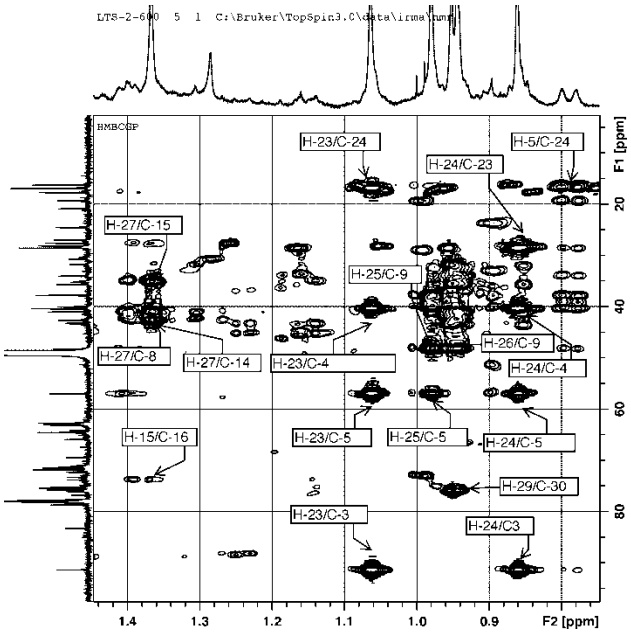

Figure 2: Detail (methyl region) of the HMBC spectrum of compound **4**.

Two oxygenated methine protons at δ_H 3.16 (dd, J = 11.4, 4.4 Hz) and 3.92 (br d, J = 5.8 Hz), which showed correlations in the HSQC spectrum with carbon signals at δ_C 91.4 and 73.7, respectively, were assigned to H-3 and H-16. Moreover, the ^{1}H NMR spectrum displayed two primary alcoholic functions [δ_H 3.68, 3.91], and [δ_H 3.35, 3.88] which were located at C-28 and C-30, respectively, on the basis of HMBC correlations from H-28 to C-17 (δ_C 40.7) and C-22 (δ_C 28.3) and from H-30 to C-19 (δ_C 43.4), C-20 (δ_C 35.6),

Table 1: ^{13}C and ^{1}H NMR data (J in Hz) of the aglycone moieties of compounds **4** and **5** (600 MHz, δ ppm, in CD_3OD).

No.	**4**		**5**	
	δ_C	δ_H[a] (J in Hz)	δ_C	δ_H[a] (J in Hz)
1	40.0	1.66, 0.99, m	40.0	1.65, 0.99, m
2	26.9	1.84, 1.75, m	27.0	1.85, 1.75, m
3	91.4	3.16, dd (11.4, 4.4)	91.4	3.16, dd (11.7, 4.3)
4	40.4	-	40.4	-
5	57.0	0.79 d (11.4)	57.0	0.79, d (11.2)
6	19.3	1.58. 1.43, m	19.3	1.57, 1.43, m
7	34.0	1.60, 1.40, m	34.0	1.59, 1.40, m
8	41.0	-	41.0	-
9	48.2	1.63, m	48.3	1.63, m
10	37.8	-	37.8	-
11	24.4	1.89 (2H), m	24.5	1.89 (2H), m
12	124.1	5.36, t (3.6)	123.7	5.35, t (3.4)
13	144.2	-	144.8	-
14	42.7	-	42.6	-
15	35.2	1.88, 1.37, m	35.0	1.90, 1.34, m
16	73.7	3.92, br d (5.8)	74.8	3.92, br d (5.7)
17	40.7	-	41.5	-
18	43.2	2.15, m	42.5	2.10, m
19	43.4	2.03, 1.36, m	43.6	2.06, 1.35, m
20	35.6	-	35.7	-
21	31.8	1.65, 1.47, m	33.9	1.59, 1.40, m
22	28.3	1.77, 1.49, m	32.2	1.62, 1.46, m
23	28.5	1.06, s	28.5	1.07, s
24	16.9	0.86, s	16.9	0.86, s
25	16.2	0.98, s	16.2	0.98, s
26	17.7	0.95, s	17.7	0.95, s
27	27.6	1.37, s	27.6	1.37, s
28	72.3	3.91, 3.68	70.2	3.20 (2H), d (9.2)
29	28.1	0.96 s	28.3	0.95, s
30	76.0	3.88, 3.35	75.9	3.89, 3.33
CH_3CO	20.9	2.03, s	-	-
	173.2	-		

[a] Multiciplity is not clear for some resonances due to overlapping.

Table 2: ^{13}C and ^{1}H NMR spectroscopic data (J in Hz) of the sugar portions of compounds **4** and **5** (600 MHz, δ ppm, in CD_3OD).

No.	**4**		**5**	
	δ_C	δ_H[a] (J, Hz)	δ_C	δ_H[a] (J, Hz)
	α-L-Ara (at C-3)		α-L-Ara (at C-3)	
1	105.1	4.52, d (5.5)	105.2	4.52, d (5.8)
2	78.7	3.92	78.6	3.91
3	73.4	3.89	73.4	3.89
4	78.3	3.98	78.3	3.98
5	64.5	4.12, dd (12.3, 4.3)	64.5	4.12, dd (12.4, 4.4)
		3.56 dd (12.3, 1.8)		3.56, dd (12.4, 1.8)
	β-D-GlcI (at C-2 Ara)		β-D-GlcI (at C-2 Ara)	
1	104.4	4.62, d (7.7)	104.4	4.62, d (7.7)
2	75.7	3.20	75.8	3.19
3	78.2	3.36	78.2	3.36
4	71.9	3.22	71.9	3.21
5	77.8	3.28	77.9	3.28
6	63.1	3.84, dd (11.9, 2.0)	63.1	3.84, dd (11.3, 2.0)
		3.63, dd (11.9, 4.9)		3.63 dd (11.3, 4.9)
	β-D-GlcII (at C-4 Ara)		β-D-GlcII (at C-4 Ara)	
1	105.6	4.48, d (7.7)	105.6	4.48, d (7.8)
2	75.5	3.26	75.4	3.23
3	78.0	3.28	78.0	3.28
4	71.8	3.30	71.8	3.29
5	77.8	3.37	77.8	3.37
6	62.7	3.86, 3.67	62.7	3.86, 3.67
	β-D-GlcIII (at C-30)		β-D-GlcIII (at C-30)	
1	105.2	4.25, d (7.7)	105.0	4.24, d (7.8)
2	75.3	3.21	75.3	3.21
3	78.1	3.28	78.1	3.27
4	71.5	3.29	71.5	3.29
5	78.0	3.36	78.0	3.36
6	62.9	3.87,dd (11.9,2.4)	62.9	3.87,dd(11.4,3.0)
		3.68		3.68

[a] Multiciplity is not clear for some resonances due to overlapping.

C-29 (δ_C 28.1). Spatial proximities observed in the ROESY experiment, between H-3 and H-23, H-3 and H-5, H-16 and H-18, H-16 and H-28, confirmed the β-orientation of the hydroxyl group at C-3 and α-orientation at C-16, respectively.

The location of the acetyl group was established at C-28 from the long-range HMBC coupling between H-28 (δ_H 3.68, 3.91) and C-1 of the acetyl (δ_C 173.2). From the above evidence, the aglycone of compound **4** was identified as 3β,16α,30-trihydroxy-olean-12-en-28-yl acetate.

The downfield shifts of C-3 (δ_C 91.4) and C-30 (δ_C 76.0) suggested that compound **4** was a bidesmosidic saponin. The sugar residues were determined to be L-arabinose and D-glucose after acid hydrolysis followed by derivatization [12]. In the ^{1}H NMR spectrum, signals corresponding to four anomeric protons were found at [δ_H 4.25 (d, J = 7.8 Hz), 4.48 (d, J = 7.7 Hz), 4.52 (d, J = 5.5 Hz), and 4.62 (d, J = 7.7 Hz)]. These were correlated by HSQC experiment to the corresponding carbon resonances at δ_C 105.2, 105.6, 105.1, and 104.4, respectively (Table 2). The remaining proton resonances within each sugar unit were established by TOCSY, DQF-COSY, and ROESY experiments. HSQC was used to assign the respective carbons. All monosaccharides were determined to be in the pyranose forms by ^{13}C NMR evidence [13]. From the relatively large H-1 coupling constants (~ 7.7 Hz) it was clear that the anomeric hydroxyls of all three glucose moieties should have a β-configuration. The value of the $J_{1,2}$ coupling constant for the anomeric proton of arabinose (5.5 Hz), which was reported not to be diagnostic on its own, together with NOE connectivities between H-1$_{ara}$, H-3$_{ara}$ and H-5$_{ara}$ [14] indicated an α-orientation of this sugar unit. Sequencing and points of attachment in the oligosaccharidic chains were deduced from HMBC experiment and ^{13}C shift differences between individual sugar residues and model compounds [13]. For the trisaccharide side chain at C-3 the following key-correlation peaks were observed in the HMBC spectrum (Figure 3): H-1$_{ara}$ (δ_H 4.52) and C-3 (δ_C 91.4), H-1$_{glcI}$ (δ_H 4.62) and C-2$_{ara}$ (δ_C 78.7), H-1$_{glcII}$ (δ_H 4.48) and C-4$_{ara}$ (δ_C 78.3). The side chain at C-30 of the aglycone was identified as β-D-glucose, with its anomeric proton at δ_H 4.25, and its anomeric carbon at δ_C 105.2. Its location was confirmed by the HMBC correlation between H-1$_{glcIII}$ and C-30 (δ_C 76.0).

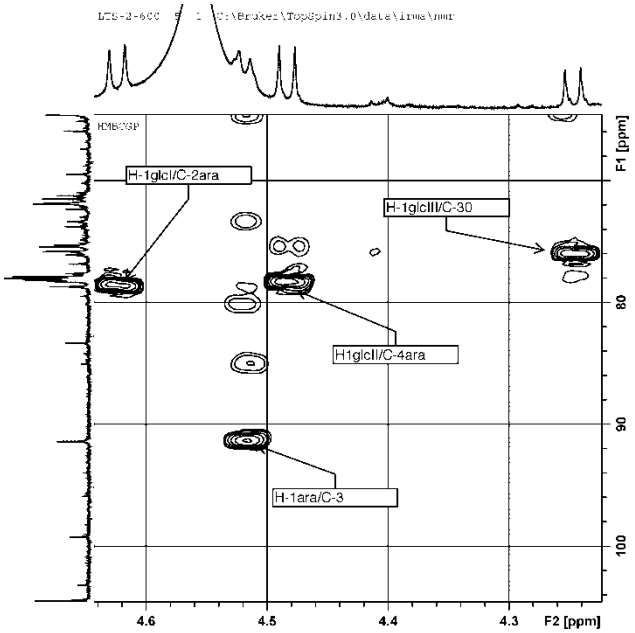

Figure 3: Detail (sugar region) of the HMBC spectrum of compound **4**.

The same conclusion with regard to the sugar sequence was also drawn from the ROESY experiment. Correlations were observed between H-1$_{ara}$ (δ_H 4.52) and H-3 (δ_H 3.16); H-1$_{glcI}$ (δ_H 4.62) and H-2$_{ara}$ (δ_H 3.92); H-1$_{glcII}$ (δ_H 4.48) and H-4$_{ara}$ (δ_H 3.98); and H-1$_{glcIII}$ and H-30 (δ_H 3.35, 3.88). Based on the above findings the structure of compound **4** was elucidated to be 30-*O*-β-D-glucopyranosyl-3β,16α,30-trihydroxy-olean-12-en-28-yl acetate 3-*O*-β-D-glucopyranosyl-(1→4)-*O*-[β-D-glucopyranosyl-(1→2)-]-*O*-α-L-arabinopyranoside. This is the first known report of compound **4**, which was named thyrsiloside A. MS, IR and NMR data comparison of compound **4** with **5**, indicated that **5** lacks an acetyl group. Thus, it was identified as davuricoside L, which has been previously reported in *L. davurica* [15].

The developed UPLC-ESI-MS/MS method was optimized for negative-ion mode, which is less dependent on the specific conditions of analysis. The chromatographic profile obtained by MRM analysis exhibited all the peaks corresponding to the compounds under investigation (Table 3). Two transitions per compound were used. For saponins **1**, **4** and **5,** one transition was the loss of formate from the formate adduct. For **5**, the second transition was the additional loss of CH_3OH from one of the terminal sugar moieties. In the case of compound **4**, the second transition was deacetylation leading to **5**. In the case of saponins **2** and **3**, the first transition was the loss of the xylopyranose moiety, and the second was the loss of the β-D-xylopyranosyl-(1→2)-β-D-glucopyranose fragment.

Table 3: Optimized settings for quantitative analysis of saponosides from *Lysimachia thyrsiflora*.

Compound	Rt [min]	Transition	Cone potential [V]	Collision energy [eV]
chloramphenicol	3.95	320.85 → 151.83	-32	22
		320.85 → 256.93	-32	10
α-hederin	6.39	795.55 → 471.39	-32	64
		795.55 → 749.69	-32	30
5	3.17	1137.64* → 1059.81	-32	42
		1137.64* → 1091.80	-32	40
4	3.80	1133.62 → 1091.82	-148	50
		1179.69* → 1133.72	-30	18
3	4.25	1061.59 → 767.53	-150	44
		1061.59 → 929.64	-150	42
2	4.97	1059.59 → 765.53	-150	44
		1059.59 → 927.64	-150	42
1	5.08	927.51 → 765.53	-114	32
		973.51* → 927.65	-30	28

* Formate adduct

The calibration curve was constructed by plotting the peak area (y) versus concentration (x) by analyzing a set of 15 external standard (α-hederin) solutions. Linearity of response was confirmed in the range 0.1164 – 1.50 μg/mL (y-intercept of the linear equation was statistically significant p<0.005; for details see Table 4).

Table 4: Calibration data for α-hederin.

Calibration curve	Correlation coefficient (R^2)	Determination coefficient (R^2)	Linear range (μg/mL)
y=0.4932x – 0.03339	0.9925	0.9850	0.1164-1.50
	LOD (μg/mL)	LOQ (μg/mL)	Shapiro-Wilk test
	0.0384	0.1164	p>0.11

After preliminary estimation of saponin concentrations in the analyzed material, appropriate dilution levels were selected and used. In order to quantify the saponin content, six biological samples, obtained from six individual plants were analyzed in triplicate. Table 5 reports the quantitative analysis results. The major constituents were triterpene saponins of the 13,28-epoxy-oleanane type (98.7%), compound **1** being the most abundant. The plant samples were collected from the same stand and so were fairly homogenous in terms of similarity due to the effect of growth conditions. To reflect plant-to-plant physiological differences, which are attributed to variations in their microenvironments or developmental stage, biological variability was quantified. The average coefficient of variation in *L. thyrsiflora* roots was 23%, and this value is similar to those obtained for other saponin accumulating plant species [16]. The RSD is also indicative of the effect of the several steps involved in the extraction/purification process, and is comparable with other studies [17].

Validation data showed good precision and intermediate precision, with a % RSD less than 8%. ANOVA test showed no statistically significant differences between different days (p>0.23). In all the deliberately varied chromatographic conditions (flow rate, column temperature, mobile phase composition), the examined saponosides were adequately resolved, and the order of elution remained unchanged.

Table 5: Concentrations of individual and total saponins in the underground parts of *Lysimachia thyrsiflora* by MRM UPLC-ESI-MS/MS.

compound	concentration[a] µg/mg dry wt	SD	RSD (%)
1	0.60	± 0.247	41.4
2	0.56	± 0.307	54.2
3	0.013	± 0.0123	91.9
4	0.031	± 0.109	34.5
5	0.077	± 0.0288	37.5
Total	1.19	± 0.274	23.1

[a]all concentrations were calculated based on peak areas of the $[M-H]^-$ selected ion chromatograms relative to the standard α-hederin; each measurement was made in triplicate for six independent plant samples; abbreviations: µg/mg dry wt, µg per mg of dry weight; SD, standard deviation; RSD, relative standard deviation of saponin concentrations measured for six independent plants.

Cytotoxicity of compounds **4** and **5** was evaluated on human cancer cell lines differing in metastatic potential: melanoma BLM and A375, prostate cancer cell DU-145 and PC-3, and glioblastoma astrocytoma (U375), and normal cells of respective origin: skin fibroblasts (HSF) and prostate cells (PNT2). The results obtained (see details in Supplementary Data) showed that for both compounds EC_{50} values were approximately 30 µg/mL. PC-3 proliferation was inhibited after 48 h at GI_{50} 24 and 20 µg/mL, respectively. Compared with the reference drug, mitoxantrone, this activity was not significant. Our previous experiments proved that compounds bearing the 13β,28-epoxy bridge are much more potent against the cell lines mentioned above [6]. Disc diffusion assay for antifungal activity revealed no significant effect at the concentrations tested.

Experimental

General experimental procedures: Optical rotations were measured in MeOH at 20ºC on a P-2000 polarimeter. IR spectra were recorded on a Nicolet iS5 spectrometer. NMR experiments (^{1}H, ^{13}C, DEPT 135, ^{1}H-^{1}H DQF-COSY, ROESY, TOCSY, HMBC, HSQC) were performed on a Bruker AVANCE III 600MHz spectrometer (Bruker, Rheinstetten, Germany), using standard pulse sequences, in CD_3OD (99.95%, Sigma-Aldrich, St. Louis, USA). ^{1}H spectra were recorded at 600.20 MHz, and ^{13}C at 150.94 MHz. All chemical shifts (δ) are given in ppm, and TMS was used as an internal standard. Coupling constants are reported in Hz. Spectra were analyzed using MestRec magnetic resonance companion version 4.4 and CARA 1.8.4 (Computer Aided Resonance Assignment). FAB-MS were recorded on a Finnigan MAT 95 mass spectrometer; glycerol as the matrix, Cs ions accelerated at 13keV. HR-ESI-TOF-MS were obtained on a Waters SYNAPT G2-S HDMS spectrometer. CC was carried out on Merck Kieselgel 60 (70 - 230 mesh). Preparative TLC was performed on commercially precoated silica gel G plates (Analtech, 500 µm). Analytical TLC was carried out on Merck silica gel 60 aluminum plates (Merck, Darmstadt, Germany) and the compounds were visualized by spraying with 5% H_2SO_4 in MeOH, followed by heating for 5-10 min at 105ºC. All solvents for extraction and chromatographic separation were of analytical grade and purchased from Chempur (Piekary Śląskie, Poland). HPLC analysis of sugar derivatives was carried out on a Dionex apparatus equipped with a PDA 100 UV-VIS detector, on a 250 x 4.6 mm i.d. Hypersil GOLD C-18 (Thermo EC) column at 35ºC with isocratic elution with 25% CH_3CN in 50 mM H_3PO_4 for 40 min, at a flow rate of 1 mL/min, and subsequent washing for 15 min. For UV spectroscopy, HPLC-grade acetonitrile, HPLC-grade acetonitrile, methanol and H_3PO_4 were from J.T. Baker (Phillipsburg, USA). Formic acid puriss. p. a. and chloramphenicol (≥98%, TLC) were from Sigma-Aldrich (St. Louis, USA). Standard α-hederin (ROTICHROM HPLC) was from Roth (Karlsruhe, Germany). Standard sugars, D-glucose, L-glucose, D-arabinose, and L-arabinose were from Sigma, and L-cysteine methyl ester hydrochloride (puriss >99%) and *o*-tolyl-isothiocyanate from Fluka. HPLC grade water was obtained from HLP 5 (HYDROLAB, Poland) apparatus and was filtered through a 0.2 µm filter before use. Bakerbond C18 POLAR+ (1000 mg, 6 mL) cartridges were from J.T. Baker (Phillipsburg, USA).

Plant material: The underground parts of *Lysimachia thyrsiflora* L. were collected from a controlled cultivation in the Garden of Medicinal Plants, Jagiellonian University, Cracow, Poland. Samples were identified by Dr Bożena Muszyńska, Department of Pharmaceutical Botany, Pharmaceutical Faculty, Medical College, Jagiellonian University, Cracow, Poland. A voucher specimen (No. KFg/2011021) is deposited at the Department of Pharmacognosy, Pharmaceutical Faculty, Medical College, Jagiellonian University, Cracow, Poland. The mixture of *L. thyrsiflora* saponins, isolated previously [5,6], was used to develop the UPLC-MS method, to optimize chromatographic conditions, and for the specificity and system suitability tests.

Extraction and isolation: The dried and powdered plant material (940 g) was extracted with $CHCl_3$ (3 times) and then exhaustively with MeOH containing 0.5% pyridine at 40ºC. The combined MeOH extract was concentrated *in vacuo*, to yield a viscous residue (120 g), which was then partitioned between *n*-BuOH and water. The *n*-BuOH-soluble fraction was evaporated to dryness below 45ºC and chromatographed on a silica-gel column (Merck Kieselgel 60; 70-230 mesh) with $CHCl_3$–MeOH–H_2O (23:12:2). Fractions were combined on the basis of TLC (SiO_2, $CHCl_3$–MeOH–H_2O 23:12:2; sulfuric acid + heating 5-10 min, 105ºC) to give 7 major fractions. The pooled saponin fraction with R_f values 0.27 and 0.2 (fraction 7, 1500 mg) was further purified by repeated prep. TLC (commercially precoated silica gel G plates; Analtech, 500 microns) using $CHCl_3$–MeOH–H_2O (23:12:2; 8:7:1). Bands were removed from plates after spraying with water, the silica was extracted with MeOH, and solvent was evaporated *in vacuo* to yield compounds **4** (25 mg) and **5** (38 mg).

Acid hydrolysis: Identification of sugar units was established after total acid hydrolysis of **4** and **5** on a TLC plate with gaseous HCl for 25 min. Two monosaccharides were identified by TLC comparison with authentic samples ($CHCl_3$-MeOH-H_2O 23:12:2, twice; visualization: aniline phthalate in *n*-BuOH + heating) as glucose and arabinose. Absolute configurations were determined according to a slightly modified method, previously reported by Tanaka *et al*. [12]. Compounds **4** and **5** (5 mg) were dissolved in 1.0 mL of a mixture of 4 M HCl in dioxane and water (1:1; v/v) and heated at 95ºC for 3 h. The sapogenins were extracted with EtOAc (3 x 3.0 mL) and the acid aqueous phases were neutralized with 2M ammonium hydride and concentrated under vacuum. After drying over P_2O_5 for 48 h, the residues were dissolved in anhydrous pyridine (1.0 mL) in oven-dried screw-capped vials purged with argon. Then, L-cysteine methyl ester hydrochloride (5 mg) was added, the vials were again purged with argon, and the mixtures were allowed to react at 60ºC for 1 h. Next, 5 µL of *o*-tolyl-isothiocyanate was added and the reaction mixtures were heated at 60ºC for an additional 1 h. Samples were directly analyzed by HPLC and the peaks were detected at 14.09 min and 14.9 min for

monosaccharide derivatives of **4**, and at 14.1 min and 15.0 min for monosaccharide derivatives of **5**. The retention times were compared with authentic sugars derivatized according to the same procedure: D-glucose (14.1 min), L-arabinose (14.9 min).

30-*O*-β-D-Glucopyranosyl-3β,16α,30-trihydroxy-olean-12-en-28-yl acetate 3-*O*-β-D-glucopyranosyl-(1→4)-*O*-[β-D-glucopyrano-syl-(1→2)-]-*O*-α-L-arabinopyranoside (4)

Amorphous white solid.
$[\alpha]_D$: - 4.5 (*c* 0.4, MeOH).
IR (KBr): 3333 (>OH), 2922 (>CH), 1737 (ester), 1714 (C=O), 1255 and 1033 (>C-O-C) cm^{-1}.
^1H and ^{13}C NMR (CD_3OD, 600 MHz) data of the aglycone moiety and the sugar portion: Tables 1 and 2, respectively.
FAB-MS (negative mode) *m/z* 1133.4 $[M - H]^-$; 971.4 $[M - H - 162]^-$, 809.3 $[M - H - 162 - 162]^-$
HR-ESI-TOF-MS $[M + Na]^+$ *m/z* 1157. 57 calcd for $C_{55}H_{90}O_{24}Na$.

Davuricoside L (5)

Amorphous white solid.
$[\alpha]_{D20}$: +13.3 (*c* 0.4, MeOH).
IR (KBr): 3319 (>OH), 2920 (>CH), 1255 and 1033 (>C-O-C) cm^{-1}.
^1H and ^{13}C NMR (CD_3OD, 600 MHz) data of the aglycone moiety and the sugar portion: Tables 1 and 2, respectively.
FAB-MS (negative mode) *m/z* 1091.3 $[M - H]^-$; 929.3 $[M - H - 162]^-$
HR-ESI-TOF-MS $[M + Na]^+$ *m/z* 1115.56 calcd for $C_{53}H_{88}O_{23}Na$.

Semiquantitative UPLC-ESI-MS/MS analysis: Preparation of standard solutions: Chloramphenicol (10 mg), as internal standard, was placed in a volumetric and the volume brought to 10 mL using MeOH. One mL of this solution was subsequently diluted to make a 100 µg/mL stock solution. α-Hederin (1 mg), as external standard, was added to a volumetric flask and the volume brought to 10 mL with MeOH to make a 100 µg/mL solution. One mL of this solution was subsequently diluted to make a 10 µg/mL stock solution. These solutions were stored at -10ºC.

Preparation of calibration samples: To 10 µL of chloramphenicol internal standard stock solution 0 – 150 µL of α-hederin reference stock solution was added and diluted with water to 1 mL making calibration samples with a concentration of chloramphenicol of 1 µg/mL and of α-hederin in the range from 0 – 1.5 µg/mL.

Plant samples preparation: Dried, finely ground plant material (0.5 g) from 6 individual plants was extracted with $CHCl_3$ (2 x 20 mL, for 2 h), and then with MeOH (2 x 20 mL, for 2 h) on a boiling water bath under reflux. The MeOH extracts were combined and the solvent removed under reduced pressure. The residues were redissolved in 8 mL of H_2O, and 2 mL aliquots were cleaned up using SPE C_{18} cartridges, preconditioned with MeOH and H_2O. The cartridge was washed first with 6 mL H_2O, then with 18 mL 30% MeOH. Finally, saponins were eluted with 18 mL MeOH. These fractions were evaporated under reduced pressure, and redissolved in 10 mL MeOH making stock sample solutions. From each stock sample solution, 20 µL, 50 µL, 100 µL and 200 µL were taken, and then 10 µL of the chloramphenicol standard solution was added and brought to 1 mL with H_2O, making a series of sample dilutions. Each dilution was analyzed by UPLC-MS/MS.

Quantification: The UPLC-MS/MS system consisted of a Waters ACQUITY® UPLC® (Waters Corporation, Milford, MA, USA) coupled to a Waters TQD mass spectrometer (electrospray ionization mode ESI-tandem quadrupole). Chromatographic separations were carried out using an Acquity UPLC BEH (bridged ethyl hybrid) C_{18} column, 2.1 × 100 mm, and 1.7 µm particle size. The column was maintained at 40°C, and eluted under linear gradient conditions from 95% to 0% of eluent A over 10 min, at a flow rate of 0.3 mL/min. Eluent A: water/formic acid (0.1%, v/v); eluent B: acetonitrile/formic acid (0.1%, v/v). Ten µL aliquots of each sample were injected in triplicate. A Waters TQD mass spectrometer was calibrated for quantitative analysis using α-hederin solution at a concentration of 10 µg/mL at a flow rate 20 µL/min, and a mixture of eluent A and B (1:1, v/v) at a flow rate of 0.28 mL/min. Optimized settings were as follows: source temperature 150°C, desolvation temperature 350°C, desolvation gas flow rate 600 L/h, capillary potential -4.00 kV, collision gas flow 0.1 mL/min. Cone potential and collision energy were individually optimized for each transition (Table 3). Nitrogen was used as both nebulizing and drying gas, and argon as collision gas. Saponosides were analyzed using the MRM (Multiple Reaction Monitoring) method. All analytical data were processed using MassLynx V4.1 software (Waters Corporation, Milford, MA, USA).

Method validation: The described method was validated for the determination of α-hederin and the mixture of saponins **1-5** by the UPLC method according to the ICH guidelines [18]. ***Specificity:*** The solution containing all investigated saponosides (mixture) was analyzed. ***System suitability:*** The system suitability parameters were defined with respect to peak resolution of examined compounds, using a mixture of **1-5**. ***Linearity:*** The linearity for α-hederin was assessed by injecting 15 separately prepared solutions covering the range of 0.10 – 1.50 µg/mL. The slope of the weighted regression line (weight 1/x), y-intercept, standard deviations of slope and intercept, correlation coefficient, R^2 value and standard error of residuals of the calibration curve were calculated. Next, to determine whether the residuals have normal distribution, the Shapiro-Wilk statistical test was used. ***Limit of detection (LOD) and limit of quantification (LOQ):*** Based on the standard error of residuals (Se) and the slope (a) of the calibration plots and following the formula LOD= 3.3Se/a and LOQ= 10Se/a, the LOD and LOQ for α-hederin were estimated. ***Precision:*** The repeatability of the method was checked by a six-fold analysis of the concentration level at 0.7 µg/mL of α-hederin solution. The same protocol was followed for 3 different days to study the intermediate precision of the proposed method. The RSD (%) of the peak area of α-hederin was calculated. Statistical significance of interday differences was tested with ANOVA. ***Robustness:*** To demonstrate the robustness of the method deliberate small changes of flow rate, content of acetonitrile and column temperature were made around the optimal values. The mobile phase flow rate was 0.30 mL/min; to study the effect of the flow rate on resolution, the flow rate was changed to 0.27 and 0.33 mL/min. The effect of the column temperature was studied at 36ºC and 44ºC (instead of 40ºC), and the mobile phase composition was changed +5% from the initial composition.

Cytotoxicity assay: The human glioblastoma-astrocytoma cell line U375 was cultured in Eagle's minimum essential medium (EMEM) with 2 mM L-glutamine, 1% non-essential amino acids (NEAA), 1 mM sodium pyruvate and10% fetal bovine serum (FBS). Prostate cancer cells DU-145 and PC3 were cultured in Dulbecco's Modified Eagle's Medium (DMEM-F12 HAM) supplemented with 10% FBS. Malignant melanoma cell line A375 was cultured in DMEM high glucose medium supplemented with 10% FBS and 2 mM L-glutamine, whereas cell line BLM was cultured in RPMI medium supplemented with 10% FBS. Normal prostate cells PNT2 were cultured in RPMI-1640 supplemented with 10% FBS. Human skin fibroblasts (HSF) were cultured in DMEM supplemented with 10% FBS. All reagents were from Sigma-Aldrich, St. Louis, MO,

USA. All cells were cultured in medium containing 100 i.u./mL penicillin, 10 µg/mL streptomycin, and 10 µg/mL neomycin, in standard conditions, a humidified atmosphere at 37ºC in the presence of 5% CO_2. Cytotoxicity assay was performed as described previously [10]. In each experiment, for a positive control, mitoxantrone (Ebewe Pharma) was used, at concentrations from 0.1 to 1 µg/mL. For each value measured, 300 cells were analyzed. Each experiment was performed in triplicate. Each variable was expressed as the mean (± SD). The statistical significance was determined using either the Student's *t*-test or the non-parametric Mann-Whithey *U*-test, with $p < 0.05$ considered to indicate significant differences.

Anti-proliferative assay: The assay was performed on prostate cancer cells DU-145 and PC3, and on normal prostate cells PNT2, cultured at conditions described above. Cells were seeded into 24-well plates at an initial density of 7.6×10^2 cells per well and incubated for 24 h at 37ºC. Next, the medium was replaced with either fresh culture medium (control), or with the same medium containing different concentrations (from 0 up to 30 µg/mL) of saponosides **4** and **5**. After incubation for various times (24, 48, 72 h) the medium was removed, the cells trypsynizated and the number of cells determined using a cell counter (Coulter Beckman ZS).

Antimicrobial assay: The potential antimicrobial properties of compounds **4** and **5** were investigated by the conventional paper disc diffusion method against the following strains: *Candida albicans*, *C. glabrata*, *C. krusei*, *C. parapsilosis*, *C. lusitaniae*, *Cryptococcus neoformans*, and *Trichophyton mentagrophytes*. All strains were the isolates of the Department of Pharmaceutical Microbiology, Pharmaceutical Faculty, Medical College, Jagiellonian University. Sabouraud broth was used as media for all strains except *C. neoformans*, which was cultured in Casitone broth. Strain suspensions in 0.85% NaCl (100 µL) from 24 h incubations were applied to 9 cm Petri dishes with Sabourand agar medium. For the compounds being tested, 1 mg was dissolved in 1 mL DMSO. Five µL aliquots of these solutions were applied to paper discs and placed onto the agar plates. The solvent was used as control. The plates were incubated at 30ºC for 24 h, and the growth inhibition was assessed based on the dimensions of the lytic zones on the plates.

Supplementary data: Details on the cytotoxicity and antiproliferative assays. ^1H, ^{13}C, DEPT, HSQC, HMBC, ROESY, TOCSY, and COSY spectra for compound **4** and **5**.

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Variation of Saponin Contents and Physiological Status in *Quillaja saponaria* Under Different Environmental Conditions

Angélica Grandón S[a]*, Miguel Espinosa B[a], Darcy Ríos L[a], Manuel Sánchez O[a], Katia Sáez C[c], Víctor Hernández S[b]. and José Becerra A[b]

[a]*Department of Silviculture*, *Faculty of Forest Science. Universidad de Concepción. 4070386*, *Concepción. Chile*

[b]*Laboratory of Chemistry of Natural Products. Faculty of Natural and Oceanographic Sciences. Universidad de Concepción. 4070386*, *Concepción. Chile*

[c]*Department of Statistics*, *Faculty of Physics and Mathematics Sciences. Universidad de Concepción. 4070386*, *Concepción. Chile*

agrandon@udec.cl

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Quillaja saponaria (Quillay), an evergreen tree found in Chile, is one of the main sources of saponins. *Quillaja* saponins have hypocholesterolaemic, anticarcinogenic, antioxidant and pesticidal properties, and are used as adjuvants for vaccines. Samples of Quillay growing at three zones in O'Higgins Region, Chile (Coastal, Central and Mountain zones) were analyzed for content of saponins and physiological status. The results revealed differences in the content of saponins depending on the zone of sample collection. The highest contents were found in samples from the Mountain zone, where the highest saponin contents were accompanied by the lowest foliar nitrogen contents, the highest antioxidant activity and the highest carotenoid contents. The results suggest a physiological and adaptive mechanism of saponins in plants to survive under unfavourable environmental conditions. The results have important implications for a theoretical basis for the design of a reasonable harvest, to avoid the cost of poor quality material, and also to provide a sustainable use and conservation of this important species. Further research on the effects of stress will improve our understanding of the saponins production and their physiological functions in plants, whereas they have generally been studied for their biological and chemical applications.

Keywords: Saponins, Triterpenes, *Quillaja saponaria*, Secondary metabolites, Glycosides.

The Chilean native tree *Quillaja saponaria* Mol. (Quillay), distributed under different geographical and climatic conditions, is one of the most important sources of triterpene saponins [1a,b]. Structurally, the *Quillaja* saponins are referred to as triterpene glycosides, consisting of an aglycone of quillaic acid (hydrophobic region) and several sugars, such as rhamnose, fucose, xylose, arabinose, glucuronic acid and galactose (hydrophilic region) (Figure 1). An extract of *Quillaja* saponins is a heterogeneous mixture, and approximately 70 saponins have been reported [2a].

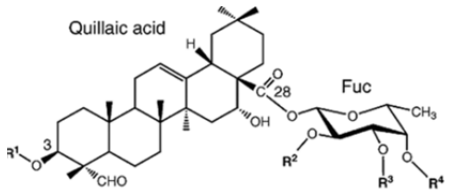

Figure 1: Basic structure of *Quillaja* saponins. The aglycone is usually the triterpene quillaic acid, with a branched trisaccharide or a disaccharide linked to the 3-position (R^1). The ester linked fucosyl residue at C-28 is substituted by an oligosaccharide in the 2-position (R^2), by a monosaccharide residue or an acyl group in the 3-position (R^3) and by an acyl group in the 4-position (R^4) [2b].

Due to their biological and chemical properties, such as antibacterial, antifungal, insecticidal, hypocholesterolaemic, anticarcinogenic, antioxidant, haemolytic, antioxidant, adjuvants for vaccines, foaming, emulsifying, surfactant and tensoactive [3a,b], they are generally considered to be part of the defensive system of the plants [1a]. *Quillaja* saponins have industrial applications as, for instance, beverage, alimentary, cosmetic, pharmaceutical, medicinal, pesticidal, photographic and mining products [1b,4]. *Quillaja* saponins are obtained by aqueous extraction of bark (traditional process) and biomass obtained from forest pruning. The industrial use of saponin causes high demand for biomass, but it is also accompanied by sub-utilization of this resource [5] due to the variation of saponin contents in Quillay. This variation has generally been attributed to genetic factors, edaphic conditions, altitude, seasonal effects, age of vegetation, and kind of vegetal tissue [6a]. However, considering the diversity of environments in which Quillay grows, it is possible that the contents of saponins and the physiological status of the trees could be influenced by the surrounding environment. The growing demand for *Quillaja* saponins requires rationalization of the harvesting of existing natural resources to avoid their improper exploitation and sub-utilization.

There has been some research on the effects of abiotic environmental factors on saponin contents, but the majority of published studies have been focused on non woody plants with short life cycles, such as *Panax quinquefolius*, *P. ginseng, Asparagus racemosus*, *Glycyrrhiza uralensis*, *Phytolacca dodecandra*, *Bupleurum chinense*, *Clematis chilensis*, *C. hexapetala*, and *Tribulus terrestris*, among others [6b]. With respect to Quillay, over the last 10 years, significant efforts have been made in qualitative and quantitative analysis of the saponins, with the aim of increasing knowledge for commercial applications [1a,3a,b]. However, much less data are available concerning variation of saponin contents in woody species under natural conditions due to the complexity involved, where two or more stressors co-occur and their effects are sometimes additive. Nevertheless, research is necessary on the influence of external conditions on saponin

accumulation in plants that should lead to a better understanding of saponin production in natural conditions. Our study examined the variation of total saponin contents and physiological status in *Quillaja saponaria* growing under different environmental conditions in the O'Higgins Region of Chile (Coastal, Central and Mountain zones). Although these zones are located at the center of the species' native range, studies from these specific regions have not previously been reported. In order to achieve these objectives, the zones were characterized at the edaphic level for available water capacity (AWC), nitrogen (N), organic matter (OM), phosphorus (P) and potassium (K). The trees were analyzed for total saponin contents, and physiological status: photosynthetic pigments, antioxidant activity, nitrogen, phosporus and potassium.

Edaphic characterization: The edaphic samples from the different zones were significantly different for OM, AWC and P. The Mountain zone showed the highest contents of K, AWC and OM. The content of OM for this zone was 13.9%, 2.9 and 1.7 times higher than the Coastal and Central zones, respectively. The AWC was 22.3% and 16.7% for Mountain and Central zones, respectively, both significantly different when compared with the Coastal zone. K content was significantly higher in the Mountain zone with 655.2 cmol/kg, 2.1 and 3.6 times greater than the Central and Coastal zones, respectively (Table 1). However, in spite of these results, this zone is characterized by presenting harsher environmental conditions than the Coastal and Central zones. The spatio-edaphic pattern of the present study identified three differentiable zones, as shown by PCA. The first and second principal components accounted for 39.9% and 29.6% of the total variation, respectively. Both components explain 70% of the variance. N explained the Coastal zone, N and P the Central zone, and OM, AW and K the Mountain zone. These specific and inherent characteristics of the zone within the Region may explain differences in the physiological status of plants, which is influenced by environmental conditions (Figure 2)

Table 1: Nitrogen (N), phosphorous (P), potassium (K), available water capacity (AWC) and organic matter (OM) from sampling zones of the O'Higgins Region. Mean values ±S.D.

Variable	Coastal zone	Central zone	Mountain zone	*P*-value
N (N-NO3) (mg/kg)	13.9 ±10.0	14.0±9.0	7.8±5.2	0.2377
P (mg/kg)	25.3 ±6.3	33.6±21.7	26.1±9.1	0.4427
K (cmol/ kg)	255.9±133.0^{b}	315.1±126.4^{b}	655.2±266.8^{a}	0.0006
AWC (%)	7.9±1.6^{b}	16.7±4.6^{a}	22.3±13.2^{a}	0.0113
OM (%)	4.8±1.0 ^{b}	8.1±3.9^{b}	13.9±4.1^{a}	<0.0001

In each column, different letters are significantly different for 95% confidence intervals (P<0.05). Fisher's LSD test.

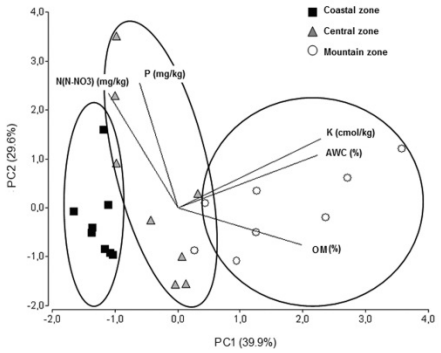

Figure 2: Biplot of soil samples distributed in Coastal, Central and Mountain zones from O'Higgins region, against values for the first two principal components, PC1 and PC2, for edaphic variables: organic matter (OM), available water (AWC), nitrogen (N), phosphorous (P) and potassium (K). Vectors represent edaphic variables that have a high loading on PC1 and PC2 and that are significantly correlated with the ordination axes ($P < 0.05$).

Physiological status: The nutritional status of Quillay originating from Coastal, Central and Mountain zones was significantly different for N and P. Samples from the Mountain zone showed the lowest N content (0.69%) and the Central zone the highest (1.04%). P was higher in the Coastal zone, with 0.12%, than the Central zone (0.09%). For K content, there was no significant difference among zones (Figure 3A). These results have been consistent across the availability of nutritional resources in each zone. Considering that the O'Higgins Region presents a scarce water pluviometric regime during the months of vegetative development, this contributes to explaining the development and growth of sclerophyll species by means of adaptive and efficient strategies for the use of water and nutritional resources in these difficult Mediterranean climates [7]. Thus the plants can mitigate the physiological process with a trade-off between growth, maintenance, storage, reproduction and defense.

The chlorophyll a and b contents were not significantly different. Carotenoids increased significantly from the Coastal to the Mountain zones, reaching a mean value of 0.34 mg/g, 2.4 and 1.6 times higher than the Coastal and Central zones, respectively (Figure 3B). The highest carotenoid content found in the Mountain zone had been associated with defensive responses in plants under stress conditions, such as water stress and/or radiation; carotenoids have antioxidant properties and a photo-protective function of the photosynthetic system against harmful photooxidative processes [8].

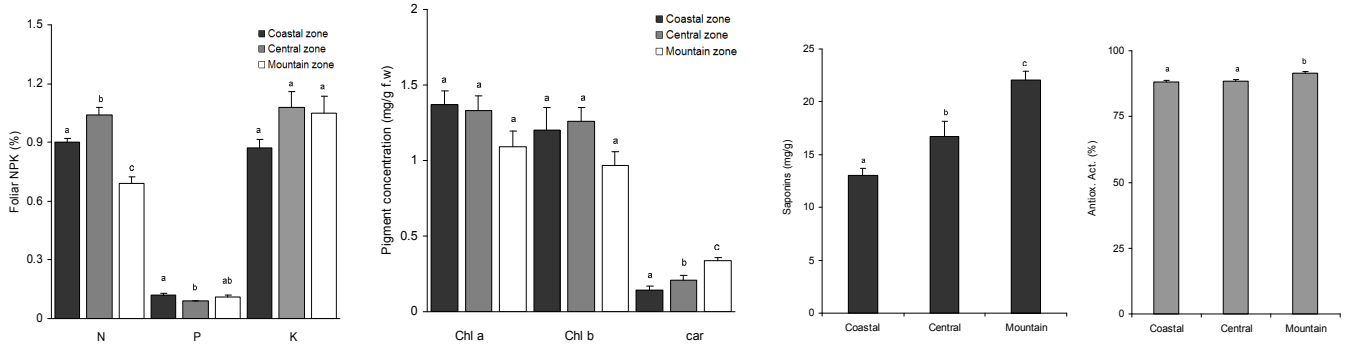

Figure 3: **A**. Foliar NPK contents in Coastal, Central and Mountain zones. Any variable not having a letter in common is significantly different at $P < 0.05$. Bars represent mean values ± S.E. **B**. Pigment concentration: chlorophyll a (Chl a), chlorophyll b (Chl b) carotenoids (car) in Coastal, Central and Mountain zones. Any variable not having a letter in common is significantly different at $P < 0.05$. Bars represent mean values ± S.E. **C**. Antioxidant activity in Coastal, Central and Mountain zones. Any zone not having a letter in common is significantly different at $P < 0.05$. Bars represent mean values ± S.E. **D**. Saponins production in Coastal, Central and Mountain zones. Any zone not having a letter in common is significantly different at $P < 0.05$. Bars represent mean values ± S.E.

The antioxidant activity was significantly higher in samples from the Mountain zone compared with those from the Coastal and Central zones (Figure 3C). This implies a defensive mechanism observed in alpine plants subjected to environmental stressors, such as solar radiation and low temperatures, which may activate the formation of chemical reactive oxygen species (e.g., hydrogen peroxide, superoxide and hydroxyl) conducive to oxidative stress [9a]. Under these conditions it has been seen that plants generate antioxidant compounds, intended to entrap radicals harmful to their cells, resulting in a broad adaptation spectrum for the plants [9b]. The high antioxidant activity of Quillay, over 85%, makes this species interesting to study for the production of antioxidants.

Saponin contents: Saponin contents of Quillay were different between zones, increasing significantly from the Coastal to the Mountain zone. The highest saponin contents were found in samples from the Mountain zone, where the content was 22.1 mg/g; 1.7 and 1.3 times higher than those of the Coastal and Central zones, respectively (Figure 3D). The saponin contents, found in the range between 1.2% and 2.2% of the dry weight, contrast with values close to 8% of soluble compounds found by San Martin and Briones [6d], and between 6.5% and 15.8% dry weight found by Copaja *et al.* [6c].

Associating saponin contents and physiological status, our study revealed, for the first time, that the highest saponin contents were accompanied by the lowest nitrogen content, and the highest antioxidant activity and carotenoids contents as a synergistic response. This finding showed that higher accumulation of saponins in Quillay would be stimulated by adverse environmental conditions. Therefore, an evaluation of quality and saponin composition are necessary in future research. The presented results suggest a physiological and adaptive mechanism of saponins in plants to survive under unfavourable environmental conditions.

This study was a first approach and contribution to the knowledge about the variation of saponin contents and physiological status in *Q. saponaria* under different environmental conditions. Further research on the effects of stress will improve our understanding of saponin production and the physiological functions of saponins in plants. Previously, they have generally been studied for their biological and chemical applications.

Experimental

Zones of study: Sampling took place at 3 zones in the O'Higgins Region: Pumanque (Coastal zone, 34º 56´S and 71º5478´ W; altitude 180 m), Roblería del Cobre de Loncha National Reserve (Central zone, 34º 12´ S and 71º 10´ W; altitude 430 m) and Río de los Cipreses National Reserve (Mountain zone, 34º 31´ S and 70º 45´ W; altitude 1250 m). Pumanque has a Mediterranean climate, with a drought period of 6-7 months and a semiarid period of 1-2 months. Annual average temperature is 14.5ºC, seasonal average temperature is 28ºC in summer and 3.7ºC in winter. The precipitation regimen varies between 500 to 700 mm annually. Roblería del Cobre de Loncha National Reserve presents a warm mild climate, with a prolonged dry season. Maximum annual temperatures oscillate between 25ºC to 28ºC and the minimum is between 10ºC to 8ºC. Río de los Cipreses National Reserve has a mild Mountain climate, with 4 months of dry warm weather in summer, and winter with rain and snow precipitation between 800 to 1500 mm annually. The temperature varies between a maximum of 19ºC to 21ºC and a minimum of 7ºC to 5ºC, with temperatures under 0ºC due to thermal oscillations in winter [10].

Sampling and nutritional analysis of soil: In every zone, 8 randomly distributed soil samples were taken from the surface, at a depth of 20 cm, near trees. One third of the soil sample was used for nutritional analysis, the second third for field capacity, permanent wilting point and available water capacity, and the last third for organic matter content; every soil sample was replicated 3 times. For nutritional analysis, 33 g of dry and sifted soil was analyzed for nitrate ($N\text{-}NO_3$), phosphorous (P) and potassium (K). $N\text{-}NO_3$ was determined through the Kjeldahl and direct Nesslerization Semichrome method. $N\text{-}NO_3$ detection was carried out by spectrophotometric absorbance measurement at a wavelength of 490 nm [11]. P was obtained by Olsen's method, extracting P in 0.5 mol/L $NaHCO_3$ at pH 8.5. Then, the extract was analyzed by colorimetry using the ethylene blue method with ascorbic acid as redactor, and detected by spectrophotometric *absorption* at *880 nm***.** K was extracted in 1 mol/L $NH_4C_2H_3O_2$ solution at pH 7.0 and detected by Atomic Spectrophotometry Emission (ASE) with either an acetylene-air or propane-air flame, measured at 766.5 nm [12].

Field capacity, permanent wilting point and available water capacity: Field capacity (FC), permanent wilting point (PWP) and percentage of soil available water capacity (AWC) were obtained as follows: The FC was determined by the pressure cooker method [13]. In brief, cylinders filled with soil were hydrated with distilled water for 24 h. Then, they were placed in a pressure cooker under 0.33 and 15 bar for 24 h, in order to obtain wet weight. All samples were later oven dried at 105ºC for 24 h to determine dry weight. The relative percentage of AWC at FC and PWP was obtained by difference between wet and dry weight at 0.33 bar and 15 bar, respectively. AWC was obtained by gravimetric weight difference between FC and PWP.

Organic matter content of soil: Organic Matter Content (OM) was determined by Weight Loss on Ignition method (WLOI) [14]. Briefly, an aliquot of 10 g soil was oven dried at 105º C for 24 h to determine dry weight. Then, the samples were calcined in a muffle furnace at 450ºC for 24 h, to obtain calcinated weight. Organic matter percentage was obtained by the relational differences of the dry and calcinated soil weight and dry soil weight.

Plant material: In September 2011, 15 trees was sampled in each study zone, with diameters between 70 to 100 cm. For each tree, 10 cm^2 of bark was extracted at breast height to determine the total saponin contents, and 300 g of foliar biomass was extracted from the middle crown for nutritional analysis, antioxidant activity and pigment quantification.

Foliar nutritional analysis: Nutritional analysis was performed for nitrogen (N), phosphorous (P) and potassium (K). N was obtained through a digestion method; 3 g dried sample was put in H_2SO_4, 98% of density 1.84 Kg/L. The NH_4 concentration was obtained by distillation of NH_3 and manual titration [15]. For P, 3 g of dried sample was passed through a 1 mm sieve. Then it was put in a crucible and placed in a muffle furnace at 500ºC for 4-8 h. Once cooled, 10 mL of HCl at 2 mol/L was added and boiled on a hot plate. The content in the crucible was filtered and P detection was determined through colorimetry using the phospho-vanadomolybdate method, measured at 466 nm. K was determined by absorption spectrophotometry and atomic emission with an air acetylene flame measured at 766.5 nm [15].

Pigment quantification: Two g of leaves were extracted in acetone/ water (8/2,v/v), and chlorophyll a, chlorophyll b, and carotenoids contents were determined by absorption spectrophotometry at 663 nm, 646 nm and 470 nm, respectively [16].

Antioxidant activity: The antioxidant activity was determined using the DPPH (1,1-diphenyl-2-picrylhydrazyl free radical) scavenging method [17]. Briefly, 2 g of leaves were extracted in 50 mL methanol: water (7/3,v/v) for 24 h at room temperature. The extract was resuspended in ethanol (500 µL), mixed with deionised water in equal volumes of DPPH (60 µM), and homogenized in a vortex mixer. Detection was performed by absorbance at 520 nm (20 min) in a spectrophotometer (model UV-VIS 2310). The antioxidant activity, based on the DPPH free radical scavenging ability of the extract, was determined through comparison of the absorbance with the control (100%) containing only DPPH.

Extraction and quantification of saponins: Two g of dried bark was extracted in 50 mL MeOH: H_2O (7/3, v/v) for 24 h at room temperature. The extract was filtered through a 0.2 micron filter and kept frozen at -18ºC, before analysis. Saponins were detected by HPLC (Shimadzu, 10 AD-VP, Japan), with a Vydac C-4 column (250 x 4.6 mm) and a 30-45% acetonitrile in water gradient, with 0.15% trifluoracetic acid. The flow rate was 1 mL min^{-1} and detection was measured at 210 nm. [6a]. The total saponin contents (dry wt) were determined by comparison with the standard calibration curve, using the standard Quillaja Liquid Ultra (QL Ultra) of Natural Response S.A. The standard was diluted to different concentrations to obtain the best linearity, with R^2=0.9.

Statistical analysis: A parametric variance analysis (ANOVA) was performed for edaphic and physiological variables, every sample being replicated 3 times (n=3). Differences were detected with Tukey multiple comparisons test. The existence of groups of variables by zone was achieved with multivariate analysis of principal components (PCA), which identified groups of samples based on the measured edaphic variables and how these relate to the observed differences between the groups classified by zone. Distributional assumptions of both normality and heterogeneity of variance was analyzed with Shapiro-Wilk and Levene tests, respectively; differences were considered statistically significant if $P < 0.05$ for the test statistic. Analyses were performed with software SAS (SAS Institute Inc, 2003) and Infostat version 2012 [18a,b].

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New Access to 7,17-seco C_{19}-Diterpenoid Alkaloids via Vacuum Pyrolysis of *N*-Deethyl-8-acetyl Derivatives

Ling Wang, Qi-Feng Chen and Feng-Peng Wang*

Department of Chemistry of Medicinal Natural Products, West China College of Pharmacy, Sichuan University, Chengdu, Sichuan, PR China, 610041

wfp@scu.edu.cn

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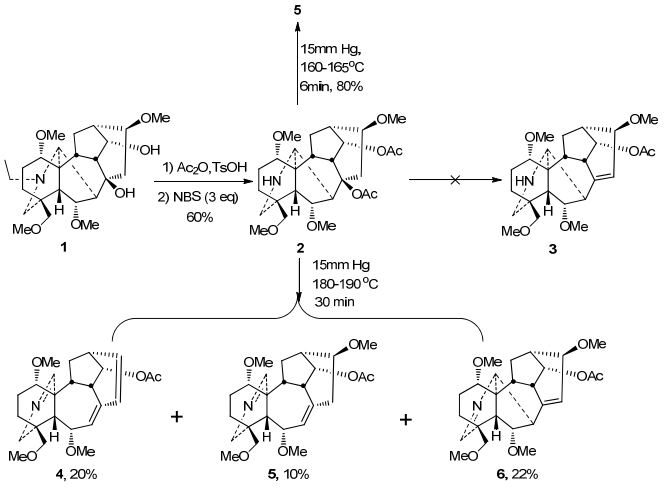
A novel access to 7,17-seco C_{19}-diterpenoid alkaloids via vacuum pyrolysis of *N*-deethyl-8-acetyl derivatives is described.

Keywords: C_{19}-diterpenoid alkaloid, Pyrolysis, 7,17-Seco C_{19}-diterpenoid alkaloid.

The C_{19}-diterpenoid alkaloids are a group of highly oxygenated, complex, natural products displaying many interesting chemical reactions and important biological activities, as well as a synthetic or structurally modified target [1,2].

In our continuing research on the chemistry of diterpenoid alkaloids, we attempted to prepare the pyro-type alkaloid **3** from **2** via pyrolysis under vacuum conditions. Very interestingly, the 7,17-seco compounds **4** and **5**, in addition to the desired compound **6**, were afforded (Scheme 1). This is a novel access to the 7,17-seco C_{19}-diterpenoid alkaloids, different from the known methods. In this paper, we wish to report this new cleavage of the C(7)-C(17) bonds in the *N*-deethyl-8-acetyl derivatives **2** and **8**.

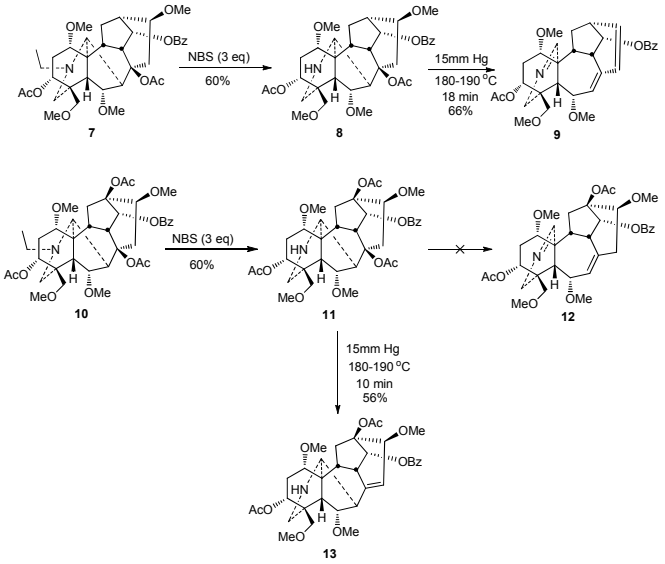
As showed in Scheme 1, treatment of diacetylchasmanine (**1**) with NBS at room temperature for 1 h afforded the desired compound **2** in 60% yield. Pyrolysis of compound **2** under vacuum conditions (15 mm Hg, 180-190°C, 30 min) gave the 7,17-seco compounds **4** (20%) and **5** (10%), as well as the pyro compound **6** (22%). It should be pointed out that pyrolysis of **2** under optimum conditions (15 mm Hg, 160-165°C, 6 min) led to the 7,17-seco compound **5** in high yield (80%).



Scheme 1. The preparation of compounds **2**, **4**, **5** and **6**

The HR-ESIMS of **4** showed a quasimolecular ion peak at *m/z* 416.2445 $[M+H]^+$ corresponding to the formula $C_{24}H_{33}NO_5$. The NMR (^1H, ^{13}C, and HMQC) data (Table 1) showed the presence of three methoxyl groups (δ_H 3.19, 3.28, 3.30, each 3H, s; δ_C 56.6 q, 57.8 q, 58.8 q), an acetyl group (δ_H 2.02 s; δ_C 171.3s, 21.2 q), a conjugated double bond (δ_C 128.8 d, δ_H 6.28, d, J = 9.6 Hz; δ_C 129.6 d, δ_H 5.87, t, J = 8.8 Hz; δ_C 131.8 d, δ_H 5.23, d, J = 6.4 Hz; δ_C 135.3 s), and an iminium moiety (δ_C 165.5 d, δ_H 7.72, d, J = 1.6 Hz). The molecular formula of **5** was established as $C_{25}H_{37}NO_6$ according to its HR-ESIMS and ^{13}C NMR data. In comparison with compound **4**, the NMR data of compound **5** showed an additional methoxyl group (δ_H 3.25, 3H, s; δ_C 56.1 q), and the absence of a double bond, leading to confirmation of the structure of **5**. The formula $C_{25}H_{35}NO_6$ of **6** was established by HR-ESIMS. The structure of **6** could be determined easily based on characteristic signals at δ_H 7.39 (1H, s) for H-19, and δ_H 5.70 (1H, d, J = 6.4 Hz) for H-15, and the absence of the 8-OAc group by comparison with **2**.

Similarly, pyrolysis (15 mm Hg, 180-190°C, 18 min) of compound **8** from **7** generated the desired 7,17-seco compound **9** in 66% yield



Scheme 2. The preparation of compounds **8**, **9**, **11**, and **13**

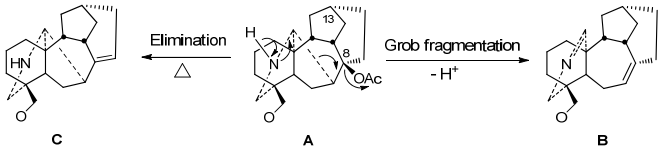
Table 1: ^{1}H and ^{13}C NMR data for compound **4** (^{1}H: 400 MHz, ^{13}C: 100 MHz, $CDCl_3$).

Position	δ_C	δ_H Mult (*J*=Hz)	^{1}H-^{1}H COSY	HMBC (H→C)
1	89.7 d	2.97 dd (11.6, 4.4)	2α, 2β	5, 10, 17, 1-OCH$_3$
2	24.4 t	1.17 m (α)	1, 2β, 3α, 3β	—
		1.75 m (β)	2α, 3β	4
3	37.8 t	1.57 m (β)	2α, 2β, 3α	1, 5, 18
		1.84 m (α)	2α, 3β	1
4	39.7 s	—	—	—
5	47.3 d	2.34 (overlapped)	6	1, 7, 17, 19
6	80.1 d	4.76 t (8.8)	5, 7	4, 8, 6-OCH$_3$
7	131.8 d	5.23 d (6.4)	6	15
8	135.3 s	—	—	—
9	42.7 d	3.08 t (4.4)	10,14	7, 12, 13, 15
10	44.0 d	2.37 (overlapped)	9, 12α	8, 14, 17
11	48.3 s	—	—	—
12	34.1 t	2.21 m (α)	10, 13	9, 11, 16
		2.35 m (β)	—	11, 16
13	36.7 d	2.72 m	12α, 14, 16	9, 10, 15
14	73.0 d	4.86 t (4.0)	9, 13	8, 16, 14- OAc
15	128.8 d	6.28 d (9.6)	16	7, 9, 13, 17
16	129.6 d	5.87 t (8.8)	13, 15	8, 14
17	165.5 d	7.72 d (1.6)	—	5
18	80.6 t	3.15 (a) ABq (8.4)	18b	3, 5
		3.95 (b)	18a	3, 19
19	58.2 t	3.42 (a) ABq (18.8)	19b	3, 5, 17
		3.54 (b)	19a	3, 17
1-OCH$_3$	56.6 q	3.19 s	—	1
6-OCH$_3$	57.8q	3.28 s	—	6
18-OCH$_3$	58.8 q	3.30 s	—	18
14-OAc	21.2 q	2.02 s	—	—
	171.3s	—	—	—

Table 2: ^{13}C NMR data for compounds **2, 5, 6, 8, 9, 11,** and **13** (100 MHz, $CDCl_3$).

Position	**2**	**5**	**6**	**8**	**9**	**11**	**13**
1	82.4 d	82.7 d	84.2 d	83.6 d	79.9 d	83.5 d	81.8 d
2	23.3 t	24.4 t	29.6 t	27.8 t	33.9 t	31.4 t	31.6 t
3	28.5 t	31.4 t	39.0 t	72.3 d	72.0 d	72.1 d	71.8 d
4	38.9 s	39.3 s	50.8 s	49.7 s	47.6 s	49.6 s	52.0 s
5	39.0 d	45.8 d	44.0 d	39.5 d	36.9 d	41.1 d	44.0 d
6	82.2 d	89.7 d	85.0 d	80.6 d	82.8 d	80.5 d	83.9 d
7	43.4 d	133.0 d	33.6 d	44.3 d	129.8 d	45.0 d	47.2 d
8	85.8 s	134.4 s	144.7 s	85.5 s	130.3 s	85.2 s	128.4 s
9	53.4 d	43.2 d	49.9 d	55.4 d	44.1 d	54.5 d	55.0 d
10	42.9 d	38.7 d	49.7 d	42.9 d	43.0 d	42.6 d	47.8 d
11	50.4 s	48.1 s	48.5 s	42.8 s	42.6 s	42.8 s	43.5 s
12	28.9 t	32.7 t	25.3 t	31.1 t	29.8 t	34.2 t	36.0 t
13	43.8 d	44.1 d	52.7 d	45.0 d	42.8 d	81.6 s	84.5 s
14	83.7 d	75.7 d	74.1 d	75.5 d	72.9 d	77.2 d	74.6 d
15	37.5 t	37.3 t	118.0 d	37.7 t	132.9 d	39.4 t	117.9 d
16	75.4 d	79.8 d	80.5 d	82.3 d	131.5 d	79.8 d	79.9 d
17	57.0 d	164.6 d	73.3 d	57.9 d	165.3 d	58.2 d	70.8 d
18	79.8 t	80.4 t	78.2 t	74.0 t	71.9 t	73.7 t	72.0 t
19	49.3 t	58.4 t	166.7 d	41.3 t	51.9 t	41.3 t	42.0 t
1-OCH$_3$	57.9 q	56.6 q	55.8 q	56.6 q	56.9 q	57.9 q	56.3 q
6-OCH$_3$	59.0 q	58.8 q	59.2 q	58.9 q	58.5 q	58.9 q	58.9 q
14-OCH$_3$	—	57.7 q	57.6 q	—	—	—	—
16-OCH$_3$	56.4 q	56.1 q	56.2 q	56.5 q	—	56.4 q	58.4 q
18-OCH$_3$	55.2 q	—	—	54.8 q	58.0 q	55.6 q	57.4 q
3- OAc	—	—	—	170.4 s	170.1 s	170.4 s	170.7 s
				21.5 q	21.1 q	21.2 q	21.8 q
8-OAc	169.1 s	—	—	169.5 s	—	169.6 s	—
	21.1 q			21.2 q		21.1 q	
13-OAc	—	—	—	—	—	177.7 s	170.1 s
						21.4 q	21.1 q
14-OAc	170.6s	171.9 s	171.9 s	—	—	—	—
	22.2 q	21.0 q	21.0 q				
14-OBz	—	—	—	166.1 s	166.4 s	166.1 s	166.7 s
				130.1 s	135.5 s	129.8 s	144.5 s
				133.0 d	129.6 d	133.2 d	132.7 d
				129.6 d×2	128.9	129.8	130.1
				128.4 d×2	d×2	d×2	d×2
					128.2	128.5	128.2
					d×2	d×2	d×2

(Scheme 2). However, treatment of compound **11**, bearing the hydroxyl group at C-13, under similar conditions (15 mm Hg, 180-190 °C, 10 min) produced only pyro compound **13** instead of the expected 7,17-seco compound **12** (Scheme 2), indicating that the hydroxyl group at C-13 in **11** was unfavorable for the cleavage of the C(7)-C(17) bond, which was difficult to interpret. The formula $C_{31}H_{37}NO_7$ of **9** was established from HR-ESIMS and ^{13}C NMR data. The NMR spectra of **9** showed the presence of a conjugated double bond (δ_C 132.9 d, δ_H 6.38, d, J = 9.2 Hz; δ_C 131.5 d, δ_H 5.95, t, J = 8.8 Hz; δ_C 129.8 d, δ_H 5.24, d, J = 6.4 Hz; δ_C 130.3 s), and an iminium moiety (δ_C 165.3 d, δ_H 7.78, s). Its structure could be determined easily by comparison of spectral data with those of compound **4**. The molecular formula for **13** was established as $C_{34}H_{43}NO_{10}$ from HR-ESIMS and ^{13}C NMR data. The structure of



Schem 3. A plausible mechanism of pyrolysis of the N-deethyl-8-acetyl derivatives of C_{19}-diterpenoid alkaloids

13 was confirmed by the characteristic signal at δ_H 5.73 (1H, d, J = 6.8 Hz) for H-15, and the absence of an 8-OAc group in the NMR spectra when compared with those of compound **11**.

A process of cleavage of the C(7)-C(17) bonds in compounds **8** and **11** was postulated (Scheme 3). As shown in Scheme 3, there were two competitive reactions, e. g., cleavage of the C(7)-C(17) bonds via Grob fragmentation (A→B) and elimilation (A→C) during pyrolysis. Importantly, the hydroxyl group at C-13 in the substrates, as in **11**, was not helpful for cleavage of the C(7)-C(17) bonds.

In conclusion, although the literature reports many methods of cleavage of the C(7)-C(17) bonds in C_{19}-diterpenoid alkaloids [3-9], most of which lead to complicated products with either low yields or difficult purification, except for the Grob fragmentation-$NaBH_4$ reduction method developed by us [10,11]. Obviously, the method reported here is simpler and more convenient than those of the Grob fragmentation-$NaBH_4$ reduction procedure.

Experimental

General methods: Optical rotations were measured in a 1.0 dm cell with a PE-314 polarimeter at 20±1°C; IR spectra were recorded on a Nicolet 200 SXV spectrometer; HRMS were obtained with a Bruker BioTOFQ mass spectrometer; ^{1}H and ^{13}C NMR spectra were acquired on a Varian INOVA-400/54 spectrometer, with TMS as internal standard; silica gel GF254 and H (Qingdao Sea Chemical Factory, China) were used for TLC and CC.

Preparation of compound 2: To a solution of diacetylchasmanine (**1**) (7.46 g, 13.90 mmol) in HOAc (100 mL) was added NBS (6.9 g, 38.84 mmol), and the mixture was allowed to stand at room temperature for 3 h prior to being poured into ice water (100 mL). The mixture was basified with conc. NH_4OH solution to pH 9 and extracted with dichloromethane (150 mL × 3). The extracts were dried (Na_2SO_4) and concentrated to give a crude product, which was subjected to CC (silica gel H, 100 g), using light petroleum/acetone/diethylamine (66:33:1) as eluent, to afford **2** as a white amorphous power (3.87 g, 55%).

Compound 2

$[\alpha]_D^{20}$: +5.0 (*c* 0.50, $CHCl_3$).

IR (KBr): 2935, 2824, 1732, 1641, 1243, 1098 cm^{-1}.

^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.97, 2.04 (each 3H, s, OAc×2), 3.01 (1H, ABq, J = 8.4 Hz, H-18), 3.22, 3.26, 3.31, 3.32 (each 3H, s, OCH$_3$×4), 3.58 (1H, ABq, J = 8.4 Hz, H-18), 4.06 (1H, d, J = 5.6 Hz, H-6), 4.81 (1H, t, J = 4.8 Hz, H-14β).

^{13}C NMR (100 MHz, $CDCl_3$): Table 2.

HRMS-ESI *m/z* $[M + H]^+$ calcd for $C_{27}H_{41}NO_8$: 508.2910; found: 508.2912.

Preparation of 4, 5, and 6: Compound **2** (570 mg, 1.12 mmol), in a round bottomed flask (50 mL), was heated at 180-190°C under 15 mm Hg for 30 min. After cooling to room temperature, the residue was chromatographed over silica gel H eluting with cyclohexane/acetone/diethylamine (30:1:0.15) to give **4** (a white

amorphous power, 110 mg, 20%), **5** (a white amorphous power, 55 mg, 10%), and **6** (a white amorphous power, 120 mg, 20%).

Compound 4

$[\alpha]_D^{20}$: +16.4 (*c* 0.50, $CHCl_3$).

IR (KBr): 2925, 1734, 1624, 1399, 1256, 1100 cm^{-1}.

^1H NMR (400 MHz, $CDCl_3$): Table 1.

^{13}C NMR (100 MHz, $CDCl_3$): Table 1.

HRMS-ESI *m/z* $[M + H]^+$ calcd for $C_{24}H_{34}NO_5$: 416.2437; found: 416.2445.

Compound 5

$[\alpha]_D^{20}$: + 24.6 (*c* 0.50, $CHCl_3$).

IR (KBr): 2927, 2823, 1732, 1637, 1371, 1249, 1103 cm^{-1}.

^1H NMR (400 MHz, $CDCl_3$) δ: 2.05 (3H, s, OAc), 3.19, 3.25, 3.28, 3.30 (each 3H, s, OCH_3×4), 4.47 (1H, t, *J* = 7.2 Hz, H-6), 4.88 (1H, t *J* = 4.4 Hz, H-14β), 5.26 (1H, d, *J* = 6.4 Hz, H-7), 7.77 (1H, s, H-17).

^{13}C NMR (100 MHz, $CDCl_3$): Table 2.

HRMS-ESI *m/z* $[M + H]^+$ calcd for $C_{25}H_{38}NO_6$: 448.2699; found: 448.2697.

Compound 6

$[\alpha]_D^{20}$: ─7.2 (*c* 0.50, $CHCl_3$).

IR (KBr): 2935, 2820, 1734, 1639, 1396, 1253, 1100 cm^{-1}.

^1H NMR (400 MHz, $CDCl_3$) δ: 2.00 (3H, s, OAc), 3.21, 3.28, 3.30, 3.34 (each 3H, s, OCH_3×4), 3.66, 3.75 (2H, ABq, *J* = 8.4 Hz, H-18), 4.20 (1H, d, *J* = 6.4 Hz, H-6), 4.65 (1H, t *J* = 4.0 Hz, H-14β), 5.70 (1H, d, *J* = 6.4 Hz, H-15), 7.39 (1H, s, H-19).

^{13}C NMR (100 MHz, $CDCl_3$): Table 2.

HRMS-ESI *m/z* $[M + H]^+$ calcd for $C_{25}H_{36}NO_6$: 446.2543; found: 446.2542.

Preparation of compound 8: To a solution of 3-acetyl-13-deoxyindaconitine (**7**) (665 mg, 1.0 mmol) in HOAc (35 mL) was added NBS (576 mg, 3.24 mmol), and the mixture was stirred at room temperature for 1 h prior to being poured into ice water (10 mL). The mixture was basified with conc. NH_4OH solution to pH 9 and extracted with dichloromethane (50 mL × 3). The extracts were dried (Na_2SO_4) and concentrated. CC (silica gel H, 10 g) of the residue, using light petroleum/acetone (2:1) as eluent, afforded compound **8** as a white amorphous power (360 mg, 60%).

Compound 8

$[\alpha]_D^{20}$: +12.8 (*c* 0.50, $CHCl_3$).

IR (KBr): 3450, 1719, 1637, 1399, 1249, 1103 cm^{-1}.

^1H NMR (400 MHz, $CDCl_3$) δ 1.38, 2.07 (each 3H, s, OAc), 3.22, 3.24, 3.26, 3.38 (each 3H, s, OCH_3×4), 4.18 (1H, d, *J* = 6.8 Hz, H-6), 7.43~8.05 (5H, OBz).

^{13}C NMR: Table 2.

HRMS-ESI: *m/z* $[M + H]^+$ calcd for $C_{34}H_{46}NO_{10}$: 628.3122; found: 628.3117.

Preparation of compound 9: Compound **8** (50 mg, 0.08 mmol), in a round bottomed flask (10 mL), was heated at 180-190°C under 15 mm Hg for 18 min. After cooling to room temperature, the residue was chromatographed over silica gel H eluting with cyclohexane/acetone (6:1) to give **9** (a white amorphous power, 26 mg, 66%).

Compound 9

$[\alpha]_D^{20}$: +48.0 (*c* 0.50, $CHCl_3$).

IR (KBr): 2934, 2889, 2821, 1718, 1645, 1278, 1102, 715 cm^{-1}.

^1H NMR (400 MHz, $CDCl_3$) δ: 2.08 (3H, s, OAc), 3.22, 3.23, 3.30 (each 3H, s, OCH_3×3), 3.80 (1H, d, *J*= 18.8 Hz, H-19), 3.03, 4.25 (2H, ABq, *J* = 8.4 Hz, H-18), 4.86 (1H, t, *J* = 7.6 Hz, H-6), 4.92 (1H, dd, *J* = 12.4, 5.2 Hz, H-3), 5.15 (1H, t, *J* = 4.0 Hz, H-14β), 5.24 (1H, d, *J* = 6.8 Hz, H-7), 5.95 (1H, t, *J* = 8.8 Hz, H-16), 6.38 (1H, d, *J* = 9.2 Hz, H-15), 7.78 (1H, s, H-17), 7.40~7.99 (5H, OBz);

^{13}C NMR: Table 2.

HRMS-ESI: *m/z* $[M + H]^+$ calcd for $C_{31}H_{38}NO_7$: 536.2648; found: 536.2649.

Preparation of compound 1: To a solution of diacetylindaconitine (**10**) (627 mg, 0.86 mmol) in HOAc (15 mL) was added NBS (528 mg, 2.98 mmol), and the mixture was allowed to stand at room temperature for 3 h prior to being poured into ice water (15 mL). The mixture was basified with conc. NH_4OH solution to pH 9 and extracted with dichloromethane (50 mL × 3). The extracts were dried (Na_2SO_4) and concentrated to give a crude product, which was subjected to CC (silica gel H, 10 g), using cyclohexane/acetone (10:1) as eluent, to afford **11** as a white amorphous power (360 mg, 60%).

Compound 11

$[\alpha]_D^{20}$: +4.8 (*c* 0.50, $CHCl_3$);

IR (KBr): 3447, 1715, 1637, 1252, 1102 cm^{-1};

^1H NMR (400 MHz, $CDCl_3$) δ: 1.27, 2.04, 2.06 (each 3H, s, OAc×3), 3.19, 3.23, 3.27, 3.39 (each 3H, s, OCH_3×4), 4.09 (1H, d, *J* = 6.0 Hz, H-6), 5.07 (1H, dd, *J* = 8.0, 6.0 Hz, H-3), 5.13 (1H, d, *J* = 4.4 Hz, H-14β), 7.44~8.09 (5H, OBz);

^{13}C NMR: Table 2;

HRMS-ESI: *m/z* $[M + H]^+$ calcd for $C_{36}H_{48}NO_{12}$: 686.3177; found: 686.3176.

Preparation of 13: Compound **11** (48 mg, 0.07 mmol), in a round bottomed flask (10 mL), was heated at 180-190°C under 15 mm Hg for 10 min. After cooling to room temperature, the residue was chromatographed over silica gel H eluting with cyclohexane/acetone (6:1) to give **13** (a white amorphous power, 26 mg, 56%).

Compound 13

$[\alpha]_D^{20}$: +36.4 (*c* 0.50, $CHCl_3$).

IR (KBr): 2931, 2821, 1735, 1640, 1245, 1098 cm^{-1}.

^1H NMR (400 MHz, $CDCl_3$) δ: 2.05, 2.06 (3H, s, OAc×2), 3.22, 3.28, 3.30, 3.46 (each 3H, s, OCH_3×4), 3.00, 4.01 (2H, ABq, *J* = 8.8 Hz, H-18), 4.29 (1H, t, *J* = 7.2 Hz, H-6), 4.89 (1H, dd, *J* = 12.8, 4.8 Hz, H-3), 5.33 (1H, d, *J* = 2.8 Hz, H-14β), 5.24 (1H, d, *J* = 6.8 Hz, H-7), 5.73 (1H, d, *J* = 6.8 Hz, H-15), 7.40~8.14 (5H, OBz).

^{13}C NMR: Table 2.

HRMS-ESI: *m/z* $[M + H]^+$ calcd for $C_{34}H_{44}NO_{10}$: 626.2965; found: 626.2969.

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Alkaloids from *Boophone haemanthoides* (Amaryllidaceae)

Jerald J. Nair[a], Lucie Rárová[b], Miroslav Strnad[b,c], Jaume Bastida[d] and Johannes van Staden[a*]

[a]*Research Centre for Plant Growth and Development*, *School of Life Sciences*, *University of KwaZulu-Natal Pietermaritzburg*, *Private Bag X01*, *Scottsville 3209*, *South Africa*

[b]*Centre of the Region Haná for Biotechnological and Agricultural Research*, *Palacký University*, *Šlechtitelů 11*, *78371 Olomouc*, *Czech Republic*

[c]*Laboratory of Growth Regulators*, *Palacký University & Institute of Experimental Botany AS CR*, *Šlechtitelů 11*, *CZ 78371 Olomouc*, *Czech Republic*

[d]*Departament de Productes Naturals*, *Facultat de Farmacia*, *Universitat de Barcelona*, *08028, Barcelona*, *Spain*

rcpgd@ukzn.ac.za

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In this study, the South African Amaryllid *Boophone haemanthoides* was examined for its phytochemical composition and cytotoxicity. In the process eight alkaloid structures, including the new compound distichaminol, were identified in bulb ethanolic extracts. Of the isolates, lycorine and distichamine exhibited strong activities against human acute lymphoblastic leukemia (CEM), breast adenocarcinoma (MCF7) and cervical adenocarcinoma (HeLa) cells with IC_{50}s ranging from 1.8 to 9.2 μM.

Keywords: Alkaloid, Amaryllidaceae, *Boophone haemanthoides*, Distichaminol.

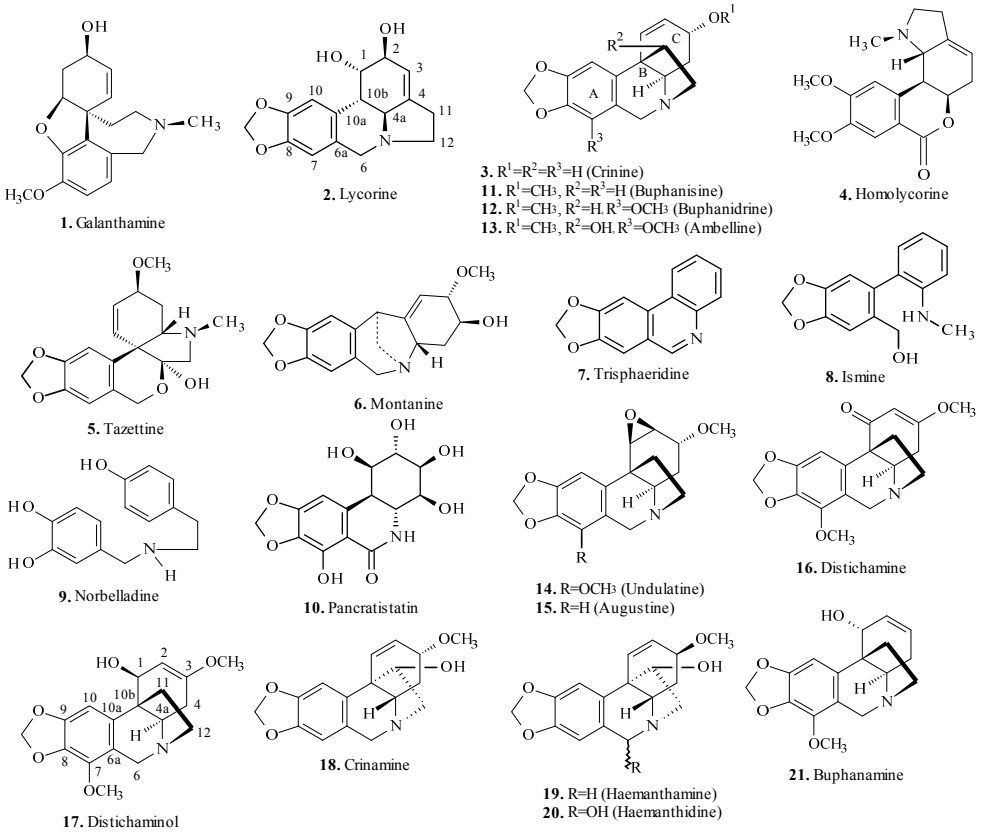
Southern Africa is a center of prominence for the family Amaryllidaceae, containing roughly a third of the global population of around 1000 species [1]. Its members are known for their horticultural and ornamental appeal with significant impact on the floriculture sector [1]. More importantly, its medicinal properties have long been realized in the traditional medicinal practices of indigenous peoples across the globe [2]. For example, the discovery of galanthamine (**1**) (scheme 1) as an Alzheimer's drug evolved out of the ethnic usage of *Galanthus* species, from which the compound was first isolated, in the Caucasus region of Eastern Europe [3]. In South Africa the Amaryllidaceae has a long history of use for medicinal purposes amongst its indigenous people which can be traced back to its original inhabitants, the San [2a,b].

The chemicals responsible for these medicinal attributes are alkaloids, structural variations of which are unique to the Amaryllidaceae [2]. Six different structural types are discernible for these compounds as represented by galanthamine (**1**), lycorine (**2**), crinine (**3**), homolycorine (**4**), tazettine (**5**) and montanine (**6**) [2]. Other less conspicuous members include degraded, oxidized and truncated variants, such as trisphaeridine (**7**) and ismine (**8**) [2]. All of these compounds are related, as a consequence of their biogenesis, from a common amino acid derived precursor, norbelladine (**9**) [2]. The pharmaceutical value of the Amaryllidaceae is epitomized by the FDA approved status granted to galanthamine (**1**) due to its potent and selective inhibitory interaction with the enzyme acetylcholinesterase (AChE), of significance in the progression of neurodegeneration associated with motor neuron diseases [3]. Following on the commercial success of galanthamine as an Alzheimer's drug, it is anticipated that further clinical candidates will emerge out of the phenanthridones {such as pancratistatin (**10**)} of the lycorine (**2**) series of alkaloids, which are known for their potent and selective antiproliferative properties [4]. Not surprisingly, therefore, AChE inhibition and cytotoxicity are two recurrent themes in the pharmacology of Amaryllidaceae alkaloids [3,4].

Lycorine (**2**) (scheme 1) is the most common alkaloid across the Amaryllidaceae and known for a broad spectrum of biological properties, including antiviral [5a], antifungal [5b], antiparasitic [5c] and anti-inflammatory [5d] activities, as well as antifeedant [5e], cell growth [5f] and AChE [5g] inhibitory effects. Furthermore, it has shown much promise as an antiproliferative agent capable of inhibiting cell division and cell elongation, as well as protein synthesis in eukaryotic cells [5h]. Its chemotherapeutic potential is best underlined by its antitumor effects in a number of cancer cell lines [6]. Furthermore, both *in vitro* and *in vivo* models of leukemia (HL-60) cells support the potency of lycorine as an anticancer agent [6d,e]. Notably, in some cell lines these effects were manifested via the apoptotic mechanistic pathway [6e-g]. In contrast, antiproliferative effects of lycorine were also exerted in apoptosis-resistant cell lines such as OE21 oesophageal cancer and SKMEL-28 melanoma cells [6h].

As mentioned above, the phenanthridone, pancratistatin (**10**), of the lycorine series has shown most promise for clinical development as an anticancer agent [7]. This potential is vindicated by the fact that pancratistatin selectively targeted mitochondria of cancer cells, with minimal effect on normal cells, initiating cell death via the apoptotic pathway, as indicated by early activation of caspase-3, followed by flipping of phosphatidyl serine [7e,f]. A stronger case could be made for commercial development of these targets based on their low interaction with the isoenzyme cytochrome P450 3A4, which is responsible for metabolism of the majority of drugs taken by humans [8]. By contrast, the crinane group, exemplified by crinine (**3**), is a broad and expanding group of compounds also known for diverse biological properties [9].

Given their close structural similarity to the lycorine series of compounds [2], cytotoxic effects have been demonstrated for several members of the crinane group [9b]. Over the past two decades there has been significant interest in diverse research aspects of the Amaryllidaceae, including areas pertaining to its



Scheme 1: Diverse alkaloid structures of the Amaryllidaceae, including major and minor group representatives **1-8**, the common biosynthetic precursor norbelladine **9** as well as the novel compound distichaminol **17** identified in the present study of *Boophone haemanthoides*.

ethnobotany, phytochemistry and pharmacology [2-9]. In addition, synthetic endeavors have targeted some of its bioactive constituents for structure-activity relationship (SAR) study purposes [4,10]. In continuation of these efforts, the South African Amaryllid *Boophone haemanthoides* was here investigated for its alkaloid composition and cytotoxic effects against a mini-panel of cancer cells. Seven alkaloids of the β-crinane series, including crinine (**3**), buphanisine (**11**), buphanidrine (**12**), ambelline (**13**), undulatine (**14**), distichamine (**16)** and distichaminol (**17)**, together with lycorine (**2**) (scheme 1) were isolated and identified by a combination of physical and spectroscopic methods. Cytotoxic effects were demonstrated for lycorine and distichamine in acute lymphoblastic leukemia (CEM), breast adenocarcinoma (MCF7) and cervical adenocarcinoma (HeLa) cells with IC_{50}s determined in the range 1.8 to 9.2 μM, while distichaminol (**17**) was uncovered as a novel alkaloid.

The African genus *Boophone* Herb. is comprised of two known species, *B. disticha* (L.f.) Herb. and *B. haemanthoides* F.M. Leight. *B. disticha* is widely distributed in Africa, ranging from Sudan in the north to the Western Cape Province in the south, while *B. haemanthoides* is a rare and threatened species with a restricted territory within the winter rainfall region of South Africa and parts of southern Namibia [11a]. Both plants are known to be widely used in traditional medicine [11b,c]. Isolation of the alkaloids from bulbs of *B. haemanthoides* is described in the Experimental section. Of the compounds isolated, lycorine (**2**), crinine (**3**), buphanisine (**11**), buphanidrine (**12**), ambelline (**13**) and undulatine (**14**) (scheme 1) are common across most genera of the Amaryllidaceae [2]. By contrast, distichamine (**16**) is unique in that its C-ring double bond is situated at C-2/C-3, as opposed to the usual C-1/C-2 positioning in most crinane compounds, with concomitant vinylization of the C-3 methoxyl group, as well as oxidation at C-1. Moreover, it has never been found outside the genus *Boophone* and as such occupies a privileged chemosystematic status within the Amaryllidaceae [11d]. A previous phytochemical study made on a wild population of *B. haemanthoides* from the Saldhana Bay area of South Africa revealed the presence of buphanidrine, buphanisine, crinine and distichamine [11d]. In the present study, bulbs of *B. haemanthoides* were collected in the Nieuwoudtville area of the Northern Cape Province of South Africa. The elucidation of the novel structure distichaminol (**17**) was based on its close structural similarity to distichamine (**16**) and buphanamine (**21**), previously described from *B. disticha* [12]. HRMS indicated a mass of 332.1491 g/mol for the $[M+1]^+$ ion of distichaminol, correct for the molecular formula $C_{18}H_{22}NO_5$ and theoretical mass 332.1498 g/mol. Furthermore, its EIMS had the molecular ion $[M]^+$ peak at *m/z* 331 with a relative abundance of 89%. Diagnostic fragment ions were detected at *m/z* 316 $[M\text{-}CH_3]^+$, 299 $[M\text{-}CH_3OH]^+$ and 281 $[M\text{-}CH_3OH\text{-}H_2O]^+$. FTIR indicated *inter alia* the presence of hydroxyl (3500-3000 cm^{-1}), aryl (1617 cm^{-1}), methoxyl (1039 cm^{-1}) and methylenedioxy (934 cm^{-1}) groups.

The ^1H and ^{13}CNMR spectra of distichaminol (**17**) were similar to those of distichamine (**16**) and only differences due to the C-1 hydroxyl group in **17** were significant (Table 1) [12b,c]. The lowfield region (δ 4.4-7.6) of the proton spectrum had four signals resonant for H-10 (δ 7.16, s), the methylenedioxy group (δ 5.86, s), H-2 (δ 4.91, dd, *J*=2.4, 1.0 Hz) and H-1 (δ 4.61, d, *J*=2.4 Hz), respectively. The corresponding carbon resonances were detected at δ 100.6 (d, C-10), 100.9 (t, O*C*H_2O), 96.8 (d, C-2) and 72.2 (d, C-1) respectively, as determined by HSQC experiments (Table 1). Two

Table 1: 1D and 2D NMR spectroscopic data for distichaminol (**17**).

Position	δ_H	δ_C	COSY	HMBC	NOESY
1	4.61 (d, 2.4)	72.2 (d)	H-2	C-2, C-3, C-4a, C-10a, C-10b, C-11	H-2, H-4a
2	4.91 (dd, 2.4, 1.0)	96.8 (d)	H-1, H-4α, H-4β	C-1, C-3, C-4, C-10b	H-1, 3-OCH_3
3	–	154.9 (s)	–	–	–
4α	2.05 (ddd, 17.6, 7.0, 1.0)	29.8 (t)	H-2, H-4β, H-4a	C-2, C-3, C-4a, C-10b	3-OCH_3, H-4β, H-4a
4β	2.31 (ddd, 17.6, 10.0, 1.0)	29.8 (t)	H-2, H-4α, H-4a	C-2, C-3, C-4a, C-10b	3-OCH_3, H-4α, H-12*exo*
4a	3.26 (dd, 10.0, 7.0)	64.6 (d)	H-4α, H-4β	C-1, C-3, C-4, C-10a, C-10b, C-11	H-1, H-4α, H-6α
6α	4.14 (d, 17.3)	57.8 (t)	H-6β	C-6a, C-7, C-10a, C-12	H-4a, H-6β, 7-OCH_3
6β	3.79 (d, 17.3)	57.8 (t)	H-6α	C-6a, C-7, C-10a, C-12	H-6α, 7-OCH_3, H-12*endo*
6a	–	117.0 (s)	–	–	–
7	–	140.3 (s)	–	–	–
8	–	140.5 (s)	–	–	–
9	–	148.7 (s)	–	–	–
10	7.16 (s)	100.6 (d)	–	C-6a, C-8, C-9, C-10a, C-10b	–
10a	–	134.0 (s)	–	–	–
10b	–	48.1 (s)	–	–	–
11*endo*	2.12 (ddd, 12.5, 9.8, 4.0)	36.3 (t)	H-11*exo*, H-12*endo*, H-12*exo*	C-1, C-4a, C-10a, C-10b, C-12	H-11*exo*, H-12*endo*, H-12*exo*
11*exo*	2.03 (ddd, 12.5, 10.8, 6.4)	36.3 (t)	H-11*endo*, H-12*endo*, H-12*exo*	C-1, C-4a, C-10a, C-10b, C-12	H-11*endo*, H-12*endo*, H-12*exo*
12*endo*	2.76 (ddd, 12.9, 9.0, 5.6)	51.3 (t)	H-11*endo*, H-11*exo*, H-12*exo*	C-4a, C-6, C-10b, C-11	H-6β, H-11*endo*, H-11*exo*, H-12*exo*
12*exo*	3.45 (ddd, 12.9, 10.0, 3.8)	51.3 (t)	H-11*endo*, H-11*exo*, H-12*endo*	C-4a, C-6, C-10b, C-11	H-4β, H-11*endo*, H-11*exo*, H-12*endo*
3-OCH_3	3.57 (s)	54.7 (q)	–	C-3	H-2, H-4α, H-4β
7-OCH_3	3.97 (s)	59.5 (q)	–	C-7	H-6α, H-6β
OCH_2O	5.86 (s)	100.9 (t)	–	C-8, C-9	–

and three bond HMBC correlations linked H-10 to C-6a, C-8, C-9, C-10a and C-10b. The C-1 carbon resonance, indicative of a hydroxy-substituted allylic methine carbon in **17**, is distinguished from that of distichamine (**16**) (δ 201.9, s) in which C-1 possesses an α,β-unsaturated ketone [12c]. Mutual COSY contours between H-1 and H-2 established their vicinal relationship. Furthermore, the small value of the coupling constant between H-1 and H-2 (*J*=2.4 Hz) indicated a dihedral angle approaching 90° for these protons, thus favouring the β-disposition for the C-1 hydroxyl group. By contrast, a *J* value of 5.6 Hz between H-1 and H-2 reflects α-orientation for the 1-hydroxy group as shown for buphanamine (**21**) [12b]. In addition, allylic coupling was established via COSY between H-2 and the methylene protons H-4α and H-4β by the small value of the coupling between them (*J*=1.0 Hz).

The oxygen and nitrogen related proton signals were resonant in the δ 2.6-4.2 region, of which the diastereotropic C-6 protons were immediately apparent based on their chemical shift, multiplicity and coupling constants. As such, H-6α (δ 4.14, d, *J*=17.3 Hz) was shifted further downfield than H-6β (δ 3.79, d, *J*=17.3 Hz) due to the former being *cis*-disposed to the nitrogen atom (Table 1). This was also true for H-12*exo* (δ 3.45, ddd, *J*=12.9, 10.0, 3.8 Hz) resonating further downfield to H-12*endo* (δ 2.76, ddd, *J*=12.9, 9.0, 5.6 Hz). HSQC analysis showed these protons to be attached to methylene carbons which resonated at δ 57.8 (t, C-6) and 51.3 (t, C-12), respectively. The remaining three-proton singlet resonance signals in this region were ascribable to the vinyl C-3 (δ 3.57) and aryl C-7 (δ 3.97) methoxy groups, while the nitrogen related methine proton H-4a was resonant at δ 3.26 (dd, *J*=10.0, 7.0 Hz). As expected, the methylene protons at C-4 and C-11 were the most shielded. An *anti*-periplanar relationship between H-4a and H-4β was established by the large value of the coupling between them (*J*=10.0 Hz). All eighteen carbons of distichaminol (**17**) were accounted for by ^{13}C and HSQC NMR and multiplicities ascertained by DEPT analysis (Table 1). Furthermore, absolute configuration of the 5,10b-ethano-bridge was arrived at by circular dichroism (CD) measurements which showed that distichaminol was qualitatively similar to β-5,10b-ethanophenanthridine compounds with a maximum at 250 nm and a minimum at 286 nm in its CD curve [13].

A mini-panel of cancer cells comprising CEM, MCF7 and HeLa cells, as well as the normal human fibroblast (BJ) cell line was engaged for the cytotoxicity screen using staurosporine and galanthamine as positive and negative controls, respectively. As determined by the Calcein AM assay [14], lycorine (**2**) decreased the survival of all three cancer cell lines in a dose-dependent manner after 72 h treatments, with IC_{50} values of 1.8, 9.2 and 8.9 μM, respectively (Table 2). This result is not surprising given that the cytotoxic properties of lycorine have been widely studied [6]. Similarly, distichamine (**16**) was noticeably active, exhibiting IC_{50}s of 5.1, 2.3 and 4.0 μM against the three cancer cells respectively, in accordance with a previous report [14]. However, both lycorine and distichamine were not selective in their activity since normal BJ cells were also affected to a significant extent by the treatment (IC_{50} 2.0 and 12.4 μM, respectively), in agreement with earlier findings [6,14]. Furthermore, the β-crinane compounds crinine (**3**), buphanisine (**11**), buphanidrine (**12**) and ambelline (**13**), all of which possess a C-1/C-2 double bond, as well as polar substitution at C-3α, were inactive against both normal and cancerous cells (IC_{50}s > 50 μM). This was also the case for the 1,2-β-epoxide undulatine (**14**), as well as the new compound distichaminol (**17**) (IC_{50}s > 50 μM against both normal and cancer cell types).

Since the identification of haemanthamine (**19**) as a cytotoxic agent over thirty years ago [5h], alkaloids of the crinane series of the Amaryllidaceae have been the subject of several cytotoxicity based studies. A recent review of the field has revealed that since 1976, seventy-one such compounds have been screened against fifty-four different cancer cell lines [9b]. The review also showed that crinamine (**18**), haemanthamine (**19**) and haemanthidine (**20**) are the most common targets in such studies of α-crinanes [9b]. Within the β-series, buphanisine (**11**) and ambelline (**13**) appear routinely across most of the cell lines screened [9b]. The low activities observed here for the β-crinane representatives crinine (**3**), buphanisine (**11**), buphanidrine (**12**), ambelline (**13**) and undulatine (**14**) are in line with previous studies made on various cell lines [9b]. With a few exceptions, α-crinanes in general superseded β-crinanes in terms of potency [9b]. For example, augustine (**15**) was the most active of the β-series with an ED_{50} of 0.6 μg/mL in both KB (human oral epidermoid carcinoma) and U373 (human glioblastoma astrocytoma) cells [9b].

In relation to the mechanistic basis of these alkaloids, the apoptotic mode of death in cancers has been demonstrated for crinine (**3**), distichamine (**16**), crinamine (**18**) and haemanthamine (**19**) [6h,14,15]. For example, McNulty *et al.* showed that up to 90% of rat hepatoma (5123 tc) cells exhibited apoptotic morphology after a 48 h treatment with the α-crinanes crinamine and haemanthamine,

Table 2: IC_{50} (μM) values obtained from Calcein AM assays using the specified cancerous and normal cell lines.

Sample [a]	Cell line, IC_{50} (μM) [b,c]			
	CEM	MCF7	HeLa	BJ
Galanthamine (**1**) [d]	> 50	> 50	> 50	> 50
Lycorine (**2**)	1.8 ± 0.1	9.2 ± 2.1	8.9 ± 2.6	2.0 ± 0.4
Crinine (**3**)	> 50	> 50	> 50	> 50
Buphanisine (**11**)	> 50	> 50	> 50	> 50
Buphanidrine (**12**)	> 50	> 50	> 50	> 50
Ambelline (**13**)	> 50	> 50	> 50	> 50
Undulatine (**14**)	> 50	> 50	> 50	> 50
Distichamine (**16**)	5.1 ± 0.6	2.3 ± 0.8	4.0 ± 0.4	12.4 ± 2.2
Distichaminol (**17**)	> 50	> 50	> 50	> 50
Staurosporine [d]	0.023 ± 0.002	0.064 ± 0.002	0.175 ± 0.007	0.002 ± 0.000

[a] All compounds were single entities as determined by TLC, HPLC and NMR analysis. [b] Cells were treated for 72 h with serial concentrations of samples. [c] Values are means of at least three independent experiments performed in triplicate, with standard deviation as indicated. [d] Staurosporine and galanthamine used as positive and negative controls, respectively.

with ED_{50}s determined at 12.5 and 15 μM, respectively [15a]. Interestingly, this activity was seen to be selective as normal human embryonic kidney (293t) cells remained unaffected by the treatment [15a]. Furthermore, haemanthamine (**19**) was shown to be active against apoptosis-sensitive human oligodendroglioma (Hs683) and mouse melanoma (B16F10) cancer cells with IC_{50}s of 7.0 and 6.8 μM, respectively [6h]. In addition, Berkov *et al.* reported that crinine (**3**) was active against a mini-panel of cancers comprising SKW-3, HL-60, HI-60/Dox and MDA-MB-231 cells with IC_{50}s ranging from 14.01 to 68.11 μM, with the best activity seen for SKW-3 cells [15d]. Further mechanistic tests via oligonucleosomal DNA fragmentation revealed the apoptotic cell death mode as responsible for its cytotoxicity [15d]. The antiproliferative activity of the rare Amaryllidaceae constituent distichamine (**16**), capable of inducing caspase-3 activated apoptosis in CEM cells, has recently been described [14].

Essential features of the crinane alkaloid cytotoxic pharmacophore which have come to the fore from these studies may be summarized as follows: i) the modulatory effect of the C-ring double bond (as seen in crinamine and crinine compared with their respective dihydro-analogues [15], as well as in distichamine in which the double bond features at C-2/C-3) [14]; ii) the effect of polar substitution at C-3, indicating that a H-bond acceptor is required at this position (as shown for crinamine, haemanthamine and distichamine, in possession of α-, β- and planar methoxy groups at C-3, respectively) [14,15]; iii) presence of a small substituent at C-11 (H or OH), the function of which does not appear to involve hydrogen bonding but is subject to steric constraints alone (evident for crinine, crinamine, haemanthamine and distichamine) [14,15]; and iv) oxidation at C-1 appears to be pivotal to activity {as in distichamine (**16**)} [14]. Furthermore, reversal of the polarity of the C-1 center in accommodating a hydroxyl group (as in distichaminol **17**) is here shown to be detrimental.

In summary, phytochemical investigation of the rare and threatened southern African Amaryllid *Boophone haemanthoides* has led to the identification of lycorine together with seven members of the crinane series, of which distichaminol was uncovered as a novel compound. This compound is unique within the crinane series of Amaryllidaceae alkaloids in that its C-ring double bond is situated in the unusual C-2/C-3 position (as opposed to the usual C-1/C-2 position), thus accommodating the C-1 hydroxyl as well as the C-3 vinylic methoxyl groups. It is only the second member of the crinane alkaloids identified with such a substitution, the other being distichamine. Distichaminol was shown to be non-toxic to both cancer and normal cells, which contributes further to the understanding of the pharmacophoric requirements for cytotoxic activity as well as apoptosis induction within the crinane series of Amaryllidaceae alkaloids.

Experimental

General: Melting points (uncorrected) were measured on a Gallenkamp melting point apparatus. Optical rotations were determined on a Perkin-Elmer 241 polarimeter installed with a λ_{589} sodium lamp. Circular dichroism (CD) spectra were obtained with a Jasco J-700 spectropolarimeter. IR spectra were measured on a Bio-Rad FTS-40 series spectrometer in dry film. EIMS were run on a Micromass Quattro Ultima spectrometer fitted with a direct injection probe (DIP) with ionization energy set at 70 eV and HRMS (ES) were performed with a Micromass Q-Tof Ultima spectrometer. ^1H, ^{13}C NMR, DEPT, COSY, NOESY, HSQC and HMBC spectra were recorded on a Bruker AV400 in $CDCl_3$ or DMSO-d_6, chemical shifts are reported in units of δ (ppm) and coupling constants (*J*) are expressed in Hz. Silica gel Merck KGaA (70-230 mesh) was used for CC and TLC silica gel 60 F_{254} for analyt. and prep. TLC (both Merck KGaA). Gel filtration was carried out on Sephadex LH-20 (Pharmacia). Spots on chromatograms were detected under UV light (254 and 365 nm) and by Dragendorff's reagent stain.

Plant material: Bulbs of *Boophone haemanthoides* were collected during the flowering season in September 2011 in the Nieuwoudtville area of the Northern Cape Province of South Africa. A voucher specimen (Snijman 588 NBG) was authenticated by Dr John C. Manning and deposited at the South African National Botanical Institute (SANBI).

Extraction and isolation of alkaloids: Powdered dried bulbs (784 g) of *Boophone haemanthoides* were extracted by stirring with EtOH for 48 h after which the solvent was evaporated under reduced pressure. The residue (61 g) was dissolved in H_2O (200 mL) and acidified to pH 4 with glacial acetic acid. After removing neutral material with Et_2O, the acidic solution was extracted with 3 x 200 mL $CHCl_3$ to provide extract A (6.1 g). Basifying the solution to pH 9 with aq. ammonia and extracting again with $CHCl_3$ (3 x 200 mL) afforded extract B (4.8 g). Finally, a $CHCl_3$-MeOH (3:2) extraction of the basic solution gave extract C (2.4 g). Extracts A, B and C were combined (13.3 g) after their TLC profiles (run in a 1:1 mixture of EtOAc-MeOH) were seen to be similar and subjected to gravity CC on silica gel by gradient elution with EtOAc and EtOAc-MeOH mixtures at a collection volume of 100 mL per flask. In this manner, 6 pooled fractions were generated after sequential TLC analysis giving FrI (0.71 g, flasks 24-26), FrII (0.81 g, flasks 27-28), FrIII (1.68 g, flasks 29-33), FrIV (2.06 g, flasks 34-41), FrV (0.56 g, flasks 42-50), FrVI (0.67 g, flasks 51-70). Further purification of fractions I-VI via a combination of Sephadex LH-20 CC (MeOH) and PTLC (MeOH-EtOAc/1:1) led to the consecutive isolation of distichamine [6.5 mg, R_f 0.54 (MeOH-EtOAc/1:1)], lycorine (**2**) [403.7 mg, R_f 0.45 (MeOH-EtOAc/1:1)], undulatine (**14**) [100.1 mg, R_f 0.44 (MeOH-EtOAc/1:1)], buphanidrine (**12**)

[222.7 mg, R_f 0.39 (MeOH-EtOAc/1:1)], ambelline (**13**) [103.2 mg, R_f 0.38 (MeOH-EtOAc/1:1)], buphanisine (**11**) [82.6 mg, R_f 0.36 (MeOH-EtOAc/1:1)], crinine (**3**) [4.3 mg, R_f 0.33 (MeOH-EtOAc/1:1)] and distichaminol (**17**) [7.8 mg, R_f 0.30 (MeOH-EtOAc/1:1)]. The physical and spectroscopic data for the known compounds closely matched those that have been published [12,16].

Distichaminol (**17**)

MP: 76-78°C.

$[\alpha]_D^{25}$: -43.5 (*c* 0.2 in $CHCl_3$).

CD$[\theta]_\lambda^{25}$: $[\theta]_{250}$ +2345, $[\theta]_{286}$ -1787 (*c* 0.2 in $CHCl_3$).

IR ν_{max}/cm^{-1} (dry film): 3500-3000 (OH), 2926, 1674, 1617 (Ar), 1469, 1376, 1315, 1270, 1211, 1127, 1039 (OCH_3), 934 (OCH_2O).

^{1}HNMR (400 MHz, $CDCl_3$): Table 1.

^{13}CNMR (100 MHz, $CDCl_3$): Table 1.

HRMS (Tof MS ES^+): calcd. 332.1498 for $C_{18}H_{22}NO_5$, found 332.1491 g/mol.

LRMS (EI) 70 eV, *m/z* (rel. int.): 331 $[M]^+$ (89), 316 $[M\text{-}CH_3]^+$ (28), 299 $[M\text{-}CH_3OH]^+$ (60), 281 $[M\text{-}CH_3OH\text{-}H_2O]^+$ (41), 270 (21), 253 (30), 242 (31), 231 (25), 218 (14), 207 (100), 191 (16), 179 (7), 165 (19), 135 (19), 115 (34).

Cytotoxicity bioassays

Cell cultures: Stock solutions (10 mmol/L) of test compounds were prepared in dimethylsulfoxide (DMSO). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), L-glutamine, penicillin and streptomycin were purchased from Sigma (MO, USA). Calcein AM was obtained from Molecular Probes (Invitrogen Corporation, CA, USA). Test cell lines comprising T-lymphoblastic leukemia CEM, breast carcinoma MCF7, cervical carcinoma HeLa and human fibroblasts BJ were obtained from the American Type Culture Collection (Manassas, VA, USA). All cell lines were cultured in DMEM medium (Sigma, MO, USA), supplemented with 10% heat-inactivated fetal bovine serum, 2 mmol/L L-glutamine, 10,000U penicillin and 10 mg/mL streptomycin. Cell lines were maintained under standard cell culture conditions (37ºC and 5% CO_2 in a humid environment), and were sub-cultured twice or thrice weekly as required according to standard trypsinization procedures [14].

Calcein AM assay: Cell line suspensions containing about 1.0×10^5 cells/mL were placed in 96-well microtiter plates and after 24 h stabilization (time zero), test compounds, which were serially diluted in DMSO, were added (4 x 20 µL aliquots). Control cultures were treated with DMSO alone, such that the final DMSO concentration in the incubation mixtures never exceeded 0.6%. Test compounds were typically evaluated at six 3-fold dilutions and the highest final concentration was generally 50 µM. After 72 h incubation, 100 µL of 2 µM Calcein AM solution (Molecular Probes, Invitrogen, CA, USA) was added and incubation was continued for a further hour. The fluorescence of viable cells was then quantified using a Fluoroskan Ascent instrument (Labsystems, Finland). The percentage of surviving cells in each well was calculated by dividing the intensity of the fluorescence signals from the exposed wells by the intensity of signals from control wells and multiplying by 100. These ratios were then used to construct dose-response curves from which IC_{50} values were calculated. Staurosporine (Sigma-Aldrich, UK) and galanthamine (Teva Czech Industries, Opava, Czech Republic) were used as positive and negative controls, respectively.

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Supinidine Viridiflorates from the Roots of *Chromolaena pulchella*

Mario A. Gómez-Hurtado[a,b], J. Martín Torres-Valencia[a,*], Rosa E. del Río[b], Gabriela Rodríguez-García[b], Virginia Motilva[c], Sofía García-Mauriño[d], Carlos M. Cerda-García-Rojas[e] and Pedro Joseph-Nathan[e]

[a]*Área Académica de Química, Universidad Autónoma del Estado de Hidalgo, Km 4.5 Carretera Pachuca-Tulancingo, Mineral de la Reforma, Hidalgo 42184, Mexico*

[b]*Instituto de Investigaciones Químico-Biológicas, Universidad Michoacana de San Nicolás de Hidalgo, Apartado 137, Morelia, Michoacán 58000, Mexico*

[c]*Facultad de Farmacia, Universidad de Sevilla, Profesor García González No. 2, Sevilla 41012, Spain*

[d]*Facultad de Biología, Universidad de Sevilla, Profesor García González No. 2, Sevilla 41012, Spain*

[e]*Departamento de Química, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, Apartado 14-740, México, D. F., 07000 Mexico*

jmartin@uaeh.edu.mx

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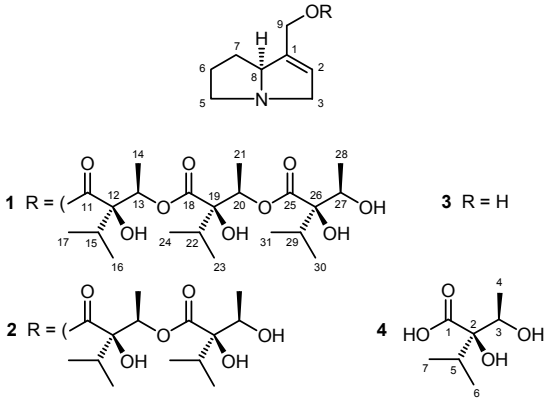
The alkaloid extract from the roots of *Chromolaena pulchella* provided two new pyrrolizidine alkaloids, elucidated as (–)-supinidine triviridiflorate (**1**) and (–)-supinidine diviridiflorate (**2**) based on their physical and spectroscopic properties. Their absolute configuration was determined by chemical correlation with (–)-supinidine (**3**) and (+)-viridifloric acid (**4**).

Keywords: *Chromolaena pulchella*, Asteraceae, Pyrrolizidine alkaloids, Absolute configuration, NMR spectroscopy.

Pyrrolizidine alkaloids (PAs), although widely distributed, are characteristic of certain genera of the Boraginaceae, Leguminosae, and Asteraceae/Compositae families [1]. Many PAs are known to produce hepatic toxicity and there are several records of livestock poisoning [1a,b]. Chemical studies of some *Chromolaena* species (Asteraceae) showed the presence of this class of natural molecules. Thus *N*-oxides of 7-angeloylretronecine, intermidine, licopsamine, echinatine, 3´-acetylrinderine, and supinine have been identified in *C. odorata* [1c]. The present work describes the isolation of the new PAs supinidine triviridiflorate (**1**) and supinidine diviridiflorate (**2**) (Figure 1) from the crude alkaloid extract of the roots of *C. pulchella*, for which we recently reported the isolation of labdanes and *ent*-clerodanes from the aerial parts [1d].



Compound **1**, a pale yellow oil, showed a $[M + 1]^+$ ion at *m/z* 572.3427 in its HRESI/APCI mass spectrum revealing the molecular formula $C_{29}H_{49}NO_{10}$ + H (calcd *m/z* 572.3435). The ^{1}H NMR chemical shift values of the alkaloid moiety were in agreement with those for a supinidine type pyrrolizidine ring system [2]. The complete ^{1}H and ^{13}C data, shown in Table 1, gave characteristic signals for a 1,2-unsaturated pyrrolizidine alkaloid with a necic acid esterified at C-9. The ^{1}H NMR spectrum of **1** showed signals assignable to a vinylic proton at δ_H 5.71 (H-2) and an AB system (J = 13.6 Hz) at δ_H 4.72 and 4.60 due to protons of the C-9 hydroxymethylene group, whereas the signal for the hydrogen atom attached to bridgehead C-8 was observed at δ_H 4.15. The signals for the methylene groups at C-3 and C-5, bearing the nitrogen atom, were observed at δ_H 3.91 and 3.37, and at 3.11 and 2.53, respectively. Moreover, signals for a trimeric α-isopropyl-α,β-dihydroxybutyric acid residue were observed as quartets at δ_H 5.35 (J = 6.2 Hz, H-13), 4.98 (J = 6.6 Hz, H-20), and 4.09 (J = 6.6 Hz, H-27) showing strong correlation in the COSY spectrum with the doublets at δ_H 1.39 (Me-14), 1.22 (Me-21), and 1.21 (Me-28), respectively. The ^{13}C and APT NMR spectra showed signals for three carbonyl carbon atoms at δ_C 174.0 (C-18), 173.6 (C-25), and 173.3 (C-11), for two vinylic carbons at 136.9 (C-1) and 126.1 (C-2), for three quaternary carbons bearing oxygen atoms at 82.6 (C-26), 81.7 (C-12), and 80.3 (C-19), for seven methine carbons, three of them bearing oxygen atoms at 76.4 (C-13), 71.5 (C-20) and 69.4 (C-27), one bearing the nitrogen atom at 71.5 (C-8), and three owing to the isopropyl groups at 33.8 (C-15), 32.7 (C-29) and 30.9 (C-22), for five methylene carbons, one of them bearing an oxygen atom at 61.9 (C-9), two bearing the nitrogen atom at 61.6 (C-3) and 56.6 (C-5), and two at 30.2 (C-7) and 25.7 (C-6), and for six methyl groups owing to three isopropyl groups at 17.3, 17.0, 16.9, 16.8, 16.6 and 15.3. Esterification at the C-9 position was confirmed by the HMBC correlation between H_2-9 and the carbonyl group C-11, while the individual assignments for the three acid residues were supported from HMBC correlations of H-13 with C-11 and C-12, of H-20 with C-18 and C-19, and of H-27 with C-25 and C-26.

Compound **2** exhibited in its HRESI/APCI mass spectrum a $[M + 1]^+$ ion at *m/z* 428.2646 in agreement with the molecular formula $C_{22}H_{37}NO_7$ + H (calcd *m/z* 428.2648). The complete ^{1}H and ^{13}C data shown in Table 1 demonstrated high structural similarities

Table 1: ^{13}C and ^{1}H NMR data for **1** and **2** (100 and 400 MHz, $CDCl_3$).[a]

	1		**2**	
Position	δ_C, mult.	δ_H, mult. (*J* in Hz)	δ_C, mult.	δ_H, mult. (*J* in Hz)
1	136.9, C		136.9, C	
2	126.1, CH	5.71, br s	125.7, CH	5.73, br s
3	61.6, CH_2	3.91, br d (15.8)	61.2, CH_2	3.95, br d (15.8)
3'		3.37, dd (15.8, 4.4)		3.40, br d (15.8)
5	56.6, CH_2	3.11, dt (9.9, 5.5)	56.6, CH_2	3.18, dt (10.0, 5.1)
5'		2.53, dt (9.9, 7.0)		2.54, dt (10.0, 7.0
6	25.7, CH_2	1.80, m	25.7, CH_2	1.81, m
7	30.2, CH_2	1.98, m	29.9, CH_2	2.04, m
7'		1.50, m		1.54, m
8	71.5, CH	4.15, br m	71.5, CH	4.25, br m
9	61.9, CH_2	4.72, br d (13.6)	62.2, CH_2	4.80, br d (13.5)
9'		4.60, br d (13.6)		4.74, br d (13.5)
11	173.3, C		174.0, C	
12	81.7, C		81.2, C	
13	76.4, CH	5.35, q (6.2)	73.9, CH	5.26, q (6.2)
14	13.1, CH_3	1.39, d (6.2)	13.9, CH_3	1.32, d (6.2)
15	33.8, CH	1.98, sept (7.0)	32.6, CH	2.09, sept (7.0)
16	17.0, CH_3	1.05, d (7.0)	16.4, CH_3	0.94, d (7.0)
17	17.3, CH_3	0.96, d (7.0)	17.1, CH_3	0.96, d (7.0)
18	174.0, C		174.3, C	
19	80.3, C		82.4, C	
20	71.5, CH	4.98, q (6.6)	69.2, CH	3.95, q (6.6)
21	14.6, CH_3	1.22, d (6.6)	17.5, CH_3	1.17, d (6.6)
22	30.9, CH	2.09, sept (7.0)	32.3, CH	2.08, sept (7.0)
23	15.3, CH_3	0.86, d (7.0)	16.4, CH_3	0.92, d (7.0)
24	16.9, CH_3	0.74, d (7.0)	16.9, CH_3	0.87, d (7.0)
25	173.6, C			
26	82.6, C			
27	69.4, CH	4.09, q, (6.6)		
28	17.2, CH_3	1.21, d (6.6)		
29	32.7, CH	2.09, sept (7.0)		
30	16.8, CH_3	0.94, d (7.0)		
31	16.6, CH_3	1.00, d (7.0)		

[a]Assigned by gHMQC and gHMBC.

with the aforementioned PA **1**, implying that compounds **1** and **2** belong to the same class of alkaloids. Compound **2** differed from **1** in the absence of the third *α*-isopropyl-*α*,*β*-dihydroxybutyric acid residue, since in the ^{1}H NMR spectrum only two methine signals for protons bearing oxygen atoms were observed at δ_H 5.26 (q, *J* = 6.2 Hz, H-13), and 3.95 (q, *J* = 6.6 Hz, H-20), whereas in the ^{13}C NMR spectrum only two carbonyl groups signals were observed at δ_C 174.0 (C-11), and 174.3 (C-18). As in the case of compound **1**, 2D NMR spectroscopy was employed to completely assign the ^{13}C and ^{1}H spectra. Alkaline hydrolysis of a mixture of compounds **1** and **2** gave the necine base (–)-supinidine (**3**) [2,3a] and the necic acid residue (+)-viridifloric acid (**4**) [3b].

Experimental

General: Optical rotation, Perkin-Elmer 341 polarimeter; IR, Perkin-Elmer 16F PC IR-FT spectrophotometer using thin films of compounds deposited on a CsI crystal; Low-resolution MS, either Agilent 1100 LC/MSD or Varian Saturn 2000 spectrometers; HRMS, Agilent LCTOF instrument; NMR, JEOL Eclipse 400 spectrometer; CC, Merck silica gel 40; TLC, silica gel 60 precoated glass plates.

Plant material: Specimens of *C. pulchella* (H.B.K.) R.M. King & H. Rob. (Asteraceae) were collected near km 61 of Morelia-Zacapu federal road 15, in the municipality of Constitución, State of Michoacán, México, during October 2005. A specimen (No. 192522) is deposited at the Herbarium of Instituto de Ecología A. C., Centro Regional del Bajío, Pátzcuaro, Michoacán, Mexico, where Prof. Jerzy Rzedowski kindly identified the plant material.

Extraction and isolation: Air-dried and powdered roots of *C. pulchella* (870 g) were extracted with MeOH (3.5 L) under reflux for 6 h. Filtration and evaporation of the extract afforded a yellow viscous oil (38.6 g) which gave a positive Dragendorff test. To this product aq. HCl 2% (300 mL) and zinc powder (40 g) were gradually added under stirring for 12 h. The acidic aqueous solution was treated with aq. KOH 5% to obtain a phase with pH 10 and then extracted with $CHCl_3$ (3 × 200 mL), dried over anhydrous Na_2SO_4, filtered, and evaporated to provide 2.6 g of residue. A portion of this (876 mg) was subjected to chromatography on silica gel 60 (20 g) using $CHCl_3$–MeOH–diethylamine (98:2:1, 96:4:1, 90:10:1, and 80:20:1). Fractions of 100 mL of each polarity were collected, monitored by TLC, and analyzed by ^{1}H NMR spectroscopy. The resulting material from each fraction was labelled as A (138 mg), B (354 mg), C (112 mg) and D (116 mg). From fractions A, C, and D fatty materials were isolated. Separation of fraction B (178 mg) by means of preparative TLC using $CHCl_3$–MeOH (9:1) as the mobile phase gave pure **1** (45 mg, R_f 0.5). Another portion of fraction B (138 mg) was purified by TLC ($CHCl_3$–MeOH–H_2O, 200:50:7) affording **2** (20 mg, R_f 0.2).

Supinidine triviridiflorate (1)

Pale yellow oil; $[\alpha]_D^{20}$: –1.5 (*c* 3.5, $CHCl_3$)
IR (film): 3518, 2972, 2937, 2878, 1725, 1454, 1386 cm^{-1}.
^{1}H and ^{13}C NMR: Table 1.
MS (EI, 70 eV): *m/z* (%) 572 $[M + 1]^+$ (40), 428 (2), 410 (4), 284 (3), 266 (19), 140 (19), 122 (100), 110 (10), 107 (6), 94 (25), 70 (38); HRESI/APCIMS: *m/z* $[M + 1]^+$ calcd for $C_{29}H_{49}NO_{10}$ + H: 572.3435; found: 572.3427.

Supinidine diviridiflorate (2)

Pale yellow oil; $[\alpha]_D^{20}$: –8.2 (*c* 2.0, $CHCl_3$).
IR (film): 3024, 2970, 2945, 1727, 1456, 1389 cm^{-1}.
^{1}H and ^{13}C NMR: Table 1.
MS (EI, 70 eV): *m/z* (%) = 428 $[M + 1]^+$ (2), 396 (2), 382 (2), 284 (28), 224 (6), 140 (19), 122 (100); HRESI/APCIMS: *m/z* $[M + 1]^+$ calcd for $C_{22}H_{37}NO_7$ + H: 428.2648; found: 428.2646.

Hydrolysis of compounds 1 and 2: A mixture of **1** and **2** (40 mg) in MeOH (3 mL) was treated with NaOH (24 mg) in H_2O (0.25 mL) and heated to reflux for 15 min in a micro-wave system working at 100 W. The mixture was treated with H_2O (10 mL) and extracted with CH_2Cl_2 (2 × 10 mL) to give (–)-supinidine (**3**) (3.5 mg) as a yellow oil, which showed $[\alpha]_D$ –9.1 (*c* 0.16, EtOH) lit. $[\alpha]_D$ –10.4 (*c* 2.64, EtOH) [3a]. The aq. phase was acidified with 2% HCl and extracted with CH_2Cl_2 (2 × 10 mL) to give (+)-viridifloric acid (**4**) (24 mg) [3b,4].

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N-Containing Metabolites from the Marine Sponge *Agelas clathrodes*

Fan Yang[a], Rui-Hua Ji[b], Jiang Li[c], Jian-Hong Gan[d] and Hou-Wen Lin[a,*]

[a]*Department of Pharmacy*, *Renji Hospital*, *Shanghai Jiao Tong University School of Medicine*, *160 Pujian Road*, *Shanghai 200127*, *P.R. China*

[b]*Department of Anesthesiology*, *Changzheng Hospital*, *Second Military Medical University*, *415 Fengyang Road*, *Shanghai 200003*, *P.R. China*

[c]*Eastern Hepatobiliary Surgical Hospital*, *Second Military Medical University*, *225 Changhai Road*, *Shanghai 200438*, *P.R. China*

[d]*College of Food Science & Technology*, *Shanghai Ocean University*, *999 Hucheng Huan Road*, *Shanghai 201306*, *P.R.China*

Fan Yang and Rui-Hua Ji contributed equally to this work.

franklin67@126.com

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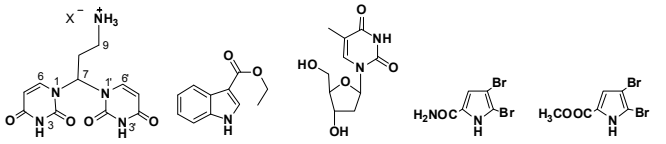
A new bisuracil analogue, 3,3-bis(uracil-1-yl)-propan-1-aminium (**1**), together with four known *N*-containing metabolites (**2–5**), were isolated from the South China Sea sponge *Agelas clathrodes*. Their chemical structures were established on the basis of spectroscopic and spectrometric analysis and comparison with known compounds. Compound **1** is an unusual naturally occurring bisuracil analogue, and compound **2** was isolated from a natural source for the first time. Compounds **2** and **4** exhibit moderate cytotoxicity against cancer cell line SGC7901.

Keywords: *Agelas clathrodes*, Bisuracilyl-substituted, *N*-containing, Marine sponge.

Marine sponges of the genus *Agelas* (order Agelasida, family Agelasidae) have provided a wide variety of natural products, such as glycosphingolipids [1a,b], bromopyrrole alkaloids [1c,d] and 9-*N*-methyladeninium diterpenoids [2a-c]. *A. clathrodes* (Schmidt, 1870) was reported to possess the *N*-containing metabolites, clathrodin [3a], clathramides [3b], (-)-agelasidine A [3c], and dispacamides [3d,e]. As part of an ongoing investigation, studies on *A. clathrodes* led to the isolation of a new bisuracilyl-substituted analogue, 3,3-bis(uracil-1-yl)-propan-1-aminium (**1**), along with four known compounds (**2-5**) (Figure 1).

Compound **1**, an amorphous solid, was deduced as $C_{11}H_{14}N_5O_4$ by HR-APCI-MS (*m/z* 280.1213 $[M]^+$; calcd. 280.1202). The ^{1}H NMR spectrum displayed signals at δ_H 5.44 (2H, d, J = 7.5 Hz) and δ_H 7.38 (2H, d, J = 7.5 Hz), which were characteristic of two symmetrical uracil moieties. The symmetry of **1** resulted in a simplified ^{13}C NMR spectrum with perfect overlap of the signals. (Table 1), including those of two quaternary carbons, three methines and two methylenes. The uracil units were confirmed by comparing the ^{13}C NMR spectral data (δ_C 164.3, 151.5, 142.1 and 100.2) with that in the literature [3f]; the signal intensities of the downfield carbons (δ_C 164.3, 151.5, 142.1 and 100.2) were all higher than those of the resonances at δ_C 72.4, 63.1 and 29.5. The ^{1}H-^{1}H COSY spectrum only showed correlation between H-5 (H-5') (δ_H 5.44) and H-6 (H-6') (δ_H 7.38). In the HMBC spectrum, the signal of H-5 correlated with those of C-4 and C-6, and HMBC cross- peaks of H-6 with C-2, C-4 and C-5 were also found. The HMBC correlation from the methine proton H-7 (δ_H 3.42) to C-6, as well as the downfield chemical shift of C-7 (δ_C 72.4) suggested that the uracil moieties were connected to C-7. In addition, the signal of H-7 further correlated with C-9. The downfield chemical shift of C-9 (δ_C 63.1) can be explained by the proximity of the aminium [3g]. On the basis of the foregoing analysis, compound **1** was determined as 3,3-bis(uracil-1-yl)-propan-1-aminium.



1 **2** **3** **4** **5**

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

Table 1: ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) data for compound **1** in DMSO-d_6.

Position	δ_H (int., mult., J in Hz)	δ_C	HMBC (H→C)
2, 2'		151.5	
4, 4'		164.3	
5, 5'	5.44 (2H, d, 7.5)	100.2	4, 6
6, 6'	7.38 (2H, d, 7.5)	142.1	2, 4, 5
7	3.42 (1H, m)	72.4	6, 9
8	2.56 (2H, ov[a])	29.5	
9	3.26 (2H, ov)	63.1	7
N-H	10.95 (ov)		

[a] Overlapped with solvent signal and deduced from HMQC correlation.

Table 2: Cytotoxic activities of compounds **1-5** on seven human cancer cell lines.

	Human cancer line, IC_{50} (μg/mL)						
Compound	A549	H1299	SGC7901	LNCAP	PC3	MDA-231	CT-26
1	26.51	>50	22.73	45.02	34.54	>50	>50
2	29.77	>50	13.24	22.67	26.91	>50	40.5
3	23.86	>50	>50	>50	>50	39.38	25.09
4	>50	>50	16.56	27.16	>50	40.47	27.9
5	>50	>50	23.72	40.9	27.17	>50	>50
5-Fu	7.01	2.5	7.51	8.93	8.17	2.38	3.83

The structures of compounds **2**–**5** were elucidated by comparison of their ^{1}H NMR, ^{13}C NMR and mass spectra with those in the literature [4]. Compounds **1**–**5** were assessed for cytotoxic activity (Table 2) against seven human cancer cell lines, including A549 (human alveolar basal epithelial), H1299 (human non-small cell lung carcinoma), SGC7901 (human gastric carcinoma), LNCAP (human prostate cancer), PC3 (human prostate cancer), MDA-231 (human breast cancer), and CT-26 (human colorectal cancer) *in vitro*. Compound **1** exhibited weak cytotoxicity to A549

(IC_{50} = 26.5 μg/mL) and SGC7901 (IC_{50} = 22.7 μg/mL). Compounds **2** and **4** displayed moderate cytotoxicity against SGC7901 (IC_{50} = 13.2 and 16.6 μg/mL).

Experimental

General: Melting point, SGW X-4 melting point apparatus; Optical rotation, JASCO P-1030 polarimeter; HR-APCI-MS, ESI-MS and EI-MS, Q-Tof micro YA019 mass spectrometer; NMR, Bruker AVANCE-400 spectrometer; HPLC, Waters 1525/2996 liquid chromatograph; CC, Sephadex LH-20 (Pharmacia) and YMC ODS-A (50 μm). Fractions were monitored by TLC (HSGF 254, Yantai, China) and compounds were visualized by heating silica gel plates after spraying them with 10% H_2SO_4 in EtOH.

Animal material: Specimens of the marine sponge *Agelas clathrodes* were collected around Yongxing Island and Seven Connected Islets in the South China Sea in June 2007, and were identified (voucher sample No. V107) by Prof. Zhi-Yong Li (Marine Biotechnology Laboratory, Shanghai Jiao Tong University, Shanghai, China).

Extraction and isolation: *A. clathrodes* (240 g, wet weight) was extracted with acetone, CH_2Cl_2, and MeOH (500 mL × 2 each) exhaustively at room temperature. The combined extracts were concentrated under reduced pressure to give a brown gum, which was partitioned between CH_2Cl_2 and H_2O to afford 2.1 g CH_2Cl_2 phase extract. This was subjected to CC on Sephadex LH-20 with CH_2Cl_2/MeOH (1:1) as eluting solvent to afford 3 fractions (*Fr. A*-*C*). *Fr. A* (670 mg) was subjected to CC on YMC ODS-A (50 μm) to give 6 subfractions (*Fr. A1*−*Fr. A6*). *Fr. A1* (382.3 mg) was further purified by HPLC (Waters 1525/2996, Symmetry Prep C18, 7 μm, 1.5 mL/min, UV detection at 260 nm) using 10% MeOH/H_2O as eluent to yield compounds **1** (1.6 mg, t_R = 14.5 min) and **3** (20.5 mg, t_R = 28.9 min). *Fr. A5* (89.6 mg) was separated by HPLC (Waters 1525/2996, Symmetry Prep C18, 7 μm, 60% MeOH/H_2O, 1.5 mL/min, UV detection at 275 nm) to yield compound **4** (13.6 mg, t_R = 43.4 min). *Fr. A6* (30.4 mg) was purified by HPLC (Waters 1525/2996, Symmetry Prep C18, 7 μm, 60% CH_3CN/H_2O, 1.5 mL/min, UV detection at 275 nm) to afford compound **5** (10.7 mg, t_R = 27.1 min). Similarly, *Fr. B* (170 mg) was subjected to chromatography repeatedly on Sephadex LH-20 and YMC ODS-A, and further purified by HPLC (Waters 1525/2996, SunFire Silica, 5 μm, 1.5 mL/min, UV detection at 210 and 280 nm) eluting with *n*-hexane/isopropanol (95:5) to give compound **2** (3.7 mg, t_R =21.9 min).

3,3-Bis(uracil-1-yl)-propan-1-aminium (**1**)
White amorphous solid. MP: 107-108°C.
$[\alpha]^{25}_D$: -2.6 (*c* 0.5, C_5H_5N).
^{1}H NMR and ^{13}C NMR (DMSO-d_6): Table 1.
HR-APCI-MS: *m/z* 280.1213 $[M]^+$ ($C_{11}H_{14}N_5O_4$, calcd. for 280.1202);

Cytotoxicity assay: Cytotoxicity was evaluated as IC_{50} by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, with 5-fluorouracil as positive control. Compounds **1–5** were solubilized in DMSO with the working concentration of test substances ranging from 1 to 100 μg/mL. Cells were inoculated into 96-well plates. After incubation for 24 h, the cells were treated with various concentrations of test substances for 48 h and then incubated with 1 mg/mL MTT at 37°C for 4 h, followed by solubilization in DMSO. The formazan dye products were measured by the absorbance at 490 nm on a microplate reader.

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Two New Compounds and Anti-complementary Constituents from *Amomum tsao-ko*

Jiahong Jin, Zhihong Cheng and Daofeng Chen*

Department of Pharmacognosy, *School of Pharmacy*, *Fudan University*, *Shanghai 201203*, *People's Republic of China*

dfchen@shmu.edu.cn

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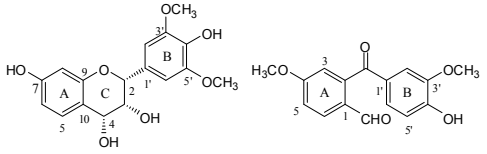
Two new compounds, (2*R*,3*R*,4*R*)-3',5'-dimethoxy-3,4,7,4'-tetrahydroxy-flavan (**1**) and 2-(4-hydroxy-3-methoxybenzoyl)-4-methoxy-benzaldehyde (**2**), together with 35 known phenolic compounds were obtained from the fruits of *Amomum tsao-ko*. Structures of the new compounds were elucidated on the basis of spectroscopic means, including 2D NMR, and high-resolution MS analysis. The isolated compounds were tested *in vitro* for their complement-inhibitory properties against the classical pathway (CP) and alternative pathway (AP). The results showed that 14 compounds exhibited anti-complementary activities against the CP and AP with CH_{50} values of 0.42 - 4.43 mM and AP_{50} values of 0.53 -1.51 mM. Preliminary mechanism studies showed that 1,7-bis(4-hydroxyphenyl)-4(*E*)-hepten-3-one (**8**) blocked C1q, C2, C3, C4, C5 and C9 components of the complement system, and hydroquinone (**15**) acted on C1q, C2, C3, C5 and C9 components.

Keywords: *Amomum tsao-ko*, Zingiberaceae, Complement inhibitor, Phenols, Diarylheptanoids, Benzenediols.

When activated inappropriately, the complement system may evoke pathologic reactions in a variety of inflammatory and degenerative diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), as well as acute respiratory distress syndrome (ARDS) [1, 2]. Therefore, inhibition of individual complement is a promising approach for the prevention and treatment of these diseases and numerous natural products have been reported to possess anti-complementary effect [3-5].

Amomum tsao-ko Crevost et Lemaire (Zingiberaceae) is an annual plant widely distributed in tropical regions of Asia. Its fruits are used under the name of "Cao-Guo" (Fructus Tsaoko) in traditional Chinese medicine (TCM) for the treatment of stomach disorders and infection of the throat, as well as a food flavor enhancer [6, 7]. In our effort to search for anti-complementary agents from TCMs, the ethanolic extract of *A. tsao-ko* fruits was found to show anti-complementary activity (CH_{50}: 0.75 ± 0.06 mg/mL, AP_{50}: 1.89 ± 0.35 mg/mL). Bioactivity-directed fractionation and isolation was thus performed with the ethanolic extract of *A. tsao-ko* fruits and led to the isolation of two new compounds, (2*R*,3*R*,4*R*)-3',5'-dimethoxy-3,4,7,4'-tetrahydroxy-flavan (**1**) and 2-(4-hydroxy-3-methoxybenzoyl)-4-methoxy-benzaldehyde (**2**) (Figure 1), together with 35 known ones (**3-37**). The isolates were tested for their *in vitro* anti-complementary activities against both the CP and AP, and the targets of the two most active compounds (**8** and **15**) on the complement activation cascade were also investigated.



1 **2**

Figure 1: Structures of **1** and **2**.

Compound **1** was obtained as a yellowish amorphous powder. Its molecular formula was determined as $C_{17}H_{18}O_7$ by HR-ESI-MS (*m*/*z* 357.0943 [*M* + Na]$^+$). Taken together with three oxygenated carbon signals at δ_C 86.6 (C-2), 72.2 (C-3) and 72.1(C-4) (Table 1), it is easily speculated that **1** possesses a flavan 3,4-diol skeleton, as is the case with melacacidin (2,3-*cis*-3,4-*cis*-flavan-3,3',4,4',7,8-hexaol) [8]. The key difference between **1** and melacacidin was that a symmetrical structure [δ_H 3.82 (3H, s, 3'-OCH_3), 3.81 (3H, s, 5'-OCH_3) and δ_H 6.68 (2H, s, H-2', 6')] was observed in ring B of **1**, while melacacidin possesses 3,4-disubstituted groups in ring B. The HMBC correlations between δ_H 6.68 (1H, s, H-2') in ring B and δ_C 86.6 (C-2) and δ_C 72.2 (C-3), and between δ_H 6.78 (1H, d, *J* = 8.3 Hz, H-5) and δ_C 72.2 (C-3) confirmed the flavanol skeleton of **1** (Figure 2A). The locations of methoxy groups at C-3'/5' and hydroxyl at C-4' were also verified by the HMBC correlations between δ_H 3.82 (3H, s, 3'-OCH_3) and δ_C 148.6 (C-3') and δ_C 149.2 (C-4'), and between δ_H 7.52 (1H, s, 4'-OH) and δ_C 149.2 (C-4'), respectively. The evidence for assignment of δ_H 7.14 (1H, s, 7-OH) was that the X-part [δ_H 6.98 (1H, d, *J*=2.0 Hz, H-8)] of the ABX system showed HMBC correlations with δ_C 110.5 (C-6), 147.5 (C-9) and δ_C 119.5 (C-10). The absolute configuration of **1** was determined as 2*R*,3*R*,4*R* by the negative *Cotton* effects at 240 nm and 274 nm in its CD spectrum [9], as well as the ROESY correlations of H- 2/H-3, and H-3/H-4 (Figure 2A). Therefore, the structure of compound **1** was determined as (2*R*,3*R*,4*R*)-3',5'-dimethoxy-3,4,7,4'-tetrahydroxy-flavan.

Compound **2** was obtained as colorless needles (PE-acetone, 5:1). The molecular formula was determined as $C_{16}H_{14}O_5$ by HR-ESI-MS (*m*/*z* 309.0736 [*M* + Na]$^+$). The ^{13}C NMR spectrum (Table 1) showed 12 carbon signals for two benzene rings (δ_C 153.1, 151.7, 148.2, 147.5, 128.9, 128.7, 126.2, 123.5, 115.4, 114.9, 111.1 and 110.7), one carbonyl at δ_C 196.3, one aldehyde at δ_C 191.2, and two overlapped methoxy groups (δ_C 55.6). The IR spectrum also showed the typical absorption band at 1612 cm^{-1} for benzene rings. The ^{1}H NMR spectrum of **2** (Table 1) showed two sets of ABX system proton signals at δ_H 6.85 (1H, d, *J* = 8.3 Hz), 7.42 (1H, d, *J* = 2.0 Hz) and 7.48 (1H, dd, *J* = 8.3, 2.0 Hz), as well as signals at δ_H 6.95 (1H, d, *J* = 8.3 Hz), 7.36 (1H, d, *J* = 2.0 Hz) and 7.40 (1H, dd,

Table 1: ^{1}H- and ^{13}C-NMR spectral data of compounds **1**[a] and **2**[b] (δ in ppm, *J* in Hz)

Position	1		2	
	δ_H (multiplicity)	δ_C	δ_H (multiplicity)	δ_C
1				148.2
2	4.66 (d, *J*=4.3)	86.6		128.7
3	4.20 (dd, *J*=4.3, 6.1)	72.2	7.36 (d, *J*=2.0)	110.7
4	3.79 (d, *J*=6.1)	72.1		153.1
5	6.78 (d, *J*=8.3)	134.1	7.40 (dd, *J*=8.3, 2.0)	126.2
6	6.82 (dd, *J*=8.3, 2.1)	110.5	6.95 (d, *J*=8.3)	115.4
7		152.6		
8	6.98 (d, *J*=2.1)	112.8		
9		147.5		
10		119.5		
1'		136.2		128.8
2'	6.68 (br. s)	104.3	7.42 (d, *J*=2.0)	111.1
3'		148.6		147.5
4'		149.2		151.7
5'		148.6	6.85 (d, *J*=8.3)	114.9
6'	6.68 (br. s)	104.3	7.48 (dd, *J*=8.3, 2.0)	123.5
1-CHO			9.75 (s)	191.2
4-OCH_3			3.82(s)	55.6
7-OH	7.14 (s)			
1'-COAr				196.3
3'-OCH_3	3.82 (s)	56.5	3.79 (s)	55.6
4'-OH	7.52 (s)		10.05 (br. s)	
5'-OCH_3	3.81 (s)	56.5		

[a] Measured in acetone-d_6 at 400 MHz for ^{1}H NMR and at 100 MHz for ^{13}C NMR.
[b] Measured in DMSO-d_6 at 400 MHz for ^{1}H NMR and at 100 MHz for ^{13}C NMR.

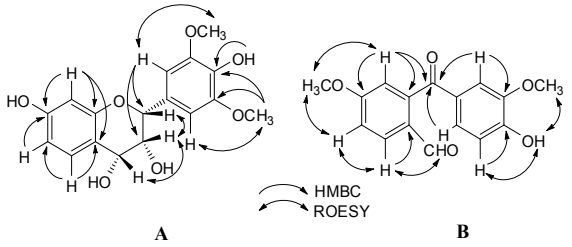


Figure 2: Key HMBC and ROESY correlations of **1** (A) and **2** (B).

2.0 Hz). The X-part of the ABX system in the A ring was deduced to be located at C-3 based on the HMBC correlations from δ_H 7.36 (1H, d, *J* = 2.0 Hz, H-3) to δ_C 148.2 (C-1), 153.1(C-4), 126.2 (C-5) and 196.3 (C-1') (Figure 2B). The methoxy group in the A ring was thus assigned at C-4 by the ROESY correlation between δ_H 7.36 (1H, d, *J* = 2.0 Hz, H-3) and the methyl signal at δ_H 3.79 (s, 3'-OCH_3). The aldehyde was assigned at C-1 by the ROESY correlation between the B-part of ABX system at δ_H 6.95 (1H, d, *J* = 8.3 Hz, H-6) and the aldehyde proton at δ_H 9.75 (1H, s, 1-CHO). Similarly, as shown in Figure 2B, the other ABX system and the locations of methoxy and hydroxyl in ring B were assigned. These two benzene rings were connected through one carbonyl group (δ_C 196.3, 1'-COAr), as indicated by the HMBC correlations between δ_H 7.36 (1H, d, *J* = 2.0 Hz, H-3), 7.42 (1H, d, *J* = 2.0 Hz, H-2'), 7.48 (1H, dd, *J* = 8.3, 2.0 Hz, H-6') and the carbonyl group (δ_C 196.3, 1'-COAr) (Figure 2B). Hence, the structure of compound **2** was established as 2-(4-hydroxy-3-methoxybenzoyl)-4-methoxy-benzaldehyde.

The 35 known phenolic compounds were identified by comparison of their ^{1}H, ^{13}C NMR and MS data with those reported in the literatures [10-20] as 4'-hydroxy-4-methoxychalcone (**3**), 4'-hydroxy-2'-methoxychalcone (**4**), 4,4'-dimethoxychalcone (**5**), 1,3-dimethoxybenzene (**6**), 4',7-dihydroxy-3',6-diprenylflavone (**7**), 1,7-bis(4-hydroxyphenyl)-4(*E*)-hepten-3-one (**8**), 4-hydroxy-2,5-dimethoxy-benzaldehyde (**9**), 3',7-dihydroxy-4'-methoxy-flavan (**10**), 2-methoxy-hydroquinone (**11**), 2',4'-dihydroxy-4- methoxy-chalcone (**12**), 5-indancarbaldehyde (**13**), 4-methoxybenzaldehyde (**14**), hydroquinone (**15**), 4-indancarbaldehyde (**16**), (-)-catechin

Table 2: Anti-complementary activities of the compounds from *A. tsao-ko* through the classical pathway (CH_{50}) and alternative pathway (AP_{50}) (mean ± SD, n=3)

Compounds	CH_{50} (mM)	AP_{50} (mM)
4'-Hydroxy-2'-methoxychalcone (**4**)	0.96 ± 0.15	NA[a]
1,7-Bis(4-hydroxyphenyl)-4(*E*)-hepten-3-one (**8**)	0.42 ± 0.15	0.66 ± 0.11
2-Methoxy-hydroquinone (**11**)	0.62 ± 0.09	0.86 ± 0.13
2',4'-Dihydroxy-4-methoxychalcone (**12**)	2.69 ± 0.42	NA
5-Indancarboxaldehyde (**13**)	1.33 ± 0.15	1.51 ± 0.38
Hydroquinone (**15**)	0.55 ± 0.11	0.53 ± 0.15
4-Hydroxy-2'-methoxychalcone (**23**)	4.43 ± 1.26	NA
Tsaokoarylone (**25**)	0.84 ± 0.07	0.72 ± 0.16
6,7-Dihydroxyindan-4-carbaldehyde (**28**)	0.66 ± 0.14	0.96 ± 0.13
3-Methoxy-catechol (**31**)	0.56 ± 0.13	0.54 ± 0.10
2-Methoxy-resorcinol (**32**)	0.64 ± 0.14	0.58 ± 0.14
4-(2-Hydroxypropyl)phenol (**34**)	0.93 ± 0.14	1.26 ± 0.18
Catechol (**36**)	0.58 ± 0.13	0.57 ± 0.15
4-Methoxy-catechol (**37**)	0.69 ± 0.16	0.64 ± 0.08
Heparin [b]	40 ± 14 [c]	97 ± 19 [c]

[a] NA: Not active. [b] Positive control. [c] μg/mL.

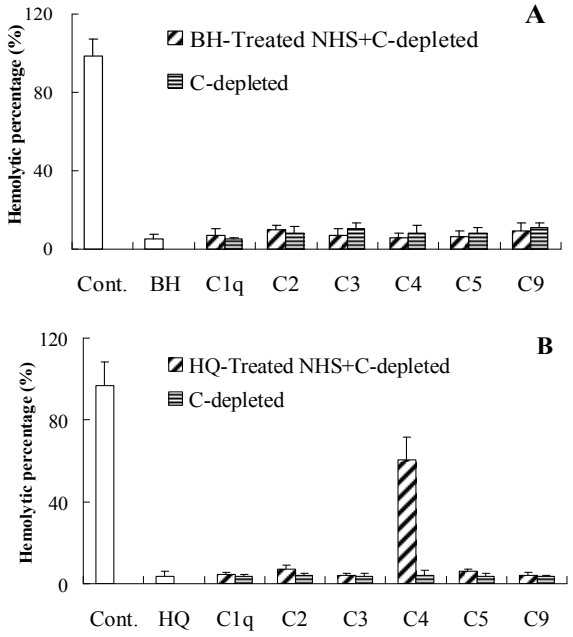


Figure 3: Targets of 1,7-bis(4-hydroxyphenyl)-4(*E*)-hepten-3-one (BH, A) and hydroquinone (HQ, B) on the complement activation cascade. BH-, HQ-treated sera were mixed with various complement-depleted (C-depleted) sera and the capacity of these C-depleted sera to restore hemolytic capacity in the CP was estimated by adding sheep antibody-sensitized erythrocytes. Cont., complement control group. Results are expressed as hemolytic percentages. Data are expressed as mean ± SD (n = 3).

(**17**), anisole (**18**), 2',4,4'-trimethoxychalcone (**19**), 4-(1-hydroxy-propyl)phenol-ethyl-4-hydroxy-(*S*)-benzenemethanol (**20**), abyssinoflavanone VII (**21**), 4-hydroxy-4'-methoxychalcone (**22**), 4-hydroxy-2'-methoxychalcone (**23**), 2-methoxy-benzaldehyde (**24**), tsaokoarylone (**25**), 3-methoxy-benzaldehyde (**26**), 3-hydroxy-4-methoxybenzaldehyde (**27**), 6,7-dihydroxy-4-indancarbaldehyde (**28**), 3-methoxy-4-hydroxy-benzaldehyde (**29**), 6-hydroxy-4-aldehydeindene (**30**), 3-methoxy-catechol (**31**), 2-methoxy-resorcinol (**32**), 3,5-dihydroxybenzoic acid (**33**), 4-(2-hydroxy-propyl)phenol (**34**), 3-hydroxybenzoic acid (**35**), catechol (**36**), and 4-methoxy-catechol (**37**),. Except for compounds **8**, **14**, **15**, **17**, **18**, **25**, **29** and **36**, all the other compounds were obtained from this species for the first time.

The isolated compounds (purity>90%, by HPLC analysis), except **33** and **35**, were evaluated for their anti-complementary activities against both CP and AP. As shown in Table 2, fourteen compounds possessed anti-complementary effects against the CP with CH_{50} values of 0.42 - 4.43 mM. On the AP, eleven isolates were found

active, with AP_{50} values of 0.53 - 1.51 mM. Compounds **8**, **15**, **31**, **32**, **36** and **37** showed good activities against both CP and AP with CH_{50} and AP_{50} values less than 0.7 mM. Derivatives of chalcone (**4**, **12** and **23**) were inactive against AP while they demonstrated inhibitory activity against CP. As the two most active compounds against the CP and AP respectively, 1,7-bis(4-hydroxyphenyl)-4(*E*)-hepten-3-one (**8**) and hydroquinone (**15**) were selected for the preliminary mechanism study. When treated with 1,7-bis(4-hydroxyphenyl)-4(*E*)-hepten-3- one (**8**), hemolytic percentages of the C1q-, C2-, C3-, C4-, C5- and C9-depleted sera were 5.04 ± 1.03%, 8.33 ± 2.98%, 9.46 ± 3.00%, 8.36 ± 3.78%, 8.21 ± 2.43% and 9.10 ± 2.19%, respectively (Figure 3A), indicating that **8** interacted with C1q, C2, C3, C4, C5 and C9. Meanwhile, hydroquinone (**15**) acted on C1q, C2, C3, C5 and C9 components (Figure 3B).

Experimental

Reagents: Sheep erythrocytes were collected in Alsevers' solution. Normal human serum (NHS) was obtained from healthy male donors (average age of 20 years). Rabbit erythrocytes were obtained from the ear vein of New Zealand white rabbits. Heparin (sodium salt, 160 IU/mg) was purchased from Shanghai Aizite Biotech Co. Ltd. (Shanghai, China). Veronal buffer saline (VBS, pH 7.4) contained 0.5 mM Mg^{2+} and 0.15 mM Ca^{2+}(VBS^{2+}), and VBS 5 mM Mg^{2+} and 8 mM EGTA (VBS-Mg-EGTA). Anti-C1q, Human (Goat); Anti-C2, Human (Goat); Anti-C5, Human (Rabbit) and Anti-C9, Human (Goat) were purchased from Merck Biosciences (Darmstadt, German). Anti-C3, Human (Goat) and Anti-C4, Human (Goat) were purchased from Shanghai Sun Biotech Co. Ltd. (Shanghai, China).

Plant material: The fruits of *A. tsao-ko* were purchased from Huayu Materia Medica Co., Ltd. (Shanghai, China) in October 2007. The plant material was verified by Dr Daofeng Chen. A voucher specimen (DFC-CG-H2003050607) has been deposited in the Herbarium of Materia Medica, Department of Pharmacognosy, School of Pharmacy, Fudan University, Shanghai, China.

Extraction and isolation: The air-dried fruits (15.0 kg) were extracted with 95% ethanol (10 L × 5 times) at room temperature. The extracts were combined and concentrated under reduced pressure, and the resulting residue (319.2 g) was resuspended in 2.0 L of water and successively partitioned with PE (60-90ºC), ethyl acetate, and *n*-butanol (each 2 L × 4 times). The *n*-butanol fraction was found to show the highest activity. Therefore, the active *n*-butanol fraction was subjected to AB-8 macroporous resin chromatography eluted with 30%, 50%, 70% and 90% aqueous ethanol to afford 4 subfractions. The subfractions obtained with 50% and 70% ethanol were found to show obvious anti-complementary activities and were thus further chromatographed over a silica gel column (CC).

The 50% EtOH subfraction (21.3 g) was subjected to CC (silica gel, PE-acetone, from 100:0 to 1:1) to yield F-1 - F-8. F-2 (1.7 g) was separated by CC (silica gel, PE-ethyl acetate, from 20:1 to 10:1) and then purified over Sephadex LH-20 (chloroform-methanol, 1:1) to afford **13** (21.2 mg). F-3 (1.2 g) was purified over CC (silica gel, PE-ethyl acetate, 10:1) to give **16** (12.7 mg), **22** (8.0 mg), **26** (4.3 mg), **34** (10.2 mg), **9** (14.3 mg) and **10** (5.8 mg). F-5 (2.5 g) was loaded onto a column of silica gel and eluted with PE-ethyl acetate, from 10:1 to 5:1, and then subjected to Sephadex LH-20 (chloroform-methanol, 1:1) and preparative TLC (chloroform-methanol, 20:1) to afford **2** (15.4 mg), **3** (4.4 mg), **4** (3.9 mg), **14** (4.1 mg), **21** (12.1 mg) and **23** (16.6 mg). F-6 (1.1 g) was purified by CC (silica gel, PE-acetone, from 5:0 to 5:1) and then subjected to gel filtration over Sephadex LH-20 (chloroform-methanol, 1:1) and preparative TLC (chloroform-methanol, 15:1) to afford **31** (8.7 mg), **32** (12.1 mg), **36** (9.5 mg), **27** (3.3 mg), **30** (4.4 mg) and **35** (3.9 mg). F-7 (1.8 g) was purified by CC (silica gel, PE-acetone, from 10:1 to 5:1) and then subjected to preparative TLC (chloroform-methanol, 10:1) to furnish **11** (15.3 mg), **15** (6.8 mg), **33** (9.3 mg) and **37** (13.8 mg).

The 70% EtOH subfraction (13.7 g) was subjected to CC (silica gel, PE-acetone, from 100:0 to 1:1) to yield F-9 - F-16. F-11 was further separated by CC (silica gel, chloroform-methanol, 10:1) and then purified over Sephadex LH-20 (chloroform-methanol, 1:1) and preparative TLC (chloroform-methanol, 15:1) to give **20** (9.9 mg), **24** (9.3 mg) and **25** (17.9 mg). F-12 (1.5 g) was chromatographed over Sephadex LH-20 (methanol) and then separated by CC (silica gel, chloroform-methanol, 15:1) to yield **1** (5.3 mg), **7** (5.1 mg), **8** (7.9 mg) and **18** (6.3 mg). F-13 (1.6 g) was chromatographed over a column of silica gel, eluting with chloroform-methanol, 15:1), and then purified over Sephadex LH-20 (methanol) to afford **12** (7.2 mg), **17** (7.7 mg), **28** (5.3 mg) and **29** (10.9 mg). F-14 (2.7 g) was purified by CC (silica gel, chloroform-methanol, 7:1) and then purified over Sephadex LH-20 (chloroform-methanol, 1:1) to afford **5** (8.3 mg), **6** (5.9 mg) and **19** (6.3 mg).

(2*R*,3*R*,4*R*)-3',5'-Dimethoxy-3,4,7,4'-tetrahydroxy-flavan (**1**)
Pale yellow powder.
MP: 182-184ºC.
$[\alpha]_D^{25}$: - 26.8 (*c* 0.02, acetone).
IR (KBr) ν_{max}: 3420, 2955, 2920, 2850, 1634, 1558, 1446, 1384, 1303 cm^{-1}.
UV (acetone): λ_{max} (log ε): 223 (5.2), 255 (3.1, sh) nm.
CD (*c* 0.1, methanol): nm($\Delta\varepsilon$): 240 (-32.1), 274 (-11.4).
^1H NMR and ^{13}C NMR (acetone-d_6): Table 1;
HR-ESI-MS: *m/z* 357.0943 ([*M* + Na]$^+$, $C_{17}H_{18}O_7Na^+$, calc. 357.0945).

2-(4-Hydroxy-3-methoxybenzoyl)-4-methoxy-benzaldehyde (**2**)
Colorless needles (PE-acetone, 5:1).
MP: 166-167ºC.
IR (KBr) ν_{max}: 3416, 1721, 1612, 1462, 1383, 1272 cm^{-1}.
UV (acetone): λ_{max} (log ε): 223 (4.6), 250 (2.7, sh) nm.
^1H NMR and ^{13}C NMR (DMSO-d_6): Table 1.
HR-ESI-MS: *m/z* 309.0736 ([*M* + Na]$^+$, $C_{16}H_{14}O_5Na^+$, calc. 309.0734).

Anti-complementary activity through the classical pathway: The anti-complementary activities through the classical pathway (CP) were measured as described previously [21]. Briefly, sensitized erythrocytes (EAs) were prepared by incubation of sheep erythrocytes (4.0×10^8 cells/mL) with equal volumes of rabbit anti-sheep erythrocyte antibody in VBS^{2+}. Samples and heparin (positive control) were individually dissolved in VBS^{2+}. Normal human serum (NHS) was used as the complement source. The 1:80 diluted serum of Guinea pig was chosen to give sub-maximal lysis in the absence of complement inhibitors. Serial dilutions of the test samples (100 µL) were preincubated with a mixture of 100 µL NHS and 200 µL VBS^{2+} at 37°C for 10 min, followed by adding 200 µL EA to the mixture and co-incubating at 37°C for 30 min. The different assay controls were incubated under the same conditions: (1) vehicle control, 200 µL EAs in 400 µL VBS^{2+}; (2) control 100% lysis, 200 µL EAs in 400 µL water; (3) sample control, 100 µL dilution of each sample in 500 µL VBS^{2+}. After reaction, the resulting mixture was centrifuged immediately, and the optical density of the supernatant was then measured at 405 nm with a spectrophotometer (Wellscan MK3, Labsystems Dragon). The

absorbance of sample (As), sample control (Asc) and 100% lysis control (Al) were obtained.

Anti-complementary activity through the alternative pathway: The anti-complementary activities through the classical pathway (CP) and alternative pathway (AP) were measured according to Klerx's method [22]. Briefly, each sample was dissolved in EGTA-VB, and serial dilutions of the samples were prepared by adding appropriate volumes of EGTA-VB. After pre-incubation of each sample (150 μL) with 1:10 diluted NHS (150 μL) at 37°C for 10 min, 200 μL rabbit erythrocytes (ERs 3.0×10^8 cells/mL) were added, followed by a second incubation at 37°C for 30 min. After reaction, the resulting mixture was centrifuged immediately, and the optical density of the supernatant was then measured at 405 nm.

Identification of the targets on the complement activation cascade: Tests to identify the targets of the complement activation cascade were conducted according to Xu's method [5]. Various dilutions of each antiserum were incubated with the same volume of NHS (1:10, v/v) at 37°C for 15 min. After centrifugation, the supernatant (200 μL) was incubated with 200 μL VBS^{2+} and 200 μL EAs, and cell lysis was measured. The antiserum dilution against the NHS hemolytic capacity was then determined. The optimal dilutions (1:1 for C3 and C4; 1:32 for C5, and 1:64 for C1q, C2, and C9, v/v) were incubated with the same volume of NHS (1:10, v/v) at 37°C for 15 min, followed by centrifugation, and the supernatants were collected and stored as complement-depleted (C-depleted) sera in aliquots at -70°C before use in hemolytic assays.

Capacity of depleted sera to lyse EAs through the CP was assessed in the presence or absence of sample-treated NHS. Sample-treated NHS was obtained by incubating an optimally diluted sample with an equal volume of 1:10 (v/v) diluted NHS at 37°C for 10 min. The examined concentrations of 1,7-bis(4-hydroxyphenyl)-4(*E*)-hepten-3-one (**8**) and hydroquinone (**15**), just sufficient to cause complete loss of hemolytic activity of 1:10 diluted NHS, were 1.12 mM and 1.24 mM. For the target complement group (the assay of capacity of various depleted sera to restore the hemolytic capacity of sample-treated serum), 200 μL EAs and 200 μL individual depleted sera of C1q, C2, C3, C4, C5 or C9 were added to 200 μL sample-treated NHS and the mixture was incubated at 37°C for 30 min. After centrifugation and measurement of the optical density of the supernatant, the percentage of hemolysis was calculated. For the assay of the individual depleted serum group, C-depleted sera were directly incubated with EAs under the same conditions, and the hemolytic activities were calculated. The controls: (1) vehicle control: 200 μL EAs in 400 μL VBS^{2+}; (2) 100% lysis: 200 μL EAs in 400 μL water; (3) complement control: 100 μL NHS (1:10, v/v) and 200 μL EAs in 300 μL VBS^{2+}; and (4) sample control, 100 μL sample in 500 μL VBS^{2+}, were incubated under the same conditions.

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Antiangiogenic Activity of Flavonoids from *Melia azedarach*

Shigenori Kumazawa[a,*], Satomi Kubota[a], Haruna Yamamoto[a], Naoki Okamura[a], Yasumasa Sugiyama[b], Hirokazu Kobayashi[a], Motoyasu Nakanishi[c] and Toshiro Ohta[a]

[a]*Department of Food and Nutritional Sciences, University of Shizuoka, 52-1 Yada, Suruga-ku, Shizuoka 422-8526, Japan*

[b]*Bioorganic Chemistry Research Group, Faculty of Fisheries, Kagoshima University, 4-50-20 Shimoarata, Kagoshima 890-0056, Japan*

[c]*Innovation and Joint Research Center, Shizuoka University, 3-5-1 Johoku, Naka-ku, Hamamatsu, Shizuoka 432-8561, Japan*

kumazawa@u-shizuoka-ken.ac.jp

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Three flavonoid glycosides, **1** (rutin: quercetin 3-*O*-rutinoside), **2** (kaempferol 3-*O*-robinobioside) and **3** (kaempferol 3-*O*-rutinoside) were isolated from the subcritical water extracts of *Melia azedarach* leaves. Strong antiangiogenic activity of these compounds was observed in the *in vivo* assay using the chorioallantoic membrane (CAM) from growing chick embryos.

Keywords: Angiogenesis, Flavonoid, Subcritical extraction, *Melia azedarach*, Quercetin, Kaempferol.

Angiogenesis is the process of new endothelial blood vessel development. It is essential for the growth, invasion and metastasis of most solid tumors and has become a valuable pharmacological target for cancer prevention and treatment [1a,b]. We performed *in vivo* experimental angiogenesis screening of plant extracts using the chorioallantoic membrane (CAM) from growing chick embryos [2]. We found that the leaf extracts of *Melia azedarach* L. (Meliaceae) have strong antiangiogenic activities. In particular, the activity of the leaf extracts obtained by using subcritical water was stronger than that of the extracts by using water or alcohol under atmospheric pressure (data not shown).

At present, subcritical water extraction is used for a variety of applications across various fields of study. Studies have shown its use in extracting phenolic compounds from grape pomace and potato [3a,b]. Recently, subcritical water has gained interest as an alternative solvent for the extraction of natural active compounds. We utilized this technique to identify effective antiangiogenesis compounds from medicinal plants.

M. azedarach is a large evergreen tree that is distributed from southeastern Asia to northern Australia. Previous phytochemical and pharmacological studies on the roots of the plant have revealed that it can yield limonoids with antifeedant effects [4a,b]. Furthermore, steroids, with cytotoxic effects, and flavonoids, with antioxidant activities, have been isolated from the leaves [5a,b]. However, to our knowledge, compounds exhibiting antiangiogenesis activities have not been isolated from *M. azedarach*.

Bioactivity-guided isolation was performed through the antiangiogenic activity of the subcritical water extracts of *M. azedarach* leaves by using the CAM assay. The isolation procedures are described in the Experimental section. We isolated and identified three flavonoid glycosides by NMR and MS analysis, **1** (rutin: quercetin 3-*O*-rutinoside), **2** (kaempferol 3-*O*-robinobioside) and **3** (kaempferol 3-*O*-rutinoside), as the active compounds from the leaf extracts of *M. azedarach*.

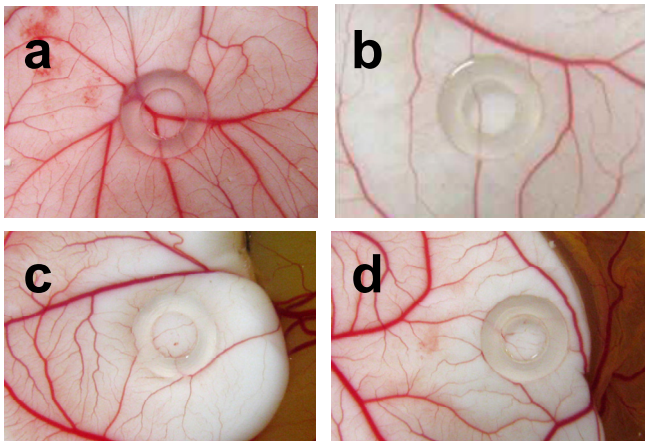

Figure 1: Effects of **1**~**3** on angiogenesis of the chorioallantoic membrane (CAM) *in vivo*. Formation and inhibition of the CAM vascular network when treated with the MeOH control (**a**), or 50 μg of **1** (**b**), **2** (**c**) and **3** (**d**) are shown.

Compound **1** is the main flavonoid in various plants and has been reported to possess a variety of pharmacological activities [6]. It has been previously identified from leaf extracts of *M. azedarach* [7]. Compound **2** has been isolated from leaves of *Alternanthera brasiliana* and shown to exhibit inhibitory effects on lymphocyte proliferation *in vitro* [8]. It has also been identified from *Gynura formosana* Kiamnra [9]. However, to date, no studies have reported the isolation of **2** from *M. azedarach*. Compound **3** has been isolated from the leaves of *M. azedarach* and reported to possess antioxidant activity [7].

Figure 1 shows the results of the antiangiogenic activities of **1**~**3** (50 μg/egg) using the CAM assay. In eggs treated with **1**~**3** (Figure 1b~d), the avascular zone was observed inside and around the silicon ring on the CAM surface. This result indicates that **1**~**3** inhibited angiogenesis, and the subsequent formation of the vascular network. Although **1**~**3** are known flavonoid glycosides, their antiangiogenic activity is reported here for the first time. We

also tested the antiangiogenic activity of **1**~**3** *in vitro* by using human umbilical vein endothelial cells (HUVECs), because we had observed the *in vitro* activity of quercetin and kaempferol, which are the aglycones of **1**~**3** [10]. However, **1**~**3** did not show any sign of the anticipated *in vitro* antiangiogenic activity (data not shown). It is reported that dietary flavonoid glycosides are subjected to deglycosidation by enterobacteria for absorption in the intestine [11]. However, the metabolism of flavonoid glycosides on the CAM surface was unclear, and hydrolysis of **1**~**3** could not be confirmed in the present study.

This study suggests that the glycosides infrom the leaves of *M. azedarach* exhibit antiangiogenic activity *in vivo*. These findings further extend the potential pharmacological effects of *M. azedarach* and could demonstrate its usefulness in cancer prevention and treatment.

Experimental

General: NMR experiments were performed on a Bruker BioSpin AVANCE III 400 MHz spectrometer (Billerica, MA, USA). Chemical shifts (δ in ppm) were referenced to the carbon (δ_C 49.0) and residual proton (δ_H 3.30) signals of CD_3OD. Low-resolution electrospray ionization (ESI) MS were recorded on an LCQ mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA).

Plant material: The leaves of *M. azedarach* were collected from Mima, Tokushima Prefecture, Japan, in July 2009. The plant was identified by Hideto Miyamoto (Medicinal Botanical Garden, University of Shizuoka, Japan). A voucher specimen was deposited at the University of Shizuoka, Japan.

Extraction and isolation: Dried leaves of *M. azedarach* (2.4 kg) were extracted with subcritical water (100°C, 6 MPa, 5 min) to give a crude extract (2.2 g). This was mixed with 1 g of silica gel (Merck, Silica gel 60, Germany) and concentrated to yield dried silica gel, which was placed on the top of a silica gel column (320 mm × 30 mm i.d.) and eluted with *n*-hexane, EtOAc and MeOH to give 19 fractions (frs.): frs. 1~6, *n*-hexane/EtOAc (1:1) eluate; frs. 7~12, EtOAc/MeOH (1:1) eluate; and frs. 13~19, MeOH eluate. A total of 200 mL of each fraction was collected and evaluated by the CAM assay. Fr. 7, which showed the highest activity, was rechromatographed by preparative HPLC on a 250 mm × 20 mm i.d. ODS column (Shiseido, Tokyo, Japan) with 0.1% TFA in CH_3CN-H_2O (18:82) at a 10 mL/min flow rate, thus yielding the active compounds **1** (2.4 mg), **2** (2.9 mg) and **3** (4.1 mg).

CAM assay: The CAM assay was performed as previously described [2]. In brief, fertilized chicken eggs were incubated at 37°C. On incubation day 3, a small window was opened in the shell, and 4 mL of the albumen was removed and further incubated. After 5-days, CAM was treated with various doses of the samples and incubated at 37°C for another 2 days. An appropriate volume of white emulsion was injected into CAM to clearly visualize the vascular network. Observations of the vascular networks were carried out to evaluate the antiangiogenic activity. Retinoic acid (5 nmol/egg) was used as the positive control.

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Application of Mixture Analysis to Crude Materials from Natural Resources (IV)[1(a-c)]: Identification of *Glycyrrhiza* Species by Direct Analysis in Real Time Mass Spectrometry (II)

Eriko Fukuda[a], Yoshihiro Uesawa[b], Masaki Baba[a] and Yoshihito Okada[a*]

[a]*Department of Natural Medicine and Phytochemistry*, *Meiji Pharmaceutical University*, *2-522-1 Noshio*, *Kiyose*, *Tokyo 204-8588*, *Japan*

[b]*Department of Clinical Pharmaceutics*, *Meiji Pharmaceutical University*, *2-522-1 Noshio*, *Kiyose*, *Tokyo 204-8588*, *Japan*

y-okada@my-pharm.ac.jp

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In order to identify *Glycyrrhiza* species by chemical fingerprinting, the bark of the roots and stolons of *Glycyrrhiza 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. This summarizes the relationship between the *p*-values of a statistical test and the magnitude of the difference in values of the samples in the groups. In this experiment, peaks that had a p value <0.05 in the t test and ≥2 absolute difference were defined as characteristic. As a result, characteristic peaks of *G. uralensis* were found at *m/z* 299, 315, 341, and 369. In contrast, characteristic peaks of *G. glabra* were found at *m/z* 323, 325, 337, 339, and 391. In conclusion, we found several characteristic peaks to distinguish *G. uralensis* and *G. glabra* by DART-MS using volcano plot. This method can be applied to identify the *Glycyrrhiza* species.

Keywords: Direct Analysis in Real Time-MS (DART-MS), *Glycyrrhiza uralrensis, Glycyrrhiza glabra*, Volcano plot, Mixture analysis.

The roots and stolons of some *Glycyrrhiza* species have been used as pharmaceuticals. In the current edition of the Japanese Pharmacopoeia (JP XVI), *G. uralensis* Fischer and *G. glabra* Linné are defined as original plant sources of "Glycyrrhizae Radix". Meanwhile, in the Chinese Pharmacopoeia, *G. inflata* Batalin is also defined [2]. These three species contain glycyrrhizic acid as a main active pharmaceutical ingredient, but other components are also medicinal. Phenolic constituents such as flavonoids and coumarins vary according to the species. Glycycoumarin, glabridin, and licochalcone A were reconfirmed as species-specific typical constituents of *G. uralensis*, *G. glabra*, and *G. inflata*, respectively [3]. Glycycoumarin is an antispasmodic ingredient [4]. Chandrasekaran *et al*. reported that *G. glabra* (almost devoid of glycyrrhizin) exhibits anti-inflammatory properties, which could be influenced in part by glabridin and isoliquiritigenin [5]. It is possible that the differences in ingredients, especially phenolic components, influence medicinal effects. Therefore, species identification is required. Identification presupposes the possibility that the appropriate species can be proposed as the situation demands in Kampo medicine.

In a previous study, we reported the identification of *G. inflata* by DART-MS. DART (Direct Analysis in Real Time) is a novel mass spectrometric ion source [6]. This method provides $[M+H]^+$ cation adducts in positive mode, resulting in fewer cleavage reactions. This point is helpful to identify the target substance in mixture states. Therefore, it is often used for qualitative analysis [7-9]. The powder of the roots and stolons of *G. inflata* can be differentiated from those of *G. uralensis* and *G. glabra* by detection of the peak that originated mainly from the $[M+H]^+$ ion of licochalcone A [1a]. On the other hand, glycycoumarin and glabridin were not identified by DART-MS analysis of the powder of the roots and stolons of *G. uralensis* and *G. glabra* under the same conditions. [1a] Compared with licochalcone A in *G. inflata*, the amounts of glycycoumarin and glabridin in the other two species are very small and below detectable limits. Thus, it is not enough to show identification of *G. uralensis* and *G. glabra* by detection of one particular peak. In order to discriminate between the two species, several characteristic peaks for each species were selected by multivariate analysis. Multivariate analysis using the DART-MS data has also been developed by means of chemometric classification [10] and identification of unknown samples [11].

According to Kuwajima *et al*., nonglycoside flavonoid constituents were mostly lost when root bark is removed [12]. The root bark of *Glycyrrhiza* species is thought to be rich in nonglycosidic flavonoids and have signature substances that are easy to detect. Therefore, the root bark of *G. uralensis* and *G. glabra* were analyzed by DART-MS. The current research examined seven samples of *Glycyrrhiza*, four of *G. uralensis*, and three of *G. glabra*, as shown in Table 1. Species identification had been determined by HPLC, based on modified conditions of Shibano *et al*. [13], before DART-MS analysis.

Table1: sample list.

Sample No.	Place of production	Species identification
Gly-3	Japan	***G. glabra***
Gly-5	Japan	***G. uralensis***
Gly-17	Kazakhstan	***G. glabra***
Gly-18	Uzbekistan	***G. glabra***
Gly-19	China	***G. uralensis***
Gly-20	Russia	***G. uralensis***
Gly-21	Mongolia	***G. uralensis***

Root bark was taken by a molding plane from three different points per sample. The root bark of each test sample was placed between the ion source and mass spectrometer detector for measurement, and subjected to streaming helium gas for 48 sec.

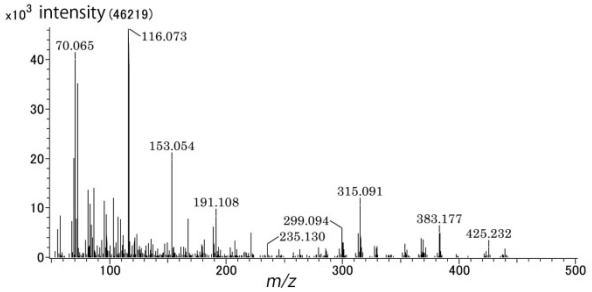

Figure 1A: DART-MS of the bark of *G. uralensis*.

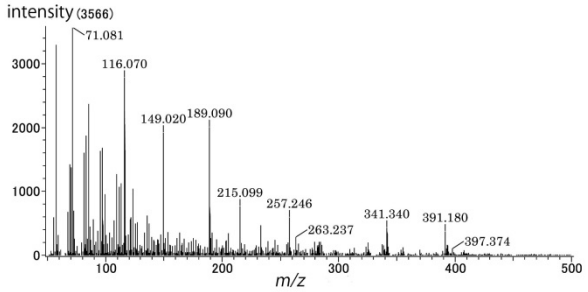

Figure 1B: DART-MS of the bark of *G. glabra*.

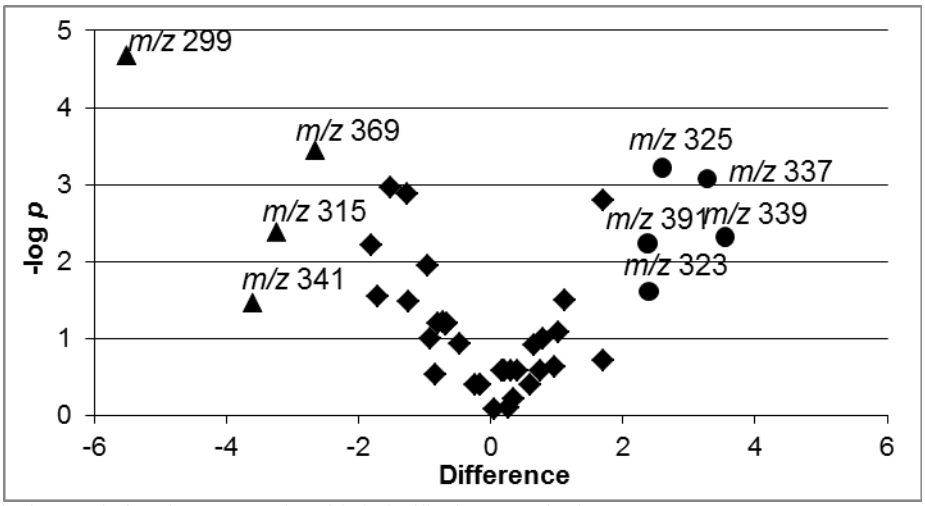

Figure 2: The volcano plot for the 43 peaks from the DART-MS data of the bark of licorice roots and stolons.
The x-axis is the difference of the average of both groups, and the y-axis is the *p*-value in -$\log_{10}p$ scale computed. Each shape represents one of 43 peaks. Using two absolute values of difference and a *p*-value of 0.05 as the cutoff threshold, four peaks from the upper left region (characteristic for *G. uralensis*; represented as ▲) and five peaks from the upper right region (characteristic for *G. glabra*; represented as ●) were selected (See Table 2).

Typical spectral data of the bark of *G. uralensis* and *G. glabra* are shown in Figure 1. In the range of *m/z* 280~450, where it is thought that secondary metabolites are greatly detected, the ions selected for the statistical analysis had abundances of at least 20% relative to the most abundant ion in that mass spectral range (20% threshold). The relative intensity of each peak was then calculated. In order to compare each sample, the data were divided by the standard deviation (SD) in the sample of the same individual, which standardizes the data (SD=1). Then, 128 out of 171 peaks which had any peak-appearance in each sample were removed. Therefore, 43 peaks were used for the analysis.

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. It is a scatter plot of the fold-change or the difference value versus the *p*-value (in -$\log_{10}p$ scale). *P*-value was calculated by the Student's *–t* test. The -$\log_{10}p$-values are plotted on the y-axis. As the intensity difference of the peaks is within normal distribution, we selected the difference values of the two for the x-axis. Value of difference is calculated as [(average of *G. glabra*)-(average of *G. uralensis*)].

Figure 2 is a volcano plot of the DART-MS data of the bark of licorice. The values of difference and *p* values between these two conditions were plotted for each peak. Peaks landing on the upper left region and the upper right region have a small *p*-value with a large absolute difference. The peaks that had a *p* value <0.05 in the *t* test and ≥2 absolute differences were selected as characteristic.

The volcano plot showed that peaks for *m/z* 299, 315, 341, and 369 are signature peaks of *G. uralensis*, and *m/z* 323, 325, 337, 339, and 391 of *G. glabra*. The peaks for *m/z* 369 and 325 were identified by LC/ESI/MS as $[M+H]^+$ ions of glycycoumarin and glabridin, respectively,.

In the case of *G. uralensis*, four characteristic peaks are found. In 9 out of 12 *G. uralensis* samples (75%), three or more of these characteristic peaks had relative intensities >20% of original DART-MS data. However, peaks that were characteristic of *G. glabra* were also detected in some *G. uralensis* samples, for example Gly19-2. (Table 3)

In the case of *G. glabra*, five characteristic peaks are found. In 7 out of 9 samples (77%), three or more characteristic peaks had relative intensities greater than 20% of original data. However, peaks that were characteristic of *G. uralensis* were also detected in some *G. glabra* samples, for example Gly17-2 (Table 3).

Although several unidentified samples remained, the detection of characteristic peaks is useful for species identification.

Gly3-1 and Gly3-3 did not detect typical peaks enough. Since they have high intensity peaks, most of the peaks under the threshold value would have been eliminated from the statistical analysis.

It is important to note that the statistical analysis performed in this study was only performed on peaks that had abundances of at least 20% relative to the most abundant ion in a given mass spectral range.

Table 2: The *p*-value and the absolute difference value for each of the nine peaks extracted.

m/z	299*	315*	341*	369*	323**	325**	337**	339**	391**
***p* value**	2.07E-05	0.004087	0.033929	0.000354	0.02424	0.000605	0.000839	0.00479	0.005785
-log $_{10}p$	4.683174	2.388613	1.469428	3.450702	1.615475	3.217991	3.076434	2.319649	2.23766
value of difference	-5.51027	-3.2431	-3.60396	-2.65802	2.391156	2.59533	3.276268	3.548244	2.367483

*Characteristic for *G. uralensis* ** Characteristic for *G. glabra*.
P-values calculated based on statistical *t*-test.
Value of difference calculated as [(average of *G. glabra*)-(average of *G. uralensis*)]

Table 3: Calculated relative intensity data of each characteristic peak and detection of the number of peaks.

m/z	299*	315*	341*	369*	323**	325**	337**	339**	391**	Own species character detection	Inverse side character detection
G. uralensis											
Gly5-1	33.24	0	100	15.04	14.58	0	0	0	0	2/4	0/5
Gly5-2	100	12.31	20.50	55.82	3.97	0	0	9.17	0	3/4	0/5
Gly5-3	100	52.12	17.59	55.68	0	0	0	9.11	0	3/4	0/5
Gly19-1	56.36	100	5.27	34.10	0	0	3.72	4.69	0	3/4	0/5
Gly19-2	100	31.66	62.54	57.01	0	0	22.91	19.92	0	4/4	1/5
Gly19-3	100	28.21	38.96	61.31	0	0	0	14.67	0	4/4	0/5
Gly20-1	100	69.85	99.78	35.70	12.34	0	0	38.59	11.29	4/4	1/5
Gly20-2	100	86.93	71.77	33.13	11.07	0	0	21.51	12.11	4/4	1/5
Gly20-3	73.15	70.90	48.91	39.54	0	0	65.96	25.18	0	4/4	2/5
Gly21-1	3.91	3.89	100	8.57	28.08	0	0	13.50	2.62	1/4	1/5
Gly21-2	13.78	15.29	100	6.97	44.04	0	6.47	6.11	11.02	1/4	1/5
Gly21-3	100	84.84	51.43	48.44	0	0	14.76	13.35	0	4/4	0/5
G. glabra											
Gly3-1	0	0	0	0	0	0	0	0	0	0/5	0/4
Gly3-2	0	0	0	0	0	0	51.85	100	25.10	3/5	0/4
Gly3-3	0	0	0	0	0	0	0	100	0	1/5	0/4
Gly17-1	0	0	23.07	0	100	50.58	83.03	42.46	18.90	4/5	1/4
Gly17-2	2.54	0	4.31	2.57	58.58	42.18	70.27	43.83	12.46	4/5	0/4
Gly17-3	0	0	0	0	99.60	48.36	100	49.26	31.28	5/5	0/4
Gly18-1	0	0	100	0	31.70	46.03	39.31	0	87.29	4/5	1/4
Gly18-2	0	0	44.40	0	62.63	56.74	78.67	38.28	79.51	5/5	1/4
Gly18-3	0	0	40.94	0	48.37	100	56.67	63.73	93.37	5/5	1/4

*Characteristic for *G. uralensis* ** Characteristic for *G. glabra*. (20% threshold)
In the case of *G. uralensis*, there are four characteristic peaks (*m/z* 299, 315, 341, 369). The number of peaks (*m/z* 299, 315, 341, 369) with relative intensities >20% in each *G. uralensis* sample (Gly5, 19, 20, 21 series) is shown in the "Own species character detection" column.
On the other hand, in *G. uralensis*, the number of peaks present characteristic for *G. glabra* with more than 20% relative intensity is shown in "Inverse side character detection" column.
In the case of *G. glabra*, five peaks were found (*m/z* 323, 325, 337, 339, 391). The number of peaks (*m/z* 323, 325, 337, 339, 391) with relative intensities >20% in each *G. glabra* sample (Gly3, 17, 18 series) is shown in the "Own species character detection" column.
On the other hand, in *G. glabra*, the number of peaks present characteristic for *G. uralensis* at more than 20% relative intensity is shown in "Inverse side character detection" column.

In conclusion, we found several characteristic peaks to identify *G. uralensis* and *G. glabra* by DART-MS using a statistical method; this can be applied to identify these two *Glycyrrhiza* species.

Experimental

Plant material: The roots and stolons of *G. uralensis* Fischer (Hokkaido, Japan) and *G. glabra* Linné (Hokkaido, Japan) were kindly supplied and identified by Dr Toshiro Shibata, the 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).

The other roots and stolons of *G. uralensis* (China, Russia and Mongolia) and *G. glabra* (Uzbekistan and Kazakhstan) 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, No.11G0005 respectively).

Species identification by HPLC: All samples were identified by HPLC analysis under similar conditions as those described by Shibano *et al.* [13]. A 5C_{18}-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: *G. uralensis*: glycycoumarin, and *G. glabra:* glabridin [3].

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).

DART mass spectrometry: All experiments were performed using a JMS-T100TD (JEOL Ltd., Japan) orthogonal TOF-MS with a DART ion source, which used helium gas at a flow rate of approximately 2.5 L/min. The gas heater was set at 250^{o}C. The mass spectrometer was operated in positive mode. Orifice 1, ring lens and orifice 2 potentials were set at 50, 10, and 5 V, respectively. The detector voltage was set to 2500 V, and the ion guide potential was 500 V. Data acquisition was taken from *m/z* 50 to 500.

LC/ESI/MS: All samples were analyzed by LC/ESI/MS to confirm that peaks for *m/z* 369 and 325 in the DART-MS were glycycoumarin and glabridin, respectively. HPLC analysis samples were diluted and injected into the LC/ESI/MS system {LCMS-2010EV system with an ESI probe (Shimadzu Co. Ltd.)}; column, COSMOSIL 3C_{18}-EB (i.d. 2.0 x 150 mm, Nacalai Tesque Inc.); guard column, OPTI-GUARD 1 mm C_{18} (Lab Lab Company Co. Ltd.). The heat block and CDL temperatures were 200^{o}C and 250^{o}C,

respectively; nebulization gas, nitrogen (flow rate, 1.5 L/min); solvent, water (0.1% formic acid) and acetonitrile (0.1% formic acid) gradient of 30% to 80% acetonitrile(0.1% formic acid) in 55 min.; flow rate, 0.2 mL/min.

Statistical analysis: The peak tables of DART-MS were imported into Microsoft Excel 2007.

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Comparison of Total Phenolic Content, Scavenging Activity and HPLC-ESI-MS/MS Profiles of Both Young and Mature Leaves and Stems of *Andrographis paniculata*

Lee Suan Chua[a*], Ken Choy Yap[a] and Indu Bala Jaganath[b]

[a]*Metabolites Profiling Laboratory*, *Institute of Bioproduct Development*, *Universiti Teknologi Malaysia*, *81310 UTM Skudai*, *Johor Bahru*, *Johor*, *Malaysia*

[b]*Biotechnology Research Centre*, *Malaysian Agricultural Research and Development Institute*, *50774 Kuala Lumpur*, *Malaysia*

chualeesuan@utm.my

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The total phenolic content and radical scavenging activity of *Andrographis paniculata* has been investigated to estimate the amount of phenolic compounds and diterpene lactones, respectively in the plant extracts. The stem extracts exhibited higher total phenolic content and scavenging activity than those of the leaf extracts from both young and mature plants. A range of 19.6–47.8 mg extract of *A. paniculata* from different parts of the plant is equivalent to the scavenging activity exhibited by one mg of standard Trolox. HPLC-ESI-MS/MS was also used to identify simultaneously the phytochemicals from the leaves and stems of both young and mature plant samples. Of the identified compounds, seven of the sixteen diterpene lactones, three of the six flavonoids, five of the six phenolic acids and two cyclic acids are reported here for the first time for this species. Multivariate statistical approaches such as Hierarchical Component Analysis (HCA) and Principle Component Analysis (PCA) have clustered the plant extracts into the leaf and stem groups, regardless of plant age. Further classification based on the phytochemical profiles revealed that mostly phenolic acids and flavonoids were from the young leaf extracts, and diterpenoids and their glycosides from the mature leaf extracts. However, the phytochemical profiles for the stems of both young and mature plants were not significantly different as presented in the dendrogram of HCA and the score plot of PCA. The marker for mature plants might be the *m/z* 557 ion (dihydroxyl dimethyl 19-[(β-D-glucopyranosyl)oxy]-19-oxo-ent-labda-8(17),13-dien-16,15-olide), whereas the *m/z* 521 ion (propyl neoandrographolide) could be the marker for leaf extracts.

Keywords: *Andrographis paniculata*, HPLC-ESI-MS/MS, Diterpene lactones, Polyphenols, Phenolic acids.

Andrographis paniculata Nees, family Acanthaceae, is an annual herbaceous plant, commonly known as "King of Bitters". It is widely grown in the tropical areas of Southeast Asia. The aerial parts (leaf and stem) of the plant have been used widely for centuries as a folklore remedy in traditional Chinese and Ayurvedic medicines for a broad range of ailments, including liver diseases, gastric disorders and the common cold [1]. This plant is the predominant constituent for at least 26 Ayurvedic formulations, as recorded in the Indian Pharmacopoeia [2-4].

Recently, many pharmacological studies have been carried out on the extracts of *A. paniculata*, mainly due to its wide application in the treatment of various infectious diseases and immune disorder symptoms. A comprehensive review of the phytochemistry, pharmacology and clinical uses of *A. paniculata* has been reported by Perumal Samy *et al.* [5]. They also reported that *A. paniculata* has very low toxicity to animals and plants. Diterpenoid lactones and flavonoids are the main chemical constituents believed to be responsible for the biological activities of the plant [6]. Till now, 50 labdane diterpenoids and 30 flavonoids have been reported from this plant [7-10]. Andrographolide is the prime active constituent, with a bitter taste, maximally found in the leaves (> 2%, w/w) [11,12]. Most phytochemical studies have been focused on the aerial part of the plant. Radhika *et al.* [13] reported three flavones from the stem of *A. paniculata*. To the best of our knowledge, no study has been carried out to differentiate the phytochemical profiles of the leaves and stems of both young and mature plants. The presence of diterpenoids, polyphenols and their derivatives in different parts of the plant at different harvest ages has not been simultaneously analyzed and compared so far.

Numerous analytical methods have been reported for the determination of andrographolide and its derivatives in the plant extracts. Of these methods, high performance liquid chromatography has been reported as the most accurate approach, since the spectrophotometric method suffers from the instability of red colored substance formation [14], and the gravimetric method gives an over estimation of andrographolide due to the co-solubility of other substances in ethyl acetate [15]. In the present study, a hyphenated system consisting of high performance liquid chromatography coupled to tandem mass spectrometry was used to determine simultaneously the major phytochemicals in the methanolic extract of *A. paniculata*. The complex chemical composition of the extract was chromatographically separated in a column by a gradient profile of a binary solvent system and then ionized by an electrospray ionization source before fragmentation and detection by a mass analyzer.

Besides the use of the high throughput analytical tool, the plant extracts were usually subjected to total phenol and antioxidant property assays. The total phenolic content is widely applied for the quantitative estimation of polyphenols such as flavones, flavanones and flavonols. The presence of diterpene lactones could be estimated from the free radical scavenging activity assay. The antiradical mechanism of diterpene lactones, particularly neoandrographolide, has been reported because of the presence of allylic hydrogens on the unsaturated lactone ring [16]. Therefore, diterpene lactones could scavenge free radicals by donating the allylic hydrogen atoms from its aglycone, either by homolytic cleavage or by deprotonation-oxidation mechanisms.

Table 1 shows the results of the TPC and DPPH activity of the leaves and stems of both young and mature plant samples. The stems had higher TPC values than the leaf samples, with the stems of the young plants exhibiting the highest TPC value of 1.64 µg GAE/mg extract. In line with the TPC results, the stem extracts also exhibited higher free radical scavenging activity than the leaf extracts. Therefore, the scavenging activity of the plant extracts might be attributed to the presence of phenolic compounds and diterpene lactones [16,17]. The scavenging activity expressed by the stem extracts was about two-fold higher than that of the leaves of mature plants. However, there was only a small degree of difference in scavenging activity between the leaves and stems of young plants. This observation also agreed with the TPC of the leaves and stems of young plants, where the degree of difference in the TPC value of both extracts was relatively small compared with the mature plant extracts.

Table 1: Total phenolic content and scavenging activities of different parts of *A. paniculata*.

[a]Extract	[b]TPC (µg GAE/mg extract)		[c]DPPH (µg TE/mg extract)		Scavenging activity index
	mean	[d]SD	mean	[d]SD	(mg extract/mg Trolox)
YL	1.43	0.0032	36.00	0.0000	28.5
YS	1.64	0.0280	40.89	0.3849	25.2
ML	1.13	0.0056	20.89	1.3878	47.8
MS	1.51	0.0000	52.89	0.3849	19.6

[a]YL, YS, ML and MS are denoted for young leaf, young stem, mature leaf and mature stem, respectively.
[b]Total phenolic content in µg of gallic acid equivalents in a mg of extract.
[c]DPPH scavenging activity in µg of Trolox equivalent in a mg of extract.
[d]Standard deviation of triplicate data.

The capacity of scavenging activity of plant extracts was also compared with the standard chemical Trolox. The comparison was carried out by using the scavenging activity index, which is defined as the amount of plant extract required to exhibit the same scavenging activity as a milligram of Trolox. It means that the lower the value of the index, the higher the scavenging activity of the plant extract. The scavenging activity index of the leaves and stems from mature plant extracts was the highest (47.8) and lowest (19.6) value, respectively. Therefore, the stems of mature plants exhibited the highest scavenging activity (52.89 µg TE/mg extract), and the leaves of mature plants the lowest (20.89 µg TE/mg extract) among the plant extracts.

A high sensitivity hyphenated liquid chromatograph integrated with a tandem mass spectrometer was used to identify phytochemicals from different parts of the methanolic *A. paniculata* extracts. Sixteen bicyclic diterpenoids, six flavonoids (2 flavanones and 4 flavones), and eight cyclic and phenolic acids were detected in this study. The compounds and their fragment ions are tabulated in Table 2. The presence of these metabolites was confirmed by referring the data to the theoretical fragments generated by MS Fragmenter 12.0 and the spectral data from literature values [18].

Table 2 also shows that bicyclic diterpenoids were mostly detected in the leaves, in particular the higher molecular weight glycosidic diterpenes from the leaves of mature plants. However, cyclic and phenolic acids were mostly detected from the leaves of young plants. It is interesting to note that andrographolide was only detected in the leaf extracts. This finding was not in line with the previous result reporting the existence of andrographolide in all parts of the plant, maximally in the leaves [19]. The contradictory finding might be due to geographical and climatic variation of plant origin. However, Arpini *et al.* [20] reported no significant difference between the chemical fingerprints of the aerial parts of plants from India and China.

In addition to the detection of nine known bicyclic diterpenoids, seven other diterpenoidal derivatives were detected from the plant grown in Malaysia (Table 2). Of the flavonoids, three (flavones) are reported for the first time for *A. paniculata*. Seven cyclic and phenolic acids are also reported for the first time for *A. paniculata*. However, the presence of these compounds needs to be confirmed, for example by nucleus magnetic resonance spectroscopy.

Hierarchical clustering analysis (HCA) was carried out based on the presence and absence of phytochemicals identified from the plant extracts, without considering the peak signal intensity of the metabolites (Figure 2). This kind of chemometric evaluation has been used by researchers from India who revealed that it is a useful complementary technique for quality determination of *A. paniculata* from different origins [21]. Significantly, the leaf extracts of both young and mature plants were in the same cluster, whereas the stem extracts were in another (Figure 2a). This observation could be explained by high similarity in the phytochemical profile of the same part of the plant, even at different plant ages. However, the dendrogram of variables segregated the identified phytochemicals into three major groups, as presented in Figure 2b. Mostly phenolic acids and flavonoids were clustered in the same group, whereas diterpenoids and their glycosides were grouped in the same class. Another group of metabolites consisted of the mixture of diterpenoids and phenols. The phytochemicals in the same class most probably are involved in a similar pathway of secondary metabolism. This is because these compounds are important secondary metabolites for plant defense. Both mevalonic acid (MVA) and methyl erythritol phosphate (MEP), and deoxyxylulose (DXP) pathways have been suggested for the biosynthetic routes to the production of andrographolide [22].

Another unsupervised multivariate analysis, principle component analysis (PCA), has been used to reduce the multidimensional mass spectral data into 12 principle components using Pareto scaling. The setting parameters were minimum spectral peak width, 0.3 Da; mass tolerance, 0.1 Da and retention time tolerance, 0.1 min. The first four components were covered for 51.1% of the total variation. In line with the pattern recognition result of HCA, the leaf and stem extracts were significantly clustered into two major groups; the positive and negative regions, respectively in the first principle component (PC1) of the score plot, regardless of the plant age (Figure 3a). In the positive region of the second principle component (PC2), the young leaf extracts were further separated from the mature leaf extracts. Thus, the plant extracts divided into three groups, namely young leaf extracts, mature leaf extracts, and young and mature stem extracts. This classification agreed with the grouping based on HCA for the identified metabolites (Figure 2b). The first group, consisting of phenolic acids and flavonoids, might be attributed to the young leaf extracts. The second group, consisting of diterpenoids and their glycosides, might be due to the mature leaf extracts. Most probably, the third group, which consisted of a diterpenoid and phenol mixture, was due to the metabolites extracted from the stems of both young and mature plants. The observation also shows that the constituents of the stem extracts share the nearest similarity in their phytochemical profiles, regardless of plant age. From the loading plot (Figure 3b) and the "compare" function of ACD software under the module of SpecManager 12.01, the marker ion for the mature plant might be *m/z* 557 (dihydroxyl dimethyl 19-[(β-D-glucopyranosyl)oxy]-19-oxo- ent-labda-8(17),13-dien-16,15-olide), whereas the *m/z* 521 ion (propyl neoandrographolide) could be the marker for the leaf extracts.

Table 2: Bicyclic diterpenoids, flavonoids, phenolic acids and cyclic acids detected from the methanolic leaf and stem extracts of both young and mature *Andrographis paniculata*.

Compound Name	Formula	MW (g/mol)	Rt (min)	Fragment ion at negative mode (*m/z*)	[a]YL	[b]YS	[c]ML	[d]MS	References
Bicyclic diterpenoids (M)									
3,19-Dihydroxy-14,15,16-trinor-ent-labda-8(17),11-diene-13-oic acid	$C_{17}H_{26}O_4$	294	13.24	293/275(-H_2O)/249(-C_2H_2)	-	-	√	-	[9]
Andrographolactone	$C_{20}H_{28}O_2$	296	13.80	295/277(-H_2O)/251(-CO_2)/233(-H_2O)/195/171/165	-	√	√	√	[26]
14-Deoxy-11,12 didehydroandrographolide	$C_{20}H_{28}O_4$	332	7.57	331/303(-CO)/287(-CO_2)/283/269/255/239/213/197/171/145/108/69	√	√	√	√	[7, 27, 28]
3,19-Dihydroxy-ent-labda-8(17),12-dien-16,15-olide	$C_{20}H_{36}O_4$	334	8.74	333/313/285(-CO)/269(-CO_2)/257/241/69	√	-	√	-	[9]
Andrographolide	$C_{20}H_{30}O_5$	350	3.49	349/331(-H_2O)/305(-CO_2)/303(-CO_2H_2)/287(-2OCH_3)/283/273(-2CH_3-CO)/265/253/243/239/245/83/67/55	√	-	√	-	[7, 27, 28]
14-Deoxy-11-hydroxyandrographolide	$C_{20}H_{30}O_5$	350	6.41	349/331(-H_2O)/305(-CO_2)/301(-2CH_3)/287(-2OCH_3)/273/267/255/253/239/213/199/171/145/133/108/83/69	-	√	-	-	[7, 29]
3,18,19-Trihydroxy-ent-labda-8(17),13-diene-16,15-olide	$C_{20}H_{30}O_5$	350	2.91	349/331(-H_2O)/305(-CO_2)/301(-2CH_3)/299/287(-2OCH_3)/283/265/253/239/217/215/199/171/145/83/67/55	√	-	√	-	[9]
Methyl methoxyl andrographolide	$C_{21}H_{32}O_7$	396	7.19	395(+CO+H_2O)/377(+CO)/349(M-H)/331(-H_2O)/303(-CO)/287(-CO_2)/255/239/213	√	√	√	√	The present study
14-Deoxy-11-hydroandrographolide dimer glycoside	$C_{46}H_{68}O_{14}$	844	5.87	843(2M+Glu-2H_2O)/349(M-H)/331(-H_2O)/287(-CO_2)/267/239/213	-	-	√	-	The present study
Neoandrographolide	$C_{26}H_{40}O_8$	480	9.13	479/317(-Glu)/289(-CO)/161(Glu-H)/113/101/85/59/57	√	√	√	-	[7, 27]
Propyl neoandrographolide	$C_{29}H_{46}O_8$	522	10.13	521(+C_3H_6))/479(M-H)/461(-H_2O)/413/401/317(-Glu)/289(-CO)/161(Glu-H)/101/85/59	√	-	√	-	The present study
Methyl methoxyl neoandrographolide	$C_{28}H_{46}O_9$	526	9.74	525(+CH_3+OCH_3)/479(M-H)/317(-Glu)/161(Glu-H)	√	√	√	√	The present study
Propyl neoandrographphplide dimer	$C_{58}H_{91}O_{16}$	1132	10.32	1131(+2CO_2)/1043(2M-H)/521(M-H)/479(-C_3H_6)/461(-H_2O)/413/317(-Glu)/161(Glu-H)/143/59	-	-	√	-	The present study
14-Deoxyandrographiside	$C_{26}H_{40}O_9$	496	8.57	495/333(-Glu)/303(-2CH_3)/285(-H_2O)/161(Glu-H))/113/101/85	-	-	√	-	[27, 29, 30]
Methyl methoxyl 14-deoxyandrographiside	$C_{27}H_{42}O_{11}$	542	8.71	541(+OCH_3+CH_3)/495(M-H)/333(-Glu)/285(-2CH_3-H_2O)/161(Glu-H)/113/101	√	-	√	√	The present study
Dihydroxyl dimethyl 19-[(β-D-glucopyranosyl)oxy]-19-oxo-ent-labda-8(17),13-dien-16,15-olide	$C_{28}H_{46}O_{11}$	558	6.42	557(+2OH+2CH_3)/493(M-H)/449(-CO_2)/331(M-Glu)/303(-CO)/287(-CO_2)/161(Glu-H)/101	-	-	√	√	The present study
Flavavone and Flavones									
2'-Hydroxy-2,4',6'-trimethoxychalone	$C_{18}H_{18}O_5$	314	11.23	313/298(-CH_3)/283(-CH_3)/255(-CO)/211(-CO_2)/183(-CO)/165/137	-	-	-	√	[31]
7-*O*-Methyldihydrowogonin	$C_{17}H_{16}O_5$	299	15.10	299/281(-H_2O)/253(-CO)/169	√	√	-	√	[32]
Dihydroskullcapflavone I	$C_{17}H_{16}O_6$	316	12.96	315/297(-H_2O)/229/155/113	-	-	-	√	[31]
7-*O*-Methylwogonin	$C_{17}H_{14}O_5$	298	14.30	297/279(-H_2O)/261(-H_2O)/183(-benzyl)	√	√	√	√	[33]
Apigenin-7-*O*-glucoronide	$C_{21}H_{18}O_{11}$	446	7.01	445/269(-Gln)/225(-CO_2)/183/149/117/85	-	-	√	-	The present study
Luteolin-7-*O*-glucoronide	$C_{21}H_{18}O_{12}$	462	7.40	461/285(-Gln)/255(-OCH_2)/241(-CO_2)/213(-CO)/187/145	-	-	√	√	The present study
Diosmetin-7-glycoside	$C_{21}H_{18}O_{12}$	462	9.10	461/299(-Glu)/269(-2CH_3)/241(-CO)/184/165/137	-	√	-	-	The present study
Cyclic and Phenolic acids									
Dihydroxyl glucosyl cyclohexane	$C_{17}H_{26}O_4$	294	1.16	293/131(-Glu)/113(-H_2O)/85(-CO)/59	√	-	√	√	The present study
Quinic acid	$C_7H_{12}O_6$	192	1.55	191/173(-H_2O)/127(-CH_3-OCH_3)/108/93/87/85/67	√	√	√	√	The present study
Coumaroylquinic Acid	$C_{16}H_{18}O_8$	338	1.74	337/191(quinic acid)/173(-H_2O)/163(coumaric acid)/119/93/85	√	-	-	-	The present study
Caffeoylquinic Acid (Chlorogenic acid)	$C_{16}H_{18}O_9$	354	1.75	353/191(quinic acid)/179(caffeic acid)/173(-H_2O)/135/127/93/85	-	-	-	√	[34]
Feruloylquinic acid	$C_{17}H_{20}O_9$	368	7.35	367/337(-COH_2)/323(-CO_2)/305(-H_2O)/293(-COH_2)/257(-2H_2O)/241/229/217/69	-	√	-	-	The present study
Caffeic glycoside	$C_{15}H_{18}O_9$	342	0.97	341/179 (-Glu)/161(Glu-H)/143(-H_2O)/113(-2CH_3)/101/59	√	-	√	-	The present study
Hydroxyl cyclohexyl chlorogenic acid	$C_{22}H_{28}O_{10}$	452	1.36	451/405(-CO_2H_2)/353(chlorogenic acid)/345/191(quinic acid)/179(caffeic acid)/165/139/59	-	-	-	√	The present study
Chlorogenic glycoside	$C_{22}H_{28}O_{14}$	516	6.80	515/353(-Glu)/255/203/191(quinic acid)/179(caffeic acid)/173(-H_2O)/135/137/93/67	√	-	-	-	The present study

[a]YL : young leaf extracts; [b]YS: young stem extracts; [c]ML: mature leaf extracts; [d]MS: mature stem extracts.

The simultaneous identification of plant metabolites using the high throughput HPLC-ESI-MS/MS approach has revealed that different parts of *A. paniculata* have different phytochemical profiles. The difference could also be seen for the same plant part at different plant ages. However, the difference was not significant for the stems of young and mature plant extracts based on HCA and PCA. Besides that, a difference has also been proven in total phenolic content and free radical scavenging activity of the plant extracts. The stem extracts of the plant were reported to have higher total phenols and radical scavenging activity than the leaf extracts. The degree of difference became significant as the plant grew. More glycosidic diterpenoid lactones were detected in the leaves of the mature plant extract. This observation was in line with the previous findings that the antioxidant property of flavonol aglycones was higher than their glycosides [23, 24].

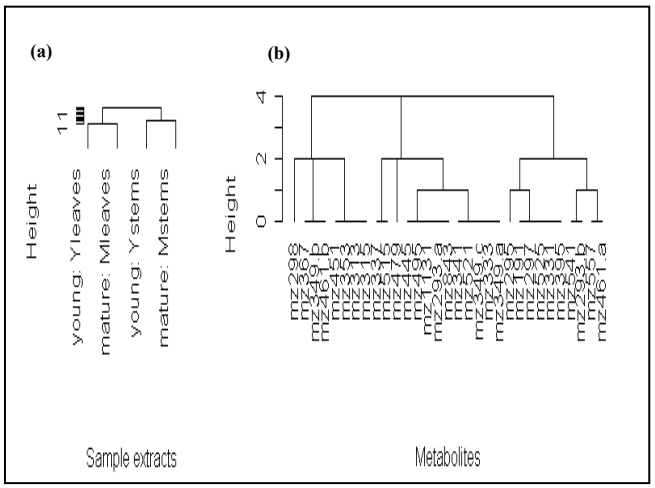

Figure 2: Hierarchical clustering analysis of plant extracts (a) and identified metabolites (b).

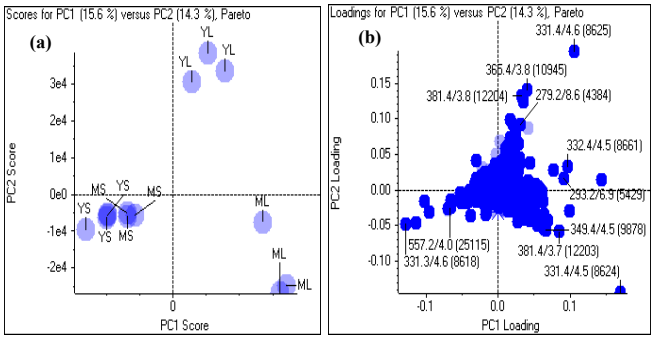

Figure 3: Score (a) and loading (b) plots of mass spectral data from various parts of plant extracts. YL: young leaves; YS: young stems; ML: mature leaves; MS: mature stems.

Experimental

Chemicals and reagents: HPLC-grade of acetonitrile and methanol were obtained from Fisher Scientific (Pittsburg, USA). Ammonium formate and formic acid were purchased from Fluka (Buchs, Switzerland). Folin-Ciocalteau reagent and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were sourced from Sigma–Aldrich (St. Louis, MO). Gallic acid (98%) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, 97%) were obtained from Acros Organics (Pittsburgh, PA). 18.2 Megaohm^{-cm} water was produced from a Barnstead NANOpure Diamond water purification system (State of Illinois, USA).

Plant material: Wild plant samples of *A. paniculata* were collected and authenticated by the Malaysian Agricultural Research Development Institute (MARDI), Selangor, Malaysia. The voucher specimen (MARDI 4770) has been deposited in the Herbarium of MARDI. The plant samples were cleaned and segregated into young (< 4 weeks old) and mature plants (> 6 weeks old). The mature plants usually start flowering when 6 to 8 weeks old. The leaves and stems were separated and air-dried indoors at room temperature for one day. The semi-dried plant samples were pulverized in liquid nitrogen and further freeze-dried for another 2 days at -80^{o}C.

Methanolic extraction of plant samples: The dried and finely powdered leaves (500 mg) and stems (500 mg) of *A. paniculata* were extracted with methanol: water (1:1, 10 mL) using a shaker at room temperature (25^{o}C). After 60 min of extraction, the extracts were filtered and stored at -20^{o}C for subsequent analysis.

Total phenolic content: The total phenolic content (TPC) of the extracts was analyzed using Folin-Ciocalteu reagent, based on the method described by Chua *et al*. [25], with minor modification. The extracts (1.5 mg/mL) were mixed with 1500 µL of Folin–Ciocalteu reagent. Sodium carbonate (100 g/L; 1200 µL) was added to the mixture after 5 min. The solution was shaken thoroughly and incubated for 2 h at 30^{o}C, before the absorbance was measured at 765 nm using a UV-Vis spectrophotometer (Perkin-Elmer Lambda 25, Waltham, MA). Gallic acid (0-200 µg/mL) was used as a standard for calibration curve preparation. The TPC was expressed as µg of gallic acid equivalents (GAE) in mg of dry plant extract. All assays were carried out in triplicate.

Free radical-scavenging activity by DPPH: The antioxidant property of *A. paniculata* extracts was determined by the DPPH (1,1-diphenyl-2-picrylhydrazyl) assay. The plant extracts (1.5 mg) were dissolved in 1 mL methanol and the solutions (0.1 mL) were added to 3 mL of DPPH• solution (1 mM). The absorbance was measured at 515 nm after 30 min of incubation at 30^{o}C. Trolox was used as positive control and prepared in a series of concentrations ranging from 0 – 600 µg/mL for a calibration curve. The ability to scavenge DPPH• was calculated using Eq. (1), where W is the dry weight of either Trolox (standard chemical) or plant extract, and $A_{control}$ and A_{sample} are the absorbance of control and sample, respectively. The equation was used to calculate the scavenging activity index. The experiment was performed in triplicate.

$$\text{Scavenging activity (mg}^{-1}\text{)} = [(A_{control} - A_{sample}) / A_{control}] / W \qquad (1)$$

HPLC–ESI-MS/MS: A hyphenated system consisted of a high performance liquid chromatograph (Agilent 1200; Agilent Technologies Inc., Santa Clara, CA) and a triple quadrupole-linear ion trap tandem mass spectrometer (Applied Biosystems 3200 Q TRAP; Life Technologies Corporation, Carlsbad, CA) was used for phytochemical separation and identification, respectively. The phytochemicals were separated using a C18 reserved phase Aqua column (2.1 x 50 mm, 5µm) from Phenomenex, Torrance, CA, and then ionized by an electrospray ionization source (ESI) before detection. The mobile phase was a binary solvent system consisting of solvent A (water with 0.1% formic acid and 5 mM ammonium formate) and solvent B (acetonitrile with 0.1% formic acid and 5 mM ammonium formate). The gradient was: 0-20 min, 10-90 % B; 20-25 min, 90 % B; 25.1-30 min, 10 % B for column equilibration before the next run. The total runtime was 30 min. The flow rate was 0.2 mL/min and the injection volume was 20 µL. All samples were filtered through a 0.2-µm nylon membrane filter prior to injection. The mass spectra were acquired from *m/z* 100–1200 with a 20-ms ion accumulation time. All mass spectrometric data were acquired in negative ionization mode. The capillary and voltage of the ESI source were maintained at 400^{o}C and 4.5 kV, respectively. All other parameters were as follows: nitrogen was used as ion source gas for nebulisation, 40 psi; for drying solvent, 40 psi; curtain gas, 10 psi; collision gas, high; declustering potential, 40 V, and collision exit energy, 10 V. The scan rate was 1000 amu/s. Data acquisition and data processing were performed using Analyst 1.4.2. The scan mode of enhanced mass spectra (EMS) was used to screen the sample profile. Enhanced product ion (EPI) scan was used to determine the characteristic ions and to confirm the presence of aglycone peaks.

Data processing and interpretation: The data processing software, MarkerView 1.2 (Applied Biosystems/MDS Sciex), was applied to

perform sample classification on triplicate mass spectra of different parts of the plants at different harvest ages by using principle component analysis (PCA). Hierarchical clustering analysis (HCA) was carried out to classify the plant extracts based on sample extracts, as well as identified phytochemicals using R version 2.11.1. The module of MS Fragmenter 12.0 and SpecManager 12.01 in Advanced Chemistry Development (ACD, Toronto, Canada) software was used to predict compound fragmentation and for data mining, respectively.

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Xanthones from aerial parts of *Hypericum laricifolium* Juss.

Irama Ramírez-González[a,*], Juan Manuel Amaro-Luis[b] and Alí Bahsas[b]

[a]*Instituto de Investigaciones, Facultad de Farmacia y Bioanálisis, Universidad de Los Andes, Mérida*
[b]*Laboratorio de Productos Naturales, Departamento de Química, Facultad de Ciencias, Universidad de Los Andes, Mérida, República Bolivariana de Venezuela*

irama@ula.ve

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From the aerial parts of *Hypericum laricifolium* Juss., twelve compounds were isolated and identified. They were the xanthones: 1-hydroxy-7-methoxy-xanthone (**1**), 1,7-dihydroxy-xanthone (**2**), 2-hydroxy-xanthone (**3**), 6-deoxyisojacareubin (**4**), 1,3-dihydroxy-6-methoxy-xanthone (**6**), and 1,5,6-trihydroxy-7-methoxy-xanthone (**7**), together with β-sitosterol, betulinic acid, vanillic acid, isoquercitrin and a mixture of quercetin and isorhamnetin. All the compounds were characterized by spectroscopic and mass spectrometric methods, and by comparison with literature data. Thisis the first report on the presence of xanthones in *H.laricifolium.* 1,3-Dihydroxy-6-methoxy-xanthone has been previously synthesized, but this is the first report of its isolation from a natural source.

Keywords: *Hypericum laricifolium*, Guttiferae, Xanthones, Flavonoids, 1,3-Dihydroxy-6-methoxy-xanthone.

The genus *Hypericum*, family Guttiferae, contains about 475 species, occurring world-wide, which have been included in 36 taxonomic sections on the basis of morphological characters [1]. We have undertaken a phytochemical study of *H. laricifolium* Juss., a sclerophyllous shrub abundant in moor habitats and whose area of distribution extends from western Venezuela to north Peru [2]. In Venezuela, it is traditionally known as "huesito" and is distributed mainly in Mérida State [3]. Several ethnobotanical uses and medicinal properties are attributed to this plant; thus, in Peru and Colombia its flowers are used as a source of natural dyes [4], and in Ecuador the leaves are used for cattle food, the stems to make charcoal, and the wood to build houses, and farming instruments [5]. In Andean traditional medicine, *H. laricifolium* is recognized for its antibacterial, antiviral and antifungal activity [6], and by its efficacy in the treatment of gastrointestinal affections [7] and psychosomatic disorders [8]. A decoction of this plant is drunk to treat cold as well as for treating skeletal pain and as a tranquilizer [5].To the best of our knowledge,there is only one report dealing with phytochemical studies of this species, collected in Ecuador, in which triterpenes, flavonoids and other phenoliccompounds were isolated [9].

Here, we present the results of a chemical study of the aerial parts of *H. laricifolium* gathered in PiedrasBlancas moor (Mérida State, Venezuela). Chromatographic separation of the dichloromethane extract resulted in the isolation of compounds **1**, **2, 3** and **4**, as well as β-sitosterol, betulinic acid and vanillic acid. Compound **1**was identified as 1-hydroxy-7-methoxy-xanthone by comparison of its NMR spectroscopic and mass spectrometric data with those reported in the literature [10a-c]. This xanthone has been reported earlier from other *Hypericum*s pecies [10a-10c]. Spectral data of compound**2** were very similar to those of compound **1.** The only notable differences were the absence of signals attributed to theC-1 methoxyl group and the appearance, in the ^{1}H NMR spectrum, of a new singlet at δ_H: 9.06, assigned to an additional hydroxyl proton (*OH-7*). Detailed analysis of its 2D-NMR spectrum led to the conclusión that **2** is 1,7-dihydroxy-xanthone, which has been previously isolated from several *Hypericum* species [10a,c, 11a,b].The compound **3**, based on ^{1}H,^{1}H-COSY and HMBC data, was identified as 2-hydroxy-xanthone [10a, 11b, 12]. Compound**4** was identified from its NMR and mass spectrometric data as 6-deoxyisojacareubin. To the best of our knowledge this xanthone has been isolated previously from only two *Hypericum* species: *H. japonicum* [11a,13] and *H. wightianum* [14].Treatment of **4** with AcO_2/pyridineyielded 1,5-di-*O*-acetyl-6-deoxyisojacareubin (**5**), which was identified on the basis of its spectral data. From the dichloromethane extract were also isolated betulinic and vanillic acids; both have been previously reported in *Hypericum* species [14-16]. From the metanol extract, the flavonoids isoquercitrin, quercetin and isorhamnetin were isolated and identified by comparison of their spectroscopic data with those reported in the literature.These threef lavonoids have been isolated from other *Hypericum* species [10b,c,17a,b].

Finally, xanthones **6** and **7** were also isolated from this extract. Analysis of ^{1}H NMR and HMBC data identified compound **6** as 1,3-dihydroxy-6-methoxy-xanthone and compound **7** as 1,5,6-trihydroxy-7-methoxy-xanthone. Compound **6** has been previously synthesized [18], but, up to now, has not been reported as a natural product. It is particularly interesting to emphasize that xanthone **6** is *a powerful inductor of apoptosis* in cells transfected with human multidrug-resistance protein 1 (MRP1) [34]. Compound **7** has been reported previously from *Harungana madagascarensis* Lam ex Poir [19a] and *Hypericum ascyron* L. [19b].

Experimental

Plant material: *Hypericum laricifolium* Juss. was collected at paramo Piedras Blancas, Municipio Autónomo Miranda, Estado Mérida, Venezuela, in December 2008. A *voucher specimen* (J. M. Amaro & I. Ramírez, N° 1645) was deposited at Herbarium MERF of the University of Los Andes (ULA).

General: Melting points, Fisher-Johns apparatus; UV, Perkin-Elmer, Lambda 3B spectrophotometer; IR, Perkin-Elmer FT-1725X spectrophotometer; 1D and 2D-NMR, Bruker-Avance DRX400; EI MS, Hewlett-Packard 5930A; TLC, silica gel PF 254; CC, silica gel 60 (70-230 mesh.).

Extraction: Dried leaves and flowers, finely pulverized (≅ 5.7 Kg), were exhaustively extracted with dichloromethane at room temperature. The extract obtained was concentrated *in vacuo* to afford brown oil (≅ 460 g). Subsequently, plant material was further extracted under reflux in a Soxhlet apparatus with methanol and by concentrating the methanolic solution a new extract (≅ 750 g) wasobtained.

Isolation of the constituents: The dichloromethane extract was subjected to vacuum CC on silica gel eluted with *n*-hexane, dichloromethane and acetone, and binary mixtures of these solvents, in order of increasing polarity. Fractions of 1 L were collected, which were concentrated *in vacuo*, analyzed by TLC and suitably combined; 20 fractions were obtained (A-T). Rechromatography of C (≅27 g) on a silica gel column eluted with mixtures of *n*-hexane-acetone (17:3) gave β-sitosterol (≅32 mg), betulinic acid (≅45 mg) and compound **1** (≅38 mg), which precipitated as an amorphous solid, homogeneous by TLC. Fraction D (≅64 g) was rechromatographed on a silica gel column, and fractions eluted with *n*-hexane-acetone (1:1; 2:3) were combined, concentrated *in vacuo* and passed through a column of Sephadex LH-20 affording compounds **2** (≅42 mg) and **4**(≅85 mg).

Concentration of fraction E (≅45 g) provided a solid residue that was partially purified by flash chromatography on silica gel developed with *n*-hexane-acetone (4:1). Final purification, which was carried out by preparative TLC on silica gel plates eluted with *n*-hexane-acetone (4:1), gave pure compound **3** (≅ 35 mg). Rechromatography of fraction F (≅18 g) on a silica gel column, using *n*-hexane-acetone (2:3) as eluant, afforded pure vanillic acid, which was crystallized from methanol as white needles (≅ 22 mg). A sample of solid, crude, methanol extract (≅30 g) was redissolved in methanol and a portion of this, which remained insoluble (≅ 5.5 g), proved to be a mixture of quercitin and isorhamnetin, which were identified by means of ^{1}H NMR analysis. The soluble portion was concentrated and subjected to chromatography on a Sephadex LH-20 column eluted with methanol. Twenty fractions were collected: Fraction 6, after concentration to dryness, gave a residue (≅ 65 mg) that was crystallized from methanol and identified by NMR as quercitrin (≅ 65 mg). In the same way, concentration of fractions 7 and 8 provided two different yellow solids, **6** (≅ 8 mg) and **7** (≅ 15 mg), respectively.

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A New Xanthone from the Pericarp of *Garcinia mangostana*

Manqin Fu[a], Samuel X. Qiu[b], Yujuan Xu[a], Jijun Wu[a], Yulong Chen[a], Yuanshan Yu[a] and Gengsheng Xiao[a,*]

[a]*Guangdong Open Access Laboratory of Agri-food Processing*, *Sericulture and Agri-food Research Institute*, *Guangdong Academy of Agicultural Sciences*, *Guangzhou 510610*, *P.R. China*

[b]*Key Laboratory of Plant Resources Conservation and Sustainable Utilization*, *South China Botanical Garden*, *Chinese Academy of Sciences*, *Guangzhou 510650*, *P.R. China*

fumanqin84@126.com

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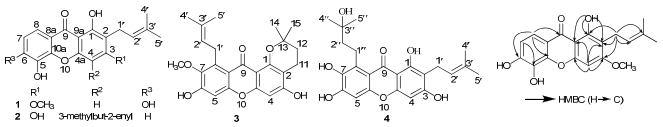
A new prenylxanthone, garcimangostanol (**1**), was isolated from the EtOAc-soluble partition of the ethanol extract of the pericarp of *Garcinia mangostana* L., along with three known compounds, namely 8-deoxygartanin (**2**), 1-isomangostin (**3**), and garcinone C (**4**). The structure of compound **1** was elucidated on the basis of its 1D, 2D NMR and MS data. Compounds **1-4** exhibited either significant or moderate cytotoxicity against MCF-7, A549, Hep-G2 and CNE human cancer cell lines *in vitro* with IC_{50} values from 4.0 ± 0.3 to 23.6 ± 1.5 μM by MTT colorimetric assay.

Keywords: *Garcinia mangostana*, Xanthone, Cytotoxicity.

Garcinia mangostana L. (family Clusiaceae), commonly known as mangosteen, is wildly distributed in Indonesia, Thailand, Myanmar and some other Southeast Asian countries. Its pericarp has long been used as anti-inflammatory agent, astringent and indigenous medicine for treatment of skin infections, wounds and diarrhea in Southeast Asia [1]. Earlier chemical studies on mangosteen pericarp revealed a series of xanthone derivatives, which showed antioxidant, anti-inflammatory, antifungal, and antitumor activities [2]. In this article, we report the isolation and structural elucidation of a new prenylated xanthone (**1**), along with three known xanthones: 8-deoxygartanin (**2**) [3a], 1-isomangostin (**3**) [3b], and garcinone C (**4**) [3c] from the EtOAc-soluble partition of the ethanol extract of the pericarp of *G.mangostana*. In addition, the cytotoxicity is also described of compounds **1-4** against human breast carcinoma (MCF-7), human lung cancer (A549), human hepatoma (Hep-G2) and human carcinoma of nasopharynx (CNE) cell lines, tested by MTT colorimetric assay [4].

Garcimangostanol (**1**), a yellow amorphous powder, has the molecular formula $C_{19}H_{18}O_6$, as deduced from the HREI mass spectrum (*m/z* 342.1099 $[M]^+$) and ESIMS (*m/z* 343 $[M + H]^+$), as well as the NMR spectral data (Table 1). The ^1H NMR spectrum indicated the characteristic signal pattern of a prenyl moiety at δ 1.64 (3H, s, H-4'), 1.78 (3H, s, H-5'), 3.34 (2H, d, *J* = 7.6 Hz, H-1'), and 5.22 (1H, m, H-2'), which was further supported by the ^{13}C NMR spectral signals at δ 17.8 (C-4'), 21.8 (C-1'), 25.8 (C-5'), 123.1 (C-2'), and 131.7 (C-3'). The ^1H NMR spectrum also revealed an intramolecularly hydrogen-bonded hydroxyl group at δ 13.31 (1H, s, OH-1), an aromatic proton at δ 6.60 (1H, s, H-4), two sets of coupled doublets at δ 7.01 (1H, d, *J* = 8.8 Hz, H-7) and 7.65 (1H, d, *J* = 8.8 Hz, H-8), and a methoxyl group [δ 4.00 (3H, s, 3-OCH_3)]. The ^1H and ^{13}C NMR chemical shifts of **1** were closely similar to those of dulxanthone-A [5], except that an aromatic proton at C-2 and a prenyl group at C-4 in dulxanthone-A were replaced by an aromatic proton at C-4 and a prenyl group at C-2 in compound **1**. This structure assignment for **1** was confirmed by its HSQC and HMBC spectra (Figure1b). The HMBC correlations from H-4 to C-4a (δ 157.0), C-9a (δ 103.3), C-2 (δ 112.0), and C-3 (δ 164.9), from H-1' (δ 3.34) to C-2', C-3', C-1 (δ 160.3), C-2, and C-3, and from 1-OH (δ 13.31) to C-1, C-2, and C-9a were indicative of the connectivity of the aromatic proton at C-4 and the prenyl group at C-2. Thus, the structure of **1** was determined as 1,5,6-trihydroxy-2-(3-methylbut-2-enyl)-3-methoxyxanthone.



(a) (b)

Figure 1:(a)Structures of compounds **1**–**4**; (b)Key HMBC correlations of compound **1**.

Table 1: ^1H and ^{13}C NMR spectroscopic data of compound **1** in acetone-d_6.

position	δ_H	δ_C
1		160.3
2		112.0
3		164.9
4	6.60(s)	90.7
5		133.3
6		152.1
7	7.01(d, *J* = 8.8)	113.6
8	7.65(d, *J* = 8.8)	117.3
9		181.3
4[a]		157.0
8[a]		114.8
9[a]		103.3
10[a]		147.0
1′	3.34(d, *J* = 7.6)	21.8
2′	5.21-5.23(m)	123.1
3′		131.7
4′	1.64(s)	17.8
5′	1.78(s)	25.8
3-OMe	4.00(s)	56.6
1-OH	13.31(s)	

Table 2: Cytotoxicity (IC_{50}, μM) of compounds **1-4** against tumor cell lines[a]

Compounds	MCF-7	A549	Hep-G2	CNE
1	14.1 ± 1.1	11.9 ± 0.8	13.5 ± 1.0	4.2 ± 0.2
2	16.8 ± 1.4	15.1 ± 1.2	12.7 ± 0.5	4.0 ± 0.3
3	18.6± 1.6	23.6 ± 1.5	15.6 ± 1.2	5.7 ± 0.3
4	7.1 ± 0.5	4.9 ± 0.8	4.3 ± 0.1	6.6 ± 0.2
Doxorubicin[a]	3.4 ± 0.1	3.7 ± 0.2	3.5 ± 0.1	2.3 ± 0.1

[a]Each value represents mean ± SD, and doxorubicin was used as a positive control.

Compounds **1-4** were tested for their cytotoxicity against MCF-7, A549, Hep-G2 and CNE cell lines by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay, in which

doxorubicin was used as a positive control. The IC_{50} values of compounds **1**-**4** on the viability of the test cancer cell lines after 72 h of incubation are presented in Table 2. The results showed that compounds **1**-**3** were moderately cytotoxic against MCF-7, A549, and Hep-G2 cell lines with IC_{50} values from 11.9 ± 0.8 to 23.6 ± 1.5 μM, and significantly cytotoxic against CNE cells with IC_{50} values of 4.2 ± 0.2, 4.0 ± 0.3, and 5.7 ± 0.3 μM, respectively. Moreover, compound **4** showed strong cytotoxicity against all the tested cell lines with IC_{50} values from 4.3 ± 0.1 to 7.1 ± 0.5 μM.

Some structural features apparently contribute to the potent inhibitory activity of xanthones, such as isopentenyl cyclization lowered the inhibitory activity (compound **3**), and the hydrated form, 3-hydroxy-3-dimethyl butanyl (compound **4**), enhanced the inhibitory activity. The remarkable cancer cell anti-proliferative activities of compounds **1**-**4** has shed some light on the active ingredients and action mechanism supportive of the beneficial properties and rationale for its use as a folk medicine.

Experimental Section

General: The following instruments were used to obtain physical data: Bruker DRX-400spectrometer (400 MHz for ^{1}H NMR, 100 MHz for ^{13}C NMR); Perkin-Elmer Lambda 35 UV-vis, WQF-410 FT-IR spectrophotometer; MDS SCIEX API 2000 LC/MS/MS, MAT95XP mass spectrometer;XT-4 Boetiusmicromelting point apparatus; CC, RP-C_{18}, Sephadex LH-20; TLC: pre-coatedsilica gel GF254;Doxorubicin and MTT were purchased from Sigma Chemical Co.(St. Louis, MO).

Plant material: The pericarp of *G. mangostana* was obtained by peeling the fruits bought from a local grocery store in Fengyang, China, which were imported from Thailand in August 2012. The plant material was identified by Prof. Yulian Wang (Anhui Science and Technology University, China). A voucher samplewas deposited at the herbarium of Anhui Science and Technology University.

Extraction and isolation: The powdered dry pericarps of *G. mangostana* (2 kg) were extracted 3 times with 95% ethanol (5 L × 3) at room temperature, for 3 days each. After evaporation of the solvent *in vacuo*, the combined crude ethanolic extract (120 g) was partitioned with EtOAc (2 L× 3) to afford the EtOAc (50 g) extract. This was subjected to a silica gel CC and eluted with light petroleum–acetone with increasing polarities (10:1−1:1) to yield 8 fractions (1-8). Fraction 3 (4.5 g) was subjected to silica gel CC using light petroleum−EtOAc (8:1-1:1) to afford **1** (15 mg). Fraction 4 (2.4 g) was subjected to Sephadex LH-20 CC and eluted with MeOH to obtain **2** (25 mg). Fraction 5 (3.5 g) was separated by ODS CC eluting with 80% MeOH, followed by silica gel using light petroleum−acetone (90:10-50:50) to yield **3** (12 mg) and **4** (30 mg).

Garcimangostanol (**1**)
Yellow amorphous powder.
MP: 183 - 185°C.
UV(MeOH): λ_{max} (log ε): 254 (4.31), 284 (3.96), 328 (4.12) nm.
IR (KBr): 3545, 1645, 1587 cm^{-1}.
^{1}H NMR and ^{13}C NMR: Table 1.
MS ((+)-ESI): *m/z* = 343 $[M+H]^+$.
HR-EI-MS: 342.1099 (100) (calcd. 342.1098 for $C_{19}H_{18}O_6$, $[M]^+$).

Cell culture: MCF-7, A549, Hep-G2, andCNE cell lines were cultured as monolayers in nutrient RPMI 1640 medium supplemented with streptomycin (100 μg mL^{-1}), penicillin (100 U mL^{-1}), and 10% heat inactivated (56 °C) fetal bovine serum (FBS) at 37°C in a 5% CO_2 humidified incubator.

Cytotoxicity assay: Cells were plated at 1 × 10^4 cell/well in 96-well microtiter plates and allowed to adhere overnight. Stock solutions of compounds (10 mM) were dissolved in dimethylsulfoxide (DMSO).The cells weretreated in triplicate with various concentrations (50, 25, 12.5, 6.25, 3.125, 0 μM) of test samples, and doxorubicin as the positive control for 48 h. At the end of the incubation time, 20 μL MTT reagent (5 mg/mL) was added to each well for 4 h, and the resulting crystals were dissolved in DMSO. Absorbance (A) was measured in an ELISA plate reader (TECAN, Austria) at a wavelength of 570 nm. Data were calculated as a percentage of inhibition using the following formula: Inhibitory ratio (%) = $(A_{control}-A_{sample})/(A_{control}-A_{blank})\times100\%$. A_{sample}, $A_{control}$ and A_{blank}refer to the absorbance of the sample, control (cells grown only in nutrient medium) and blank (no cells), respectively. IC_{50} concentration was defined as the concentration of an agent inhibiting cell survival by 50%, compared with a vehicle-treated control.

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Isolation of a Phomoxanthone A Derivative, a New Metabolite of Tetrahydroxanthone, from a *Phomopsis* sp. Isolated from the Mangrove, *Rhizhopora mucronata*

Yoshihito Shiono[a], Takehiro Sasaki[a], Fumiaki Shibuya[a], Yukito Yasuda[a], Takuya Koseki[a] and Unang Supratman[b]

[a]*Department of Food, Life, and Environmental Science, Faculty of Agriculture, Yamagata University, Tsuruoka, Yamagata 997-8555, Japan*

[b]*Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran, Jatinangor 45363, Indonesia*

yshiono@tds1.tr.yamagata-u.ac.jp

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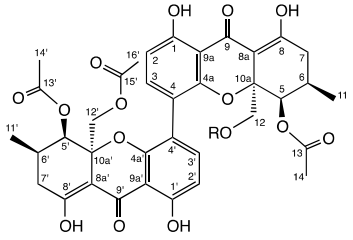
From the rice culture of *Phomopsis* sp. IM 41-1, isolated from the mangrove plant, *Rhizhopora mucronata*, two dimeric tetrahydroxanthones, phomoxanthone A (**1**) and 12-*O*-deacetyl-phomoxanthone A **(2)** were obtained. Their structures were determined by spectroscopic and spectrometric methods. Both compounds exhibited antimicrobial activities.

Keywords: Endophyte, Mangrove, *Phomopsis* sp., Phomoxanthone, Antimicrobial activity.

The interaction of endophytes, fungi that exist in a symbiotic relationship within the intracellular space of plants without causing apparent harm to their host plants, has received considerable attention. Endophytes produce bioactive substances that are known to enhance growth of the host plant and provide protection against pathogens and herbivores. These fungi have proven to be a promising source for the production of structurally novel and pharmacologically active secondary metabolites [1]. Our previous investigation of endophytic fungi isolated from plants in Japan resulted in the isolation of some bioactive and structurally unique metabolites [2]. In our continuing efforts to discover biologically active compounds among the secondary metabolites of endophytes, we have investigated the fungal strain *Phomopsis* sp. IM 41-1 isolated from *Rhizophora mucronata,* a mangrove plant collected from a forest in Muara Angke, Jakarta, Indonesia. *R. mucronata* (local name: Bakau Hitam) has found use in folk medicine for treatment of diarrhea, dysentery, fever, malaria, and leprosy [3]. Examination of the rice culture of *Phomopsis* sp. IM 41-1 resulted in the isolation of two dimeric xanthone-type compounds, **1** and **2**. In this report, we describe the characteristics and fermentation products of the producing strain, as well as the isolation, structure elucidation and biological characterization of the two compounds.

Steamed unpolished rice was used as the substrate for the cultivation of the endophyte, *Phomopsis* sp. IM 41-1. The culture medium was extracted with methanol. The extract was concentrated, and the aqueous residue extracted with ethyl acetate. The EtOAc layer was subjected to silica gel column chromatography eluting with a *n*-hexane–EtOAc gradient. Purification of the compounds was guided by their characteristic coloration on TLC. Further chromatographic studies yielded two pure compounds: phomoxanthone A (**1**) [4] and a new phomoxanthone A derivative (**2**).

Compound **2** was obtained as a yellow powder, and its molecular formula was determined to be $C_{36}H_{36}O_{15}$ by HRESITOFMS. The IR



1 : R = -^{15}C(O)^{16}CH$_3$
2 : R = H

Figure 1: Structures of compounds **1** and **2**.

spectrum of **2** exhibited absorptions due to hydroxyl, carbonyl, and aromatic functions, whereas the UV spectrum showed the characteristic absorption pattern of a phomoxanthone derivative (λ_{max} 340 and 221 nm). The NMR spectrum of **2** also showed two sets of signals at the same position that were either completely overlapped or separated. The ^{13}C NMR and DEPT experiments revealed the presence of 36 carbons: four methylene, eight methine, five methyl, and 19 quaternary (sp^2 carbons also included three esters and two carbonyls). The ^{1}H NMR spectral data revealed the presence of five methyl groups [δ_H 1.07 (total 6H, d, J = 6.0 Hz, Me-11, 11') and 2.10 (total 9H, s, Me-14, 14' and 16')], of which three were singlets and two doublets; four aromatic protons [δ_H 6.47 (1H, d, J = 8.0 Hz, H-2), 6.51 (1H, d, J = 8.0 Hz, H-2'), 7.40 (1H, d, J = 8.0 Hz, H-3), 7.41 (1H, d, J = 8.0 Hz, H-3')], two oxymethylenes [δ_H 3.55 (1H, d, J = 12.2 Hz, H-12), 4.10 (1H, d, J = 12.2 Hz, H-12), 4.18 (1H, d, J = 12.5 Hz, H-12'), 4.50 (1H, d, J = 12.5 Hz, H-12)], two oxymethines [δ_H 5.56 (1H, s, H-5) and 5.75 (1H, s, H-5')] and two exchangeable downfield protons [δ_H 11.8 (1H, s, OH-1), 11.9 (1H, s, OH-1'), 13.1 (1H, s, OH-8) and 14.1 (1H, s, OH-8')]. These signals were assigned based on the COSY spectrum and compared with the ^{1}H NMR signals of **1**. These results suggested that **2** was a nonsymmetrical dimer similar to **1**. Analysis of the 1D and 2D NMR data of **2** suggested that it

Analysis of the 1D and 2D NMR data of **2** suggested that it consisted of one monomer identical to that of **1**, but with the other one lacking the substitution pattern of the acetoxyl moiety. The plane structure of **2** was determined as shown in Fig. 2 by HMBC analysis. NOE experiments (Fig. 2) exhibited key correlations from H_3-11 to H-5, from H-12 to H-6, from H_3-11' to H-5' and from H-12' to H-6', implying the same relative configuration as **1**. Finally, the ^{1}H and ^{13}C NMR spectra of **1** corresponded with those of the acetylated product obtained by acetylation of **2** with Ac_2O/pyridine, providing further evidence for the structure of **2**. The relative configuration at C-5, C-6, and C-10a in the acetylation product of **2** was considered to be the same as that in **1**, because protons and carbons at C-5, C-6, C-7, and C-12 in the acetylation product of **2** had chemical shifts and coupling constants quite similar to those of **1**. Based on these results, the structure assigned to **2** was 12-*O*-deacetyl-phomoxanthone A (**2**) (Fig. 1). In addition, the possibility of **2** being an artifact of **1** produced during the separation could be excluded since **1** was stable for one week in MeOH and silica gel.

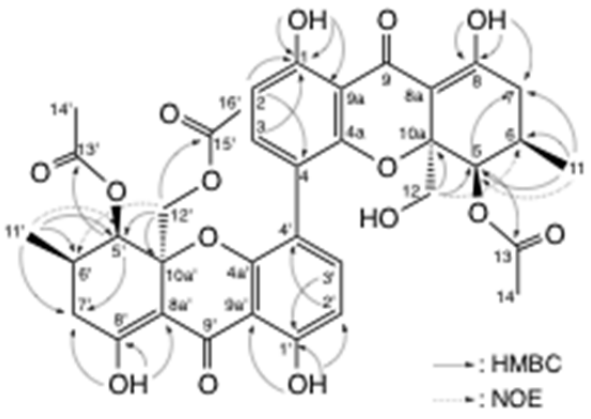


Figure 2: Selected HMBC and NOE correlations observed for **2**.

The activities of compounds **1** and **2** against Gram-positive and Gram-negative bacteria and fungal strains were evaluated using the agar diffusion method (Table 1). Both compounds exhibited moderate activity against *Botrytis cinerea*, *Sclerotinia sclerotiorum*, *Diaporthe medusaea*, and *Staphylococcus aureus* at a concentration of 30 μg/disk. Both compounds lacked activity against the Gram-negative bacterium *Pseudomonas aeruginosa.* Although the synthetic products are very effective in the assay system, the antimicrobial activity of compounds **1** and **2** produced by *Phomopsis* sp. IM 41-1 could play an important role in the protection of the host plant from degradation and disease caused by pathogens.

Table 1: Antimicrobial activities of compounds **1** and **2**

Microorganisms	**1**[a]	**2**[a]	Control [b]
Trichoderma harzianum NBRC 33016	ND	ND	15
Verticillium dahliae Klebahn NBRC 9470	ND	ND	20
Sclerotinia sclerotiorum de Bary NBRC 103652	13	12	15
Diaporthe medusaea Nitschke NBRC 30895	11	ND	10
Botrytis cinerea Persoon NBRC 100717,	11	11	20
Staphylococcus aureus NBRC 13276	9	9	32
Pseudomonas aeruginosa ATCC 15442	ND	ND	18

[a]Diameter of the inhibition areas (mm) using the plate diffusion assay (30 μg of each tested compound soaked in a 8 mm filter disk). [b] The positive control, thiabendazole was used for *T. harzianum*, *V. dahliae*, *S. sclerotiorum*, *D medusaea*, and *B. cinerea*, penicillin G (1.3 μg/disk) for *S. aureus*, and chloramphenicol (10 μg/disk) for *P. aeruginosa*. ND: not detectable.

Previous published reports have investigated biological activities of these compound types, including antimicrobial, antimalarial, antitubercular and cytotoxic activities. Although phomoxanthones A and B exhibited some activity, *O*-5,12,5',12'-tetradeacetyl-phomoxanthone A did not show any in these tests [4, 5]. These previous reports suggested that the acetylated moieties had a significant effect on biological activities, whereas the activity of **1** was linked to its lipophilicity. In the present study, the differences in the antimicrobial activities between **1** and **2** were small. Further pharmacological studies of **1** and **2** are currently in progress to better reveal the effect of the acetylation.

Experimental

General: Optical rotation values were measured with a Horiba SEPA-300 polarimeter, and IR and UV spectra were respectively recorded with Jasco J-20A, Shimadzu UV mini-1240, and Jasco J-20A spectrophotometers. Mass spectra were obtained with Jeol JMS-700 and Synapt G2 mass spectrometers. NMR data were recorded on a Jeol EX-400 spectrometer at 400 MHz for ^{1}H and 100 MHz for ^{13}C. Chemical shifts are given on a δ (ppm) scale with TMS as an internal standard. ^{1}H, ^{13}C, DEPT, COSY, HMQC and HMBC spectra were recorded using standard Jeol standard pulse sequences.

Isolation procedure: The fungal strain *Phomopsis* sp. IM 41-1 was cultivated on sterile steamed unpolished rice (total 1000 g, 20 g / Petri dish x 50) at 25˚C for 4 weeks. The rice was extracted with MeOH (5 L), and the extract concentrated. The resulting aqueous concentrate (0.5 L) was partitioned with *n*–hexane (0.5 L), and EtOAc (1.5 L). Each fraction was concentrated to dryness to yield the *n*–hexane (600 mg), EtOAc (500 mg) and aqueous soluble residues (1.5 g). Purification of the EtOAc soluble fraction was monitored on TLC plates by the characteristic intense blue coloration with 10% vanillin in H_2SO_4. The EtOAc soluble fraction was chromatographed on a silica gel column using stepwise elution with *n*-hexane: EtOAc (100:0-0:100) and EtOAc: MeOH (50:50-0:100), respectively, to afford fractions 1-1 to 1-13. Fractions 1-3 and 1-4 were combined and further chromatographed on a silica gel column using $CHCl_3$: EtOAc (80:20) to yield phomoxanthone A (**1**, 30.0 mg) and 12-*O*-deacetyl-phomoxanthone A (**2**, 19.0 mg).

12-*O*-Deacetyl-phomoxanthone A (2)

Yellow amorphous powder.

$[\alpha]_D^{20}$: +40.0 (*c* 0.65, $CHCl_3$).

UV/Vis (MeOH) nm (log ε) : 340 (4.2), 221 (4.0).

IR (KBr): 3421, 2927, 1747, 1608, 1437, 1373, 1230, 1049, 883 cm^{-1}.

^{13}C NMR (100 MHz, $CDCl_3$): δ_C 17.5 (Me-11)[a], 17.6 (Me-11')[a], 20.7 (C-14) [b], 20.8 (C-14')[b], 20.9 (C-16')[b], 27.6 (C-6), 27.7 (C-6'), 33.3 (C-7, 7'), 65.2 (C-12), 65.5 (C-12'), 70.2 (C-5), 70.4 (C-5'), 80.4 (C-10a') [c], 82.4 (C-10a)[c], 100.3 (C-8a) [d], 100.8 (C-8a') [d], 106.3 (C-9a) [e], 106.4 (s, C-9a')[e], 107.9 (C-2), 108.2 (C-2'), 117.7 (C-4), 117.9 (C-4'), 140.1 (C-3, 3'), 157.1 (C-4a)[f], 157.4 (C-4a')[f], 159.3 (C-1), 159.5 (C-1'), 170.3 (C-13) [g], 170.4 (C-13') [g], 170.6 (C-15') [g], 177.8 (C-8), 177.9 (C-8') and 187.8 (s, C-9, 9').

^{1}H NMR (400 MHz, $CDCl_3$): δ_H 1.07 (total 6H, d, *J* = 6.0 Hz, Me-11, 11'), 2.10 (total 9H, s, Me-14, 14', 16'), 2.36-2.47 (overlapped signals, H-6, 6', 7, 7'), 3.55 (1H, m, H-12), 4.10 (1H, d, *J* = 12.2 Hz, H-12), 4.18 (1H, d, *J* = 12.5 Hz, H-12'), 4.50 (1H, d, *J* = 12.5 Hz, H-12'), 5.56 (1H, s, H-5'), 5.75 (1H, s, H-5), 6.47 (1H, d, *J* = 8.0 Hz, H-2), 6.51 (1H, d, *J* = 8.0 Hz, H-2'), 7.40 (1H, d, *J* = 8.0 Hz, H-3), 7.41 (1H, d, *J* = 8.0 Hz, H-3'), 11.8 (1H, s, OH-1), 11.9 (1H, s, OH-1'), 14.0 (1H, s, OH-8) and 14.1 (1H, s, OH-8').

[a-g] Assignments may be interchangeable.

Acetylation of 2: 12-*O*-Deacetyl-phomoxanthone A (**2**, 5 mg) in pyridine (2 mL) was acetylated with acetic anhydride (2 mL) at

water and extracted with EtOAc (5 mL x 3). The organic layer was washed with saturated NaCl and dried over Na_2SO_4 and concentrated *in vacuo* to give a residue, which was purified by silica gel CC to yield the acetate (4 mg) as an amorphous substance. The ^1H and ^{13}C NMR data of this compound were identical to those obtained for natural phomoxanthone A (**1**).

^1H NMR (400 MHz, $CDCl_3$): δ_H 1.90 (6H, s, Me-16, 16'), 1.00 (6H, d, *J* = 5.6 Hz, Me-11, 11'), 2.05 (6H, s, Me-14, 14'), 2.34 (4H, m, H-6, 6', 7, 7'), 2.45 (2H, m, H-7, 7'), 4.17 (2H, d, *J* = 12.7 Hz, H-12, 12'), 4.26 (2H, d, *J* = 12.7 Hz, H-12, 12'), 5.38 (2H, s, H-5, 5'), 6.57 (2H, d, *J* = 8.6 Hz, H-2), 7.34 (2H, d, *J* = 8.6 Hz, H-3, 3'), 11.52 (2H, s, OH-1, 1'), 14.10 (2H, s, OH-8, 8').

^{13}C NMR (100 MHz, $CDCl_3$): δc 17.5 (C-11, 11'), 20.4 (C-16, 16'), 20.6 (C-14, 14'), 27.8 (C-6, 6'), 33.3 (C-7, 7'), 64.5 (C-12, 12'), 70.3 (C-5, 5'), 80.3 (C-10, 10'), 100.1 (C-8a, 8a'), 106.0 (C-9a, 9a'), 109.3 (C-2, 2'), 115.3 (C-4, 4'), 141.2 (C-3, 3'), 153.5 (C-4a, 4a'), 161.5 (C-1, 1'), 169.6 (C-13, 13'), 170.2 (C-15, 15'), 177.4 (C-8, 8'), 187.7 (C-9, 9').

HR-ESI-TOF-MS: *m/z* $[M+Na]^+$ calcd for $C_{38}H_{38}O_{16}Na^+$: 773.2058; found: 773.2040.

Antimicrobial activity: The test microorganisms were *Pseudomonas tolaasii* NBRC 103163, *Trichoderma harzianum* NBRC 33016, *Verticillium dahliae* Klebahn NBRC 9470, *Botrytis cinerea* Persoon NBRC 100717, *Sclerotinia sclerotiorum* (Libert) de Bary NBRC 103652, *Diaporthe medusaea* Nitschke NBRC 30895, *Staphylococcus aureus* NBRC 13276 and *Pseudomonas aeruginosa* ATCC 15442. Antimicrobial assays were carried out by the paper disk diffusion method using a published protocol [6].

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Anti-allergic Inflammatory Effects of Cyanogenic and Phenolic Glycosides from the Seed of *Prunus persica*

Geum Jin Kim[a], Hyun Gyu Choi[a], Ji Hyang Kim[a], Sang Hyun Kim[b], Jeong Ah Kim[c*] and Seung Ho Lee[a*]

[a]*College of Pharmacy*, *Yeungnam University*, *Gyungsan*, *Gyungbuk*, *712-749 Korea*
[b]*School of Medicine*, *Kyungpook National University*, *Daegu*, *702-701 Korea*
[c]*College of Pharmacy*, *Research Institute of Pharmaceutical Sciences*, *Kyungpook National University*, *Daegu*, *702-701 Korea*

jkim6923@knu.ac.kr (J.A.Kim), seungho@yu.ac.kr (S.H.Lee)

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A methanol extract of the seed of *Prunus persica* (Rosaceae) was found to inhibit histamine release in human mast cells. Activity-guided fractionation of the methanol extract yielded three cyanogenic glycosides (**1-3**) and other phenolic compounds (**4-8**). To evaluate their anti-allergic and anti-inflammatory activities, the isolates (**1-8**) were tested for their inhibitory effects on histamine release and on the gene expressions of tumor necrosis factor (TNF)-α and interleukin (IL)-6 in human mast cells. Of these, phenolic glycosides **7** and **8** suppressed histamine release and inhibited the pro-inflammatory cytokines TNF-α and IL-6. These results suggest that isolates from *P. persica* are among the anti-allergic inflammatory principles in this medicinal plant.

Keywords: *Prunus persica*, Cyanogenic glycosides, Phenolic glycosides, Anti-allergic inflammatory activities.

Allergic inflammation affects target cells, such as epithelial, fibroblast, vascular, and airway smooth muscle cells, which become an important source of inflammatory mediators [1a]. Mast cells appear to play a critical role in initiating allergic inflammation, since they are directly activated by allergens via an immunoglobulin E (IgE)-dependent mechanism [1a,1b]. Activated mast cells lead to degranulated histamine, and release of pro-inflammatory and chemotactic cytokines [1b]. Several proinflammatory cytokines have been implicated in allergic diseases, including interleukin (IL)-1β, IL-6, TNF (tumor necrosis factor)-α, and granulocyte-macrophage colony-stimulating factor (GM-CSF), and these may be important for amplifying allergic inflammatory responses [1a,1b].

Prunus persica (L.) Batsch (Rosaceae) is a deciduous tree that bears an edible fruit. Its seeds are used in traditional medicine to treat amenorrhea and rheumatoid arthritis [2a]. Phytochemical studies on this plant have reported various cyanogenic glycosides, glycerides, sterols, and fatty acids [2b-2d]. Furthermore, pharmacological studies on the seeds of *P. persica* have shown they have multiple activities, which include anti-cancer, anti-oxidant, hepatoprotective, antinociceptive, and anti-coagulant, and inhibitory effects on platelet aggregation [2d-2f]. In a previous study, we described the anti-allergic inflammatory effect of the ethanol extract of fruits of *P. persica* in human mast cells [3]. Activity-guided fractionation of the methanol extract led to the isolation of three cyanogenic glycosides, prunasin (**1**), amygdalin (**2**), neoamygdalin (**3**) and five phenolic compounds, dehydroconiferyl alcohol (**4**), dehydroconiferyl alcohol-4'-*O*-β-D-glucopyranoside (**5**), dehydroconiferyl alcohol-10-*O*-β-D-glucopyranoside (**6**), vanilloloside (**7**) and lacticolorin (**8**). Compounds **5**, **7** and **8** were isolated for the first time from this plant.

It is known that the inhibitory effect of mast cells on histamine release plays an important role in the anti-allergic effect [1a]. Therefore, we examined *in vitro* histamine release inhibitory activity using assays of compound 48/80-induced histamine release from human mast cells and pro-inflammatory cytokines inhibitory activity. As shown in Figure 2, compared with the

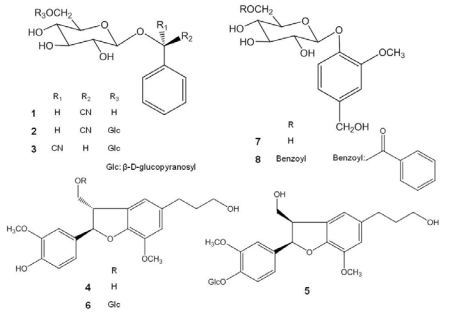

Figure 1: Chemical structures of compounds **1-8** isolated from *Prunus persica.*

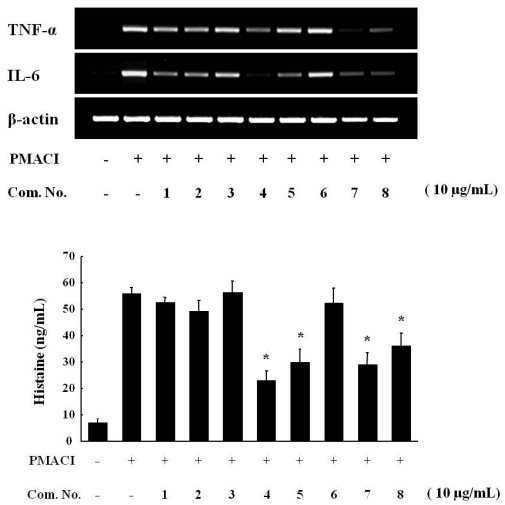

Figure 2: Anti-allergic inflammatory effects of compounds **1-8** isolated from *P. persica* on human mast cells. HMC-1 cells were pre-incubated with compounds **1-8** for 30 min prior to incubation with PMA (20 nM) and calcium ionophore A23187 (1 μM). (A) Effect of compounds **1-8** on the expression of pro-inflammatory cytokines. Extraction and analysis of mRNA was performed as described in Experimental. (B) Effect of compounds **1-8** on histamine release in mast cells. Histamine content was measured using a spectrometer. Each bar represents the mean ± SEM of three independent experiments. *Significant difference at $P < 0.05$.

phorbol-12-myristate 13-acetate plus calcium ionophore A23187 (*PMACI*) control, compounds **4**, **5**, **7** and **8** significantly inhibited histamine release and pro-inflammatory cytokines.

Experimental

Plant material: Seeds of *Prunus persica* were purchased from Yak-Ryung-si market in Daegu, Korea and were identified by Dr Seung Ho Lee at the College of Pharmacy, Yeungnam University. A voucher specimen (YU00163) has been deposited in the Natural Product Laboratory at the College of Pharmacy, Yeungnam University, Korea.

Extraction and isolation: The seeds of *P. persica* (1.2 kg) were pulverized and extracted with MeOH (5.0 L × 3) for one week at room temperature. The solution was evaporated to dryness to obtain 85.0 g of extract. This was loaded onto a silica gel column and eluted with a step-wise gradient mixture of ethyl acetate (EtOAc) and MeOH (1:0 to 0:1) to yield 13 sub-fractions (Fr.3-1 ~ Fr.3-9). Fr.3 (678.7 mg) was further divided into 9 sub-fractions (Fr.1-1 ~ Fr.1-9) using silica gel chromatography and eluting with a step-wise gradient mixture of methylene chloride (MC) and acetone (100:0 to 40:60). Fr.3-5 (75.9 mg) was purified by preparative HPLC (MeOH-H_2O, 3:7 to 7:3; 6 mL/min) to afford (-) dehydroconiferyl alcohol (**4**) (t_R 29.8 min, 4.5 mg) [4a]. Fr.4 (8.5 g) was loaded onto silica gel and eluted with a mixture of MC and MeOH (100:0 to 85:15) to yield 6 sub-fractions (Fr.4-1 ~ Fr.4-6). Purification of Fr.4-2 (113.5 mg) using preparative HPLC (MeOH-H_2O, 40:60 to 65:35; 6 mL/min) yielded (-) lacticolorin (**8**) (t_R 37.4 min, 3.0 mg) [4f]. Fr.5 (2.1 g) was separated on silica gel using a gradient mixture of MC and MeOH (10:0 to 5:5) to afford 9 sub-fractions (Fr.5-1 ~ Fr.5-9). Fr.5-7 (139.9 mg) was further purified by preparative HPLC with a gradient mixture of MeOH and H_2O (from 1:9 to 5:5, 6 mL/min) to afford (+) neoamygdalin (**3**, t_R 23.2 min 69.4 mg) [4b]. Fr.5-8 (755.5 mg) was also purified by preparative HPLC (MeOH-H_2O, 3:7 to 4:6; 6 mL/min) to give (-) dehydroconiferyl alcohol-10-*O*-β-D-glucopyranoside (**6**, t_R 30.8 min, 1.2 mg) [4d]. Fr.6 (15.2 g) was subjected to silica gel vacuum liquid chromatography, and eluted with a gradient mixture of $CHCl_3$/MeOH/H_2O [9:1:0.05, 9:1.5:0.05, 9:2:0.1, 9:3:0.1, 0:10:0 (each 2 L)] to yield (-) amygdalin (**2**, 3.0 g) [4b] and 4 sub-fractions (Fr.6-1 ~ Fr.6-4). Fr.6-3 was fractionated by silica gel chromatography using a gradient of $CHCl_3$ and MeOH (10:0 to 7:3) to yield (-) prunasin (**1**, 50.0 mg) [4b] and 7 sub-fractions (Fr.6-3-1 ~ Fr.6-3-7). Fr.6-3-7 (458.6 mg) was further purified by preparative HPLC (MeOH-H_2O, 1:9 to 6:4; 6 mL/min) to afford (-) vanilloside (**7**, t_R 27.9 min, 5.7 mg) [4e] and (-) dehydroconiferyl alcohol-4'-*O*-β-D-glucopyranoside (**5**, t_R 57.9 min, 21.2 mg) [4c].

Histamine assay: The histamine content was measured by the *o*-phthaldialdehyde spectrofluorometric procedure, as previously described [1b]. The serum was separated from the released histamine by centrifugation at 400 × *g* for 5 min at 4°C. The fluorescent intensity was measured at an excitation wavelength of 355 nm and an emission wavelength of 450 nm using a Perkin-Elmer Fluorescence Spectrometer LS-50B (Norwalk, CT, USA).

Real-time polymerase chain reaction (PCR): Quantitative real-time PCR was carried out using the Thermal Cycler Dice TP850 (Takarabio Inc., Shiga, Japan) according to the manufacturer's protocol. Total RNA was isolated from ear tissues of each group. The conditions for PCR were similar to those previously described. Briefly, 2 μL of cDNA (100 ng), 1 μL of sense and antisense primer solution (0.4 μM), 12.5 μL of SYBR Premix Ex Taq (Takarabio Inc.), and 9.5 μL of D_2O were mixed together to obtain a final 25 μL reaction mixture in each reaction tube. The primers used for PCR were (IL-6 F 5'-AAAGAGGCACTGGCAGAAAA, R 5'-ATCTGAGGTGCCCATGCTAC, TNF-α F 5'-CCTGTAG CCCACGTCGTAGC, R 5'-TTGACCTCAGCGCTGAGTTG). The amplification conditions were 10 s at 95°C, 40 cycles of 5 s at 95°C and 30 s at 60°C, 15 s at 95°C, 30 s at 60°C, and 15 s at 95°C. The normalization and quantification of mRNA expression was performed using the TP850 software supplied by the manufacturer.

Statistical analysis: Statistical analyses were performed using SAS statistical software (SAS Institute, Cary, NC, USA). Treatment effects were analyzed using ANOVA followed by Dunnett's multiple range tests. Significance was set at $P < 0.05$.

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Isolation, Synthesis and Biological Evaluation of Phenylpropanoids from the Rhizomes of *Alpania galanga*

Sumit S Chourasiya[a,b], Eppakayala Sreedhar[a], K. Suresh Babu[a]*, Nagula Shankaraiah[b], V. Lakshma Nayak[c], S. Ramakrishna[c], S. Sravani[d] and M.V. Basaveswara Rao[e]

[a]*Division of Natural Product Chemistry*, *CSIR- Indian Institute of Chemical Technology*, *Hyderabad-500007*, *India*

[b]*Department of Medicinal Chemistry*, *National Institute of Pharmaceutical Education and Research (NIPER)*, *Hyderabad-500037*, *India*

[c]*Division of Medicnal Chemistry &Pharmacology Division*, *CSIR-Indian Institute of Chemical Technology*, *Hyderabad-500 607*, *India*

[d]*Pharmacognosy Division*, *Manipal University*, *Manipal-576 104*, *India*

[e]*Department of Chemistry*, *Krishna University*, *Machilipatnam*, *India*

suresh@iict.res.in

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Bioactivity guided investigation of the DCM: MeOH (1:1) extract from the rhizomes of *Alpinia galanga* led to the isolation of phenylpropanoids (**1-9**) and their structures were established by ^{1}H NMR, ^{13}C NMR, IR and LC-MS/MS. These compounds have been evaluated for their *in vitro* anticancer activity against the human cancer cell lines A549 (lung cancer), Colo-205 (colon cancer), A431 (skin cancer), NCI H460 (lung cancer), PC-3 (prostate cancer), and HT-29 (colon cancer). Compounds **4** and **9** showed potent anticancer activity (ranging from 1.3–19.7 µg/mL) against all the tested cancer cell lines. In addition, an asymmetric synthesis of acetoxychavicol acetate (**1**) and *trans-p*-coumaryl alcohol (**4**) has been accomplished in six steps starting from readily available *p*-hydroxybenzaldehyde for the first time. Grignard reaction and Sharpless kinetic resolution reactions were utilized as the key steps to install the basic core.

Keywords: *Alpania galanga*, Phenylpropanoids, Cytotoxic activity, Stereoselective synthesis.

Cancer is one of the most serious threats against human health in the world. Over the past few decades, extensive research has led to the development of a plethora of chemotherapeutic agents; however, none of these agents are capable of completely eliminating cancer [1]. The limitations of current anticancer drugs, increased incidence, and rapid development of drug resistance [2] have highlighted the need for the discovery of new anticancer agents, preferably with novel mechanisms of action. In this respect, the plant kingdom is now recognized as a source of promising chemopreventive agents [3]. They can be viewed as evolved privileged structures with potent biological activities and biologically pre-validated leads which have played a highly significant role in the discovery and development of new drugs for the treatment of human diseases. This is especially true in the cancer field, where about half the drugs are of natural origin and include two of the most important agents, taxol and taxotere.[4]

As part of pharmacological-phytochemical integrated studies of medicinal plants from the Indian flora [5], we are investigating chemical constituents of plants belonging to the Zingiberaceae family, as well as their cytotoxic activity [6]. In a continuing investigation, phytochemical studies were carried out on *Alpinia galanga*, a tree that grows commonly in the southern part of India, where it is popularly known as Kulanjan. This plant is reported to exhibit antileishmanial [7], antifungal [8], hypoglycemic [9], gastro-protective [10], and anticancer activities [11].

As part of an ongoing effort to discover potential leads from Indian medicinal plants for development of anticancer therapeutics, we observed potent, significant, cytotoxic activity in the DCM: methanol (1:1) extract of *Alpania galanga* (L.) Willd. The active extract was subjected to bioassay-guided separation that resulted in the isolation of phenyl propanoid derivatives (**1-9**).

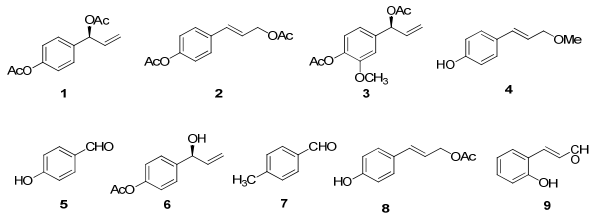


Figure 1: Isolated compounds from the rhizomes of *Alpania galanga*.

Herein, we report the isolation, structure elucidation and cytotoxicity evaluation of phenylpropanoids (**1**–**9**), along with the synthesis of *trans-p*-coumaryl alcohol (**4**) and acetoxychavicol acetate (**1**) by using the Grignard reaction and Sharpless kinetic resolution as the key steps. In addition, we also prepared a series of ester analogues by using different acids and assessed them for their cytotoxic activity. To the best of our knowledge, this is the first report on the asymmetric synthesis of phenylpropanoid ester derivatives

The concentrated DCM: MeOH (1:1) extract was chromatographed on silica gel, and the resultant fractions were subjected to bioassay for cytotoxic activity against a panel of cancer cell lines. Repeated column chromatography of the bioactive fractions resulted in the isolation of nine compounds. The compounds were identified as (1'*S*) -1'-acetoxychavicol acetate (**1**) [12,13], *p*-coumaryl diacetate (**2**) [14], (1'*S*)-1'-acetoxyeugenol acetate (**3**) [12,13], *trans-p*-coumaryl alcohol (**4**) [10], *p*-hydroxybenzaldehyde (**5**) [14],

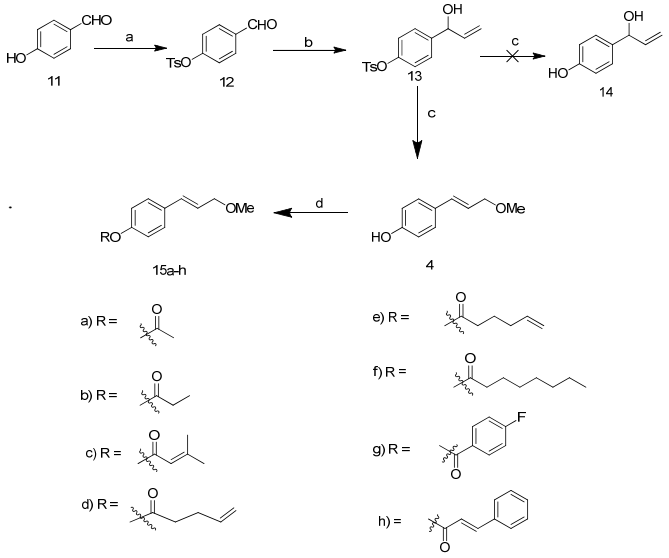
1'*S*-1'-hydroxychavicol acetate (**6**) [10], *p*-methyl benzaldehyde (7), *trans*-*p*-hydroxycinnamyl acetate (**8**) [10], and *trans*-*o*-hydroxy-cinnamaldehyde (**9**) from ^{1}H and ^{13}C NMR data comparison with those reported in literature.

The isolated phenylpropanoids (**1-9**) were evaluated for their anticancer activity against a panel of six human cancer cell lines, A549 (lung cancer), Colo-205 (colon cancer, A431 (skin cancer), NCI H460 (lung cancer), PC-3 (prostate cancer), and HT-29 (colon cancer) by employing MTT assay [6]. Doxorubicin was used as the reference drug. The results, summarized in Table 1, are expressed as IC_{50} values. The *in vitro* screening results revealed that these compounds exhibit promising anticancer activity with IC_{50} values ranging from 1.9 to 13.5 µg/mL against different cancer cell lines. The most active compounds, **4** and **9**, gave IC_{50} values ranging from 1.8–13.4 and 5.6–19.9 µg/mL, respectively.

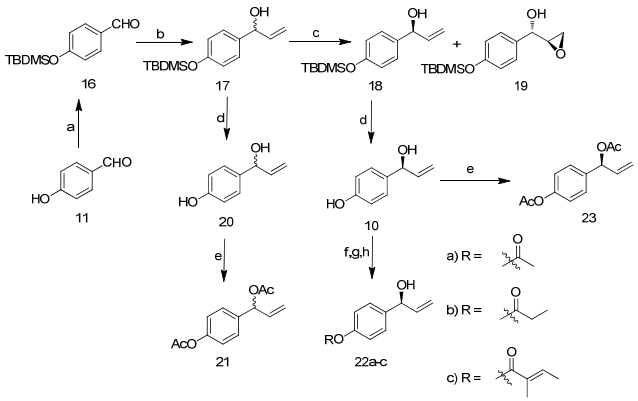
Table 1: Cytotoxicity (IC_{50}, µg/mL) of the isolated compounds (**1-9**).

Compounds	Cell lines IC_{50} µg/mL					
	A549	**Colo-205**	**A431**	**NCI H460**	**PC-3**	**HT-29**
1	77.6	50.1	19.9	60.5	31.6	33.1
2	172.3	128.0	100.4	83.1	100.0	146.8
3	79.1	108.1	91.2	97.2	72.4	42.6
4	1.9	3.4	1.3	4.4	13.5	4.4
5	39.9	14.8	12.9	21.4	30.9	25.7
6	58.9	77.8	95.5	66.0	56.0	36.3
7	39.8	37.1	33.1	37.1	22.4	47.5
8	115.2	34.9	46.8	59.1	21.4	165.5
9	9.8	14.8	19.9	5.2	17.7	5.6
Doxorubicin	1.1	1.2	0.37	0.4	1.4	1.1

Synthesis of 4 and its derivatives: Prompted by the biological properties of **4**, the present study was devised to synthesize new derivatives by using the above lead compounds and investigating their cytotoxic properties. As shown in Scheme 1, the synthesis began with 4-hydroxy benzaldehyde 11, which was converted to its tosyl ester [15] **12** in 71% yield. Grignard reaction of **12** in the presence of vinyl magnesium bromide furnished racemic alcohol **13**, the tosyl deprotection [15] of which, in the presence of methanol under reflux conditions, yields 4-hydroxy phenyl methyl ether phenylpropanoid core **4** via allylic rearrangement[16]. Using this compound as a starting material, we made several derivatives by esterifying [17] with aliphatic and aromatic acids in the presence of EDCI and DMAP pyridine to afford corresponding esters **15a-h**.



Scheme 1: (a) tosyl chloride, TEA, r.t, 2 h, 71%; (b) vinyl magnesium bromide, THF, 0°C, 45 min, 60%; (c) methanol, K_2CO_3, reflux, 2 h, 60%; (d) different acids, DMAP, EDCI, pyridine, r.t., 12 h, 65–85%.



Scheme 2: (a) TBDMSCl, imidazole, CH_2Cl_2, r.t.,1 h, 91%; (b) vinyl magnesium bromide, THF, 0°C, 45 min, 91%; (c) (–) DIPT, $Ti(iOPr)_4$, DCM, –20°C, 12 h, 51%; (d) TBAF, THF, 0°C, 1 h, 68%; (e) acetic anhydride, TEA, CH_2Cl_2, r.t., 1 h, 70% ; (f) acetic anhydride, TEA, CH_2Cl_2, r.t., 30 min, 70% (for **22a**); (g) propionic anhydride, TEA, CH_2Cl_2, r.t., 30 min, 73% (for **22b**);(h) tiglic acid, DMAP, EDCI, pyridine, r.t.,16 h,74% (for **22c**).

Stereoselective synthesis of 22a-c and 21: Further, we have also extended our efforts to the synthesis of **21** and **22a-c**. As shown in Scheme 2. Synthesis began with 4-hydroxy benzaldehyde (**11**), which was converted to its TBDMS ether **16** [16] in 91% yield. Grignard reaction [16] of **16** with vinyl magnesium bromide furnished racemic alcohol **17**, which was resolved by Sharpless kinetic resolution [18] to give the required *S* alcohol **18** and epoxide **19**. The deprotection of the TBDMS group [16] in **17** and **18** with TBAF afforded phenylpropanoid cores **20** and **10**. Finally, the phenylpropanoid core **20** was coupled [17] with acetic acid in the presence of TEA to afford **21** in 71% yield.

Selective acetylation of the phenolic hydroxyl of **10** leading to monoacetate **22a-c** was accomplished with acetic acid and propanoic acid in the presence of triethylamine to afford **22a** (70%) and **22b** (73%), and with tiglic acid in the presence of EDCI and DMAP in pyridine to afford **22**c (74%).

Biological activity: All the synthesized derivatives were tested *in vitro* against a panel of cancer cell lines [6]. To our disappointment, introduction of the ester moiety at C-4 (phenolic OH) was not a good site to modify since none of the derivatives showed any activity. The lack of activity of the synthetic derivatives cannot be explained simply by reaction mechanism. Either the binding affinity or selectivity of the structures of the compounds to the reactive site of the target (enzyme) must play a key role. At this point, it may be that the hydroxyl group remains the optimum functional group to exhibit activity. These early conclusions point the way for further more focused studies aimed at the design and synthesis of more potent and selective analogues as biological tools and potential drug candidates.

Conclusion: In conclusion, we have isolated nine phenylpropanoid derivatives and their cytotoxic properties were investigated. The results of our study indicated that (*E*)-3-methoxyprop-1-enyl) phenol (**4**) showed potent activity comparable with the standard drug doxorubicin. Based on these results, we also synthesized (1'*S*) -1'-acetoxychavicol acetate (**1**) and *trans*–*p*-coumaryl alcohol (**4**), and their derivatives using the Grignard and Sharpless asymmetric reactions as key steps.

Experimental

General: Melting points were recorded on a Fisher Johns apparatus and are uncorrected. FABMS were obtained from a VG Auto spec-M instrument, IR spectra from a Nicolet spectrometer, and ^{1}H NMR

and ^{13}C NMR spectra from Varian 200 and 400 MHz and Bruker 300 MHz spectrometers, using TMS as internal standard. The solvents used were all of AR grade and were distilled under positive pressure in a dry nitrogen atmosphere where necessary. TLC was performed on Merck silica gel 60 F_{254} plates. Visualization was by spraying with 5% H_2SO_4 solution followed by heating. CC was performed on silica gel (60-120 mesh) purchased from Merck, Mumbai, India.

Plant material: The rhizomes of *Alpinia galangal* was collected from the forest of Tirumala in Chitoor Dist. (Andhra Predesh, India) in January 2010 and identification was made by Prof. Dr K. Madhava Chetty, Department of Botany, Sri Venkateswara University, Tirupati. A voucher specimen of the plant is deposited in the herbarium, Department of Botany, with an accession number 546.

Extraction and isolation: The active chloroform extract (1 g) of *Alpinia galanga* was fractionated by CC with ethyl acetate-*n*-hexane. Initially, the column was started with 1% ethyl acetate: *n*-hexane, and then with increasing polarity of solvent (2%, 4%, 8%, 10%, 15%, 20%, 25%, and 30%); 9 compounds were obtained: (*S*)-4-(1-acetoxyallyl)phenyl acetate (**1**) (20 mg) [12,13], (*E*)-3-(4-acetoxyphenyl)allyl acetate (**2**) (25 mg) [14], (*S*)-1-(4-acetoxy-3-methoxyphenyl)allyl acetate (**3**) (20 mg) [12,13], (*E*)-4-(3-methoxyprop-1-en-1-yl)phenol (**4**) (20 mg) [10] (50 mg), 4-hydroxybenzaldehyde (**5**) (15 mg) [14], (*S*)-4-(1-hydroxy-allyl)phenyl acetate (**6**) (18 mg) [10], 4-methylbenzaldehyde (10 mg) (**7**), (*E*)-3-(4-hydroxyphenyl)allyl acetate (**8**) (22 mg) [10], and (*E*)-3-(2-hydroxyphenyl)acrylaldehyde (26 mg) (**9**).

4-(1-Hydroxyallyl) phenyl 4-methylbenzenesulfonate (12): The 4-hydroxybenzaldehyde (**11**) (10 g, 80.96 mmol) was dissolved in DCM (100 mL). To the stirred solution, TEA (8.27g, 81.96 mmol) was added at 0°C, followed by *p*-toluene sulfonyl chloride (15.57g, 81.96 mmol). Then the reaction temperature was raised to 25°C and stirred for 2 h. The reaction was diluted with water (50 mL) and the layers separated. The organic layer was further washed with water (2×30 mL), followed by saturated aq. $NaHCO_3$ (30 mL) and brine (30 mL). The combined organic layers were dried with Na_2SO_4, filtered and concentrated under reduced pressure. The crude residue was purified by flash chromatography using Hex: EA (85:15) to give tosylated *p*-hydroxybenzaldehyde (**12**).

White crystalline solid.
Yield: 71%.
^1H NMR (300 MHz, $CDCl_3$): δ 2.46 (s, 3H, CH_3), 7.16 (d, 2H, *J* = 8.4 Hz, Ar H), 7.32 (d, 2H, *J* = 8.12 Hz. Ar H), 7.71 (d, 2H, *J* = 8.3 Hz, Ar H), 7.83(d, 2H, *J* = 8.4 Hz, Ar H), 9.96 (s, 1H, CHO).
^{13}C NMR (75 MHz, $CDCl_3$): δ 21.6 (CH_3), 123.0 (C), 128.4 (C), 129.9 (C), 131.2 (C), 131.9 (C), 134.7 (C), 145.8 (C), 153.8 (C), 190.6 (C=O).
ESI-MS**:** *m/z* 304.0 (M^+)

4-(1-Hydroxyallyl)phenyl 4-methylbenzenesulfonate (13): To a solution of **12** (7 g, 25.37 mmole) in dry THF (70 mL) under a nitrogen atmosphere at 0°C was added, dropwise, 30.43 mL of a 1M solution of vinyl magnesium bromide in THF. The reaction was stirred for 45 min. at r.t. After completion (TLC), the reaction was quenched with saturated NH_4Cl solution at 0°C and extracted with ethyl acetate (2×50 mL). The combined organic layers were washed with brine, dried over Na_2SO_4 and the solvent was evaporated to dryness. The crude product was purified by CC using Hex: EA (90:10) as eluent to give vinyl alcohol **13**.
Light yellow viscous liquid.
Yield: 60%.
^1H NMR (300 MHz, $CDCl_3$): δ 2.46 (s, 3H, CH_3), 5.17 (dd, 2H, *J* = 10.4, 4.5 Hz, =CH_2), 5.31 (d, 1H, *J* = 17.3Hz , CH-OH), 5.89-6.0 (m,1H, CH=), 6.94 (d, 2H, *J* = 8.3 Hz, ArH),7.25-7.31 (m, 4H Ar H), 7.70 (d, 2H, *J* = 8.3 Hz, Ar H).
^{13}C NMR (75 MHz, $CDCl_3$): δ 21.8 (CH_3), 74.3 (C-OH), 115.8 (C), 122.3 (C), 127.4 (C), 128.4 (C), 129.6 (C), 139.7 (=CH_2), 141.8 (CH=), 145.6 (C), 150.8 (C).
ESI-MS: *m/z* 304.0 (M^+).

(E)-4-(3-Methoxyprop-1-en-1-yl) phenol (4): To a stirred solution of vinyl alcohol **13** (0.1 g, 0.3486 mmol) in methanol (bottle grade 5 mL) was added K_2CO_3 (0.24 g, 1.745 mmol), and the mixture was heated at reflux for 2 h. The reaction mixture was cooled to r.t., and checked by TLC. The mixture was acidified with 1 N HCl until the pH of the mixture reached 2. The combined aq/methanol solution was extracted with ethyl acetate (2×15 mL). The organic extracts were washed with brine (10 mL), dried with Na_2SO_4, filtered and concentrated *in vacuo*. The crude product was purified by CC using Hex: EA (80:20) to give **4**.

Light yellow viscous liquid.
Yield: 60%.
^1H NMR (300 MHz, $CDCl_3$): δ 3.36 (s, 3H, OCH_3), 4.05 (d, 2H, *J* = 6.0 Hz, CH_2-OMe), 5.67 (brs, 1H, OH), 6.01-6.11 (m, 1H =CH), 6.48 (d,1H, *J* = 15.8 Hz, CH=), 6.73 (d, 2H, *J* = 8.4 Hz, Ar H), 7.19 (d, 2H, *J* = 8.4 Hz, Ar H).
^{13}C NMR (75 MHz, $CDCl_3$) δ: 57.6 (OCH_3), 73.3 (CH_2-O), 115.5 (C), 122.8 (C), 127.8 (C), 129.0 (C), 132.9 (C), 155.6 (C).
EI-MS: *m/z* 1640.0 (M^+).

General procedure for the preparation of compounds 15a-15h: EDCI (140 mg, 0.35 mmol) and DMAP (4.45 mg, 0.036 mmol) were added to a solution of tiglic acid in pyridine at 0°C. The reaction mixture was stirred at room temperature to dissolve the EDCI completely. After complete dissolution, a solution of compound **4** (0.04 g, 0.1829 mmol) in pyridine was added. The reaction was monitored by TLC to check the completion of the reaction (16 h). The reaction mixture was concentrated to remove pyridine. The reaction was quenched with aqueous 1 N HCl and extracted with DCM (2 × 20 mL). The product was purified by CC using *n*-hexane–EA (85:15) to give **15a-15h** in good yields.

(*E*)-4-(3-Methoxyprop-1-en-1-yl)phenyl acetate (15a)
Pale yellow liquid.
Yield: 72%.
IR (KBr): 2926, 1760, 1602, 1197 cm^{-1}.
^1H NMR (300 MHz, $CDCl_3$): δ 2.26 (s, 3H,CH_3CO), 3.36 (s, 3H, OCH_3), 4.05 (d, 2H, *J* = 6.0 Hz, CH_2O), 6.05-6.20 (m, 1H,=CH), 6.48 (d, 1H, *J* = 15.8 Hz, CH=) 7.00 (d, 2H, *J* = 7.9 Hz, Ar H), 7.34 (d, 2H, *J* = 7.9 Hz, Ar H).
^{13}C NMR (75 MHz, $CDCl_3$): δ 21.0 (CH_3), 57.8 (OCH_3), 72.8 (CH_2O), 115.5 (C), 121.6 (C), 126.1 (C), 127.2 (CH), 134.3 (CH), 155.0 (C), 168.7 (C=O).
ESI-MS: $C_{12}H_{14}O_3$, *m/z* 206.0 (M^+), 246 (M^++40).

(*E*)-4-(3-Methoxyprop-1-en-1-yl)phenyl propionate (15b)
Pale yellow liquid.
Yield: 82%.
IR (KBr): 2922, 1758, 1598, 1346 cm^{-1}.
^1H NMR: (300 MHz, $CDCl_3$): δ 1.26 (t, 3H, *J* = 7.9 Hz, CH_3), 2.56 (q, 2H, *J* = 7.5 Hz,CH_3CH_2), 3.36 (s, 3H, OCH_3), 4.05 (d, 2H, *J* = 6.0 Hz, -CH_2), 6.05 (m, 1 H, =CH), 6.48 (d, 1H, *J* = 15.8 Hz, CH=), 6 .73 (d, 2H, *J* = 8.4 Hz, Ar H), 7.19 (d, 2 H, *J* = 8.4 Hz, Ar H).

^{13}C NMR (75 MHz, $CDCl_3$): δ 8.93 (CH_3), 27.6 (CH_2), 57.8 (OCH_3), 72.8 (CH_2O), 121.5 (C), 123.6 (C), 126.1(C), 127.2 (CH), 130.5 (CH), 148.3 (C), 172.8 (C=O).
ESI-MS: $C_{13}H_{16}O_3$, *m/z* 220.0 (M^+), 243(M^+ +23).

(*E*)-4-(3-Methoxyprop-1-en-1-yl)phenyl 3-methylbut-2-enoate (15c)
Pale yellow liquid.
Yield: 80%.
IR (KBr): 2924, 1739, 1601, 1123 cm^{-1}.
^{1}H NMR (300 MHz, $CDCl_3$): δ 2.0 (s, 3H, CH_3), 2.21(s, 3H, CH_3), 3.36 (s, 3H, OCH_3), 4.05 (d, 2H, *J* = 6.0 Hz, CH_2O), 5.88 (s, 1H, =CHCO), 6.10- 6.20 (m, 1 H, =CH), 6.48 (d, 1H, *J* = 15.8 Hz, CH=), 7 .03 (d, 2H, *J* = 8.4 Hz, Ar H), 7.35 (d, 2 H, *J* = 8.4 Hz, Ar H).
^{13}C NMR (75 MHz, $CDCl_3$) δ: 27.1 (CH_3), 29.4 (CH_3), 58.0 (OCH_3), 72.3 (CH_2-O), 115.1 (C), 122.0 (C), 125.9 (CH), 131.5 (CH), 155.4 (C), 162.9 (C=O).
ESI-MS: $C_{15}H_{18}O_3$, *m/z* 246.0 (M^+) , 269 (M^++23).

(*E*)-4-(3-Methoxyprop-1-en-1-yl)phenyl pent-4-enoate (15d)
Pale yellow liquid.
Yield: 85%.
IR (KBr): 2925, 1756, 1601, 1167 cm^{-1}.
^{1}H NMR (300 MHz, $CDCl_3$): δ 2.49 (q, 2H, *J* = 6.8 Hz,=CH-CH_2), 2.63 (t, 2H, *J* = 7.8 Hz, CH_2-CO), 3.36 (s, 3H, OCH_3), 4.05 (d, 2H, *J* = 5.85 Hz, CH_2-CH_3), 5.06 (dd, 2H, *J* = 10.74 Hz, $CH_{2=}$), 5.87-5.92 (m, 1H, CH_2=CH-), 6.18-6.25 (m, 1H, =CH), 6.54 (1H, d, *J* = 16.5 Hz, CH=), 6.9 (d, 2H, *J* = 7.8 Hz, Ar H), 7.35 (d, H, *J* = 8.7 Hz, Ar H).
^{13}C NMR (75 MHz, $CDCl_3$): δ 29.6 (CH), 33.5 (CH), 57.9 (OCH_3), 72.8 (OCH_2), 115.8 (C), 121.5 (C), 126.1 (C), 127.2 (CH), 131.4 (CH), 134.4 (CH), 136.2 (CH), 150.3 (C), 171.3 (C=O).
ESI-MS: *m/z* 246.0 (M^+), 269 (M^++23).

(*E*)-4-(3-Methoxyprop-1-en-1-yl)phenyl hex-5-enoate (15e)
Pale yellow liquid.
Yield: 75%.
IR (KBr): 2923, 1759, 1506, 1166 cm^{-1}.
^{1}H NMR (300 MHz, $CDCl_3$): δ 1.84 (q, 2H, *J* = 7.5 Hz, CH_2=CH-C$\underline{H}_2$), 2.18-2.28 (m, 2H, -C$H_2$$CH_2$CO), 2.54 (t, 2H, *J* = 7.5, CH_2-CO) 3.36 (s, 3H –CH_3), 4.05 (dd, 2H, *J* = 1.5, 0.85 Hz,-CH_2-OCH_3), 5.76 (m, 1 H, CH_2=C$\underline{H}$), 6.18 (m, 1H, =CH) 6.54 (1H, d, *J* = 16.5 Hz, CH=), 7.0 (d, 2H, J = 8.3 Hz Ar H), 7.35 (d, 2H, *J* = 8.3 Hz, Ar H).
^{13}C NMR (75 MHz, $CDCl_3$) δ: 23.9 (CH_2), 32.9 (CH_2), 33.5 (CH_2), 57.9 (OCH_3), 72.8 (OCH_2), 121.5 (C), 126.1 (C), 127.3 (CH), 131.3 (CH), 134.0 (CH), 137.2 (CH), 150.3 (C), 171.3 (C=O).
ESI-MS: *m/z* 260.0 (M^+), 283(M^++23).

(*E*)-4-(3-Methoxyprop-1-en-1-yl)phenyl octanoate (15f)
Pale yellow liquid.
Yield: 85%.
IR (KBr): 2925, 1761, 1607, 1215 cm^{-1}.
^{1}H NMR (300 MHz, $CDCl_3$): δ 0.9 (t, 3H, *J* = 4.5 Hz, CH_3), 1.29-1.33 (m, 6H, -CH_2) 1.61-1.7 (m, 2H, -CH_2), 2.25-2.30 (m, 2H, -CH_2), 2.50-2.55 (m, 2H, CH_2-CO), 3.36 (s, 3H OCH_3), 4.05 (d, 2H, *J* = 6.0 Hz CH_2-OCH_3), 6.18 (m, 1H, =CH), 6.54 (1H, d, *J* = 16.5 Hz CH=), 7.0 (d, 2H, *J* = 8.3 Hz Ar H), 7.35 (d, 2 H, *J* = 8.3 Hz Ar H).
^{13}C NMR (75 MHz, $CDCl_3$): δ 14.0 (CH_3), 22.5 (CH_2), 24.8 (CH_2), 28.8 (CH_2), 29.6 (CH_2), 31.6 (CH_2), 34.5 (CH_2), 57.9 (OCH_3), 72.8 (OCH_2), 121.5 (C), 125.9 (C), 127.3 (C), 131.3 (CH), 134.4 (CH), 150.3 (C), 171.3 (C=O).
ESI-MS: *m/z* 290.0 (M^+), 313(M^++23).

(*E*)-4-(3-Methoxyprop-1-en-1-yl)phenyl 4-fluorobenzoate (15g)
Pale yellow liquid.
Yield: 82%.
IR (KBr): 2925, 1735, 1601, 1265 cm^{-1}.
^{1}H NMR (300 MHz, $CDCl_3$): δ 3.36 (s , 3H, OCH_3), 4.05 (d, 2H, *J* = 6.0 Hz, CH_2-OCH_3), 6.12-6.25 (m, 1 H, =CH), 6.40 (d, 1H, *J* = 16.0 Hz, CH=), 6.59 (d, 2H, *J* = 16.0 Hz, Ar H), 6.83 (d, 2H, *J* = 16.0 Hz, Ar H), 7.09 (d, 2 H, *J* = 8.3 Hz, Ar H),7.83 (d, 2H, *J* = 16.0 Hz, Ar H).
^{13}C NMR (75 MHz, $CDCl_3$): δ 57.9 (OCH_3), 72.9 (OCH_2), 121.6 (C), 126.2 (C), 127.4 (C), 131.2 (C), 131.3 (CH), 131.8 (CH), 150.0 (C), 164.1 (C=O), 167.8 (C).
ESI-MS: *m/z* 286.0 (M^+), 287(M^++1).

4-((*E*)-3-Methoxyprop-1-en-1-yl)phenyl cinnamate (15h)
Pale yellow liquid.
Yield: 74%.
IR (KBr): 2921, 1728, 1601, 1137 cm^{-1}.
^{1}H NMR (300 MHz, $CDCl_3$): δ 3.34 (s, 3H, OCH_3), 4.06 (d, 2H, *J* = 5.7 Hz, -CH_2-OCH_3), 6.38 (d, 1H, *J* = 5.2 Hz, CH=), 6.57 (d, 1H, *J* = 5.2 Hz, =CH), 7.09 (d, 1H, *J* = 5.2 Hz, C*H*=CH-C=O), 7.3 - 7.48 (m, 10H Ar H).
^{13}C NMR (75 MHz, $CDCl_3$): δ 57.8 (CH_3), 72.8 (OCH_2), 117.2 (C), 121.6 (C), 126.6 (C), 127.3 (C) 127.9 (C), 128.2 (C), 128.8 (C), 130.3 (CH), 131.3 (CH), 134.2 (CH), 144.5 (CH), 146.5 (C), 150.1 (C), 165.2 (C=O).
ESI-MS (*m/z*): 317 (M^++23).

4-{(Tert-butyldimethylsilyl)oxy}benzaldehyde (16): About 10 g of *p*-hydroxybenzaldehyde (**11**) was dissolved in 100 mL of dry DCM and to the stirred solution was added imidazole portion wise at 0°C. After being stirred for 20 min at 0°C, TBDMSCl was added and the mixture was stirred for 1 h at r.t. The reaction was completed in 1 h. Then the reaction mixture was quenched with cold water and the compound extracted with chloroform. The organic layer was washed with brine and dried over sodium sulfate. The organic layers were combined and evaporated in a rotary evaporator. The crude product was purified by silica gel CC using *n*-hexane: EtOAc (85:15) to give **16** [16] as a white crystalline solid (91%).

1-4-{(tert-butyldimethylsilyl)oxy}phenyl}prop-2-en-1-ol (17): To a solution of **16** (10 g, 42.37 mmole) in dry THF (100 mL) under a nitrogen atmosphere at 0°C was added, dropwise, 50.48 mL of a 1M solution of magnesium bromide in THF. The reaction was allowed to stir for 45 min at r.t. After completion (TLC), the reaction was quenched with saturated NH_4Cl solution at 0°C and extracted with ethyl acetate (2×50 mL). The combined organic layers were washed with brine, dried over Na_2SO_4 and the solvent was evaporated to dryness. The crude product was purified by CC using Hex: EA (90:10) as eluent to give vinyl alcohol (**17**)[16] as light yellow viscous liquid.(60%).

4-(1-Hydroxyallyl) phenol (20): To a stirred solution of **17** (2 g, 6.8 mmol) in LR grade THF at 0°C was added TBAF, dropwise, and the reaction was stirred for 1 h. The progress of the reaction was monitored by TLC. After completion (1 h), the reaction mixture was concentrated to remove THF and the oil was dissolved in EA and extracted with 1 N HCl (20 mL), washed with brine, dried over Na_2SO_4 and concentrated. The product was purified by silica gel CC using *n*-hexane: EtOAc (90:10) to give **20** [16] as a yellow oily liquid (68%).

4-(1-Acetoxyallyl) phenyl acetate (21): To a solution of **20** (1 g, 6.5 mmol) in DCM (10 mL), under a nitrogen atmosphere at r.t., was added, dropwise, triethylamine (2.32 mL, 16.392 mmol), followed by acetic anhydride (1.52 mL, 16.2 mmol). The reaction mixture

was stirred for 1 h at 0°C. After completion (TLC), the reaction was quenched with ice at r.t., extracted with $CHCl_3$ (2× 25 mL), the combined organic layers washed with brine, dried over Na_2SO_4 and the solvent evaporated to dryness. The crude product was purified by CC using Hex: EA (90:10) to give compound **21**[16] as a light yellow oily liquid (70%).

(S)-1-(4-{(Tert-butyldimethylsilyl)oxy}phenyl)prop-2-en-1-ol (18) and (1S)-(4-{(tert-butyldimethylsilyl)oxy}phenyl)(oxiran-2-yl)methanol (19): Completely evacuated, flame dried, powdered 4 Å molecular sieves (10 g) and (-)-DIPT (2.10 g, 9.38 mmol) were placed in a 250 mL two necked r.b flask and dissolved in dry DCM (40 mL). To the stirred mixture a solution of Ti $(iOPr)_4$ (2.22 g, 7.82 mmol) was added at -20°C, followed by (1.44 g, 9.38 mmol) cumene hydroperoxide at an interval of 20 min. After being stirred for 20 min, a solution of compound **17** (4.12 g, 15.64 mmol) in DCM (10 mL) was added, dropwise. The reaction mixture was allowed to stir for 12 h. The reaction was monitored by TLC. After completion (12 h), the reaction was quenched by a solution containing 4 g of NaOH dissolved in 40 mL of saturated NaCl at 0°C. Stirring continued for 3 h at r.t. before the mixture was filtered through Celite and the filtrate concentrated. The product was purified by silica gel CC using *n*-hexane: EtOAc (90:10) to give **18** and *n*-hexane: EtOAc (75:25) to give **19**.

Compound 18
Syrupy liquid.
Yield: 48%.
$[\alpha]^{20}_D$: -15 (*c* 0.1, $CHCl_3$).
^{1}H NMR (300 MHz, $CDCl_3$): δ 0.18 (s, 6H, dimethyl), 0.98 (s, 9H, ter butyl), 5.09-5.18 (m, 2H, $=CH_2$), 5.31 (d, 1H, *J* = 16.9 Hz, -C*H*OH), 5.94-6.05 (m, 1H, CH=), 6.90 (d, 2H, *J* = 8.4 Hz, Ar H), 7.17 (d, 2H, *J* = 8.4 Hz, Ar H).
^{13}C NMR (75 MHz, $CDCl_3$): δ -4.5 (C-Si), 17.8 (CH_3Si), 25.6 (*C*H_3C), 75.1 (C-OH),114.3 (C),120.0 (C), 127.5 (C), 135.2 (CH_2), 140.3 (CH), 154.8 (C).
ESI-MS (*m/z*): 287 (M^+ +23).

Compound 19
A syrupy liquid.
Yield: 47%.
$[\alpha]^{20}_D$: -100 (*c* 0.1, $CHCl_3$).
^{1}H NMR (300 MHz, $CDCl_3$): δ 0.18 (s, 6H,CH_3), 0.98 (s, 9H, ter butyl), 2.70-2.73 (m, 1 H,CH-O), 2.90-2.93 (m, 1 H, CH-O), 3.13-3.16 (m, 1H, CH-O), 4.84 (d, 1H, *J* = 2.26 Hz,C*H*-OH), 6.73 (d, 2H, *J* = 8.4 Hz, Ar H), 6.9 (d, 2 H, *J* = 8.4 Hz, Ar H).
^{13}C NMR (75 MHz,$CDCl_3$): δ: -4.69 (C-Si), 18.14 (CH_3), 25.6 (CH_3), 43.5 (-CH-0),70.46 (CH-OH),120.1 (C),127.6 (C), 132.0 (C), 155.6 (C).
ESI-MS: $C_{15}H_{24}O_3Si$, *m/z* 281 (M^++1) , *m/z* 303 (M^++23).

(S)-4-(1-Hydroxyallyl) phenol (10): To a stirred solution of compound **18** (2g, 6.8 mmol) in LR grade THF at 0°C was added TBAF, dropwise, and the reaction was stirred for 1 h. After completion of the reaction (TLC), the mixture was concentrated to remove THF, and the oil was dissolved in EA and extracted with 1 N HCl (20 mL), washed with brine, dried over Na_2SO_4 and concentrated. The product was purified by silica gel CC using *N*-hexane: EtOAc (90:10) to give **10**.
Yellow oily liquid.
Yield: 68%.
$[\alpha]^{20}_D$: -15 (*c* 0.1, CH_3OH).
^{1}H NMR (300 MHz, $CDCl_3$): δ 5.0-5.18 (m, 2H, $=CH_2$), 5.25(d, 1H, *J* = 15.6 Hz-C<u>H</u>-OH), 5.98-6.1 (m, 1 H, CH=), 6.73 (d, 2H, *J* = 8.4 Hz, Ar H), 7.19 (d, 2 H, 8.4 Hz, Ar H).
^{13}C NMR (75 MHz, $CDCl_3$): δ: 64.6 (CH), 106.4 (C), 113.6 (C), 119.1 (CH_2), 124.6 (CH), 148.5 (C).

(S)-4-(1-Acetoxyallyl)phenyl acetate (23):To a stirred solution of compound **10** (0.05g, 0.33 mmol) in DCM (10 mL) under nitrogen at r.t. was added triethylamine (2.32 mL, 16.392 mmol), followed by acetic anhydride (51.48 mg, 0.39 mmol), dropwise. The reaction mixture was stirred for 1 h at 0°C. After completion (TLC), the reaction was quenched with ice at r.t. and extracted with $CHCl_3$ (2×10 mL). The combined organic layers were washed with brine, dried over Na_2SO_4 and the solvent was evaporated to dryness. The crude product was purified by CC using Hex: EA (90:10) to give compound **23** [16] as **a** light yellow oily liquid (70%).

General procedure for the preparation of compounds 22a & 22b: To a stirred solution of compound **10** (0.05 g, 0.33 mmol) in DCM (10 mL) under a nitrogen atmosphere at r.t. was added triethylamine (2.32 mL, 16.392 mmol).To this solution, acetic anhydride (51.48 mg, 0.39 mmol) was added, dropwise. The reaction mixture was allowed to stir for 30 min. at 0°C to achieve selective acylation of the phenolic OH. After completion (TLC), the reaction was quenched with ice at r.t. and extracted with $CHCl_3$ (2×10 mL). The combined organic layers were washed with brine, dried over Na_2SO_4 and the solvent evaporated to dryness. The crude product was purified by CC using Hex: EA (90:10) to give compounds **22a** and **22b.**

(*S*)-4-(1-Hydroxyallyl) phenyl acetate (22a)
Light yellow oily liquid.
Yield: 70%.
$[\alpha]^{20}_D$: -65 (*c* 0.1,$CHCl_3$).
IR (KBr): 3019, 1765, 1741, 1606 cm^{-1}.
^{1}H NMR (300 MHz, $CDCl_3$): δ 2.29 (s, 3H, OAc), 5.22-5.31 (dd, 2H, $=CH_2$), 5.3 (d, 1H, C<u>H</u>-OH), 5.9-6.02 (m, 1H, CH=) 7.04 (d, 2H, *J* = 8.4, Ar H), 7.3 (d, 2H, *J* = 8.4Hz, Ar H).
^{13}C NMR (75 MHz, $CDCl_3$): δ 21.0 (CH_3), 75.14 (CH-OH), 116.9 (C), 121.5 (C), 128.3 (C), 135.9 (CH_2), 136.3 (CH), 150.3 (C), 169.2 (C=O).
ESI-MS: $C_{11}H_{12}O$, *m/z* 192 (M^+).

(*S*)-4-(1-Hydroxyallyl)phenyl propionate (22b)
Light yellow oily liquid.
Yield: 73%.
$[\alpha]^{20}_D$: -105 (*c* 0.1, $CHCl_3$).
IR (KBr): 2922, 1758, 1729, 1640, 1346 cm^{-1}.
^{1}H NMR (300 MHz, $CDCl_3$): δ 1.09 (t, 3H CH_3), 2.53 (q, 2H, CH_2), 5.22-5.31 (dd, 2H, $=CH_2$), 5.32 (d, 1H, *J* =17.34Hz, C*H*-OH),5.90-6.10 (m,1H, CH=),7.04 (d, 2H, *J* = 8.4, Ar H), 7.3 (d, 2H, *J* = 8.4 Hz, Ar H).
^{13}C NMR (75 MHz, $CDCl_3$): δ 9.5 (CH_3), 21.04 (CH_2),75.1 (CH-OH), 116.9 (C), 121.5 (C), 128.3 (C), 135.9 (CH_2), 136.3 (CH), 150.3 (C), 169.2 (C=O).
ESI-MS: $C_{12}H_{14}O_3$, *m/z* 206 (M^+).

(S, Z)-4-(1-Hydroxyallyl)phenyl 2-methylbut-2-enoate (22c): To a stirred solution of tiglic acid (1.09 mmol) in pyridine at 0°C, EDCI (140 mg, 0.35 mmol) and DMAP (4.45 mg, 0.036 mmol) were added. The reaction mixture was stirred at room temperature and, after complete dissolution of EDC, a solution of compound **10** (0.04 g, 0.1829 mmol) in pyridine was added. The reaction was allowed to stir for 16 h and then the mixture was concentrated to remove pyridine. The reaction was quenched with aqueous 1 N HCl and extracted with DCM (2 × 20 mL). The combined organic layers were washed with brine, dried over Na_2SO_4 and evaporated to

dryness. The product was purified by CC using *n*-hexane: EtOAc (85:15) to give **22c**.
Yellow liquid.
Yield: 74%.
$[\alpha]^{20}_{D}$: -25 (*c* 0.1, $CHCl_3$).
IR (KBr): 3445, 2922, 1729, 1640, 1346 cm^{-1};
^{1}H NMR (300 MHz, $CDCl_3$): δ 1.90 (d, 3H, J=7.1Hz,CH_3), 1.94 (s, 3H, CH_3), 2.15 (brs 1H OH), 5.31 (dd, 2H, =CH_2), 5.32 (d, 1H, *J* = 17.3 Hz, C*H*-OH), 7.04 (d, 2H, *J* = 8.4, Ar H), 7.3 (d, 2H, *J* = 8.4 Hz, ArH).
^{13}C NMR (75 MHz, $CDCl_3$): δ 12.1 (CH_3), 14.6 (CH_3), 74.8 (CH-OH), 114.7 (C), 115.2 (C), 121.7 (C), 127.3 (C), 127.4 (CH), 139.4 (CH), 150.4 (C), 166.5 (C=O).
ESI-MS: $C_{14}H_{16}O_3$, *m/z* 232 (M^+), 255 (M^++23).

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Rosmarinic Acid Interaction with Planktonic and Biofilm *Staphylococcus aureus*

Lívia Slobodníková[a], Silvia Fialová[b], Helena Hupková[a] and Daniel Grančai[b]

[a]*Comenius University in Bratislava, Faculty of Medicine, Institute of Microbiology, Sasinkova 4, 811 08 Bratislava, Slovakia*

[b]*Comenius University in Bratislava, Faculty of Pharmacy, Department of Pharmacognosy and Botany, Odbojárov 10, 832 32 Bratislava, Slovakia*

fialova@fpharm.uniba.sk

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The subject of study was the evaluation of antibacterial activities of rosmarinic acid (RA) on clinical *Staphylococcus aureus* strains obtained from catheter-related infections. Minimal inhibitory (MIC) and minimal bactericidal concentrations (MBC) of RA were tested by broth microdilution assay. Biofilm-eradication activity was detected on 24-hour biofilm in microtiter plates using a regrowth technique; activity on biofilm formation was measured by a microtiter plate method after RA application to bacterial samples after 0, 1, 3 and 6 hours of biofilm development. RA had antimicrobial activity on all tested strains in concentrations from 625 to 1250 $\mu g.mL^{-1}$ (MICs equal to MBCs). No biofilm-eradication activity on 24-hour biofilm was observed in the tested range of concentrations (from 156 to 5000 $\mu g.mL^{-1}$). Subinhibitory RA concentrations suppressed the biofilm production, when applied at early stages of its development. Concentrations lower than subinhibitory stimulated the biofilm mass production in a concentration- and time-dependent manner. Considering our results, RA could be a candidate for a topical antimicrobial agent with killing activity on planktonic forms of bacteria and suppressing activity in the early stages of biofilm development, but probably not for the therapy of catheter-related infections as a sole agent.

Keywords: Rosmarinic acid, Antibacterial activity, *Staphylococcus aureus*, Catheter-related infections, Biofilm.

Phenolic acids are important phenols produced in plants, for which they have diverse functions including participation in plant-microbe interaction or symbiosis [1]. One of the most common phenolic acids, widely found in the Lamiaceae family, is rosmarinic acid (RA), an ester of caffeic acid and 3, 4-dihydroxyphenylacetic acid, synthetized in the plant tissue from the amino acids L-phenylalanine and L-tyrosine [2]. RA is an important secondary metabolite in many medical plants that have been used in traditional medicine for more than 2000 years. It is the main bioactive compound in some of them [3] and many plant extracts containing RA are used as phytotherapeutical preparations [4].

Natural polyphenols and related substances could play an important role in the human defense system, e.g. as antimicrobial, antioxidant, or anticancer agents [2,4]. RA and its derivatives possess promising biological activities, such as neuroprotective, cardioprotective and chemopreventive effects, as well as antioxidant, astringent, anti-inflammatory and antimicrobial activity [4-7]. RA is a biologically active, non-toxic substance with antioxidant activity stronger than that of α-tocoferol (vitamin E) [4]. The anti-inflammatory activity of RA was proved by its inhibition of lipoxygenase and cyclooxygenase, which resulted in the inhibition of the arachidonic acid pathway [2].

Antibacterial activity of RA has been shown against Gram-positive and certain Gram-negative bacteria [8,9]; however, up till now there are no available data either about RA activity on clinical bacterial strains from hospital infections, or about its interaction with biofilm forms of bacterial growth. *Staphylococcus aureus*, a frequent skin colonizer, is one of the most important pathogens with respect to catheter-related infections, characteristic of biofilm formation [10]. The increasing frequency of polyresistant, mostly nosocomial strains, necessitates research on new antimicrobial agents, active both against planktonic and biofilm forms of microbial growth; to such agents may belong substances of herbal origin as well. This study is, therefore, devoted to research of RA *in vitro* activity on clinical *S. aureus* strains from catheter-related infections.

Antimicrobial activity of rosmarinic acid on planktonic bacteria: The suppressive activity of RA on several microbial species has already been published. Previous works on antimicrobial activity of RA were provided using both Gram-positive and Gram-negative bacteria. Mencherini *et al.* detected major bacteriostatic effects of RA against the Gram-positive bacteria, *S. aureus* and *S. epidermidis*. In the case of Gram-negative bacteria, yeasts, and molds, the MICs were 20-times higher [3]. Klancnik *et al.* examined the antimicrobial activity of RA against *Bacillus cereus*, *S. aureus*, *Salmonella infantis*, *Campylobacter jejuni* and *C. coli* (MICs on agar dilution were 6 mg/mL, 10 mg/mL, 1.25 mg/mL, 1.25 mg/mL, and 1.25 mg/mL, respectively) [11]. In our study, the MIC and MBC of RA were measured by a standardized broth microdilution method, using both susceptible and polyresistant *S. aureus* clinical isolates, including MRSA strains. Without respect to the antimicrobial susceptibility patterns of the strains, RA proved active against all of them, in concentrations from 625 to 1250 $\mu g.mL^{-1}$ (Table 1). The MIC values were equal to the MBCs.

Antibiofilm activity of rosmarinic acid: Microorganisms growing in biofilm are protected against many external factors, including antimicrobial agents [12]. The minimal biofilm eradicating concentrations of many antibiotics can be several times higher than MICs, measured in the planktonic form of growth, which may disable the clinical use of many of these antibiotics in the therapy of biofilm-related infections [13]. Even if many data are available on biofilm-inhibitory activity of various plant extracts, or molecules of plant origin [14-17], data about the real biofilm eradicating activity (i.e. total killing of bacteria in already established biofilm) are missing. Data about RA interaction with the biofilm form of

Table 1: Rosmarinic acid activity against *S. aureus* strains (tested in Mueller-Hinton broth).

Strain	Origin	Antimicrobial susceptibility						RA MIC/MBC
		OXA	GEN	CIP	VAN	COT	MLSB	[μg.mL^{-1}]
1	CVC	R	S	R	S	S	+	625
2	CVC	R	S	R	S	S	+	625
3	CVC	S	S	R	S	S	-	625
4	CVC	R	S	R	S	S	+	625
5	CVC	S	S	R	S	S	+	625
6	CVC	R	S	R	S	S	+	625
7	CVC	R	R	R	S	S	+	625
8	CVC	S	S	R	S	S	+	625
9	WDC	S	S	S	S	S	+	625
10	WDC	S	S	S	S	S	-	1250
11	WDC	S	S	S	S	S	-	625
12	WDC	S	S	S	S	S	-	1250
13	WDC	S	S	S	S	S	-	1250
14	RSC	R	S	R	S	R	+	1250
15	RSC	R	S	R	S	S	+	625
16	RSC	S	S	S	S	S	+	1250
ATCC 43300		R						625
ATCC 29213		S						625

CVC – central venous catheter; WDR - wound drainage catheter; RSC - respiratory suction catheter; OXA – oxacillin; GEN – gentamicin; CIP – ciprofloxacin; VAN – vancomycin; COT – cotrimoxazole; MLSB – resistance to macrolides, lincosamides and streptogramines-B; RA – rosmarinic acid; MIC – minimal inhibitory concentration; MBC – minimal bactericidal concentration; S – susceptible; R - resistant

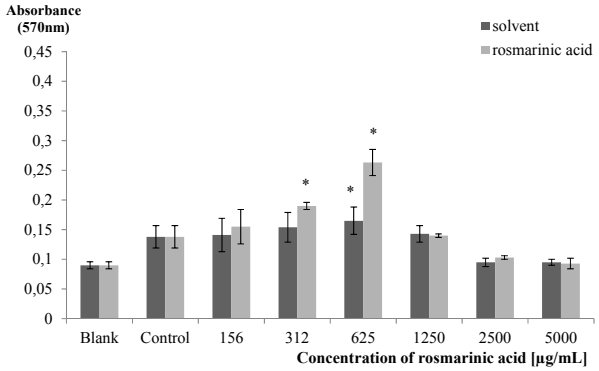

Figure 1: Impact of rosmarinic acid on biofilm formation by *Staphylococcus aureus* after 24 hours cultivation (strain 11); the values are mean ± SD, * $P <$ 0.05, versus control (bacteria in medium alone).

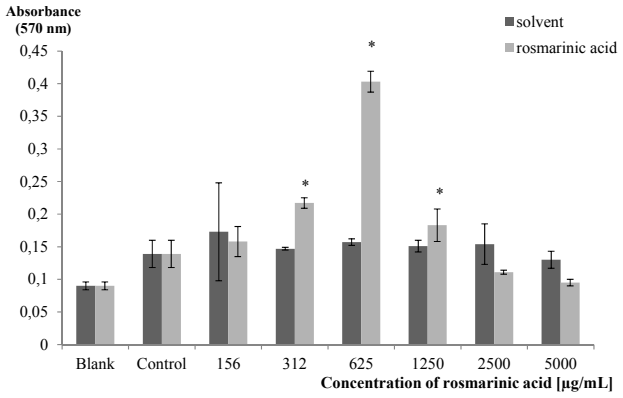

Figure 2: Impact of rosmarinic acid on biofilm formation by *Staphylococcus aureus* after 24 hours cultivation (strain 13); the values are mean ± SD, * $P <$ 0.05, versus control (bacteria in medium alone).

bacteria in the available literary sources are lacking as well. Therefore, the next step in our study was detection of RA antibiofilm activity. Despite our expectations, we could not see any biofilm eradication activity of RA in our test system (24 hour biofilm on the surface of polystyrene microtiter wells; detection of regrowth of the surviving bacteria), even when the highest RA concentration used in the study (5000 μg.mL^{-1}) exceeded the detected MICs (from 625 to 1250 μg.mL^{-1}; Table 1) by two- to three-times.

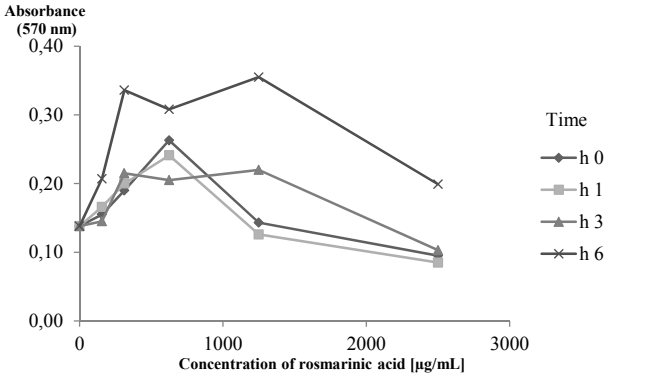

Figure 3: Concentration- and time-dependent activity of rosmarinic acid on biofilm formation by *Staphylococcus aureus* (strain 11).

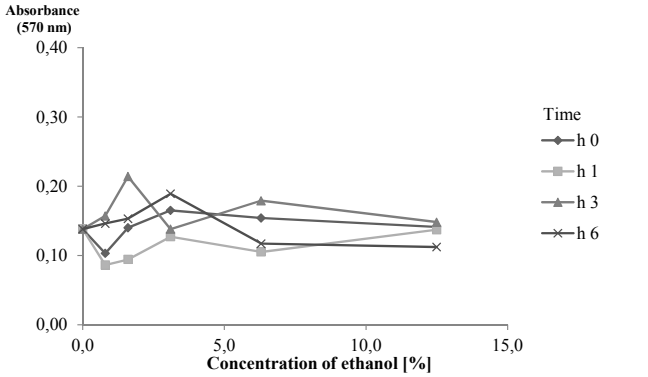

Figure 4: The corresponding ethanol solvent activity on biofilm formation by *Staphylococcus aureus* (strain 11).

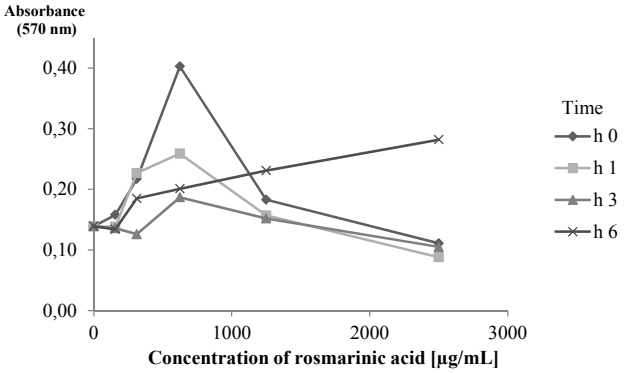

Figure 5: Concentration- and time-dependent activity of rosmarinic acid on biofilm formation by *Staphylococcus aureus* (strain 13).

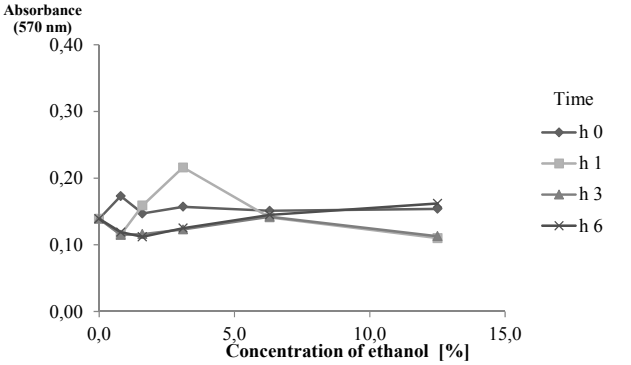

Figure 6: The corresponding ethanol solvent activity on biofilm formation by *Staphylococcus aureus* (strain 13).

Various plant extracts or pure substances of herbal origin have been screened for biofilm-reducing activity (for review see Taraszkiewicz *et al*. [16]), with several promising results. The biofilm suppressing activity may be mediated by interfering with microbial attachment mechanisms [18], by interfering with autoinducer molecules or their receptors (quorum quenching activity) [19], or by specific suppression of genetic determinants of biofilm matrix synthesis [15]. RA impact on biofilm formation in our study was tested in very rich medium supporting the biofilm production (Tryptose-Soy Broth supplemented with 1% glucose – TSB-S). As the composition of medium substantially influences the measured MIC values, one- to two-dilutions higher MICs were achieved in this richer medium (from 2500 to 5000 $\mu g.mL^{-1}$; Figures 1 and 2 for strains 11 and 13) in comparison with MIC values obtained in the Mueller-Hinton medium (MH), used for MIC, MBC, and biofilm-eradication activity testing (from 625 to 1250 $\mu g.mL^{-1}$; Table 1). Unfortunately, MH medium is not effective in supporting biofilm production, so it was not possible to use it in the testing of the RA biofilm-reducing activity. The sub-inhibitory concentrations of RA (MIC/2, i.e. 1250 $\mu g.mL^{-1}$ or 2500 $\mu g.mL^{-1}$, in TSB-S) showed a mild biofilm-suppressing activity, but the concentrations lower than sub-inhibitory ($\leq$ MIC/4, i.e. $\leq$ 625 $\mu g.mL^{-1}$ or $\leq$ 1250 $\mu g.mL^{-1}$ in TSB-S) stimulated biofilm mass production (Figures 1 and 2).

When evaluating the role of the time interval between seeding the microtiter wells with bacteria and the moment of RA application, a time-, concentration- and strain-dependent pattern was observed (Figures 3 and 5). Generally, the highest biofilm-inhibitory activity was observed at the very early stages of biofilm production (1 to 3 hours). On the other hand, the highest biofilm-stimulating activity was observed when applied to 6-hour biofilm (Figures 3 and 5). An exception was strain 13 at the application time of 6 hours, when the highest stimulatory effect was observed in higher concentrations (2500 $\mu g.mL^{-1}$ in TSB-S) (Figure 5). This strain had a higher MIC (1250 $\mu g.mL^{-1}$ in MH medium, and 5000 $\mu g.mL^{-1}$ in TSB-S) and probably more developed biofilm after 6-hour cultivation than strain 11 (Figures 3 and 5).

Except for the biofilm stimulation by the RA ethanol solution in low concentrations, a stimulatory activity was detected by the ethanol solvent itself (Figures 4 and 6), but lower than in the case of RA. This phenomenon has already been described by several authors, who detected biofilm stimulation by low concentrations of antibiotics [20], alcohol [21], or even plant extracts [22, 23]. It seems that RA also belongs to the xenobiotic class, which can induce the bacterial defense response to environmental stress if applied in rather low concentrations. It is also possible, that ethanol and RA acted synergistically in the process of biofilm building by the *S. aureus* strains used in our study.

Rosmarinic acid in the present study showed effective killing activity on all of the tested *S. aureus* strains and decreased the biofilm production at sub-inhibitory concentrations, when applied at the early stages of its formation. Based on the obtained results, RA could be a candidate for a potential antimicrobial agent with killing activity on planktonic forms of bacteria and suppressing activity in the early stages of biofilm development. However, lack of RA effect on already developed biofilm does not support our hypothesis about its use in the therapy of catheter-related infections as a sole agent.

Experimental

Bacterial strains: Sixteen clinical *S. aureus* strains isolated from infections associated with central venous, wound, and respiratory suction catheters were used in the study (Table 1). Two collection strains (ATCC 29213 and ATCC 43300) were included as well. The strains were preserved in Brain-Heart Infusion with 20% glycerol at -20°C. Before analysis, they were inoculated on blood agar and cultivated overnight at 37°C.

Antimicrobial susceptibility: The antimicrobial susceptibility to oxacillin, gentamicin, ciprofloxacin, vancomycin, cotrimoxazole, erythromycin and clindamycin was tested by the disk-diffusion method [24], and interpreted according to the CLSI guidelines [25]. Commercial antibiotic discs (OXOID, UK) and Mueller-Hinton agar medium (OXOID, UK) were used in the tests. Resistance to oxacillin/methicillin was confirmed by the Penicillin Binding Protein 2a (PBP2a) latex agglutination test (OXOID, UK).

Rosmarinic acid: Pure RA (Sigma-Aldrich, Germany) was dissolved in 50% ethanol, diluted 1:1 with twice concentrated medium, and afterwards serial geometric dilutions from 5000 to 156 $\mu g.mL^{-1}$ were prepared. Mueller-Hinton Broth (OXOID, UK) was used for antimicrobial activity testing on planktonic bacteria and for biofilm eradication activity, and Tryptose-Soy Broth (OXOID, UK) supplemented with 1% of glucose for detection of impact on biofilm production. The final concentrations of solvent (ethanol) in the samples ranged from 12.5 to 0.8%.

Rosmarinic acid antimicrobial activity: The planktonic form of bacterial growth was tested for minimal inhibitory (MIC) and minimal bactericidal concentrations (MBC) of RA by broth microdilution assay according to CLSI [26].

Biofilm eradication activity: The antibiofilm activity of RA was tested on 24 h biofilm prepared on the surface of microtiter wells in Tryptose-Soy Broth supplemented with 1% glucose according to Stepanovic *et al.* [27]. The medium containing biofilm-free bacteria was discarded and the wells were washed 3-times with PBS in order to remove bacteria not associated with biofilm. Rosmarinic acid in concentrations > MBC was added to washed biofilms in the wells. After 24 h incubation at 35°C, the RA was drained out, the wells washed and fresh medium added. The biofilm eradication activity was detected by the regrowth method [28], after additional 24 h cultivation at 35°C.

Impact on biofilm formation: The activity of RA on biofilm formation was tested in microtiter plate wells in Tryptose-Soy Broth with 1% of glucose. Rosmarinic acid was added to the bacteria in the microtiter plate wells at 0, 1, 3, and 6 h after inoculation. The intensity of biofilm production was measured spectrophotometrically at 570 nm (MRX Microplate Reader, DYNEX Technologies, USA) after fixation with methanol, staining with 0.5% crystal violet and elution with 99% ethanol [27]. Medium free bacteria was used as a blank sample. All tests were performed in 3 parallel samples. In each test run, samples containing the solvent in corresponding concentrations were tested. Bacterial suspensions in RA- and solvent-free medium were used as controls.

Statistical analysis: The results were statistically evaluated by the unpaired t-test, using two-tailed *P* value.

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metabolites and determination of anti-glycation and antimicrobial activity of selected species of *Mentha* L. and *Lycopus* L. leaves.

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New Butenolide and Pentenolide from *Dysidea cinerea*

Phan Van Kiem[a,*], Nguyen Xuan Nhiem[a], Ngo Van Quang[c], Chau Van Minh[a], Nguyen Hoai Nam[a], Nguyen Thi Cuc[a], Hoang Le Tuan Anh[a], Bui Huu Tai[a], Pham Hai Yen[a], Nguyen Xuan Cuong[a], Nguyen Phuong Thao[a], Nguyen Thi Hoai[d], Nan Young Kim[b], Seon Ju Park[b] and Kim Seung Hyun[b,*]

[a]*Institute of Marine Biochemistry*, *Vietnam Academy of Science and Technology (VAST)*, *18 Hoang Quoc Viet*, *Caugiay*, *Hanoi*, *Vietnam*

[b]*College of Pharmacy*, *Yonsei Institute of Pharmaceutical Sciences*, *Yonsei University*, *Incheon 406-840*, *Korea*

[c]*Institute of Chemistry*, *VAST*, *18 Hoang Quoc Viet*, *Caugiay*, *Hanoi*, *Vietnam*

[d]*Faculty of Pharmacy*, *Hue University of Pharmacy and Medicine*, *Hue*, *Vietnam*

phankiem@yahoo.com; kimsh11@yonsei.ac.kr

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Two new butenolide and pentenolide derivatives, dysideolides A-B, were isolated from the marine sponge *Dysidea cinerea*. Their structures were determined by the combination of spectroscopic and chemical methods, including 1D- and 2D-NMR spectroscopy, and CD spectra, as well as by comparing with the NMR data reported in the literature.

Keywords: *Dysidea cinerea*, Dysideidae, Dysideolide A, Dysideolide B, Marine sponge, Circular dichroism.

The marine sponge *Dysidea cinerea* Keller (Dysideidae) is distributed in the Red Sea, Western India, and Zanzibar. Marine sponges are a prolific source of anti-HIV proteins and study on chemical components of *D. cinerea* has led to the isolation of avarol and avarone derivatives [1a]. Several biological activities, such as anti-cytotoxic activity and inhibition of human immunodeficiency virus type 1 reverse transcriptase [1b,c], have also been reported. In the course of study of the chemical constituents of *D. cinerea*, butenolide and pentenolide derivatives were isolated (Figure 1).

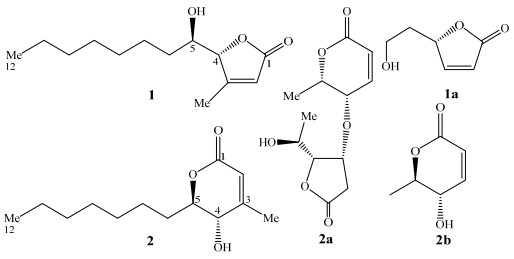

Figure 1: Structures of compounds **1** – **2** and reference compounds.

Compound **1** was isolated as colorless oil and its molecular formula was determined as $C_{13}H_{22}O_3$ from the ion at *m/z* 225.1490 $[M–H]^-$ in the HR ESI MS (Calcd. for $C_{13}H_{21}O_3$: 225.1496). The ^{1}H NMR spectrum of **1** showed: one olefinic proton at δ_H 5.84, two oxymethine protons at δ_H 3.86 and 4.91, and one tertiary methyl group at δ_H 2.10. The ^{13}C NMR and DEPT spectra of **1** revealed 13 carbon signals, including one carbonyl (δ_C 176.3), one quaternary (δ_C 170.6), three methine (δ_C70.0, 88.9, and 118.0), six methylene (δ_C 23.7, 27.2, 30.4, 30.6, 33.0, and 34.9), and two methyl carbons (δ_C 14.0 and 14.4) (Table 1). The ^{1}H and ^{13}C NMR data of **1** were very similar to those of 4,10-dihydroxy-10-methyl-dodec-2-en-1,4-olide, except for the position of the hydroxyl group at C-5 [2]. The long range correlations between H-4 (δ_H 4.91) and C-1 (δ_C 176.3), C-2 (δ_C 118.0), C-3 (δ_C 170.60, and 3-Me (δ_C 14.0); between 3-Me (δ_H 2.10) and C-2 (δ_C 118.0), C-3 (δ_C 170.6), and C-4 (δ_C 88.9) in the HMBC spectrum (Figure 2) suggested that compound **1** had an α,β-unsaturated lactone and the methyl group was located at C-3. Furthermore, the hydroxyl group at C-5 was confirmed by the HMBC correlation between H-5 (δ_H 3.86) and C-3 (δ_C 170.6), C-4 (δ_C 88.9), and C-6 (δ_C 34.9), and by COSY correlations between H-4 (δ_H 4.91) and H-5 (δ_H 3.86); and between H-5 (δ_H 3.86) and H-6 (δ_H 1.62). The absolute configuration of **1** was determined by circular dichroism. The CD spectrum of **1** showed a positive peak at 207 nm, consistent with those of compound **1a** (a positive peak at 203 nm) [3a]. Therefore, the stereochemistry at C-4 of **1** was proved to be *R*. The remaining configuration at C-5 was determined by the advanced Mosher's method [3b]. Treatment of **1** with (*S*)- and (*R*)-*R*-methoxy-*R*-(trifluoromethyl)phenylacetyl (MTPA) chlorides using catalytic DMAPafforded (*S*)- and (*R*)-MTPA esters (**1s** and **1r**), respectively. Analysis of the proton chemical shift differences between its (*S*)- and (*R*)-MTPA esters showed negative $\Delta\delta_H$ signs for H-2(-0.03), H-4 (-0.06), and 3-Me (-0.03),and positive signs for H-5(+0.03), and H-12 (+0.02). This distribution of Δδ signs enabled assignment of the *R* configuration to C-5. Consequently, compound **1** was elucidated to be (4*R*,5*R*)-4,5-dihydroxy-3-methyldodec-2-en-1,4-olide, and named dysedeolide A.

Compound **2** was isolated as colorless oil and its molecular formula was determined to be $C_{13}H_{22}O_3$ by the HRESIMS ion at *m/z* 225.1491 $[M–H]^-$ (Calcd. for $C_{13}H_{21}O_3$: 225.1496). The ^{1}H NMR spectrum of **2** showed: one olefinic proton at δ_H 5.78, two oxymethine protons at δ_H 4.07 and 4.22, and one tertiary methyl group at δ_H 2.03. The ^{13}C NMR and DEPT spectra of **2** revealed 13 carbon signals, including one carbonyl (δ_C 163.5), one quaternary (δ_C 158.9), three methine (δ_C 69.4, 81.8, and116.7), six methylene (δ_C22.6, 24.9, 29.1, 29.3, 31.7, and 32.7), and two methyl carbons (δ_C14.1 and 19.1). The ^{1}H- and ^{13}C NMR data of **2** were very similar to those of dysideolide A, except for the butenolide group replaced by a penteolide group. The HMBC correlations between H-5 (δ_H 4.22) and C-3 (δ_C 158.9), C-4 (δ_C 69.4), and C-7 (δ_C 24.9); between 3-Me (δ_H 2.03) and C-2 (δ_C 116.7), C-3 (δ_C158.9), and C-4 (δ_C 69.4) suggested that compound **2** had an α,β-unsaturated-δ-lactone and the methyl group was at C-3. In addition, the hydroxyl

Table 1: NMR spectral data for compounds **1**– **2**.

Pos.	1		2	
	δ_C [a,c]	δ_H [a,d] (mult., *J* in Hz)	δ_C [b,c]	δ_H [b,d] (mult., *J* in Hz)
1	176.3	-	163.5	-
2	118.0	5.84 (s)	116.7	5.78 (s)
3	170.6	-	158.9	-
4	88.9	4.91 (s)	69.4	4.07 (d, 7.6)
5	70.0	3.86 (t, 6.4)	81.8	4.22 (dt, 3.6, 7.6)
6	34.9	1.62 (m)	32.7	1.67 (m), 1.75 (m)
7	27.2	1.37, 1.51	24.9	1.41, 1.55
8	30.6	1.31	29.3	1.27
9	30.4	1.31	29.1	1.27
10	33.0	1.29	31.7	1.23
11	23.7	1.30	22.6	1.25
12	14.4	0.88 (t, 6.5)	14.1	0.86 (t, 6.5)
3-Me	14.0	2.10 (s)	19.1	2.03 (s)

[a]CD_3OD, [b]$CDCl_3$, [c]100 MHz, [d]400 MHz, Assignments were made by HMQC, HMBC, and COSY experiments.

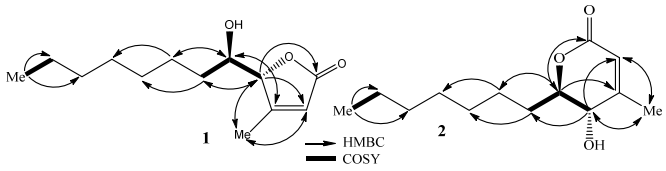

Figure 2: Key HMBC and COSY correlations of **1** – **2**.

group at C-4 was confirmed by the HMBC correlations between H-4 (δ_H 4.07) and C-2 (δ_C 116.7), C-5 (δ_C 81.8), and C-6 (δ_C 32.7), and COSY correlations between H-4 (δ_H 4.07) and H-5 (δ_H 4.22) and H-6 (δ_H 1.67 and 1.75). The absolute configuration at C-5 of **2** was fixed by CD measurements and comparison with **2a** [3c]. The CD spectrum of **2** showed a positive peak at 219 nm, while compound **2a** showed a negative peak at 221 nm (5*S* configuration) [3c]. Based on this evidence, the stereochemistry at C-5 was proved to be *R*. Moreover, the coupling constant between H-4 and H-5 in the hexacyclic ester of **2**, $J_{4\text{-}5}$ = 7.6 Hz, confirmed the *threo* of the both protons H-4 and H-5 by comparing coupling constant of two *erythro* protons of **2a**, $J_{4\text{-}5}$ = 3.0 Hz [3c] and *threo* protons of **2b**, $J_{4\text{-}5}$ = 8.8 Hz [3d]. Based on the above evidence, compound **2** was elucidated as (4*S*,5*R*)-4,5-dihydroxy-3-methyldodec-2-en-1,5-olide, and named dysideolide B.

Experimental

General: The NMR spectra were recorded on an Agilent 400-MR spectrometer using TMS as internal standard. The HRESIMS were obtained using an AGILENT 6550 iFunnel Q-TOF LC/MS system. Optical rotations were determined on a Jasco DIP-370 automatic polarimeter. Circular dichroism spectra were determined on a Chirascan^{TM} CD spectrometer. Preparative HPLC was carried out using an AGILENT 1200 HPLC system. CC was performed using either silica-gel (Kiesel gel 60, 70-230 mesh and 230-400 mesh, Merck) or YMC RP-18 resin (30 - 50 µm, Fuji silisa Chemical Ltd.), and TLC by using pre-coated silica-gel 60 F254 (0.25 mm, Merck) and RP-18 F254S plates (0.25 mm, Merck).

Sponge material: *D. cinerea* was collected in Lang Co beach, Vietnam during August 2011, and identified by Dr Do Cong Thung, Institute of Marine Resources and Environment. A voucher specimen (DC1108) was deposited at the Herbarium of the Institute of Marine Biochemistry, VAST.

Extraction and isolation: Freeze dried tissue of *D. cinerea* (1.0 kg) was cleaned to remove sodium chloride and then extracted 3 times with MeOH under reflux for 15 h to yield 60 g of a dark solid extract. This was suspended in water and partitioned with $CHCl_3$ to obtain $CHCl_3$ (DC1, 20.0 g) and water extracts (DC2, 40 g) after removal of the solvents *in vacuo*. The DC1 extract (20.0 g) was chromatographed on a silica gel column eluting with a gradient of *n*-hexane – acetone (40 : 1 → 0 : 1, v/v) to obtain 5 sub-fractions, DC1A (3.2 g), DC1B (4.5 g), DC1C (3.3g), DC1D (3.1 g), and DC1E (5.0 g). The sub-fraction DC1B (4.5 g) was chromatographed on a silica gel column eluting with *n*-hexane – EtOAc (10 : 1, v/v) to give 3 smaller fractions, DC1B1 (1.0 g), DC1B2 (1.5 g), and DC1B3 (1.7 g). Fraction DC1B2 was chromatographed on an YMC RP-18 column eluting with MeOH – water (5 : 1, v/v) to give 3 fractions, DC1B2A (450 mg), DC1B2B (200 mg), and DC1B2C (400 mg). Fraction DC1B2B was chromatographed by HPLC using J'sphere ODS H-80, 250 mm × 20 mm, and 35% acetonitrile in water at a flow rate of 3 mL/min to yield **1** (7.0 mg) and **2** (6.0 mg).

Dysideolide A (**1**)
Colorless oil.
$[\alpha]_D^{25}$: +45 (*c* 0.1, MeOH);
CD $[\theta]_{207}$: + 2.2 × 10^4 (*c* 5.0 ×10^{-4}, MeOH).
^{1}H and ^{13}C NMR (CD_3OD): Table 1.
HRESIMS found *m/z*: 225.1490 $[M - H]^-$ (Calcd. for $C_{13}H_{21}O_3$: 225.1496).

(*S*)-MTPA ester of 1 (**1s**)
^{1}H NMR (500 MHz,$CDCl_3$):δ_H 5.86 (H-2), 4.79 (H-4), 4.61 (H-5), 0.89 (H-12), and 2.13 (3-Me).

(*R*)-MTPA ester of 1 (**1r**)
^{1}H NMR (500 MHz, $CDCl_3$):δ_H 5.89 (H-2), 4.85 (H-4), 4.58 (H-5), 0.87 (H-12), and 2.16 (3-Me).

Dysideolide B (**2**)
Colorless oil.
$[\alpha]_D^{25}$: +35 (*c* 0.1, MeOH).
CD $[\theta]_{219}$ + 2.2 × 10^4 (*c* 5.0 ×10^{-4}, MeOH).
^{1}H and ^{13}C NMR ($CDCl_3$, 400 MHz): Table 1.
HRESIMS found *m/z*: 225.1491 $[M - H]^-$ (Calcd. for $C_{13}H_{21}O_3$: 225.1496).

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A New Cyclopeptide from Endophytic *Streptomyces* sp. YIM 64018

Xueqiong Yang[a,1], Yabin Yang[a,1], Tianfeng Peng[a], Fangfang Yang[a], Hao Zhou[a], Lixing Zhao[b], Lihua Xu[b] and Zhongtao Ding[a,*]

[a]*Key Laboratory of Medicinal Chemistry for Natural Resource, Ministry of Education, School of Chemical Science and Technology,Yunnan University, Kunming 650091, China*

[b]*Yunnan Institute of Microbiology, Yunnan University, Kunming 650091, China*

[1]The authors contributed equally to this paper

ztding@ynu.edu.cn

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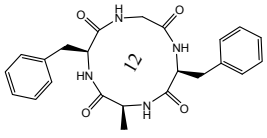
One new cyclopeptide, cyclo(L-Phe-L-Ala-L-Phe-Gly), named as vinaceuline (**1**) and three known cyclodipeptides, cyclo (Phe-Gly), cyclo (Phe-4-hydroxyl-Pro) and cyclo (Phe-Ile) were isolated from broth culture of endophytic *Streptomyces* YIM 64018 associated with *Paraboea sinensis*. The planar structure of the new compound was assigned on the basis of 1D and 2D NMR spectroscopic techniques, while the absolute configurations of the amino acid residues were determined by application of the advanced Marfey method. Cyclotetrapeptides are rarely found as Streptomycete metabolites.

Keywords: *Streptomyces* sp., Cyclopeptide, Vinaceuline.

Streptomycetes are believed to be a rich source of new and useful compounds, and up to 40% of known microbial metabolites are derived from this group. Endophytes are also considered as important natural sources of a variety of secondary metabolites [1,2]. New cyclopeptides with antimicrobial and anticancer activities have been reported from different bacterial genera [3,4]. In this work, chemical investigation of the endophytic *Streptomyces* sp. (YIM64018) was carried out for its antimicrobial activity against *Escherichia coli*. One new cyclopeptide and three known cyclodipeptides were isolated, and their structures were determined as cyclo(L-Phe-L-Ala-L-Phe-Gly) (**1**), named as vinaceuline, cyclo (Phe-Gly)[5], cyclo (Phe-4-hydroxyl-Pro)[5] and cyclo (Phe-Ile)[6] (Figure 1).



1

Figure 1: Structure of compound **1**.

The molecular formula of compound **1** was determined as $C_{23}H_{26}N_4O_4$ from its DEPT spectrum and HRESI-MS (*m/z* 423.2069 for $[M+H]^+$), with 13 degrees of unsaturation. The ^{13}C NMR spectrum showed the presence of four amide CO at δ 168.8, 168.2, 167.1 and 166.5, three CH at δ 57.1, 56.8 and 51.1, three CH_2 at δ 44.8, 40.6 and 40.3, and one CH_3 at δ 20.5. The ^1H NMR spectrum showed the presence of four amide NH at δ 9.35 (1H, br s), 9.18 (2H, br s), and 9.06 (1H, br s), three methine protons at δ 4.63 (1H, br s), 4.58 (1H, br s), and 4.17 (1H, t, J = 5.7Hz), three methylene signals at δ 3.95, 3.52 (2H), 3.45 (2H), and 3.28 (2H), one methyl group at δ 1.09 (3H, d, J = 7.0Hz). These facts indicated that **1** is a cyclotetrapeptide. The amino acid units were identified by ^1H-^1H COSY, HSQC and HMBC experiments as two phenylalanine groups (Phe), one alanine group (Ala) and one glycine group (Gly) (Figure 2).

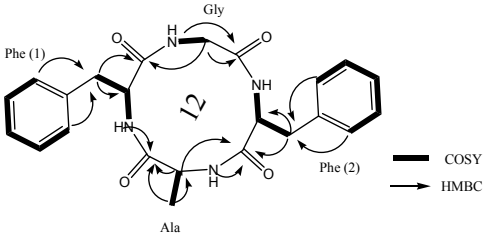


Figure 2: Key HMBC and ^1H-^1H COSY correlations of compound **1**.

The sequence of the amino acid residues in **1** was established by analysis of the HMBC (Figure 2) and ROESY data. The HMBC spectrum showed correlations between δc 168.2 (Phe (1)-CO) and δ_H 3.95 (αH- Gly), δc 168.8 (Ala-CO) and δ_H 9.18 NH (Phe (1)), and δc 167.1 (Phe (2)-CO) and δ_H 4.17 (αH- Ala). The ROESY spectrum also revealed correlations between δ_H 9.35 NH {(Phe (2)} and 9.06 NH (Gly), and 9.18 NH (Ala). This evidence indicated the presence of a tetrapeptide fragment as -Gly-Phe-Ala-Phe-. The appearance of fragments of Gly-Phe or Phe-Gly, Phe-Ala or Ala-Phe in ESI-MS analyses at *m/z* 205 $[M+H]^+$ and 219 $[M+H]^+$ also confirmed this proposed linkage pattern. To determine the absolute configurations of the amino acid constituents in **1**, the advanced Marfey method was applied [7]. All derivatives were identified by their retention times and molecular weights. The configuration of the Phe unit was determined by comparing the retention times of LD-derivatized hydrolysate (27.94 min and 39.49 min) with the L-derivatized hydrolysate (27.86 min). The configuration of the Ala unit was determined by comparing the retention times of LD-derivatized hydrolysate (14.25 min and 20.65 min) with the L-derivatized hydrolysate (14.52 min). Therfore the structure of compound **1** was determined as cyclo (Gly-L-Phe-L-Ala-L-Phe) and was named vinaceuline (**1**).

Three known compounds were determined as cyclo (Phe-Gly), cyclo (Phe-4-hydroxyl-Pro) and cyclo (Phe-Ile) by spectroscopic and ESIMS analysis, and by comparison with references [5-6].

Experimental

General experimental procedures: Silica gel (100-200, 200-300 mesh; Qingdao Marine Chemical Group Co.) and Sephadex LH-20 (GE Healthcare Co) were used for CC. 1D and 2D NMR spectra were obtained on Bruker DRX-600 MHz and Bruker DRX-500 MHz instruments with TMS as internal standard. MS were recorded with Agilent G3250AA, AutoSpec Premier P776 and Waters Xevo TQ-S mass spectrometers.

Biological material and cultivation of actinomycetic strain: The bacterial strain was isolated using trehalose-proline medium from the plant *Paraboea sinensis*, and was assigned as *Streptomyces vinaceus* by characterization and complete 16S rRNA gene sequence. The strain has been preserved at Yunnan Institute of Microbiology, Yunnan University, China. This bacterium was cultivated on a 40 L scale using 1L Erlenmeyer flasks containing 250 mL of the seed medium (yeast extract 0.4%, glucose 0.4%, malt extract 0.5%, decavitamin 0.01%, pH 7) for 3 days and the fermentation medium (soluble starch 1%, glucose 1%, peptone 0.5%, NaCl 0.4%, 0.05% K_2HPO_4, $MgSO_4{\cdot}7H_2O$ 0.05%, $CaCO_3$0.2%, pH 7.0) for 5 days at 28°C on a rotary shaker (250 rpm).

Extraction and isolation of compounds: The fermentation broth (40L) containing *Streptomyces* sp. YIM 64018 was filtered, the filtrate extracted with EtOAc and the solvent removed under vacuum. The EtOAc extract (11 g) was separated into 4 fractions by CC on silica gel (200–300 mesh), eluting stepwise with $CHCl_3$/MeOH gradient ($CHCl_3$, $CHCl_3$/MeOH: 10:1, v/v, $CHCl_3$/MeOH: 3:1, v/v, MeOH). The second fraction (eluted with $CHCl_3$/MeOH: 10:1, v/v) was separated by CC on silica gel eluting stepwise with a $CHCl_3$/MeOH gradient (from 30:1 to 2:1, v/v) to produce 5 fractions (Fr.1~Fr.5). Fr.2 was further purified by repeated CC on silica gel eluting stepwise with a $CHCl_3$/MeOH gradient (from 20:1 to 1:1, v/v) and Sephadex LH-20 (MeOH) to afford **1** (4.6 mg), cyclo (Phe-Gly) (6.2 mg), cyclo (Phe-4-hydroxyl-Pro) (2.7 mg), and cyclo (Phe-Ile) (5.1mg).

Acid hydrolysis and advanced Marfey analysis: A 1.0 mg sample of compound **1** was subjected to acid hydrolysis at 110°C for 24 h with 6 N HCl (1.0 mL), and then the hydrolysates were evaporated to dryness and resuspended in acetone (100 μL). To each half portion (50 μL) was added 20 μL $NaHCO_3$ (1 M) and either 100 μL 1-fluoro-2,4-dinitrophenyl-5-L-leucinamide or 1-fluoro-2,4-dinitrophenyl-5-D-leucinamide (L-FDLA or D-FDLA, 1% in acetone), and the mixture was heated at 40°C for 1.5 h. The reaction mixtures were cooled, neutralized with 2 N HCl (10 μL), dried and dissolved in 50% aqueous MeCN. About 5 μL of each solution of FDLA derivatives was analyzed by LC/MS.

The analysis of the L- and LD-FDLA (mixture of D- and L-FDLA) derivatives was performed using an Agilent Eclipse XDB-C_{18} column (4.6×150 mm, 5 μm) maintained at 40°C. Aqueous MeCN containing 0.01 M methanoic acid was used as mobile phase with a linear gradient elution mode (MeCN, 30-50% for 50 min) at a flow rate of 1.0 mL/min. A Waters Xevo TQ-S mass spectrometer was used for detection in ESI (negative) mode. The capillary voltage was kept at 2.5 KV, and the ion source at 350°C. Nitrogen gas was used as a sheath gas at 400 L/h. A mass range of m/z 100-1000 was scanned in 0.2 s.

Compound 1

white crystals ($CHCl_3$).

$[\alpha]_D^{25}$ 21.3 (*c* 0.1, DMSO).

^{1}H NMR (C_5D_5N, 500 MHz) δ: 9.35 (1H, br s, Phe(2)-NH), 9.18 (1H, br s, Phe(1)-NH), 9.18 (1H, br s, Ala-NH), 9.06 (1H, br s, Gly-NH), 7.44 (2H, d, *J* = 8.0 Hz, Phe (1)-H-2,6), 7.43 (2H, d, *J* = 8.0 Hz, Phe (2)-H-2, 6), 7.25 (2H, d, *J* = 8.0 Hz, Phe (1)-H-3, 5), 7.23 (2H, d, *J* = 8.0 Hz, Phe (2)-H-3, 5), 7.22 (1H, d, *J* = 8.0 Hz, Phe (1)-H-4), 7.20 (1H, d, *J* = 8.0 Hz, Phe (2)-H-4), 4.63 (1H, br s, Phe (1)-αH), 4.58 (1H, br s, Phe (2)-αH), 4.17 (1H, q, *J* = 5.7Hz, Ala-αH), 3.95 (1H, dd, *J* = 3.0, 17.0 Hz, Gly-αH), 3.52 (1H, m, Gly-αH), 3.45 (2H, m, Phe (1,2)-βH), 3.28 (2H, m, Phe (1,2)-βH), 1.09 (3H, d, *J* = 7.0Hz, Ala-βH).

^{13}C NMR (C_5D_5N, 125 MHz) δ: 168.8 (Ala-CO), 168.2 (Phe(1)-CO), 167.1 (Phe(2)-CO), 166.5 (Gly-CO), 136.9 (Phe (1)-C-1), 136.7 (Phe (2)-C-1), 130.7 (Phe(1)-C-2,6), 130.5 (Phe(2)-C-2,6), 128.5 (Phe(1)-C-3,5), 128.5 (Phe(2)-C-3,5), 127.0 (Phe(1)-C-4), 126.9 (Phe(2)-C-4). 57.1 (Phe (1)-αC), 56.8 (Phe (2)-αC), 51.1 (Ala-αC), 44.8 (Gly-αC), 40.6 (Phe (1)-βC), 40.3 (Phe (2)-βC), 20.5 (Ala-βC).

HRESI-MS: *m/z* $[M+H]^+$ calcd for $C_{23}H_{27}N_4O_4$: 423.2026; found: 423.2069.

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Involvement of Trypsin-Digested Silk Peptides in the Induction of RAW264.7 Macrophage Activation

Kyoung-Ho Pyo[a], Min-Ki Kim[a], Kwang-Soon Shin[b], Hyang Sook Chun[c] and Eun-Hee Shin[a,d,*]

[a]*Department of Parasitology and Tropical Medicine, Seoul National University College of Medicine and Institute of Endemic Diseases, Seoul National University Medical Research Center, Seoul 110-799, Republic of Korea*
[b]*Department of Food science and Biotechnology, Kyonggi University, Suwon-si, 443-760, Republic of Korea*
[c]*Deparment of food Science & Technology, Chung-Ang University, Ansung 456-756, South Korea*
[d]*Seoul National University Bundang Hospital, Seongnam 463-707, Republic of Korea*

ehshin@snu.ac.kr

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The activation of macrophages by trypsin-digested silk peptides was investigated by considering CD11b and CD40 expression in the RAW264.7 cell, a murine macrophage. Silk protein hydrolysates were digested with trypsin, following by centrifugal purification using the Centriprep 30k concentrator. Trypsin-digested total silk peptides and its centrifugal fractions were tested for macrophage activation *in vitro.* The functional peptide of fractionated silk peptides was examined by LC/MS/MS analysis. Trypsin-digested and fractionated silk peptides of more than 30 kDa induced an increase in the activation markers CD11b and CD40 in RAW264.7 cells. These results are supported by morphological changes reflecting an increase in the number of dendrites in activated cells. The fractionated silk peptides examined by LC/MS/MS contained partial peptides of *Bombyx mori* fibroin. These results suggest that the activation of RAW264.7 macrophages may be induced not by sericin-derived peptides but by fibroin-derived ones.

Keywords: Silk peptide, Trypsin, Fibroin, Immune response, Macrophage, CD11b, CD40.

Bombyx mori is a domesticated silkworm species, and its cocoon silk has been an important textile source [1a]. In recent years, there has been a renewal of interest in the usefulness of silk as a bioactive material [1a,b]. Silk protein produced by *B. mori* consists of two major proteins: fibroin and sericin [1c]. Previous studies have emphasized the biological usefulness of sericin because of its moisture-absorbing, antioxidant, and wound-healing properties [1d, 2a]. In contrast, previous studies have focused on fibroin for its use in tissue engineering scaffolds, wound dressings, and vascular grafts [1d,2b,c]. Recent studies have demonstrated some bio-efficacy of silk fibroin, including its ability to reduce blood pressure, its anti-tumor activity, and for treatment of atopic dermatitis [3,4a,b]. In addition, silk protein has been used to enhance the immune response, ameliorate the progression of skin lesions resembling atopic dermatitis, and facilitate insulin-releasing activity through the induction of β-cell activity in C57BL/KsJ-db/db mice [4b,c,5a].

Recently, an increase in the immune response in mice treated with silk protein has been demonstrated, namely an antiprotozoal effect on *Toxoplasma gondii,* a zoonotic protozoan [4c]. The protective immune response to *T. gondii* usually entails the activation of macrophages as well as T cells [5b]. A macrophage is an antigen-presenting cell (APC) and plays an important role in innate immunity and helper T-cell immune responses [5c,6]. The activation of macrophages to microbial ligands results in an increase in surface molecules such as CD11b and CD40 [5c,6]. CD11b is an activation marker of macrophages and is expressed on the surface of monocytes, granulocytes, macrophages, and natural killer cells. CD40 is a co-stimulatory molecule in APCs and is an essential mediator of T-cell activation for the secretion of IL-12 and TNF-α [5c,6]. In this regard, the present study identifies the specific fraction in silk peptides not only for the activation of macrophages as effector cells for an innate immune response but also APCs for an immune response mediated by T cells. For this purpose, this study examines two biomarkers on the surface of the RAW264.7 cell, a murine macrophage —CD11b and CD40—which increase during the activation of macrophages.

With LPS treatment, the expression of CD11b and CD40 in RAW264.7 cells increased by 58.9% and 86.6%, respectively (Figure 1A and 1F). For an analysis of the synergistic effect of LPS and silk peptides (TSP) on the expression of CD11b and CD40, LPS and TSP were co-treated during cell culture. The results indicate that CD11b expression was slightly lower in co-treated cells (49.7%) than in LPS (58.9%), suggesting no synergetic effect of LPS and TSP on CD11b expression. By contrast, TSP treatment induced a 24.2% increase in CD11b expression in RAW264.7 cells. Similarly, FSP>30 treatment induced a 26.3% increase in CD11b expression, and FSP<30 treatment, a 3.8% increase. In terms of CD40 expression, TSP treatment induced a 30.9% increase in CD40 expression, and FSP>30 treatment, a 36.5% increase. LPS treatment induced an 86.6% increase in CD40 expression, and co-treatment of LPS and TSP, a 77.9% increase. There was no synergistic effect of LPS and TSP, and FSP<30 had no effect on CD40 expression (Figure 1F-J).

For an analysis of morphological changes in RAW264.7 cells after silk peptide treatment, the cells were cultured with either peptides (TSP, FSP>30, or FSP<30) or LPS for 24 h in six-well plates (Figure 2). When RAW264.7 cells were not stimulated, they had no dendrites, which increase in activated macrophages (Figure 2A). However, those cells treated with LPS had many dendrites at 24 h after culture (Figure 2B). Similarly, the addition of either TSP or FSP>30 induced the production of many dendrites (Figure 2D and 2E).

By contrast, FSP<30 treatment did not induce the formation of dendrites (Figure 2F), and the morphology was similar to that of normal RAW264.7 cells (Figure 2A).

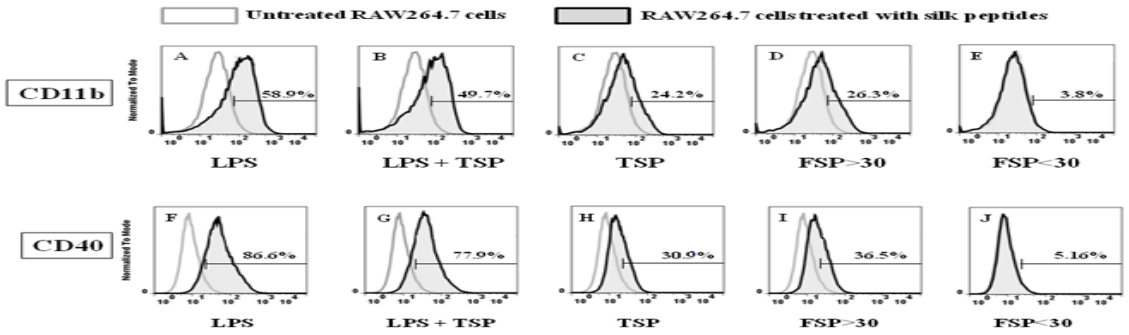

Figure 1: CD11b and CD40 expression in RAW264.7 cells after treatment with silk peptides. Activation markers of macrophages—CD11b and CD40—were examined through a FACS analysis after cultivation for 24 h. (A & F) Designated LPS stimulation; (B & G) LPS + TSP; (C & H) TSP only; (D & I) FSP>30; (E & J) FSP<30.

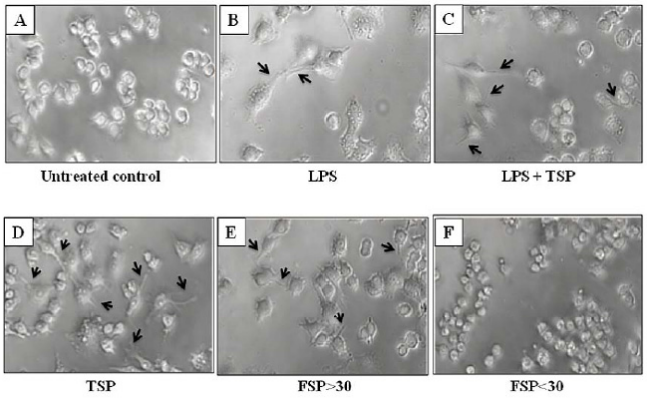

Figure 2: Morphological changes in RAW264.7 cells after treatment with silk peptides. RAW264.7 cells were incubated for 24 h with LPS, LPS+TSP, FSP>30, or FSP<30. Then the cells were observed under a microscope for dendrites in RAW264.7 cells treated with each of the aforementioned cell stimulants. Arrows indicate macrophage dendrites. Cell images were taken at original magnification x 400.

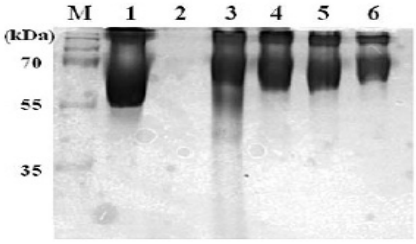

Figure 3: Verification of residual enzyme activity after the digestion of silk protein hydrolysates with trypsin. Silk protein hydrolysates were digested with trypsin, followed by enzyme inactivation at 95℃ for 30 min. For an analysis of residual trypsin activity, BSA was added to the digestion mixture. In addition, proteolysed samples were loaded on a 15% polyacrylamide gel. M: Marker; Lane 1: BSA; Lane 2: BSA+Trypsin; Lane 3: BSA+Heat-inactivated trypsin; Lane 4: BSA+TSP (1:1); Lane 5: BSA+TSP (1:2); Lane 6: BSA+TSP (1:5).

Trypsin was used for the proteolysis of silk protein to augment the absorption of ingested proteins. For an analysis of residual trypsin activity, BSA was used (Figure 3). Lane 1 shows intact BSA (63 kDa), and lane 2, trypsin-digested BSA. Lane 3 shows undegraded BSA when heat-inactivated trypsin was added. Here, heat inactivation reduced the proteolytic activity of trypsin.

For an analysis of residual trypsin activity after the proteolysis of silk protein, the reaction product of silk protein with trypsin was mixed with BSA for further proteolysis in the ratio of 1:1, 1:2, or 1:5 for BSA to the reaction mixture of trypsin and silk peptides. The results indicate no further proteolysis of BSA in the reaction mixture of trypsin and silk peptides (lanes 4, 5, and 6). This implies that there was no residual trypsin activity in trypsin-digested silk peptides and thus there was no relationship between residual trypsin

Table 1: LC-MS/MS analysis of silk peptide fraction of FSP>30.

LC-MS/MS data	Sequences	score
Fibroin [Bombyx mandarina]	DASGAVIEEEITTK	90
Putative reading frame (fibroin) [Bombyx mori]	DASGAVIEEQITTK	89
Aspartic endopeptidase Pep1	MVVFSKVTAAVFGLATIASAAPAPPTRK	80
Hypothetical protein	KKVSLNVWLALK	78
PREDICTED: similar to hCG1980490	EAGPPR	74
PREDICTED: similar to hCG1980490	ILQLPGAPDCPLLRVLAGKAEGAEEAGSLP WTVTQMLEGNGSVSLLLR	74
Fibroin	DIDDGK	73
Fibroin	SIAILNVQEILK	73
CRISPR system CASCADE complex protein CasC	QKALEVVKALGLK	71
Secretion protein HlyD	RGGGASGVHGASGARAASGAHGSGASPQ QAQP	70

activity and the activation of RAW264.7 cells. The silk peptide fraction of FSP>30 was concentrated using Centriprep 30K and analyzed by LC-MS/MS. Peptide sequences were aligned and counted by plot scores by using MASCOT (Matrix Science, www.matrixscience.com). Ten peptides were selected from the list of top hits (Table 1). The scores ranged from 70 to 90, and other putative peptides such as Pep1, hCG, CasC, and HlyD as well as fibroin-derived peptides were also detected (Table 1). However, because the results of the MALDI-TOF analysis indicate a portion of some peptides, *B. mori* fibroin-derived sequences DIDDGK and SIAILNVQEILK were considered as a main ingredient of FSP>30. Here no sericin fragment was found in silk peptides of FSP>30. Although this result does not clearly suggest that the two targeted sequences are functional peptides, fibroin-derived peptides may be major peptides in FSP>30.

Previous studies have emphasized the usefulness of silk protein in textiles [1a]. However, there has been some concern over its biomedical and biomaterial applications such as cosmetics and functional foods because of its safety and functional characteristics [1b-d,2a,b, 3,4a-c,5a]. In this regard, silk protein has been known to have a favorable effect on diabetes, blood pressure, anti-tumor activity, skin lesions resembling atopic dermatitis, and antiprotozoal activity [3,4a-c,5a]. A recent study examined the ability of silk protein to induce immunity to infection or enhance the immune system by activating immune cells [4c]. When BALB/c mice ingested silk protein hydrolysates, they were immune to *T. gondii,* an intracellular protozoan, and this protective immunity was characterized by increases in $CD4^+$- and $CD8^+$-T cells, and a sharp increase in IFN-γ [4c]. These results suggest that silk protein can be used as a natural compound for enhancing immunity [4c]. As a result, there has been growing interest in the role of silk protein as a functional material for various applications in the biological, biomedical, and biotechnology fields.

Silk protein consists of fibroin and sericin, which have distinct characteristics and biological functions [7]. Fibroin is a glycoprotein composed of two equimolar protein subunits of 370 kDa and 25 kDa covalently linked by disulfide bonds [7]. Sericin contains 18 amino acids, including essential amino acids. Sericin accounts for approximately 20-30 % of total cocoon weight [7], and, therefore, *B. mori* silk protein hydrolysates prepared from $CaCl_2$ treatment contain 70-80% fibroin and 20-30% sericin. In a recent study, enzymatic degradation was used to extract bioactive peptides from silk protein [7,8,9a]. Collagenase IA, α-Chymotrypsin, Protease XIV, Protease P, Alkylase, Alkaline protease, Trypsin, Protease N, Alcalase2.4L, As 1.398, and Neutrase have been used for the enzymatic degradation of silk protein. Among these, trypsin is a serine protease found in the digestive system of many vertebrates, where it hydrolyses proteins [9b]. Trypsin has been found to cleave the Arg-Gly bond toward the insulin β-chain at pH 8.0 [10a]. Although the enzymatic hydrolysis of silk protein has been attempted using various enzymes, trypsin is a safe endogenous enzyme used in the food industry. Tryptic hydrolysis has been well analyzed in the food science field to improve the quality of food products [10b]. In the present study, trypsin was used to increase the absorption of silk peptides by pre-performing the digestion process in the digestive tract. The results of the computer-based cleavage site prediction of endopeptidases with PeptideCutter (http://www.expasy.org/tools/peptidecutter/) [11a] indicate that the tryptic hydrolysis of fibroin produced peptides of various sizes from 146 Da to 325,525 Da. By contrast, the tryptic hydrolysis of sericin produced peptides ranging from 146 Da to 7,065 Da (data not shown). For the identification of the size-based fraction of silk peptides inducing an immune response, the fraction based on 30 kDa was obtained using the Centriprep 30k concentrator. A recent study found an increase in the immune enhancement of silk protein hydrolysates, namely an increase in T-cell responses [4c]. More specifically, the initial immune response mediated by T cells was induced and accelerated by APCs such as macrophages and dendritic cells. Macrophages play a critical role in innate as well as acquired immunity and can induce protective cellular immunity to *T. gondii* infection. In this regard, we used the RAW264.7 cell, a murine macrophage, to investigate the silk peptide fraction and examine the immune response because trypsin-digested silk peptides can activate macrophages and increase the expression of specific activation markers in macrophages.

LPS can activate macrophages through TLR4 (Toll-like receptor 4) [11b]. Macrophages activated with LPS can increase the expression of CD11b and CD40. In this regard, RAW264.7 cells can express the activation markers CD11b and CD40 in response to LPS [5a,b,6]. In the present study, LPS alone, and LPS with TSP induced a similar increase in CD11b and CD40 expression in RAW264.7 cells. This indicates no synergistic effect of silk peptides and LPS on CD11b and CD40 expression. TSP and FSP>30 induced 24.2% and 26.3% increases, respectively, in CD11b expression. However, FSP<30 induced only a negligible (3.8%) increase in CD11b expression. Similarly, TSP and FSP>30 induced 30.9% and 36.5% increases, respectively, in the expression of CD40, a co-activation marker. Morphological changes in RAW264.7 cells reflected the result in CD11b and CD40 expression. That is, when RAW264.7 cells were not stimulated, they had no dendrites, which generally increase in activated macrophages. However, RAW264.7 cells with LPS treatment had many dendrites at 24 h after treatment. Similarly, TSP and FSP>30 induced the production of many dendrites. However, FSP<30 did not induce dendrite formation. These results indicate that TSP and FSP>30 may activate macrophages through a mechanism different from that of LPS because of a lack of any synergistic effect of LPS and TSP. When macrophages are activated, surface molecules such as TLRs, integrin, and activating receptors increase to recognize pathogens, and there is an increase in cell motility [11b]. Although the mechanism underlying the activation of RAW264.7 cells by silk peptides remains unclear, this study's results suggest that silk peptides may activate cells not through TLR 4 but through other activation receptors. As another mechanism, trypsin is a factor that can activate PAR2 (protease-activated receptor 2) and induce superoxide anion production through degranulation by eosinophils [11c]. However, trypsin activity was checked in this study through a BSA degradation test, and the endotoxin level was determined using the LAL test kit (data not shown). The results demonstrate no relationship between both these factors and the immune activation of RAW264.7 cells. In addition, immune activation by TSP treatment was consistent with the effect of FSP>30. Further, according to the LC-MS/MS analysis, FSP>30 contained fibroin-derived peptides. As discussed earlier, the tryptic degradation of sericin was divided into small peptides of less than 30 kDa, and the results for anti-tumor activity and blood pressure [3,4a] suggests that fibroin, a major ingredient in FSP>30, may be a key factor in the activation of RAW264.7 cells. The present study suggests that the activation of RAW264.7 macrophages may be induced not by sericin-derived peptides but by fibroin-derived ones. In this regard, future research should precisely identify the active peptides.

Experimental

Preparation of trypsin-digested silk peptides: Hydrolyzed silk protein was prepared from cocoons of the silkworm *B. mori* [4c]. The hydrolysates were sterilized by boiling at 90°C ± 3°C. For the additional trypsin digestion of the hydrolysates, trypsin was dissolved in DW and reacted at 37°C for 12 h with the hydrolysates by adjusting at the final trypsin concentration of 0.25%. After the digestion, the trypsin enzyme was inactivated by boiling at 95°C for 30 min. Trypsin-digested total silk peptides (TSP) were concentrated and freeze-dried.

Cell culture and the* in vitro *treatment of silk peptides: RAW264.7 cells were obtained from ATCC and maintained in DMEM (WelGENE Inc., Daegue, Korea) with 10 % FBS (Gibco, Carlsbad, CA, USA), 1% antibiotics/antimycotics (Gibco), 10 mM HEPES, 2 mM L-glutamine, 2 mM sodium bicarbonate, and 5 x 10^{-5} M 2-mercaptoethanol. Cultured RAW264.7 cells were counted and seeded at 1.0 x 10^6 cells/well onto six-well plates. For the *in vitro* experiment, 5 experimental groups were considered: LPS, LPS + trypsin-digested total silk peptides (TSP), TSP, fractionated silk peptides of more than 30 kDa (FSP>30), and fractionated silk peptides of less than 30 kDa (FSP<30). The concentration of LPS was 10 μg/mL, and that of the other stimulants 0.83 mg/mL for both TSP and fractionated silk proteins (FSP>30 and FSP<30). RAW264.7 cells were cultured *in vitro* for 24 h and then harvested using cold PBS to prevent cell activation by chemical or mechanical stimulation.

Fluorescence-activated cell sorter analysis: Harvested RAW264.7 cells were washed with DMEM without FBS by centrifugation at 1,200 rpm at 4°C for 5 min. The cell pellet was suspended in FACS (fluorescence-activated cell sorter) buffer (1% BSA and 0.1% NaN3). An anti-CD16/32 antibody (eBioscience, San Diego, USA) was applied and incubated for 10 min to block Fc receptors. FITC-conjugated anti-mouse CD11b (eBioscience) and PE-conjugated anti-mouse CD40 (eBioscience) were used for the FACS staining of RAW264.7 cells. Both anti-CD11b-FITC and anti-CD40-PE were diluted to 1: 100 with FACS buffer. The number of stained cells

among 2 x 10^4 cells was determined by FL-1 (FITC) and FL-2 (PE) filters in the FACSCalibur flow cytometer (BD, USA).

Fractionation of silk peptides using Centriprep 30K: One hundred mg of the silk peptide (TSP) was dissolved in 10 mL of DDW and fractionated by the Centriprep 30K concentrator (Millipore, Bedford, MA, USA). Ultrafiltration was performed at 1,500 x *g* at 4°C for 10 min. The concentrated samples were dissolved in DDW up to 10 mL and centrifuged again for 5 min. This process was repeated, and the final sample (FSP>30) was sterilized using a 0.45 μm syringe-driven filter unit (Millipore, MA, USA). Small peptides (FSP<30) were collected from the Centriprep filtered fraction and filtered using a 0.45 μm syringe filter (Millipore).

Morphological observation of RAW264.7 cells activated by silk peptides: RAW264.7 cells were cultured with LPS, LPS+TSP, TSP, FSP>30, or FSP<30 in six-well plates. After 24 h, the morphology of the cells was observed using a microscope equipped with a video camera. The cells were observed at x400 magnification.

Residual enzyme activity after the trypsin proteolysis of silk peptides: Silk protein hydrolysates were digested with trypsin (Novozymes, Bagsvaerd, Denmark) at 37°C for 12 h for proteolysis of silk protein. The reaction was stopped by incubating at 95°C for 30 min. For the determination of residual enzyme activity at the end of the enzyme reaction, the reaction product between silk protein and trypsin was incubated with 15 μg of BSA at 37°C for 12 h for the final reaction ratio of 1:1, 1:2, or 1:5 in BSA to the silk peptide reaction product. The final reaction sample was loaded on a 15% SDS-polyacrylamide gel based on SDS-PAGE.

In-gel digestion for LC/MS/MS: Ten μg of the protein sample was separated by 12% SDS-PAGE (mini-PROTEAN, Bio-Rad, Richmond, CA, USA), and the gel was stained with Coomassie Brilliant Blue R-250. In-gel digestion was conducted based on prescribed methods [12]. The gel was fractionated into 6 parts according to their molecular weight. Each part of the gel was digested with trypsin (0.1 μg) for 16 h at 37°C after the reduction and alkylation of cystein in the protein. Digested peptides were extracted using an extraction solution (50 mM ammonium bicarbonate, 50% acetonitrile, and 5% trifluoroacetic acid). Digested peptides were resolved in 10 μL of sample buffer containing 0.02% formic acid and 0.5% acetic acid.

1-DE and LC-MS/MS using LCQ mass spectrometry: Peptide samples (10 μL) were concentrated using a MGU30-C18 trapping column (LC Packings, Sunnyvale, CA). Then the peptides were eluted from the column and directed onto a 10 cm×5 μm i.d. C18 reverse phase column at a flow rate of 120 nL/min. For an additional analysis, peptides were eluted by a gradient of 0~65% acetonitrile for 120 min. Each full MS (m/z range of 300 to 2,000) scan was followed by 3 MS/MS scans of the most abundant precursor ions in the MS (with dynamic exclusion enabled). For protein identification, MS/MS spectra were searched by MASCOT (Matrix science, www.matrixscience.com) and determined by the database of the *B. mori* genome sequence.

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Low-Volatile Lipophilic Compounds in Needles, Defoliated Twigs, and Outer Bark of *Pinus thunbergii*

Alexander V. Shpatov[a], Sergey A. Popov[a,*], Olga I. Salnikova[a], Ekaterina A. Khokhrina[a], Emma N. Shmidt[a] and Byung Hun Um[b]

[a]*Novosibirsk Institute of Organic Chemistry*, *Lavrentyev Ave. 9*, *Novosibirsk*, *630090*, *Russia*
[b]*Natural Products Research Center*, *Korea Institute of Science and Technology – Gangneung Institute*, *290 Daejeon-dong*, *Gangneung 210-340*, *Gangwon-do*, *Republic of Korea*

spopov@nioch.nsc.ru

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Despite a long history of the use of *Pinus thunbergii* for technical, medicinal, agricultural, and other purposes, the composition of low-volatile metabolites in the used parts of the plant has been poorly investigated. We report here on the distribution of lipophilic extractive compounds in different parts of the shoot system (needles, defoliated twigs, outer bark) of *P. thunbergii* studied by GC/MS. The highest and lowest contents of lipophilic substances were found in defoliated twigs and in outer bark correspondingly. Acid compounds in the extract of needles comprised mainly labdane type diterpenoids (*trans*-communic acid), while in the extracts of defoliated twigs and outer bark the acids were represented predominantly by abietane type compounds (neoabietic, dehydroabietic, abietic, levopimaric and palustric acids). The major neutral components of the extract of needles were 10-nonacosanol, labdanoids (18-hydroxy-13-*epi*-manoyl oxide, *trans*-communol), and β-sitosterol. In the case of the extract of defoliated twigs, labdanoids (18-hydroxy-13-*epi*-manoyl oxide, *trans*-communol, 13-*epi*-torulosol), serratane triterpenoids (3β-methoxyserrat-14-en-21-one), and β-sitosterol were the main neutral constituents, whereas serratanoids (3β-methoxyserrat-14-en-21-one) alone dominated among the neutral compounds of the outer bark extract. Most of the neutral components and the labdane type acids were detected for the first time in organs and tissues of *P. thunbergii*. The distribution of lipophilic metabolites in the studied parts of *P. thunbergii* shoot system may be applied for chemotaxonomy purposes. Diversified accumulation of extractive substances in different organs of the plant should be taken into account for isolation of specific components from the pine raw material.

Keywords: *Pinus thunbergii*, Phytochemical study, Extractive compounds, Diterpenes, Triterpenes, Labdanoids, Serratanoids, GC/MS.

Pinus thunbergii Parl. (fam. Pinaceae, gen. *Pinus*, subgen. *Pinus*, sect. *Pinus*, subsect. *Pinus*) is a two-needle pine species naturally growing in coastal areas of southern Korea and Japan up to elevations of about 1000 m. Various parts of the plant have been used in traditional and folk medicines and cuisine. Needles and sprouts of *P. thunbergii* are used as ingredients in preparation of a healthy drink preventing arteriosclerosis, hypertension, and aging [1]. Water soluble compounds from the bark of the plant showed antioxidant activity [2], as well as skin whitening and wrinkle smoothing effects [3]. Ethanol and methanol extracts of the pine needles were shown to inhibit α-amylase [4] and be active against influenza viruses [5]. Essential oil from needles possesses antimicrobial activity [6], while essential oils from bark, needles, and wood exhibit growth inhibitory activity to microalgae [7]. Among all the *P. thunbergii* extractives, only the composition of the needle essential oil has been thoroughly investigated [8,9]. However, high-volatile essential oil components, predominantly mono- and sesquiterpenoids, comprise a minor part of the pines' lipophilic extractives [9,10]. Certain data on extractive compounds from different parts of *P. thunbergii* have been reported. The chemical composition of the pine oleoresin, including mono-, sesqui-, and diterpenoids, was briefly characterized [11]. Resin acids from the plant cone extracts were studied several decades ago [12]. Some leaf wax components, estolides, were isolated and their structures elucidated [13], and the structures of polyprenols from the pine needles were determined [14]. A number of phenolic compounds were identified in wood and bark of *P. thunbergii* [15]. Total phenolic and proanthocyanidin contents of the water extract of pine bark was ascertained [2]. The diterpene hydrocarbon, cembrene (thunbergene), was first isolated from the root turpentine oil of *P. thunbergii* [16]. Data on the high-volatile components and several groups of extractive compounds of *P. thunbergii* are available, but the composition of the low-volatile lipophilic metabolites in needles, twigs, and especially bark of the plant remains generally undisclosed.

The goal of this work was a comparative study of the low-volatile lipophilic components of the needles, defoliated twigs, and outer bark of *P. thunbergii* from a single natural population, employing a GC/MS method. The distribution of lipophilic compounds in the shoot system of *P. thunbergii* will help to elaborate isolation methods for valuable phytochemicals from the plant raw material. On the other hand, chromatographic profiling of low-volatile secondary metabolites could be useful for chemotaxonomy of the genus *Pinus*.

Table 1: Yields of extracts from needles **(N)**, defoliated twigs **(T)**, and outer bark **(B)** of *P. thunbergii*, yields of fractions of acids **(A)** and neutral compounds **(NC)** from the extracts, and ratio of the fraction yields.

	Extract[a]	Acids **(A)**[b]	Neutral compds. **(NC)**[b]	Ratio of acids/neutral compds. **(A/NC)**
Needles [9]	0.3 (essential oil)[c]			
Needles **(N)**	3.7	64.8	23.8	2.7
Defoliated twigs **(T)**	5.3	62.9	18.1	3.5
Outer bark **(B)**	1.1	31.1	31.9	1.0

[a]Yields (%, w/w) of extracts relative to the air-dried raw material. [b]Yields (%, w/w) of fractions relative to the extract. [c]Yield (%, v/w) of essential oil relative to the fresh (8.5% moisture) raw material.

Air-dried needles, defoliated twigs, and outer bark of *P. thunbergii* were exhaustively extracted with *n*-hexane. The obtained extracts were fractionated according to the general procedure [17] into neutral compounds (**NC**) and acids (**A**). Yields of the extracts and the resulting fractions are shown in Table 1. The yields of

Table 2: Yields of fractions of acids after saponification **(S)** and unsaponifiable neutral compounds **(U)** from neutral compounds **(NC)** of *P. thunbergii* extract, and ratio of the fraction yields.

	Acids after saponification **(S)**[a]	Unsaponifiable neutral compds. **(U)**[a]	Ratio of acids/unsaponifiable neutral compds. **(S/U)**
Needles **(N)**	23.0	56.0	0.4
Defoliated twigs **(T)**	45.0	19.0	2.4
Outer bark **(B)**	34.0	55.0	0.6

[a]Yields (%, w/w) of fractions relative to the neutral compound fraction.

Table 3: Composition of acid fractions (as methyl esters) from extracts of *P. thunbergii* needles **(N-A)**, defoliated twigs **(T-A)**, and outer bark **(B-A)** by GC/MS[a].

Compound	*RI*	N-A	T-A	B-A
Palmitic acid methyl ester	1933	0.4		
Linoleic acid methyl ester	2099		0.2	1.3
Linoleic acid methyl ester + oleic acid methyl ester (major)	2106		1.0	
Oleic acid methyl ester + α-linolenic acid methyl ester (major)	2106	0.9		2.1
Stearic acid methyl ester	2135			0.4
9β(H)-9,10-Secodehydroabietic acid methyl ester	2171			0.4
9α(H)-9,10-Secodehydroabietic acid methyl ester	2191			0.3
8,15-Isopimaradien-18-oic acid methyl ester	2194	0.3		
Communic acid methyl ester isomer I (major) + 13-*epi*-manoyl oxide-18-oic acid methyl ester[b]	2225	1.8		
13-*epi*-Manoyl oxide-18-oic acid methyl ester[b]	2226		0.9	
Pimaric acid methyl ester	2235	1.1	1.2	3.8
Communic acid methyl ester isomer II	2243	0.9		
Manoyl oxide-18-oic acid methyl ester[b]	2248		0.6	
***trans*-Communic acid methyl ester**	**2251**	**43.8**	**4.7**	
Sandaracopimaric acid methyl ester	2252			2.0
Isopimaric acid methyl ester	2293	0.9	1.8	2.2
Levopimaric acid methyl ester (major) + palustric acid methyl ester	**2302**	**1.7**	**19.0**	
Arachidic acid methyl acid	2332		0.5	2.5
Dehydroabietic acid methyl ester	**2339**	**7.2**	**10.1**	**30.5**
Imbricataloic acid methyl ester	**2372**	**4.7**	**3.0**	
Abietic acid methyl ester	**2384**	**5.9**	**7.2**	**8.0**
Unidentified [M^+=312]	2438			2.6
Neoabietic acid methyl ester	**2443**	**0.5**	**19.0**	
13-Oxopodocarp-8(14)-en-18-oic acid methyl ester	2450		0.4	1.1
Imbricatoloic acid methyl ester	**2456**	**5.1**	**2.7**	
Dihydroagathic (mercusic) acid dimethyl ester	2463	1.3	0.5	0.4
15-Oxopimaric acid methyl ester	2491	0.7	0.4	
Unidentified [M^+=332]	2501		2.4	
Unidentified [M^+=332] + abieta-7,13,15-trien-18-oic acid methyl ester (major) + unidentified [M^+=330]	2499	0.6		
Abieta-7,13,15-trien-18-oic acid methyl ester	2503		5.1	0.9
Unidentified [M^+=312] (major) + unidentified [M^+=344]	2507			1.3
15-Hydroxydehydroabietic acid methyl ester	2536	1.2		
15-Hydroxydehydroabietic acid methyl ester + behenic acid methyl ester	2537		4.3	10.0
Behenic acid methyl ester	2537	1.8		
Unidentified [M^+=332]	2572		1.8	0.7
7-Oxodehydroabietic acid methyl ester	2585			4.2
7-Oxodehydroabietic acid methyl ester (major) + unidentified [M^+≥316] + unidentified [M^+=348]	2585	0.8	0.8	
Unidentified [M^+≥314] + unidentified [M^+=330]	2595		2.4	1.5
Unidentified [M^+=330] + unidentified [M^+=348]	2599	6.8		
Eicosandioic acid dimethyl ester + unidentified [M^+=370]	2663			0.4
Unidentified [M^+=330] + unidentified [M^+=348]	2674	4.8	3.1	0.5
Lignoceric acid methyl ester	**2735**	**0.5**	**2.7**	**8.6**
15-Hydroxy-7-oxodehydroabietic acid methyl ester	2768			0.6
Docosandioic acid dimethyl ester	2866			0.7
Cerotic acid methyl ester	2938		0.4	2.2
Unidentified [M^+=394] + tetracosandioic acid dimethyl ester (major)	3079			0.4
n-Octadecyl ferulate methyl ether				0.5
n-Tetracosyl ferulate methyl ether				0.9
Total		***93.6***	***96.2***	***91.2***

[a]Content of compounds is given in %; only major unidentified components are tabulated.
[b]Compounds with tentative configuration of stereogenic centers at atoms C-4 and C-13.

extractives decreased in the order: defoliated twigs **(T)** > needles **(N)** > outer bark **(B)**. Neutral compounds (**NC**) containing large amounts of fats and waxes were saponified to give unsaponifiable neutral compounds (**U**) and acids (**S**). Yields of fractions **S** and **U** are shown in Table 2. The amount of acids after saponification of the neutral compounds from defoliated twigs **(T-S)** was significantly higher than those in the case of needles **(N-S)** and outer bark **(B-S)**, thus indicating the larger percentage of waxes, fats, and other esters in the defoliated twig extractives.

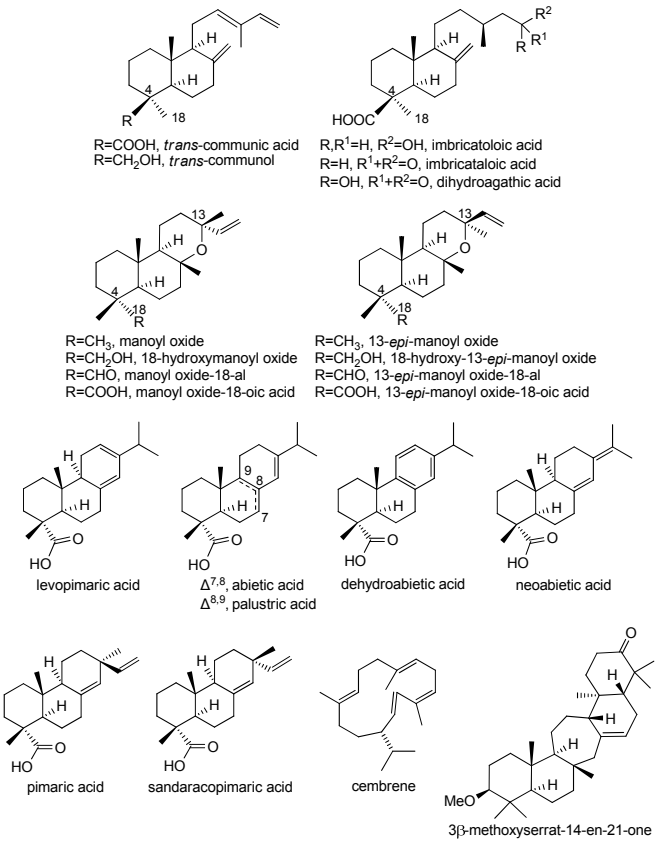

Figure 1: Structures of selected di- and triterpenoids from *P. thunbergii* needles, defoliated twigs, and outer bark.

The fractions **A**, **NC**, **S**, and **U** were analyzed using GC/MS (acid fractions were methylated with diazomethane prior to analysis). Data on the composition of fractions **A** and **U** are given in Tables 3 and 4, respectively.

The major component of the acid fraction from the needle extract **(N-A)** was the labdanoid, *trans*-communic acid (43.8%), while contents of other diterpene acids were considerably lower {dehydroabietic (7.2%), abietic (5.9%), imbricatoloic (5.1%) acids}. The total percentage of fatty acids was *ca.* 4%. On the contrary, in the analogous fraction from the defoliated twig extract **(T-A)** the main acids were of the abietane type {neoabietic (19.0%), levopimaric together with palustric (19.0%), and dehydroabietic acids (10.1%)}. The content of labdane type acids {*trans*-communic (4.7%), imbricataloic (3.0%), and imbricatoloic (2.7%) acids} in this fraction was significantly inferior to that of fraction **N-A**. The fraction **T-A** was richer in fatty acids (*ca*. 7%) than **N-A**. Analogous to fraction **T-A**, the major components among the acids from the extract of the outer bark **(B-A)** were abietanoids {dehydroabietic (30.5%) and abietic (8.0%) acids}, while very minor amounts of labdane type acids {dihydroagathic acid together with dihydroagathic acid monomethyl ester (0.4%)} were detected. Fraction **B-A** contained a significant amount of fatty acids (*ca.* 23%), the dominant one being lignoceric acid (9.1%). In the order of the fractions **N-A**, **T-A**, and **B-A**, the percentages of labdane type acids (*trans*-communic, imbricataloic, imbricatoloic, and dihydroagathic acids) decreased, while the percentages of dehydroabietic, abietic, pimaric, and isopimaric acids increased. The maximal amounts of neoabietic and levopimaric together with palustric acids were found in fraction **T-A** compared with the other acid fractions. The percentage of saturated C_{20}, C_{22}, C_{24}, and C_{26} fatty acids increased in the series **N-A, T-A, B-A**, though the

Table 4: Composition of unsaponifiable neutral compound fractions from extracts of *P. thunbergii* needles **(N-U)**, defoliated twigs **(T-U)**, and outer bark **(B-U)** by GC/MS[a].

Compound	*RI*	N-U	T-U	B-U
n-Octadecane	1800			0.5
Cembrene (thunbergene)	1927			0.5
Manoyl oxide	1988	1.0	1.1	
n-Eicosane	2000			0.4
13-*epi*-Manoyl oxide	2009	2.4	1.2	
Dehydroabietane + manool (major)	2054		0.7	
Abieta-7,13-diene	2079		0.3	
Phytol	2121	2.6		
cis-Abienol (major) + unidentified [M^+>277]	2149		1.0	
Pimarinal	2162		0.8	0.5
Manoyl oxide-18-al[b]	2195		0.8	
Unidentified [M^+=286]	2220	1.3		
Unidentified [M^+=286] + 13-*epi*-manoyl oxide-18-al[b]	2217		2.2	
Sandaracopimarinol	2252		1.1	
Sandaracopimaric acid methyl ester	2252	0.4		
Dehydroabietal	2269		1.0	
18-Hydroxymanoyl oxide[b]	2295		2.7	
Abietal	2305		1.1	
***trans*-Communol**	**2317**	**3.8**	**3.7**	
18-Hydroxy-13-*epi*-manoyl oxide[b]	**2321**	**4.0**	**8.0**	
Dehydroabietic acid methyl ester	2342	0.4	1.3	2.1
13-*epi*-Torulosol	**2359**		**3.6**	
Dehydroabietol	2368		0.6	
Neoabietal	2374		1.4	
Abietic acid methyl ester	2384	0.3		0.7
Abietol	2399		0.9	
iso-Agathlal	2457		1.0	
Unidentified [M^+=332] + neoabietol	2461		1.0	
Abieta-7,13,15-trienoic acid methyl ester	2497		0.4	
n-Pentacosane	2500		0.4	
15-Hydroxydehydroabietic acid methyl ester	2528		0.7	
n-Heptacosane + unidentified [M^+=?]	2700	0.9	1.7	
1-Tetracosanol	2708			1.2
10-Nonacosanone	3089	3.1		
3,5-Stigmastadiene	3091			0.7
10-Nonacosanol	**3110**	**38.0**	**3.0**	
α-Tocopherol	3138	1.1		
Unidentified [M^+=?] + [M^+=434]	3210	5.1	0.8	
Campesterol	3229	1.0	1.2	0.7
β-Sitosterol	**3323**	**15.8**	**19.8**	**14.3**
Unidentified [M^+=450]	3337	2.6		
5α-Stigmastan-3-one	3372			1.2
Stigmast-4-en-3-one	3456			2.7
3β-Methoxylanosta-9(11)-dienol	3486			0.8
Unidentified [M^+=410]	3499			3.5
Unidentified [M^+=440]	3553			4.5
Serratanoids [M^+=454] + [M^+=426]	3627			6.2
3β,21-Dimethoxyserrat-14-ene	3669		3.0	5.0
29-Nor-3β-methoxyserrat-14-en-21-one	3677		0.9	5.8
3α-Methoxyserrat-14-en-21-one	3687		0.3	1.4
3β-Methoxyserrat-14-en-21-one	**3741**		**15.7**	**27.5**
3β-Methoxyserrat-14-en-21β-ol			2.0	1.9
Serrat-14-en-3,21-dione			1.9	4.6
Unidentified [M^+=440]			2.6	2.5
High-volatile compounds (not tabulated)		***11.9***	***6.3***	***6.9***
Total		***95.4***	***96.2***	***95.9***

[a]Content of compounds is given in %; only major unidentified components are tabulated.
[b]Compounds with tentative configuration of stereogenic centers at atoms C-4 and C-13.

content of unsaturated fatty acids was insignificant in all acid fractions. The prevalent component of the acids from the pine needles, *trans*-communic acid, was reported among the dominant compounds in oleoresin and a cone extract of *Pinus luchuensis* (subsect. *Pinus*) [11,18], a species which is very close to *P. thunbergii* in terms of botanical classification. Predominance of levopimaric with palustric acids over abietic acid in the fraction **T-A** indicates additionally that the analyzed samples of plant material were taken from healthy trees, non-infected with the invasive pine wood nematode, *Bursaphelenchus xylophilus,* widespread in East Asia nowadays [19].

The fraction of unsaponifiable neutral compounds from the needle extract **(N-U)** contained *ca.* 12% of high-volatile compounds (mostly sesquiterpenoids), *ca.* 12% of diterpenoids, and *ca.* 17% of sterols. The major components in the fraction **N-U** were 10-nonacosanol (38.0%), β-sitosterol (15.8%), labdane type alcohols, 18-hydroxy-13-*epi*-manoyl oxide (4.0%) and *trans*-communol (3.8%). The total content of mono- and sesquiterpenoids in the unsaponifiable neutral compounds from the twig extract **(T-U)** was only *ca.* 6%, whereas the percentages of diterpenoids and triterpenoids made up *ca.* 37% and *ca.* 21%, respectively. The main components were β-sitosterol (19.8%), the triterpenoid 3β-methoxyserrat-14-en-21-one (15.7%), and the labdanoids (18-hydroxy-13-*epi*-manoyl oxide (8.0%), *trans*-communol (3.7%), and 13-*epi*-torulosol (3.6%)). The greatest variety of diterpene aldehydes and alcohols was found in fraction **T-U.** The fraction of bark unsaponifiable compounds **(B-U)** comprised high-volatile components (*ca.* 7%), diterpenoids (*ca.* 4%), sterols (*ca.* 20%), and a large amount of lanostane and serratane type triterpenoids (*ca.* 62%). The dominant compounds in the fraction were 3β- methoxyserrat-14-en-21-one (27.5%) and β-sitosterol (14.3%). Compared with the parent **NC** fractions, the content of β-sitosterol in the fractions **N-U** and **T-U** was *ca.* 4 times higher, while in the fraction **B-U** only *ca.* 2 times. The fraction **T-U** contained maximal amounts of diterpenoids, while **B-U** was particularly rich in triterpenoids.

The content of fatty acids in the fraction of acids after saponification of the neutral compounds from needles **(N-S)** was *ca.* 34%, the major ones being lauric, oleic, linoleic, and palmitic acids (*ca.* 20% in total). In the analogues fraction obtained from the extract of defoliated twigs **(T-S)** the dominant acids, linoleic, oleic, behenic, and lignoceric acids, made up to 66%. At the same time, the fraction of acids after saponification of the neutral compounds from outer bark **(B-S)** contained mostly saturated C_{20}-C_{26} even fatty acids (*ca.* 61% in sum), the major one being lignoceric acid (*ca.* 31%). The composition of the *P. thunbergii* extractive substances resembles that for *P. densiflora* [20], another two-needle pine from subsect. *Pinus*, growing in the same region. The principal differences in the composition of lipophilic extractives in the case of these two geographically and systematically related species enclose the following features. First, all the bicyclic labdane type acids (*trans*-communic, imbricataloic, imbricatoloic, dihydroagathic acids) from the *P. thunbergii* extracts possessed the same (4*S*)-stereoconfiguration, whereas the analogous metabolites produced by *P. densiflora* were found to have the (4*R*)-configuration. Secondly, despite the fact that cembrene (thunbergene) was first found in roots of *P. thunbergii*, only minor amounts of it could be detected among the extractives of the outer bark. On the contrary, cembrane type diterpenoids are abundantly produced in the vegetative organs of *P. densiflora*.

The distribution was studied of low-volatile lipophilic compounds in needles, defoliated twigs, and outer bark of *P. thunbergii*. Acid compounds in the extract of needles comprised mainly labdane type diterpenoids (*trans*-communic acid), while in the extracts of defoliated twigs and outer bark the acids were represented predominantly by abietane type compounds (neoabietic, dehydroabietic, abietic, levopimaric and palustric acids). The major neutral components of the needle extract were 10-nonacosanol, labdanoids (18-hydroxy-13-*epi*-manoyl oxide, *trans*-communol), and β-sitosterol; in the case of the extract of defoliated twigs, they were labdanoids (18-hydroxy-13-*epi*-manoyl oxide, *trans*-communol, 13-*epi*-torulosol), serratane triterpenoids (3β-methoxyserrat-14-en-21-one), and β-sitosterol; whereas serratanoids (3β-methoxyserrat-14-en-21-one) alone dominated among the neutral compounds of the outer bark extract. Most of the neutral components and the labdane type acids were detected for the first time in organs and tissues of *P. thunbergii*. The low-volatile lipophilic compounds found in the studied parts of the pine extend the scope of its secondary metabolites detected earlier in essential oils.

Experimental

Plant material: Foliated twigs and outer bark of *P. thunbergii* Parl. were collected in September 2010 near Gangneung city, Republic of Korea. The specimens were identified by Dr E.A. Korolyuk (Central Siberian Botanical Garden (CSBG), Novosibirsk). Voucher specimens ("Korea, near Gangneung city, 09. 2010. Collect. Popov S.") are deposited with the Central Siberian Botanical Garden (Novosibirsk; NS).

Drying, extraction, and fractionation: Foliated twigs of *P. thunbergii* were separated to give needles and defoliated twigs. Needles, defoliated twigs, and outer bark were dried to constant weight (residual moisture content ~5%) in a drying cabinet at 30-50ºC for 24 h. Dried plant material was cut into pieces of 10-15 mm and extracted with *n*-hexane in a Soxhlet apparatus for 24 h. The extracts were concentrated in vacuum, diluted with MeOBu-*t* and fractionated to give acids (**A**) and neutral compounds (**NC**) by treatment of ether solutions of the initial extracts with 1% NaOH aqueous solution. Portions of the **NC** fractions were saponified by boiling in 5% KOH aqueous ethanol solution and separated to give acids (**S**) and unsaponifiable neutral compounds (**U**). Portions of the acid fractions (**A, S**) were converted to methyl esters by treatment with a diethyl ether solution of diazomethane [21].

GC-MS analysis: GC-MS was performed using a Hewlett-Packard instrument with a HP 5890 Series II gas chromatograph with HP 5971 (EI, 70 eV) mass-selective detector and a HP-5ms capillary column (30 m × 0.25 mm internal diameter, film thickness 0.25 μm); carrier gas was He at a flow rate of 1 mL/min; programmed column temperature was at 50°C for 2 min, from 50°C to 300°C at 4°C/min, and 300°C for 30 min (or from 50°C to 320°C at 4°C/min, and 320°C for 30 min when an analyte contained triterpenoids); vaporizer temperature was 280°C; ion-source temperature was 175°C, and scan rate was 1.2 scans/s in the mass range 30-650 amu. Linear retention indices (*RIs*) of the components were calculated as described in [22] by interpolation of *RIs* of *n*-alkanes C_7-C_{40}. The components were identified: 1) by comparing their mass spectra with reference MS from commercial libraries (Wiley 7n and NIST 02), home-made library, literature data [22,23], and ones of authentic compounds; and 2) by comparing their calculated *RIs* with those of authentic standards and the data published in the literature [22]. The contents (%) of compounds were determined from peak areas in chromatograms without using correction coefficients.

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Lipid Constituents of the Edible Mushroom, *Pleurotus giganteus* Demonstrate Anti-Candida Activity

Chia-Wei Phan[a,b], Guan-Serm Lee[a,b], Ian G. Macreadie[c], Sri Nurestri Abd Malek[a,b], David Pamela[a,d] and Vikineswary Sabaratnam[a,b]

[a]*Mushroom Research Centre*, *Institute of Biological Sciences*, *Faculty of Science*, *University of Malaya*, *50603 Kuala Lumpur*, *Malaysia*

[b]*Institute of Biological Sciences*, *Faculty of Science*, *University of Malaya*, *50603 Kuala Lumpur*, *Malaysia*

[c]*School of Applied Sciences*, *RMIT University*, *Victoria 3083*, *Australia*

[d]*Department of Anatomy*, *Faculty of Medicine*, *University of Malaya*, *50603 Kuala Lumpur*, *Malaysia*

viki@um.edu.my

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Different solvent extracts of *Pleurotus giganteus* fruiting bodies were tested for antifungal activities against *Candida* species responsible for human infections. The lipids extracted from the ethyl acetate fraction significantly inhibited the growth of all the *Candida* species tested. Analysis by GC/MS revealed lipid components such as fatty acids, fatty acid methyl esters, ergosterol, and ergosterol derivatives. The sample with high amounts of fatty acid methyl esters was the most effective antifungal agent. The samples were not cytotoxic to a mammalian cell line, mouse embryonic fibroblasts BALB/c 3T3 clone A31. To our knowledge, this is the first report of antifungal activity of the lipid components of *Pleurotus giganteus* against *Candida* species.

Keywords: *Pleurotus giganteus*, *Candida*, yeast, Antifungal, Medicinal mushroom, Fatty acid, Fatty ester methyl ester, Ergosterol.

Fungal infections are problematic for human health and are responsible for high rates of morbidity and mortality worldwide. Species of *Candida* are the dominant cause of opportunistic mycoses and among them, *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis* and *C. krusei* account for 95–97% of all Candida infections [1,2]. *C. albicans* and *C. tropicalis* are susceptible to polyenes, flucytosine, azoles and echinocandins, while *C. glabrata* is either susceptible or resistant to fluconazole[3]. Furthermore, *C. krusei* displays decreased susceptibility to amphotericin B, as well as fluconazole. Considering the increasing incidence of drug-resistant *Candida* infections, the search for more effective anti-Candida agents as an alternative to synthetic ones is needed. The interest in natural products from medicinal plants as a source of anti-Candida agents has grown dramatically. A wide variety of plant extracts have been reported to have anti-*C. albicans* activity. Examples include propolis, *Punica granatum* (pomegranate), *Streblus asper* (Siamese rough bush), *Vitis vinifera* (common grape vine), and tea tree oil from *Melaleuca alternifolia* [4].

Medicinal mushrooms are relatively less researched for their antifungal properties. However, in the last five years, there has been a renewed interest in using mushrooms as antimicrobial agents. *Lentinula edodes* (shiitake), *Boletus edulis* (Penny bun), *Pleurotus ostreatus* (oyster mushroom), *Coprinus comatus* (shaggy mane), *Astraeus hygrometricus* (earthstar mushroom), and *Cordyceps militaris* were shown to exhibit antifungal activity against *C. albicans* [5–8]. *Pleurotus giganteus* (Zhudugu, Dabeijun, morning glory mushroom), a saprobic mushroom, is one of the largest mushroom which grows on soil either as solitary or gregarious fruiting bodies [9]. This mushroom has gained popularity in China for its culinary properties. The medicinal properties of this mushroom are less known. We have previously reported the hepatoprotective and neuronal stimulating effects of *P. giganteus* [10,11]. In this study the antifungal activities of different solvent extracts of this mushroom were evaluated. The extracts prepared with different solvents had different profiles of fatty acids, and fatty acids have been shown to demonstrate antimicrobial activities [12].The main metabolites / components in the extracts were analysed by GC-MS. As a preliminary *in vitro* toxicity assessment, the *P. giganteus* extracts were also investigated for cytotoxicity to mouse embryonic 3T3 fibroblast cells.

Table 1: Activity of different extracts of *Pleurotus giganteus* against *Candida* species.

Candida strains	Untreated	Solvent extracts (µg/mL)								
		Methanol			Ethyl acetate			Aqueous		
		25	50	100	25	50	100	25	50	100
Candida albicans WM1172	++++	++++	+++	++	+	-	-	++++	++++	++++
Candida albicans ATCC90028	++++	+++	+++	+	+	-	-	++++	++++	+++
Candida dubliniensis	++++	++++	+++	-	+	-	-	++++	++++	+++
Candida glabrata CBS138	++++	++++	++	-	+	-	-	++++	++++	++++
Candida glabrata ATCC90030	++++	++++	++++	-	+	-	-	++	+++	+++
Candida krusei ATCC6258	++++	+++	+++	+	-	-	-	++++	+++	+++
Candida pseudotropicalis	++++	+++	+++	++	-	-	-	++++	++++	++++
Candida tropicalis WM30	++++	+++	++++	++	+	-	-	++++	++++	++++

Strains were grown with different mushroom extracts at the concentrations shown for two days on YEPD media. Growth was scored from "-" to "++++", indicating no growth to strong growth.

The anti-Candida activity of methanol, ethyl acetate and aqueous extracts of *P. giganteus* against all yeast species tested are summarised in Table 1. *Candida* species showed strong growth (denoted as "++++") when extracts were not added to the medium. The aqueous extract had minimum or no inhibitory activity against all *Candida* spp. The ethyl acetate extract completely inhibited the growth of all *Candida* spp. when tested at 50 and 100 µg/mL. Thus, the ethyl acetate extract was fractionated to identify the active component/s responsible for the antifungal activity. Sub-fractions A to H were obtained and the minimum inhibitory concentration (MIC) values against all the tested yeasts are given in Table 2. The MIC values for all the *Candida* spp. tested, ranged from 2.0 ± 1.0 to 10.3 ± 2.5 µg/mL for sub-fraction A; and 9.3 ± 2.3 to 34.3 ± 10.8 µg/mL for sub-fraction B; respectively. The MIC values of

Table 2: Activity of the sub-fractions of ethyl acetate extracts against Candida species.

Candida strains	Sub-fractions from ethyl acetate extract (MIC[a])								IC_{50} (mM)	
	A	B	C	D	E	F	G	H	Fluconazole	Amphotericin B
Candida albicans WM1172	7.3 ± 1.5	16.0 ± 6.1	26.6 ± 11.6	*	*	*	*	*	1.0	0.6
Candida albicans ATCC90028	7.0 ± 1.0	22.6 ± 11.5	40.6 ± 16.6	*	*	*	*	*	2.0	0.7
Candida dubliniensis	8.2 ± 3.7	31.6 ± 7.8	37.0 ± 7.0	*	*	*	*	*	24.0	2.1
Candida glabrata CBS138	8.1 ± 1.5	12.5 ± 2.4	28.3 ± 6.6	*	*	*	*	*	>10.0	1.3
Candida glabrata ATCC90030	9.2 ± 1.6	29.4 ± 7.0	41.3 ± 7.6	*	*	*	*	*	>10.0	1.2
Candida krusei ATCC6258	10.3 ± 2.5	37.2 ± 5.0	*	*	*	*	*	*	8.0	0.4
Candida pseudotropicalis	3.8± 1.4	9. 3 ± 2.3	23.0 ± 11.0	*	*	*	*	*	9.0	0.6
Candida tropicalis WM30	2.0 ± 1.0	34.3 ± 10.8	*	*	*	*	*	*	<1.0	1.2

Results were from three independent experiments performed in triplicate. [a]MIC is expressed in μg/mL. * : >50 μg/mL

sub- fraction C varied from 23.0 ± 11.0 to >50 μg/mL; whereas the MIC values for sub-fractions D-H were all >50 μg/mL. Overall, sub-fraction A showed the lowest MIC value for all *Candida* spp. tested.

Sub-fractions A and B were further analysed by GC-MS. Both samples were pale yellow-colored oils with a distinct odor. Constituents of sub-fractions A and B are listed in Table 3. Twelve compounds were identified in sub-fractions A and B. Sample A was characterized by high amounts of fatty acid methyl esters, namely: methyl palmitate, ethyl palmitate, methyl linoleate, methyl oleate, methyl stearate, and ethyl oleate. Sample B contained fatty acids (palmitic acid and oleic acid), fatty acid methyl esters (methyl linoleate and methyl oleate), ergosterol, ergosta-5,7,9 (11),22-tetraen-3β-ol, ergost-5,8(14)-dien-3-ol, and γ-ergostenol.

Table 3: Chemical composition of lipids in sub-fractions A and B of .*P. giganteus*.

Constituents	RT (min)	Percentage (%)	Quality
Sub-fraction A			
Methyl palmitate	20.50	14.8	99
Ethyl palmitate	21.81	1.2	98
Methyl linoleate	23.70	19.8	99
Methyl oleate	23.80	39.3	99
Methyl stearate	24.26	3.3	99
Ethyl oleate	24.99	12.3	99
Sub-fraction B			
Methyl palmitate	20.49	0.2	95
Palmitic acid	21.28	14.4	99
Methyl linoleate	23.68	0.4	93
Methyl oleate	23.79	1.0	93
Oleic acid	24.61	31.7	99
Ergosta-5,7,9(11),22-tetraen-3β-ol	39.83	2.2	90
Ergosterol	40.33	24.4	98
Ergost-5,8(14)-dien-3-ol	40.51	10.2	87
γ-Ergostenol	41.32	3.7	94

The methanol, ethyl acetate, and aqueous extracts were not toxic to 3T3 fibroblasts cells and the IC_{50} values were more than 2 mg/mL (Fig. 1). Meanwhile, cell viability (%) decreased steadily with increasing concentrations of sub-fractions A and B at levels up to 500 μg/mL. The IC_{50} value of sub-fraction A was 352 μg/mL and the R^2 value was 0.9609. For sub-fraction B, the IC_{50} was 362 μg/mL with the R^2 value recorded at 0.9552.

To our knowledge, this is the first report on the antifungal activity of the lipid components of *P. giganteus*. It has been reported that crude extracts of *P. ostreatus* and *C. comatus* inhibited the growth of *C. albicans* [6]. However, the MICs were much higher (up to 1 mg/mL) when compared with this study, which recorded an MIC of

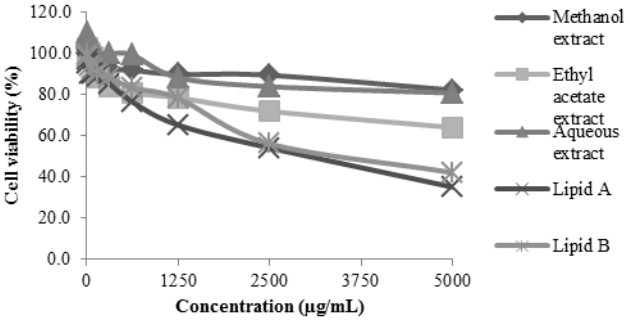

Figure 1: Cell viability of embryonic fibroblast cells after treatment with various extracts of *P. giganteus*.

100 μg/mL. The sub-fractions A and B were shown to contain several bioactive components. Since they are blends of fatty acids and fatty acid methyl esters, they do not act on specific targets in the fungal cells, and fungal resistance may be unlikely to occur. Furthermore, fatty acids and their methyl esters were reported to have fungicidal activity to *C. albicans, C. krusei*, *C. tropicalis* and *C. parapsilosis* [13]. The entities might play crucial roles in lipophilic or hydrophilic effects on the cell wall and membrane, hence affecting the distribution of the lipids in the cells [14]. Moreover, ergosterol present in the sample could disrupt the ergosterol biosynthesis pathway in the yeast, causing growth inhibition or cell death. This was further supported by a study of Irshad *et al.* [15], who reported that ergosterol-rich *Cassia fistula* oil significantly decreased the *in vivo* ergosterol content in the *Candida* cell wall.

In this study, the sub-fractions A and B were not cytotoxic to mouse fibroblasts at the concentrations tested (Fig. 1). Animal testing is becoming less popular and is gradually being replaced by *in vitro* methods for toxicity assessment of pharmaceutical products. In conclusion, *P. giganteus* lipids are promising natural products to be further explored as antifungal agents against *Candida* species.

Experimental

Mushroom: The fruiting bodies of *Pleurotus giganteus* (Berk) Karunarathna & K.D. Hyde were obtained from Nas Agro Farm, Selangor, Malaysia. A voucher specimen (KLU-M 1227) was deposited in the Herbarium in the University of Malaya.

Chemicals: Fluconazole and amphotericin B were purchased from Sigma Co. (St. Louis, MO, USA). The stocks were prepared in dimethyl sulfoxide (DMSO) prior to bioassays. [3-(4,5-Dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT), was also obtained from Sigma. Methanol (MeOH), ethyl acetate (EtOAc), *n*-hexane and acetone were from Merck (Darmstadt, Germany).

Extracts preparation: The fresh fruiting bodies of *P. giganteus* were sliced, freeze-dried and ground to a fine powder (500 g). The mushroom powder was extracted with 80% MeOH to yield a MeOH extract (115 g, 23.0%). This (125 g) was further partitioned in EtOAc-H_2O (100 mL: 100 mL) to give an EtOAc-soluble extract (6.96 g, 6.05%) and a H_2O extract (74.2 g, 64.52%).

Fractionation of extract: The EtOAc extract (5.00 g) was further fractionated by CC over silica gel. The extract was eluted with *n*-hexane containing increasing concentrations of acetone to obtain 8 fractions (A to H) based on similarity of spots on TLC.

Cell culture: Mouse embryonic fibroblasts were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10%, v/v, heat-inactivated fetal bovine serum (PAA), 100 U/mL penicillin and 100 µg/mL streptomycin. The cells were routinely passaged every 2-3 days and incubated at 37°C and 5%, v/v, CO_2 in a humidified atmosphere.

Cytotoxicity: The crude MeOH and fractionated EtOAc extracts were dissolved in DMSO (10 mg/mL) as stock solutions. The H_2O extract (10 mg/mL) was stocked in sterilised distilled water. The cytotoxic effects of varying concentrations of MeOH, EtOAc and H_2O extracts, as well as the fractions A-H in DMSO to 3T3 fibroblast cells were tested by the established colorimetric MTT assay [16]. The absorbance was measured at 550 nm using a microplate reader. The IC_{50} is the concentration of extract or fraction that reduced fibroblast cell growth by 50%.

Anti-yeast activity: *Candida albicans* WM1172, *C. albicans* ATCC90028, *C. dubliniensis*, *C. glabrata* CBS138, *C. glabrata* ATCC90030, *C. krusei* ATCC6258, *C. pseudotropicalis*, and *C. tropicalis* WM30 were used in this study. The yeast inhibition assay was performed according to the method of Macreadie *et al.* [17]. The yeast strains were grown in YEPD (1% yeast extract, 2% peptone, 2% glucose). If required, media were solidified by the addition of 1.5% agar. Yeast inocula (100 µL) with a starting optical density at A_{595} of 0.02-0.04 were added to each well of a 96-well microplate (Orange Scientific, Braine-l'Alleud, Belgium). Mushroom extracts were then added as two-fold serial dilutions commencing with a 100 µg/mL concentration. Fluconazole (0.1 mM) and amphotericin B (1.0 mM) were used as positive controls. A growth control DMSO solvent alone was also included. The microplate was incubated in a microplate shaker at 35ºC. After 2 h and 4 h incubation, the A_{595} was recorded using a microplate reader (Sunrise™, Tecan, Austria). Each sample was assayed in triplicate. The lowest concentration of extracts that inhibited growth of *Candida* spp. is the minimum inhibitory concentration (MIC).

Gas chromatography-mass spectrometry (GCMS): GCMS analysis was performed on sub-fractions A and B using Network Gas Chromatography system (Agilent Technologies 6890N) equipped with an Inert Mass Selective Detector (Agilent Technologies 5975) (70eV direct inlet) on a HP-5ms (5% phenyl methyl siloxane) capillary column (30 m × 250 µm × 0.25 µm) initially set at 150ºC, then increased at 5ºC per min to 300ºC and held for 10 min. Helium was used as carrier gas at a flow rate of 1 mL per min. The total ion chromatogram obtained was autointegrated by chemstation and the constituents were identified by comparison with the accompanying mass-spectra database (Wiley 9th edition with NIST 11 Mass Spectral Library, USA) wherever possible.

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Effect of Trehalose Addition on Volatiles Responsible for Strawberry Aroma

Mirela Kopjar[a*], Janez Hribar[b], Marjan Simčič[b], Emil Zlatić[b], Tomaž Požrl[b] and Vlasta Piližota[a]

[a]*Faculty of Food Technology, F. Kuhača 20, 31000 Osijek, Croatia*
[b]*Biotechnical Faculty, Jamnikarjeva 101, 1000 Ljubljana, Slovenia*

mirela.kopjar@ptfos.hr

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Aroma is one of the most important quality properties of food products and has a great influence on quality and acceptability of foods. Since it is very difficult to control, in this study the effect of addition of trehalose (3, 5 and 10%) to freeze-dried strawberry cream fillings was investigated as a possible means for retention of some of the aroma compounds responsible for the strawberry aroma. In samples with added trehalose, higher amounts of fruity esters were determined. Increase of trehalose content did not cause a proportional increase in the amount of fruity esters. However, results of our research showed that trehalose addition did not have the same effect on both γ-decalactone and furaneol.

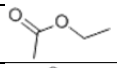
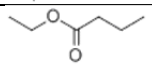
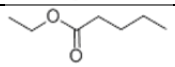
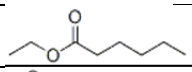
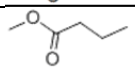
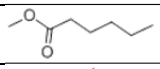
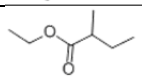
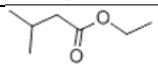
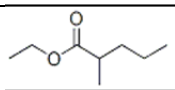
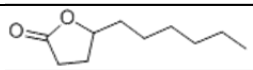
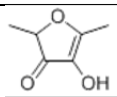
Keywords: Aroma compounds, Trehalose addition, Freeze-dried strawberry cream filling.

Next to color and texture, aroma is one of the most important quality properties, and thus its stability in different foods has been of increasing interest due to its strong relationship with consumer's acceptability of foods. However, it is very difficult to control. Different factors, like processing, storage, addition of ingredients and packaging materials, can cause modifications of overall aroma by either reducing aroma compound intensity or producing off-flavor components. Incorporation of small amounts of aroma compounds into foods can greatly influence the final product quality, cost and consumer satisfaction. The food industry is continuously developing ingredients, processing methods, and packaging materials to improve aroma preservation and delivery [1]. Strawberry is a very delicious fruit, growing in nearly all countries of the world. Due to its typical, very attractive aroma and color, strawberry has always been a favored raw material for production of different fruit product such as jams, strawberry cream fillings, jelly, and its aroma has often been analysed. Volatile components of strawberries have been extensively studied and more than 360 volatiles are assumed to be involved in strawberry aroma. A complex mixture of esters, aldehydes, alcohols and sulfur compounds mainly determines strawberry aroma, but esters are quantitatively and qualitatively the most important class of volatiles [2]. During preparation of fruit products, different additives are used influencing food product physicochemical properties, aroma, texture and color. Carbohydrates are known to enclose volatile compounds [2-6], thus in this study the influence of trehalose on selected aroma compounds was investigated. Trehalose (α,α-trehalose) is a disaccharide formed by an α-1,1 linkage of two d-glucose molecules. It is a non-reducing sugar that is not easily hydrolysed by acid, and the glycosidic bond is not cleaved by α-glucosidase. The molecular formula and weight are $C_{12}H_{22}O_{11}$ and 342.31, respectively [7,8]. Due to its natural functions and technical qualities, trehalose could be applied in the food, cosmetic and medical industries [9-11]. Influence of trehalose addition on overall aroma, color and texture in strawberry cream filling was investigated in our previous article [5].

The objective of this study was to give broader insight of the influence of trehalose addition and its amount on selected fruity esters, γ-decalactone and furaneol (the compounds that are the most responsible for strawberry aroma) in strawberry cream fillings. Properties and structure of selected compounds are presented in Table 1.

In samples without aroma addition (Figure 1), ethyl acetate and ethyl butanoate were detected, while ethyl pentanoate and ethyl hexanoate were not. In samples with trehalose addition, higher amounts of ethyl acetate and ethyl butanoate were observed in comparison with samples without trehalose addition. The highest amounts of both esters were observed when 5% trehalose was added, while samples with 10% trehalose addition had the lowest amount of these esters. With 3% trehalose addition, a sharp increase in the amounts of methyl butanoate and methyl hexanoate was observed. The highest amount of these two esters was observed when 5% of trehalose was added. In samples with the highest amount of trehalose addition (10%), in both cases, a decrease in amount was observed, but still the amount of methyl esters was higher than in samples without trehalose addition. Ethyl 2-methyl butanoate was detected in samples without trehalose addition, while ethyl 3-methyl butanoate was detected only in the samples with trehalose addition. Addition of trehalose caused an increase in the ethyl 2-methyl butanoate amount. Samples with addition of 3 and 5% of trehalose had almost the same amount, while addition of 10% of trehalose did not cause retention of ethyl 2-methyl butanoate. Increase in the amount of γ-decalactone and furaneol was not observed with trehalose addition; actually, with trehalose addition, the amount of γ-decalactone was lower. In samples with aroma addition more aroma compounds were detected (Figure 2). In samples with 3% of trehalose addition, ethyl acetate was determined in the highest amount, but with further addition, a reverse effect occurred. In samples with addition of 5 and 10% of trehalose lower amounts of this ester were observed than in samples without trehalose addition. In the case of ethyl butanoate, the highest amount was observed in samples with 5% of trehalose addition, while for ethyl pentanoate and ethyl hexanoate the highest amount was observed with 3% of trehalose addition. Methyl hexanoate was not detected and methyl butanoate was recorded only in samples with 5 and 10% of trehalose addition.

Table 1: Investigated strawberry aroma compounds and their properties.

Aroma compound	CAS number	MW	Vapour pressure mm/Hg	logP (o/w)	Description	Structural formula
Ethyl acetate	141-78-6	88.11	112.0	0.71	Etherial, fruity, sweet, grape and rum-like	
Ethyl butanoate	105-54-4	116.16	12.8	1.85	Fruity, juicy, pineapple, cognac	
Ethyl pentanoate	539-82-2	130.18	4.75	2.3	Sweet, fruity, acidic, pineapple, apple, green, berry and tropical	
Ethyl hexanoate	123-66-0	144.21	1.66	2.83	Sweet, fruity, pineapple, waxy, fatty and estery with a green banana nuance	
Methyl butanoate	623-42-7	102.13	31.1	1.24	Pungent, etherial, fruity, perfumey and fusel with a fermented, cultured, creamy undernote of fruity apple sweet banana pineapple	
Methyl hexanoate	106-70-7	130.13	3.95	2.3	Fruity, pineapple, ether	
Ethyl 2-methyl butanoate	7452-79-1	130.18	7.85	2.12	Fruity, estery and berry with fresh tropical nuances	
Ethyl 3-methyl butanoate	108-64-5	130.18	7.85	2.12	Sweet, diffusive, estery, fruity, sharp, pineapple, apple, green and orange	
Ethyl 2-methyl pentanoate	39255-32-8	144.21	2.91	2.65	Fruity, green, melon and waxy with a fatty nuance	
γ-decalactone	706-14-9	170.25	0.00512	2.72	Fresh, oily, waxy, peach, coconut, buttery, sweet	
Furaneol	3658-77-3	128.13	0.032	0.34	Sweet, slightly burnt brown caramelised, cotton candy with a savoury nuance	

Data were obtained from http://www.thegoodscentscompany.com and http://www.chemicalbook.co

The ethyl 2-methyl butanoate amount increased with increase of trehalose amount and the highest effect was with the addition of 5% trehalose. The behavior of ethyl 3-methyl butanoate was very interesting and had a different tendency from ethyl 2-methyl-butanoate. Samples without and with 3 and 10% trehalose addition had the same amount of ethyl 3-methyl butanoate, while the sample with 5% trehalose addition had a higher amount of this aroma compound. The tendency of ethyl 2-methyl pentanoate was not observed for any other ester. With addition of trehalose there was a decrease in the ethyl 2-methyl pentanoate amount. Decrease of γ-decalactone and furaneol amounts occurred in samples with trehalose addition.

Comparison of samples with native strawberry aroma (without addition of aroma) with samples with addition of strawberry aroma showed that the initial concentration of aroma compounds was very important, but also a different trend was observed in samples with trehalose addition in these two types of samples. As a consequence of previous studies [12,13], a possible explanation for the different behavior of aroma compounds in samples without and with strawberry aroma addition could be competitive and cooperative effects of the aroma compounds. Results illustrated in Figure 3 were obtained by statistical analysis (using CA and MANOVA/ANOVA) and from them it is evident that the amount of aroma compounds in samples depended on their initial concentration, as well as on the amount of trehalose addition.

The real mechanism of trehalose action is still not known. Three theories have been put forward to explain the mechanism of the action of trehalose: 1) water replacement hypothesis, 2) glass transformation hypothesis, and 3) chemical stability hypothesis. The ability of sugar molecules to bind protectively onto the surface of molecular structures has been ascribed also to their ability to form hydrogen bonds, the so called 'water replacement hypothesis'. Unlike most other disaccharides, trehalose has no direct internal hydrogen bonds. All four internal bonds are indirectly connected via the two water molecules that form part of the native dihydrate structure. This arrangement gives to the molecule an unusual flexibility around the disaccharide bond, which may allow trehalose to fit more closely to the irregular surface of macromolecules than other, more rigid disaccharides, in which the rings are directly hydrogen bonded to each other [9]. The greater bioprotective action of trehalose on biological structures in comparison with sucrose and maltose was connected with its greater destructuring effect on the tetrahedral H-bond network of water [14]. The trehalose system showed the smallest partial molar volume value, indicative of a more packed conformation, together with a greater partial volume increase with temperature, which indicates a greater structural sensitivity in contrast to sucrose and maltose solutions. From a biological point of view, these findings could imply a greater ability of trehalose to encapsulate biomolecules in more rigid and packed structures and hence a greater bioprotector effectiveness of trehalose in respect to sucrose [15].

Formulation of food products is very important since the composition of the matrix strongly affects the quality of the foods, and its stability. Retention of aroma compounds is a very complex phenomenon, as can be seen from our results. It depends on matrix composition and interactions between volatile and non-volatile constituents of the matrix, as well as on the structure of aroma compounds and their initial concentration. Small modifications (like replacement of certain amount of sucrose with trehalose and addition of strawberry aroma) of food matrix composition greatly affect the retention of the aroma compounds in strawberry cream

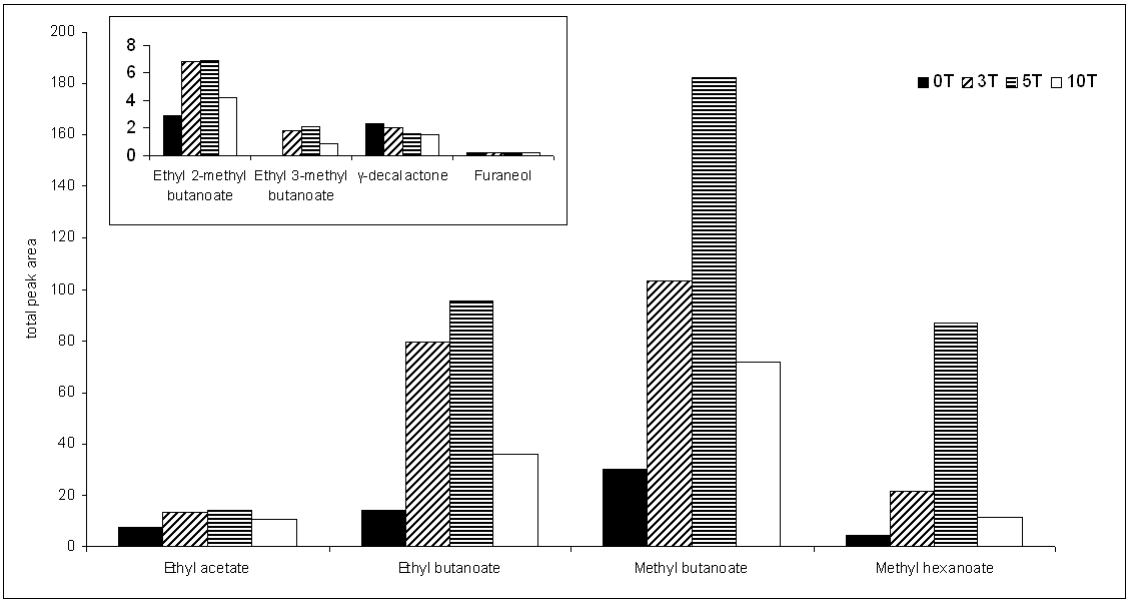


Figure 1: Total peak areas of aroma compounds in freeze-dried samples without aroma addition.

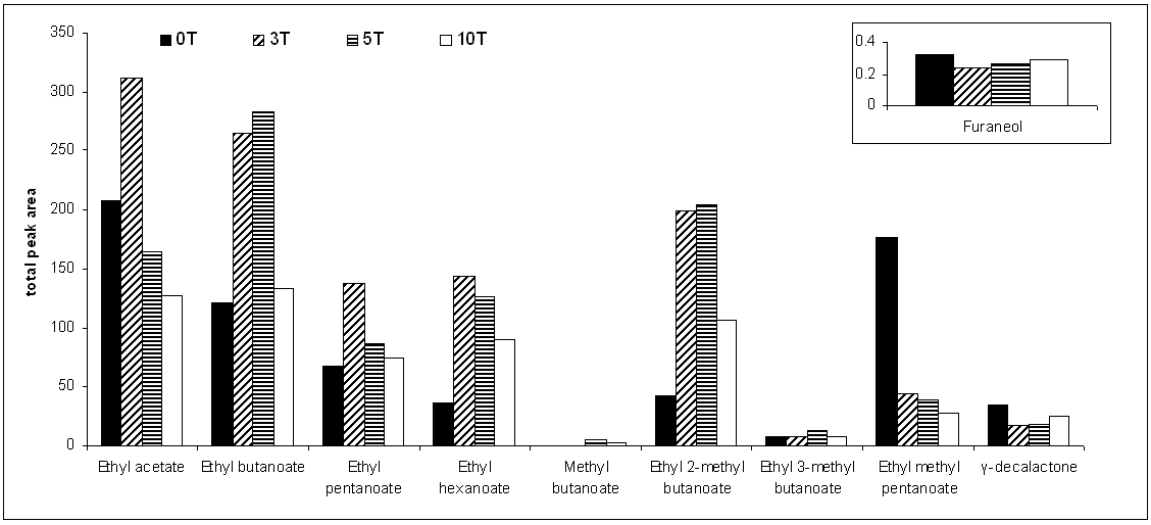


Figure 2: Total peak areas of aroma compounds in freeze-dried samples with aroma addition.

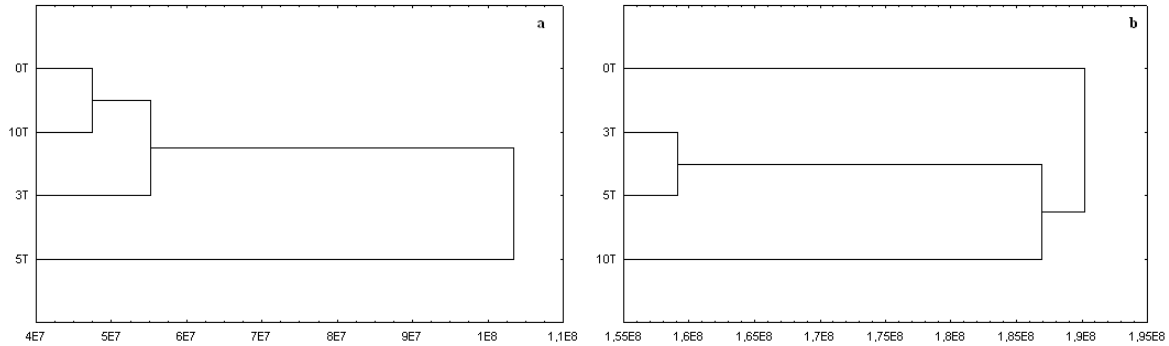


Figure 3: Dendrogram of cluster analysis of aroma compounds in samples without aroma addition (a) and with aroma addition (b).

Experimental

Sample preparation: Raw material for preparation of strawberry cream fillings without and with trehalose addition (3, 5 and 10% as sucrose replacement) were obtained from the food company Fructal d.d. (Ajdovščina, Slovenia), where ingredients (commercial frozen strawberry puree, starch, vegetable fat, sucrose, glucose syrup, sorbitol) were mixed together according to an industrial recipe. The total solids of the mixture of ingredients were 40%. Samples of strawberry cream fillings were prepared by freeze-drying of mixture of ingredients with 40% of total solids until 76% of total solids were achieved. Freeze drying of samples was performed in a Christ

Freeze Dryer (Gamma 2–20, Germany). This required a drying time of 50 h. The following conditions were used: freezing temperature –20°C; temperature of sublimation from –20 to 0°C, and vacuum of 0.630 mbar. The temperature of an isothermal desorption was from 0 to 20°C under the vacuum of 0.01 mbar. Strawberry cream fillings were prepared without and with addition of strawberry aroma (as prepared in industry). Aroma was added after strawberry cream filling preparation. Samples were homogenised on a metallic plate and, after that, cream fillings were left 10 days to stabilise at room temperature. Sampling for aroma compounds evaluation was conducted before addition of strawberry aroma and after 10 days of sample stabilisation. Samples were prepared in triplicates.

Determination of aroma compounds: GC-MS analyses were carried out for evaluation of the fruity esters, γ-decalactone and furaneol (Table 1). Extraction of volatiles was carried out with a microextraction needle 85 μm Carboxen/PDMS (Supelco) at 50°C for 40 min. For analyses, a 6890N instrument (Agilent, SAD) equipped with a MS 5971A detector (Hewlett Packard, SAD) was used. Compounds were analyzed on a ZB-WAX column (60 m x 0.32 mm x 0.5 μm, Phenomenex). The temperature programming was as follows: 5 min at 40°C, temperature gradient 4°C/min, and final temperature 230°C for 5 min. The carrier gas was helium with a flow rate of 1 mL/min at 40°C. Desorption of adsorbed volatiles was carried out by exposing the fiber in the injector port of the GC at 270°C for 5 min. For thermal desorption, the splitless injection mode was used and the split valve was opened after 0.5 min. Mass spectra were obtained with 70 eV electron impact ionization, while the mass spectrometer was continuously scanning *m/z* 30–300. Determination of the analysed compounds was confirmed by retention times of single compounds and from bibliographic data. The results were expressed as total peak area.

Statistical analyses: For evaluation of the influence of strawberry aroma addition and trehalose amount, total peak area of GC determinations of all investigated aroma compounds were taken into account and data were evaluated using General ANOVA/MANOVA (one-way ANOVA) and Cluster Analysis (CA). Statistical analyses were carried out using software program STATISTICA 8 (StatSoft, Inc, USA).

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Pogostemon hirsutus Oil, Rich in Abietane Diterpenes

Ramar Murugan[a*], Gopal Rao Mallavarapu[b], Veerappan Sudha[a] and Pemaiah Brindha[a]

[a]*Centre for Advanced Research in Indian System of Medicine*, *School of Chemical and Biotechnology*, *SASTRA University*, *Tirumalaisamudram*, *Thanjavur 613 401*, *India*
[b]*602*, *A-Block*, *Renaissance Temple Bells*, *Opp. ISKCON Temple*, *Yeshwantpur*, *Bangalore 560 022*, *India*

ramarmurugan@yahoo.com

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Volatile oil extracted from the leaves of *Pogostemon hirsutus* Benth., a rare species from Anamalai hills of the Western Ghats of Tamil Nadu, India was analyzed by GC-FID and GC-MS. Fifty-four compounds amounting to 90.8% of the oil were identified. The oil was rich in monoterpenes and diterpenes. The major components of the oil were α-pinene (11.5%), sabinene (8.5%), β-pinene (5.2%), abietatriene (16.3%), dehydroabietal (3.5%) and dehydroabietol (21.0%). Abietane diterpenes are reported for the first time in the genus *Pogostemon* of Lamiaceae with six abietanes representing 42.6% of the oil.

Keywords: *Pogostemon hirsutus*, Lamiaceae, Essential oil, Diterpenes, Abietatriene, Dehydroabietol.

The genus *Pogostemon* Desf. of Lamiaceae is represented by 97 species globally and 54 species in India [1a]. *Pogostemon cablin* (Blanco) Benth. is the only species in this genus commercially cultivated for its essential oil, known as patchouli, which is extensively used in perfumery. As part of our search for indigenous wild *Pogostemon* species whose essential oils can be utilized in perfumery, we have studied and reported the chemical constituents of the essential oils of three indigenous and endemic *Pogostemon* species such as *P. heyneanus* Benth., *P. travancoricus* Bedd. var. *travancoricus* and *P. speciosus* Benth. [1b,c,d]. In continuation of our studies on the bioprospecting of *Pogostemon* species, we have now investigated the volatile constituents of the leaves of *P. hirsutus* collected from the Anamalai hills of the Western Ghats, India. The aim of this study was to explore the possibility of commercial utilization of the wild aromatic plants of the Western Ghats based on the chemical composition of the essential oils and olfactive characteristics.

Pogostemon hirsutus was previously considered to be endemic to Sri Lanka, but later it was discovered from the Anamalai hills of the Western Ghats and reported as a new distributional record for India [1e]. It is a small hirsute herb, growing about a feet high, in humid, rocky slopes in high altitude grassland above 2000 m altitude. Both fresh and dried leaves are slightly aromatic. The inflorescence is simple, terminal spike with white flowers. A detailed description along with a photograph is given in our earlier report [1e].

Hydro-distillation of the dried leaf of *P. hirsutus* yielded 0.4% of essential oil on dry weight basis. The oil was yellow with a strong earthy odor. GC analysis of the oil showed more than 100 peaks. GC-FID and GC-MS analyses of the oil enabled the identification of 54 compounds representing 90.8% of the oil. The identified compounds with their RIs and relative concentration in the oil are listed in Table 1. The oil was rich in monoterpenes and diterpenes and particularly dominated by abietanes. The concentration of sesquiterpenes was minimum, which constituted less than 8% of the oil. The major components of the oil were α-pinene (11.5%), sabinene (8.5%), β-pinene (5.2%), abietatriene [1,2,3,4,4a,9,10,10a-octahydro-1,1,4a-trimethyl-7-(1-methylethyl)-,(4a*S*-*trans*)-phenanthrene] (16.3%), dehydroabietal [1,2,3,4,4a,9,10,10a-octahydro-1,4a-dimethyl-7-(1-methylethyl)-,(1*R*-(1α,4aβ,10aα))-1-phenanth-

Table 1: Composition of the essential oil of *P. hirsutus*.

Compound	RI		Area	Method of
	Observed	literature	%	identification
α-Thujene	930	930	1.3	GC, GC-MS
α-Pinene	940	939	11.5	GC, GC-MS
Camphene	955	954	0.5	GC, GC-MS, PE
Sabinene	980	975	8.5	GC, GC-MS, PE
β-Pinene	982	979	5.2	GC, GC-MS, PE
β-Myrcene	992	991	0.1	GC, GC-MS, PE
p-Cymene	1024	1024	1.0	GC, GC-MS, PE
Limonene	1030	1029	0.3	GC, GC-MS, PE
γ-Terpinene	1062	1059	0.1	GC, GC-MS
n-Octanol	1070	1068	0.1	GC, GC-MS, PE
Terpinolene	1088	1088	0.2	GC, GC-MS
Linalool	1097	1096	0.2	GC, GC-MS, PE
cis-Verbenol	1139	1141	0.1	GC, GC-MS
trans-Pinocarveol	1141	1139	0.3	GC, GC-MS
trans-Verbenol	1146	1144	0.2	GC, GC-MS
Pinocarvone	1162	1164	0.1	GC, GC-MS
Terpinen-4-ol	1179	1177	0.8	GC, GC-MS, PE
Napthalene	1182	1178	0.2	GC, GC-MS, PE
cis-Pinocarveol	1185	1184	0.1	GC, GC-MS
α-Terpineol	1190	1191	0.2	GC, GC-MS, PE
Myrtenal	1196	1195	0.1	GC, GC-MS
p-Cymen-7-ol	1290	1290	0.2	GC, GC-MS
trans-Pinocarvyl acetate	1299	1298	0.2	GC, GC-MS
cis-Pinocarvyl acetate	1310	1312	0.2	GC, GC-MS
α–Cubebene	1350	1348	0.1	GC, GC-MS
α-Ylangene	1373	1375	0.1	GC, GC-MS
α-Copaene	1378	1376	2.5	GC, GC-MS
β-Cubebene	1386	1388	0.1	GC, GC-MS
β-Elemene	1390	1390	0.1	GC, GC-MS
(*E*)-Caryophyllene	1418	1418	0.1	GC, GC-MS
α-Guaiene	1437	1439	0.1	GC, GC-MS
α-Selinene	1495	1498	0.1	GC, GC-MS
trans-β-Guaiene	1500	1502	0.3	GC, GC-MS
β-Sesquiphellandrene	1517	1522	0.1	GC, GC-MS
δ-Cadinene + *trans*-Calamenene	1523	1524	1.4	GC, GC-MS
α-Cadinene	1540	1538	0.2	GC, GC-MS
(*E*)-Nerolidol	1561	1563	0.3	GC, GC-MS
Caryophyllene oxide	1582	1583	0.4	GC, GC-MS
Cubenol	1646	1649	0.1	GC, GC-MS
α-Cadinol	1652	1652	0.1	GC, GC-MS
Pogostone	1702	1702	0.8	GC, GC-MS
Isopimara-9(11),15-diene	1903	1905	2.8	GC, GC-MS
Pimara-8(14),15-diene	1953	1949	1.1	GC, GC-MS
Sclarene	1971	1974	0.1	GC, GC-MS
Kaur-15-ene	2000	1997	1.0	GC, GC-MS
Abietatriene	2054	2056	16.3	GC, GC-MS
Abieta-8,13-diene	2090	2087	0.2	GC, GC-MS
p-(3,4-Dihydro-2,2,4-trimethyl-2*H*-1-benzopyran-4-yl)-phenol	2111	--	1.5	GC-MS
Abita-8(14),13(15)diene	2155	2154	0.7	GC, GC-MS
13α-Methyl,13-vinylpodocarp-7-en-3α-ol	2160	--	2.5	GC-MS
Sclareol	2222	2223	0.6	GC, GC-MS
Dehydroabietal	2280	2275	3.5	GC, GC-MS
Abietal	2311	2313	0.9	GC, GC-MS
Dehydroabietol	2368	2368	21.0	GC, GC-MS
		Total	90.8	

RI - Retention Index; PE - peak enrichment on co-injection with standard.

rene carboxaldehyde] (3.5%) and dehydroabietol [1,2,3,4,4a,9,10,10a-octahydro-1,4a-dimethyl-7-(1-methylethyl)-, (1*R*-(1α,4aβ,10aα))-1-phenanthrenemethanol] (21.0%).

The composition of *P. hirsutus* oil is quite different from that of the oil of *P. cablin* (patchouli oil) which is widely used in perfumery and flavours. Patchouli oil contains mainly sesquiterpenes and oxygenated sesquiterpenes and the main component being patchouli alcohol. On the contrary, the oil of *P. hirsutus* contained mostly monoterpenes and diterpenes. Patchouli alcohol was not detected in the oil. However, pogostone which is one of the important constituents of patchouli oil was found in the essential oil of *P. hirsutus*. The main component of *P. hirsutus* oil was dehydroabietol, which is reported to possess antiplasmodial activity and found to inhibit the growth of chloroquine-sensitive as well as chloroquine-resistant strains of *Plasmodium falciparum* that causes malarial fever [2].

Diterpenes are widespread in conifers. However, Asteraceae and Lamiaceae are the two families in angiosperms rich in diterpenes. They have been used in chemotaxonomic studies in conifers and in some families in angiosperms especially in Lamiaceae [3-8]. Abietanes are a large class of tricyclic diterpenoids, commonly found in conifers and in some angiosperms. In Lamiaceae, they have been reported in genera such as *Coleus*, *Hyptis*, *Lepechinia*, *Nepeta*, *Plectranthus*, *Rabdosia*, *Salvia* and *Stachys* [3,5,8,9]. However, abietanes have not been reported so far in the genus *Pogostemon*. In the present study, six abietane compounds representing 42.6% of the essential oil of *P. hirsutus* were identified. Therefore, the high content of abietanes in the essential oil of this species is significant.

Experimental

Plant material: Leaf sample of *P. hirsutus* was collected from Akkamalai of Valparai region of Anamalai hills of the Western Ghats, Tamil Nadu, India in the month of January. Herbarium specimens (*R. Murugan 21*) were prepared for identification and deposited in the Herbaria of Madras Christian College (MCCH), Chennai and Institute of Ayurveda and Integrative Medicine (FRLH), Bangalore, India. The species was identified and confirmed by matching with the authentic herbarium specimens including *Type specimens* deposited in the Herbaria of the Botanical Survey India (CAL), Kolkata and the Royal Botanic Gardens (K), London.

Extraction of oil: The collected leaf sample was dried in the shade for about three weeks and subjected to hydro-distillation in a Clevenger-type apparatus for about 8 hours. Yellow coloured essential oil was obtained and dried over anhydrous Na_2SO_4. The oil was stored in refrigerator at 5°C until analyzed.

Analyses: The GC-FID and GC-MS analyses of the essential oil were done by the method adopted in our previously published report [1d]. Component identification was done by comparison of the retention indices (RIs) of the GC peaks obtained using homologous series of *n*-alkanes (C_8-C_{25}) with those of compounds reported in literature [10] and also by comparing the mass spectra of the peaks with standards reported in literature and matching the mass spectra of the compounds available in NIST and Wiley libraries. Peak enrichment (PE) on co-injection with available authentic standards was also done to confirm the identification. Peak area percentages were calculated from FID response without the use of correction factors.

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Combined Analysis of the Root Bark Oil of *Cleistopholis glauca* by Chromatographic and Spectroscopic Techniques

Zana A. Ouattara[a,b], Jean Brice Boti[b], Coffy Antoine Ahibo[b], Félix Tomi[a], Joseph Casanova[a] and Ange Bighelli[a]*

[a]*Université de Corse-CNRS, UMR 6134 SPE, Equipe Chimie et Biomasse, Route des Sanguinaires, 20000 Ajaccio, France*

[b]*Laboratoire de Chimie Organique Biologique, UFR-SSMT, Université Félix Houphouët Boigny-Abidjan, BPV 34 Abidjan, Côte d'Ivoire*

ange.bighelli@univ-corse.fr

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The composition of root bark oil from *Cleistopholis glauca* Pierre ex Engler & Diels growing wild in Ivory Coast was investigated by GC (in combination with retention indices) and ^{13}C NMR spectroscopy after partition of hydrocarbons and oxygenated compounds on silica gel. Thirty-one compounds have been identified. *C. glauca* produces a sesquiterpene-rich oil, patchoulenone (33.5%), cyperene (9.5%) and germacrene D (6.6%) being the main components. Special attention was paid to the identification and quantification of germacrene C (a heat-sensitive compound) and δ-elemene, which were achieved by a combination of GC(FID) and ^{13}C NMR spectroscopy. The composition of *C. glauca* root bark and leaf oils differed drastically.

Keywords: *Cleistopholis glauca*, Root bark oil, Essential oil composition, Germacrene C, Patchoulenone.

Cleistopholis glauca Pierre ex Engler & Diels (*Annonaceae*) is native to central Africa, growing wild in rainforest and in secondary bush, as well as along waterways. It is widespread in Cameroon, Central African Republic, Gabon, Congo-Kinshasa (actually Democratic Republic of Congo) and Angola. It was introduced thirty years ago in the Petit Yapo forest (South-Eastern Ivory Coast). *C. glauca* is a tree up to 35m high and up to 80 cm in diameter, with a cylindrical bole straight trunk covered with a fibrous bark. The bark of *C. glauca* is used in the manufacture of African huts, and fibers make excellent strings. In decoction, it is used as an emetic. The macerated bark of stems and roots is used as a vermifuge in Central African Republic [1,2].

Structural elucidation has been reported of compounds isolated from solvent extracts of *C. glauca*: oligorhamnosides [3,4]; lipids [5]; terpenes [4,5]; a phenyl propanoid [6]; and a furanic derivative [6]. The chemical composition of *C. glauca* leaf oil has been recently investigated by our group [7]. Oil samples isolated from leaves collected from individual trees were analyzed by gas chromatography (GC) using a flame ionisation detector (FID), and the compounds were characterized by a combination of retention indices (RI) and ^{13}C NMR spectroscopy. *C. glauca* produced a sesquiterpene-rich leaf oil, germacrene D (16.4-46.5%) and (*E*)-β-caryophyllene (8.0-26.2%) being the major components. In a few samples, monoterpene hydrocarbons were present in appreciable amounts: myrcene (up to 39.7%), β-pinene (up to 24.8%) and α-pinene (up to 16.4%).

In the present paper, we report on the chemical composition of a root bark oil sample from *C. glauca*, investigated by a combination of chromatographic and spectroscopic techniques and that differed drastically from the leaf oils of the same species. Identification and quantitative determination, by a combination of GC (FID) and ^{13}C NMR spectroscopy, of a heat sensitive compound, germacrene C, was investigated in detail.

Water distillation of root bark from *C. glauca* collected in Petit Yapo forest produced an essential oil with a yield of 0.04%, w/w, *vs* fresh material, much lower than that obtained from leaves (0.16-0.19%) [7]. The root bark oil was first analyzed by GC, in combination with retention indices (RI) on two capillary columns of different polarity and by ^{13}C NMR spectroscopy following a computerized method developed in our laboratory [8-10]. The experimental procedure may be summarized as follow: the ^{13}C NMR chemical shift values of the experimental spectrum are matched against the spectra of reference compounds compiled into two computerized libraries, the first one built with spectra recorded in our laboratory, the second one built with ^{13}C NMR data reported in the literature. Combined analysis by GC (RI) and ^{13}C NMR of the essential oil (EO) allowed the identification of the major components and demonstrated that *C. glauca* root bark oil was a sesquiterpene-rich oil. However, various minor components remained unidentified and some discrepancies appeared between the GC (FID) and NMR analyses, concerning particularly the content of δ-elemene, a compound that may be produced by the plant or that may result from thermal rearrangement of germacrene C, a heat-sensitive compound. We demonstrated that such a rearrangement may occur during GC (FID) and GC-MS analyses [11]. Therefore, the EO was fractionated over a silica gel chromatography column (CC) into a hydrocarbon fraction (FH) and an oxygenated compounds-containing fraction (FO); both fractions were analyzed, once again, by GC (RI) and by ^{13}C NMR (Table 1).

The major component was patchoulenone (33.5%) (Figure 1), a tricyclic sesquiterpene ketone first isolated from the oil of *Cyperus rotundus* L. [12] and whose ^{13}C NMR data have been reported later [13]. Sesquiterpene hydrocarbons were well represented by cyperene (9.5%) and germacrene D (6.6%). Camphene (5.2%) was the main monoterpene hydrocarbon and bornyl acetate (4.5%) the major oxygenated monoterpene. Other sesquiterpene hydrocarbons and oxygenated sesquiterpenes usually produced by aromatic plants, such as alismol (2.4%), δ-cadinene (1.7%), *epi*-cubenol (1.3%),

Table 1: Components of *Cleistopholis glauca* root oil.

	Components	RIa	RIp	EO	FH	FO	Identification
1	α-Pinene	931	1024	0.9	0.8	-	RIa, RIp ^{13}C NMR
2	Camphene	944	1072	5.2	4.2	-	RIa, RIp ^{13}C NMR
3	Myrcene	980	1164	0.2	0.3	-	RIa, RIp *^{13}C NMR*
4	Limonene*	1020	1204	0.3	0.5	-	RIa, RIp *^{13}C NMR*
5	1,8-Cineole*	1020	1211	0.9	-	1.2	RIa, RIp ^{13}C NMR
6	(*E*)-β-Ocimene	1038	1252	0.2	0.3	-	RIa, RIp *^{13}C NMR*
7	Bornyl acetate	1269	1580	4.5	-	7.7	RIa, RIp ^{13}C NMR
8	δ-Elemene#	1336	1472	2.3	5.4	-	RIa, RIp ^{13}C NMR
9	Germacrene C#	-	-	1.6	3.7	-	^{13}C NMR
10	α-Copaene	1376	1494	0.2	0.5	-	RIa, RIp *^{13}C NMR*
11	β-Elemene	1388	1591	1.5	3.8	-	RIa, RIp ^{13}C NMR
12	Cyperene	1402	1530	9.5	23.9	-	RIa, RIp ^{13}C NMR
13	(*E*)-β-Caryophyllene	1412	1598	0.6	2.2	-	RIa, RIp *^{13}C NMR*
14	β-Copaene	1428	1591	1.1	2.7	-	RIa, RIp ^{13}C NMR
15	*trans*-α-Bergamotene	1433	1586	0.2	0.5	-	RIa, RIp *^{13}C NMR*
16	α-Humulene	1451	1669	0.2	0.5	-	RIa, RIp *^{13}C NMR*
17	Rotundene	1459	1638	0.7	1.6	-	RIa, RIp ^{13}C NMR
18	1β*H*,7α*H*,10β*H*-Guaia-4,11-diene*	1474	1663	1.8	3.5	-	RIa, RIp ^{13}C NMR
19	γ-Muurolene*	1474	1688	0.5	1.2	-	RIa, RIp ^{13}C NMR
20	Germacrene D*	1477	1709	6.6	15.7	-	RIa, RIp ^{13}C NMR
21	α-Amorphene*	1477	1692	0.3	0.4	-	RIa, RIp *^{13}C NMR*
22	β-Selinene	1482	1714	0.3	0.6	-	RIa, RIp *^{13}C NMR*
23	Hinesene	1492	1725	0.9	2.3	-	RIa, RIp ^{13}C NMR
24	δ-Cadinene	1515	1757	1.7	4.3	-	RIa, RIp ^{13}C NMR
25	Germacrene B	1552	1827	1.0	2.4	-	RIa, RIp ^{13}C NMR
26	Patchoulenone	1604	2098	33.5	-	58.8	RIa, RIp ^{13}C NMR
27	Alismol	1612	2270	2.4	-	4.2	RIa, RIp ^{13}C NMR
28	*epi*-Cubenol	1629	2061	1.3	-	2.3	RIa, RIp ^{13}C NMR
29	α-Cadinol	1639	2242	0.5	-	1.3	RIa, RIp ^{13}C NMR
30	Juvenile hormone III	1862	2495	1.8	-	3.2	RIa, RIp ^{13}C NMR
31	10-oxo-(2*E*,6*E*)- Methylfarnesoate	1891	2579	0.9	-	1.5	RIa, RIp ^{13}C NMR

Order of elution and percentages are given on apolar column, those with an asterisk* excepted, percentage on polar column. RIa, RIp = Retention indices on apolar (BP-1) and polar (BP-20) column, respectively. FH and FO: fraction containing hydrocarbons and oxygenated compounds, respectively. #: Percentages calculated by combination of GC (FID) and ^{13}C NMR. *^{13}C NMR (italic)*: components identified by NMR in a CC fraction.

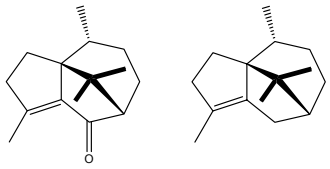

Figure 1: Structure of patchoulenone (left) and cyperene (right).

β-copaene (1.1%), (*E*)-β-caryophyllene (0.6%) and α-cadinol (0.5%), were identified by comparison of their RIs on two columns of different polarity and their ^{13}C NMR data with those of reference compounds compiled in our laboratory-built library.

Identification of some other components was achieved by comparison of the chemical shift values of the EO spectrum with literature data. For example, two compounds bearing the farnesane skeleton were identified as 10-oxo-(2*E*,6*E*)-methyl-farnesoate (**31**, 0.9%) and juvenile hormone III (**30**, 1.8%, 10,11-epoxy-3-methylenedodeca-1,6(*E*)-diene), an insect hormone rarely found in plants. The NMR data of the first one fit with those reported by Seidel *et al* [14], who isolated this compound from a stem bark extract of *C. glauca*. The carbon chemical shifts of juvenile hormone fit perfectly with those reported by Mori and Mori [15], who synthesized both enantiomers of that molecule. Recently, we reported the occurrence of this hormone in the leaf and trunk bark oils from *Cleistopholis patens* [11].

In parallel, it should be mentioned that 1β*H*,7α*H*,10β*H*-guaia-4,11-diene **18** (1.8%) was identified by comparison of its ^{13}C NMR chemical shifts (in the CC fraction FH and in the EO) with those reported by Blay *et al*. [16], who obtained the compound by synthesis. This compound has been simultaneously reported by our groups for the first time as a natural compound in the present work and in *Xylopia rubescens* leaf oil [17]. Similarly, hinesene (**23**) was identified by comparison of its NMR data and RI (apolar) with those reported by Joulain and König [18].

Special mention should be attributed to the identification and quantification of δ-elemene and germacrene C. The sesquiterpene hydrocarbon δ-elemene (**8**) was identified by GC (FID) and by ^{13}C NMR spectroscopy. However, its relative percentage attributed by GC (FID) (3.9% in the EO, 9.1% in the CC fraction FH) was over-evaluated with respect to the intensities of the corresponding signals in the ^{13}C NMR spectra. In agreement with our previous investigations on the composition of *C. patens* trunk bark oil [11], the ^{13}C NMR spectra of the EO and the FH fraction of CC exhibited the 15 signals of δ-elemene [18] and the 15 signals of germacrene C (**9**). It should be mentioned that the ^{13}C NMR data of germacrene C were reported in C_6D_6 [19]. Therefore, the spectrum of FH was recorded in C_6D_6 and in $CDCl_3$ and the chemical shift values of germacrene C in $CDCl_3$ are reported in the experimental part. As previously reported [20], germacrene C thermally rearranges to δ-elemene following a [3.3] sigmatropic reaction (Figure 2). Under our experimental chromatographic conditions, the content of δ-elemene evaluated by GC (3.9% in the EO, 9.1% in the CC fraction) corresponded in fact to the sum of δ-elemene and germacrene C. Combining GC percentages of δ-elemene with the ratio of both compounds, evaluated by comparing the mean intensities of their respective signals, allowed a better quantitative determination of δ-elemene and germacrene C: 5.4% and 3.7%, respectively in the CC fraction, 2.3% and 1.6%, respectively in the EO. In parallel we observed the presence of β-elemene (1.5%) and germacrene B (1.0%), while germacrene A and γ-elemene were not detected by NMR.

In conclusion, the root bark of *C. glauca* produced a sesquiterpene-rich oil, whose composition was dominated by patchoulenone and cyperene. Neither compound has been identified as a component of *C. glauca* leaf oil that contained mainly germacrene D (16.4-46.5%) and (*E*)-β-caryophyllene (8.0-26.2%). Combined analysis by GC (RI) and by ^{13}C NMR spectroscopy allowed the correct identification and quantification of germacrene C, a compound that thermally rearranges to δ-elemene during GC analysis.

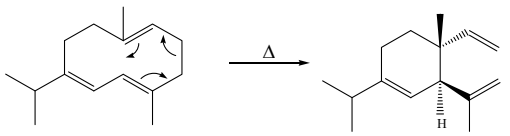

Figure 2: Thermal rearrangement of germacrene C to δ-elemene.

Experimental

Plant material: Root bark of *C. glauca* was collected in Petit Yapo forest, near Adzopé (100 km north of Abidjan), in January 2011. Plant material was authenticated by Pr L. Aké Assi, from the Centre National Floristique (CNF, Abidjan, Ivory Coast).

Essential oil isolation and fractionation: The bark (1200 g) was submitted to hydrodistillation in a Clevenger-type apparatus for 3 h. The oil was decanted, weighed (0.530 g), and stored in a glass vial at 5°C prior to analysis. An aliquot of the EO (190 mg) was chromatographed on silica gel (ICN, 250-500 µm, 10 g). Two fractions were eluted with *n*-pentane (FH, 67 mg) and with diethyl oxide (FO, 97 mg). It should be pointed out that part of the monoterpene hydrocarbons was lost during the removal of solvent under reduced pressure.

GC analysis: GC analysis was carried out with a Perkin-Elmer Clarus 500 Autosystem apparatus equipped with 2 flame ionisation detectors (FID), and fused capillary columns (50 m x 0.22 mm i.d., film thickness 0.25 µm), BP-1 (polymethylsiloxane) and BP-20 (polyethylene glycol). Carrier gas, helium; linear velocity, 0.8 mL/min. The oven temperature was programmed from 60°C to 220°C at 2°C/min and then held isothermal (20 min). Injector temperature: 250°C (injection mode: split 1/60). Detector temperature: 250°C. The relative proportions of the essential oil constituents were expressed as percentages obtained by peak area normalization, all relative response factors being taken as one.

^{13}C NMR analysis: ^{13}C NMR spectra were recorded on a Bruker AVANCE 400 Fourier Transform spectrometer operating at 100.63 MHz for ^{13}C, equipped with a 5 mm probe, in deuterated chloroform ($CDCl_3$), with all shifts referred to internal tetramethylsilane (TMS). Spectra were recorded with the following parameters: pulse width (PW), 5 µs (flip angle 45°); acquisition time, 2.7 s for 128 K data table with a spectral width (SW) of 24000 Hz (240 ppm); digital resolution 0.183 Hz/pt. The number of accumulated scans was 3,000 for the oil sample and fractions of chromatography (about 50 mg of essential oil in 0.5 mL of $CDCl_3$).

Identification of individual components: The oil sample and both CC fractions were analyzed by GC (RI) and ^{13}C NMR. Every component was identified by NMR, at least in one fraction of CC. Identification of most individual components was based: (i) on comparison of their GC retention indices (RI) on apolar and polar columns, determined relative to the retention times of a series of *n*-alkanes with linear interpolation ('Target Compounds' software of Perkin-Elmer), with those of either authentic compounds or literature data [18], (ii) by ^{13}C NMR spectroscopy, following the methodology developed and computerized in our laboratories, using home-made software, (see text) [8-10]. A few compounds have been identified by comparison of their ^{13}C NMR chemical shift values with those reported in the literature (see text).

^{13}C NMR data of germacrene C ($CDCl_3$): δ 145.3 C, 141.4 C, 129.5 C, 127.1 C, 124.9 CH, 121.6 CH, 39.9 CH_2, 39.8 CH_2, 36.6 CH, 31.6 CH_2, 27.5 CH_2, 22.3 CH_3, 22.0 CH_3, 20.9 CH_3, 16.5 CH_3.

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Bioactivities and Compositional Analyses of *Cinnamomum* Essential Oils from Nepal: *C. camphora*, *C. tamala*, and *C. glaucescens*

Prabodh Satyal[a], Prajwal Paudel[a], Ambika Poudel[b], Noura S. Dosoky[a], Kiran Kumar Pokharel[c] and William N. Setzer[a,*]

[a]*Department of Chemistry*, *University of Alabama in Huntsville*, *Huntsville*, *AL 35899*, *USA*
[b]*Department of Chemistry*, *Tribhuvan University*, *MMAMC Campus*, *Biratnagar*, *Nepal*
[c]*National Herbarium and Plant Laboratories*, *Kathmandu*, *Nepal*

wsetzer@chemistry.uah.edu

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This work examines the biological activity of essential oils of *Cinnamomum camphora* leaves, *C. glaucescens* fruit, and *C. tamala* root from Nepal. The oils were screened for phytotoxic activity against lettuce and perennial ryegrass, brine shrimp lethality, and antibacterial, antifungal, cytotoxic, insecticidal, and nematicidal activities. *C. camphora* leaf essential oil was phytotoxic to lettuce, antifungal to *Aspergillus niger*, and insecticidal, particularly toward midge and butterfly larvae, fruit flies, and fire ants. *C. camphora* oil was also toxic to brine shrimp and human breast tumor cells. *C. glaucescens* fruit essential oil showed notable nematicidal activity, as well as termiticidal and mosquito larvicidal activity. The root essential oil of *C. tamala* was toxic to mosquito larvae and fire ants.

Keywords: Allelopathy, Insecticidal, Larvicidal, Nematicidal, Antimicrobial.

Cinnamomum, a genus of evergreen aromatic trees belonging to the family Lauracaeae, is comprised of approximately 250 species [1]. In this study three species: *Cinnamomum camphora* (L.) J. Presl, *C. tamala* (Buch.-Ham.) T. Nees & Nees, and *C. glaucescens* Hand.-Mazz. from Nepal were studied for chemotypical variation in the volatile components, which were subsequently analyzed for possible biological activity.

C. camphora is primarily native to Japan and has been cultivated in the Himalayan region to produce camphor commercially, which is used in the pharmaceutical as well as the flavor industry [2,3]. Traditionally in the Ayurvedic system, *C. camphora* has been used to treat bronchitis, cold, congestion, diarrhea, dysentery, edema, flu, gas, metabolic and heart problems, as well as various gynecological problems [2b]. In addition, the Yunani, "Greek", medicinal system also uses *C. camphora* for a cephalic tonic, cardiac treatment, and also as an expectorant [2b]. *C. camphora* extracts have exhibited cytotoxic [3], anti-inflammatory and anti-oxidant activities [4], and termiticidal [5], acaricidal [6], antifungal [7], and anthelmintic [8] activities.

C. tamala, commonly known as "tezpat", is widely distributed throughout tropical and subtropical regions of Australia, the Pacific region, South America and the Himalayan region of Asia [9], and typically grows at altitudes between 900-2500 m [10]. Morphologically, *C. tamala* is a perennial or small evergreen tree, attaining 8-12 m in height and a girth of 150 cm with the leaves ranging from 12-20 cm long, 5-8 cm broad, and with white flowers [11]. Pharmacologically the plant has been reported to exhibit antihyperglycemic, antidiarrheal, antihyperlipidemic, anti-inflammatory, acaricidal, hepatoprotective, gastroprotective, antioxidant, antibacterial, α-amylase-inhibitory, and immunomodulatory activities [12]. The bark of *C. tamala* is used in Nepalese traditional medicine to treat intestinal disorders and control nausea, while the leaf is used as a spice and to control diarrhea [13]. Leaves of *C. tamala* are used in India to repel stored grain pests [14].

C. glaucescens, commonly known as "sugandh kokila", is native to the Himalayan range in Nepal and India [15], although it is also found in the plains and hilly regions at altitudes below 2500 m. Morphologically, *C. glaucescens* buds are enclosed in overlapping scales with fruits about 3 cm long, the base enclosed in a cup 10-12 mm across, while the leaves are alternate, between 7-10 cm long with elliptical shape, a well-marked midrib, and 4-5 pairs of lateral veins. *C. glaucescens* is extensively used by local people as a spice, in medicine, and in consumption of tobacco [16]. Therapeutically, *C. glaucescens* has been traditionally used as a demulcent and stimulant and has shown analgesic, antiseptic, astringent, and carminative properties [16]. Furthermore, *C. glaucescens* seed is used for treatment of the common cold, cough, toothache and taenias [16]. Seed paste is applied to treat muscular swellings [16]. Seed oil has also been demonstrated to treat muscular spasm, joint pain and body aches [16]. *C. glaucescens* fruit essential oil has been assessed for insecticidal, oviposition deterrent and antifungal activities [17].

Essential oil compositions: The essential oils were obtained in 1.3%, 0.9%, 1.0%, and 1.5% yields for *C. camphora* (#15) leaf, *C. camphora* (#72) leaf, *C. tamala* root, and *C. glaucescens* fruits, respectively. The chemical compositions of the oils are summarized in Table 1. *C. camphora* (#15) leaf oil was dominated by camphor (36.5%), camphene (11.7%), and limonene (9.0%), with lesser amounts of sabinene (6.3%) and β-pinene (6.3%), while the *C. camphora* (#72) leaf oil contained almost exclusively camphor (98.0%). Currently, there are five different chemotypes observed worldwide for *C. camphora*: camphor, linalool, 1,8-cineole, nerolidol, and borneol [18]. Specifically, camphor content varied from a Japanese sample with 51.5% to a Chinese oil sample with 83.9% and an Ivory Coast leaf oil containing 74% camphor [19].

C. tamala root essential oil was also found to be rich in camphor (35.0%), followed by linalool (10.6%), *p*-cymene (8.5%), *o*-cymene (6.8%), and 1,8-cineole (6.1%). A survey of the literature reveals

Table 1: Chemical compositions of *Cinnamomum camphora* leaf essential oil, *C. glaucescens* fruit essential oil, and *C. tamala* root essential oil from Nepal.

RI[a]	Compound	Percent Composition[b]			
		C. camphora		*C. glaucescens*	*C. tamala*
		#15	#72		
922	Tricyclene	0.7	—	—	0.1
933	Methyl hexanoate	—	—	tr[c]	—
934	α-Thujene	7.7	—	0.4	0.7
941	α-Pinene	4.7	tr	2.6	4.7
953	Camphene	11.7	0.2	0.1	2.0
957	Thuja-2,4(10)-diene	—	—	tr	tr
962	Benzaldehyde	—	—	0.2	0.1
976	Sabinene	6.3	—	5.7	0.5
980	β-Pinene	6.3	0.1	2.8	4.1
993	Myrcene	1.2	—	0.1	1.1
1000	δ-2-Carene	tr	—	—	tr
1005	α-Phellandrene	—	—	tr	—
1007	δ-3-Carene	tr	—	—	0.3
1016	α-Terpinene	—	—	0.3	—
1025	*p*-Cymene	1.8	—	0.3	8.5
1028	*o*-Cymene	1.8	—	0.2	6.8
1034	Limonene	9.0	—	—	—
1035	1,8-Cineole	2.7	0.2	24.8	6.1
1043	Salicylaldehyde	—	—	—	0.3
1060	γ-Terpinene	tr	—	1.0	—
1063	*cis*-Sabinene hydrate	tr	—	tr	tr
1068	*cis*-Linalool oxide (furanoid)	tr	—	0.2	0.3
1076	*trans*-Linalool oxide (furanoid)	—	—	—	0.3
1076	*trans*-Dihydrorose oxide	tr	—	—	—
1079	*p*-Cresol	—	—	tr	—
1087	Terpinolene	—	—	0.3	—
1087	Fenchone	0.1	—	—	—
1093	6,7-Epoxymyrcene	tr	—	—	—
1097	α-Pinene oxide	tr	—	—	—
1102	Perillene	0.2	—	—	—
1102	Linalool	0.6	—	3.7	10.6
1113	*endo*-Fenchol	—	—	tr	—
1121	*cis*-*p*-Menth-2-en-1-ol	—	—	0.1	—
1126	Methyl octanoate	—	—	tr	—
1138	*trans*-Pinocarveol	—	—	0.1	—
1139	*trans*-Sabinol	—	—	0.1	—
1144	*trans*-Verbenol	—	—	tr	—
1146	Camphor	36.5	98.0	—	35.0
1157	Sabina ketone	—	—	tr	—
1162	Pinocarvone	—	—	0.1	—
1162	Isoborneol	—	—	0.1	tr
1165	Borneol	0.2	1.4	—	0.8
1166	Pinocarvone	0.2	—	—	0.2
1168	δ-Terpineol	—	—	0.9	—
1170	*cis*-Linalool oxide (pyranoid)	—	—	tr	tr
1179	Terpinen-4-ol	1.4	tr	4.8	1.8
1182	*p*-Methylacetophenone	0.1	—	tr	tr
1184	Thuj-3-en-10-al	—	—	tr	—
1186	Cryptone	—	—	0.1	0.3
1188	*p*-Cymen-8-ol	0.2	—	—	—
1192	α-Terpineol	1.5	0.2	7.4	2.2
1196	Myrtenal	0.3	—	—	0.2
1197	Estragole (= Methyl chavicol)	—	—	—	0.1
1198	Myrtenol	0.3	—	0.3	0.1
1203	*cis*-Piperitol	—	—	tr	tr
1207	*trans*-Piperitol	tr	—	—	tr
1209	Verbenone	0.1	—	0.1	0.1
1217	*trans*-Carveol	0.2	—	tr	0.1
1226	*cis*-Carveol	0.1	—	—	—
1227	Nerol	tr	tr	0.2	0.1
1235	Ascaridole	—	—	tr	—
1236	Neral	tr	—	tr	—
1237	Cuminal	0.1	—	0.1	0.1
1239	*o*-Anisaldehyde	—	—	—	0.1
1242	Carvone	—	—	—	0.1
1242	Carvone	0.2	—	tr	—
1251	Piperitone	0.1	—	tr	0.1
1255	Geraniol	—	—	0.1	tr
1260	(2*E*)-Decenal	—	—	—	tr
1269	(*E*)-Cinnamaldehyde	—	—	—	0.4
1270	Geranial	—	—	tr	tr
1281	Neryl formate	—	—	tr	—
1283	Safrole	tr	—	—	—
1285	*neoiso*-3-Thujanol acetate	—	—	tr	—
1286	Bornyl acetate	—	—	—	0.8
1290	*p*-Cymen-7-ol	0.1	—	0.1	0.1
1299	Terpinen-4-ol acetate	—	—	—	0.1
1301	Carvacrol	—	—	—	tr
1302	Methyl (*Z*)-cinnamate	—	—	0.5	—
1305	(*E*)-Cinnamyl alcohol	—	—	—	0.2
1321	Methyl geranate	—	—	tr	—
1332	8-Hydroxy-neo-menthol	—	—	tr	—
1337	δ-Elemene	—	—	—	tr
1349	α-Cubebene	tr	—	tr	0.1
1366	Neryl acetate	—	—	—	tr
1371	Hydrocinnamyl acetate	—	—	—	0.1
1376	α-Copaene	0.2	—	0.3	1.3
1381	*trans*-Soberol	0.3	—	—	—
1382	*trans*-*p*-Menth-6-ene-2,8-diol	—	—	—	tr
1387	Geranyl acetate	—	—	—	0.1
1388	Methyl (*E*)-cinnamate	—	—	40.5	tr
1391	β-Cubebene	0.2	—	—	0.8
1394	β-Elemene	0.1	—	—	0.2
1419	(*E*)-Caryophyllene	0.1	tr	tr	1.0
1428	Carvone hydrate	0.1	—	0.3	tr
1430	β-Copaene	—	—	—	tr
1438	Coumarin	—	—	tr	0.6
1439	Aromadendrene	0.2	—	—	tr
1451	(*E*)-Cinnamyl acetate	—	—	—	1.2
1454	α-Humulene	0.1	tr	tr	1.2
1461	Alloaromadendrene	0.1	—	—	—
1462	9-*epi*-(*E*)-Caryophyllene	—	—	—	tr
1478	γ-Muurolene	tr	—	—	0.1
1483	Amorpha-4,7(11)-diene	—	—	—	0.1
1485	Widdra-2,4(14)-diene	—	—	—	tr
1487	β-Selinene	tr	—	—	tr
1493	*trans*-Muurola-4(14),5-diene	—	—	—	tr
1496	*epi*-Cubebol	tr	—	0.1	0.2
1497	Bicyclogermacrene	—	tr	—	—
1502	α-Muurolene	tr	—	—	0.1
1507	Germacrene A	—	—	—	tr
1515	γ-Cadinene	tr	—	—	tr
1518	Cubebol	0.1	—	0.1	0.1
1524	*trans*-Calamenene	tr	—	—	—
1525	δ-Cadinene	—	—	0.1	0.3
1529	(*E*)-*o*-Methoxycinnamaldehyde	—	—	—	tr
1538	α-Cadinene	—	—	—	tr
1543	α-Calacorene	—	—	—	tr
1550	Elemol	—	tr	—	—
1554	(*Z*)-Caryophyllene oxide	—	—	—	0.1
1558	Germacrene B	—	—	—	tr
1565	β-Calacorene	—	—	—	tr
1579	Spathulenol	—	tr	tr	0.2
1585	Caryophyllene oxide	0.4	tr	0.3	1.3
1592	Viridiflorol	tr	—	tr	—
1595	Cubeban-11-ol	tr	—	—	—
1604	Ledol	tr	—	tr	—
1609	Humulene epoxide II	0.3	—	0.1	0.4
1623	α-Corocalene	—	—	—	tr
1629	1-*epi*-Cubenol	tr	—	tr	0.1
1634	Caryophylla-4(12),8(13)-dien-5-ol	—	—	—	0.1
1643	τ-Muurolol	tr	—	tr	0.1
1646	α-Muurolol (= Torreyol)	—	—	—	tr
1650	β-Eudesmol	—	—	tr	—
1655	α-Cadinol	—	—	tr	—
1656	Selin-11-en-4α-ol	—	tr	—	0.1
1659	*cis*-Calamenen-10-ol	—	—	tr	0.1
1667	*trans*-Calamenen-10-ol	—	—	tr	tr
1670	14-Hydroxy-9-*epi*-(*E*)-caryophyllene	—	—	tr	0.1
1673	Cadalene	—	—	tr	tr
1678	Mustakone	—	—	tr	—
1722	(2*Z*,6*E*)-Farnesol	—	—	tr	—
1741	(2*E*,6*E*)-Farnesal	—	—	tr	—
2037	Kaur-16-ene	—	—	—	0.2
	Total Identified	98.5	100	99.4	99.4

[a] RI = "Retention Index" determined on an HP-5ms column with respect to a homologous serious of *n*-alkanes.
[b] Raw percentages based on total ion current without standardization.
[c] tr = "trace" (< 0.05%).

that this is the first study of *C. tamala* root essential oil. However, previous leaf essential oil studies have shown eugenol and cinnamaldehyde to be major components, with Indian samples reporting 81.7% to 78% eugenol [20,21]. However, other studies showed linalool (50.3-33.7%, 19.7%, and 15.7-15.3%) and cinnamaldehyde (12.7-8.7%, 52.8%, and 41.2-55.2%) as the main components of the leaf oil of *C. tamala*, while eugenol was found in trace amounts [22-24].

Hydrodistilled *C. glaucescens* fruit (berries) essential oil was found to contain primarily methyl (*E*)-cinnamate (40.5%) and 1,8-cineole

(24.8%). Smaller quantities of α-terpineol (7.4%), sabinene (5.7%), terpinen-4-ol (4.8%), and linalool (3.7%) were also present. In another study from Nepal, the fruit oil of *C. glaucescens* showed a qualitatively similar composition with major components being 1,8 cineole (13%), methyl (*E*)-cinnamate (14%), and α-terpineol (7%) [25]. In contrast, a *C. glaucescens* fruit essential oil from India showed no methyl cinnamate, but a large percentage of 1,8-cineole (43.6%) [17]. In comparison, leaf oil from India of *C. glaucescens* contained elemicin (92.9%) and methyl eugenol (4.9%) as major components [15].

Allelopathic activity: The allelopathic activities of Nepalese *Cinnamomum* essential oils were analyzed in terms of inhibition of seed germination, as well as inhibition of seedling growth against a representative dicot (lettuce, *Lactuca sativa*) and a representative monocot (perennial ryegrass, *Lolium perenne*). The allelopathic activities of *C. camphora*, *C. tamala*, and *C. glaucescens* essential oils are summarized in Table 2. *Lactuca sativa* seed germination was notably inhibited by *C. camphora* (#15) essential oil (IC_{50} = 149 μg/mL), as well as its major component, camphor (IC_{50} = 239 μg/mL). Although neither *C. tamala* nor *C. glaucescens* appreciably affected germination of either *L. sativa* or *L. perenne*, the major component of *C. glaucescens* oil, methyl (*E*)-cinnamate did inhibit *L. perenne* germination (IC_{50} = 610 μg/mL).

L. sativa seedling growth was more sensitive to the *Cinnamomum* essential oils than *L. perenne*. *C. camphora* essential oil significantly inhibited *L. sativa* radicle growth at 250 μg/mL, which can be attributed to the major component, camphor. *C. glaucescens* essential oil and methyl (*E*)-cinnamate were inhibitory to both *L. sativa* and *L. perenne* at the concentrations tested. *C. tamala* oil inhibited seedling growth of *L. sativa* at 500 μg/mL and *L. perenne* at 1000 μg/mL, likely due to the high concentration of camphor in this essential oil.

Antimicrobial activity: Essential oils from the three species were analyzed for antibacterial and antifungal activities, while the major components were also tested to determine the compounds responsible for the results (Table 3). Of the three essential oils tested, only *C. camphora* showed significant antifungal activity against *A. niger* (MIC = 20 μg/mL). The antifungal activity is hypothesized to be due to synergism among the essential oil components rather than to any one of the constituents. Camphor (36.5%), limonene (9.0%), and β-pinene (6.3%) all exhibited MICs of 156 μg/mL against *A. niger* (Table 3). *C. camphora* oil also showed marginal activity against *B. cereus* and *S. aureus*, with a MIC = 313 μg/mL. *C. camphora* essential oils have been previously noted to have antifungal [26-28] and antibacterial activities [18,29], in agreement with the antimicrobial activities of Nepalese *C. camphora*. *C. tamala* essential oil was also somewhat active against *A. niger* (MIC = 156 μg/mL).

Cytotoxicity and brine shrimp lethality: All three essential oils exhibited *in-vitro* cytotoxic activity against MCF-7 cells, and these activities also correlate with brine shrimp lethality (Table 3). Anderson and co-workers had previously reported a correlation between brine shrimp lethality and cytotoxic activity [30]. Most of the essential oil components tested also showed cytotoxicity to MCF-7 cells, as well as brine shrimp lethality.

Insecticidal activity: The *Cinnamomum* essential oils were screened for insecticidal activity against mosquito (*Culex pipiens*) and midge (*Chaoborus plumicornis*) larvae, cabbage white butterfly (*Pieris rapae*) larvae, termites (*Reticulitermes virginicus*), fruit flies (*Drosophila melanogaster*), and red imported fire ants (*Solenopsis invicta* × *richteri*) (Table 3). All three essential oils showed mosquito larvicidal activities comparable with or better than previously reported essential oils, for example, common myrtle (*Myrtus communis*) [31], bay laurel (*Laurus nobilis*) [32], amyris (*Amyris balsamifera*) [33], and spearmint (*Mentha spicata*) [34], against *Culex pipiens*. Consistent with the mosquito larvicidal activity, the essential oils and components showed larvicidal activity against *Chaoborus* larvae as well. *C. camphora* leaf oil had previously shown such activity against *Culex quinquefasciatus* [35] and *Aedes aegypti* [36].

C. camphora leaf oil was the most active essential oil against cabbage butterfly larvae, fruit flies, and fire ants (LC_{50} = 186, 153, and 176 μg/mL, respectively). *C. camphora* leaf oil had previously shown insecticidal activity against fire ants (*Solenopsis invicta*) [37], the rice weevil (*Sitophilus oryzae*), the broadbean weevil (*Bruchus rufimanus*) [38], *C. camphora* oil also showed insect repellent activity against these stored grain beetles [38] as well as adult *Aedes aegypti*, *Anopheles stephensi*, and *Culex*

Table 2: Allelopathic activity of *Cinnamomum camphora* leaf, *C. glaucescens* fruit, and *C. tamala* root essential oils and essential oil components on lettuce (*Lactuca sativa*) and perennial ryegrass (*Lolium perenne*).

Material (Concentration, μg/mL)	Germination Inhibition (%)		Seedling Growth (% of Controls)			
	Lactuca sativa	*Lolium perenne*	*Lactuca sativa* radicle	*Lactuca sativa* hypocotyl	*Lolium perenne* radicle	*Lolium perenne* hypocotyl
C. camphora oil (1000)	98.3[a]	31.7[b]	69.4[m]	37.9[m]	>100[s]	>100[s]
C. camphora oil (500)	71.7	25.0	81.6[q]	82.9[r]	>100[s]	>100[s]
C. camphora oil (250)	66.7	18.3	80.2[p]	>100[g]	>100[s]	>100[s]
C. glaucescens oil (2000)	50.0[c]	45.0[d]	46.4[m]	45.5[m]	52.2[m]	4.8[m]
C. glaucescens oil (1000)	35.0	30.0	54.7[m]	51.6[m]	60.4[m]	28.0[m]
C. glaucescens oil (500)	30.0	18.3	82.0[n]	66.2[m]	83.0[p]	72.6[p]
C. glaucescens oil (250)	15.0	15.0	87.0[p]	76.8[m]	90.9[r]	73.3[q]
C. tamala oil (2000)	98.3[e]	63.3[f]	0.0[m]	0.0[m]	60.6[m]	21.0[m]
C. tamala oil (1000)	41.7	16.7	86.6[p]	64.7[m]	70.1[m]	88.1[r]
C. tamala oil (500)	23.3	10	96.0[r]	77.8[m]	93.0[r]	99.1[s]
C. tamala oil (250)	18.3	6.7	97.7[s]	79.4[m]	97.8[s]	99.7[s]
Methyl (*E*)-cinnamate (2000)	48.3[g]	100[h]	46.9[m]	46.9[m]	—	—
Methyl (*E*)-cinnamate (1000)	20.0	93.3	63.3[m]	52.0[m]	16.9[m]	2.4[m]
Methyl (*E*)-cinnamate (500)	1.7	30.0	89.8[p]	75.8[m]	42.2[m]	10.6[m]
Methyl (*E*)-cinnamate (250)	—	20.0	—	—	45.4[m]	28.4[m]
Camphor (1000)	91.7[i]	50.0[j]	19.9[m]	4.8[m]	15.8[m]	3.5[m]
Camphor (500)	91.7	43.3	21.1[m]	9.7[m]	17.3[m]	5.3[m]
Camphor (250)	58.3	35.0	51.6[m]	19.3[m]	41.5[m]	7.8[m]
α-Pinene (4000)	6.67	13.3	>100[s]	99.6[s]	90.0[s]	>100[s]
β-Pinene (4000)	13.3	18.3	84.1[q]	58.8[m]	77.0[q]	11.5[m]
β-Pinene (2000)	1.7	25.0	98.6[s]	85.1[s]	82.5[s]	39.4[m]

[a] IC_{50} = 218 μg/mL; [b] IC_{50} > 1000 μg/mL; [c] IC_{50} = 1620 μg/mL; [d] IC_{50} > 1773 μg/mL; [e] IC_{50} = 1084 μg/mL; [f] IC_{50} = 1630 μg/mL; [g] IC_{50} = 1828 μg/mL; [h] IC_{50} = 610 μg/mL; [i] IC_{50} = 239 μg/mL; [j] IC_{50} = 1000 μg/mL.

Significance vs. controls: [m] $P < 0.001$, [n] $P \approx 0.005$ [o] $P \approx 0.01$, [p] $P \approx 0.05$, [q] $P \approx 0.2$, [r] $P \approx 0.5$, [s]not significant.

Table 3: Bioactivity screening of *Cinnamomum camphora* leaf, *C. glaucescens* fruit, and *C. tamala* root essential oils and major essential oil components.

Bioassay	*C. camphora* leaf oil	*C. glaucescens* fruit oil	*C. tamala* root oil	Camphor	1,8-Cineole	*p*-Cymene	Limonene	Linalool	Methyl cinnamate	α-Pinene	β-Pinene	α-Terpineol
Artemia salina lethality (LC_{50}, µg/mL)	2.5±2.5	20.1±2.6	28.1±3.0	21.7±9.9	26.9±13.1	4.7±1.0	6.3±3.4	36.0±3.9	20.7±3.1	21.4±5.7	15.7±6.1	> 100
MCF-7 cytotoxicity (% kill at 100 µg/mL)	71.2±26.8	55.1±4.8	41.2±13.3	100	100	100	90.78±9.6	18.0±9.8	0	68.3±4.9	78.0±4.2	100
Antimicrobial (MIC, µg/mL)												
Bacillus cereus	313	625	625	313	313	1250	625	625	1250	1250	1250	625
Staphylococcus aureus	313	313	625	1250	625	1250	313	156	1250	1250	1250	1250
Escherichia coli	1250	625	625	1250	1250	625	625	625	625	1250	1250	1250
Pseudomonas aeruginosa	625	313	625	1250	1250	625	1250	1250	625	1250	1250	1250
Aspergillus niger	19.5	313	156	625	625	313	1250	625	625	156	156	313
C. elegans nematicidal (LC_{50}, µg/mL)	574±118	151±51	1320±130	> 2500	> 2500	289±118	85.1	> 2500	138±28	> 2500	> 2500	> 2500
Insecticidal (LC_{50}, µg/mL)												
Chaoborus plumicornis (larvae)	61.5±25.0	186±54	116±53	188±22	342±177	79.6	92.8	278	nt	97.4	97.4	140
Culex pipiens (lavae)	46.4	21.5	17.2	7.2	150	nt	nt	nt	nt	nt	nt	nt
Pieris rapae (larvae)	186	820	653	nt	nt	nt	nt	nt	nt	nt	nt	nt
Reticulitermes virginicus	340	34	1075	66	1080	nt	158	nt	nt	nt	nt	nt
Drosophila melanogaster	153±50	348±83	245±25	117±58	160±81	18.4±4.0	249±62	28.0±7.3	167±67	220±54	260±123	383±184
Solenopsis invicta × *richteri*	176±53	325±90	191±59	286±23	375±74	518±56	1285±80	613±65	333±39	> 1000	> 1000	282±33

[a] nt = not tested

quinquefasciatus mosquitoes [39], and the red bud borer, *Resseliella oculiperda* [40]. Camphor was shown to be the insecticidal component in this present work and in a previous study on stored grain insect pests [41].

C. glaucescens fruit oil was the most active *Cinnamomum* oil against termites with an LC_{50} = 34 µg/mL. Several essential oils have shown termiticidal activity [42-47], but differences in assay protocols preclude comparisons of bioactivities.

Nematicidal activity: *C. glaucescens* fruit essential oil exhibited the strongest nematicidal activity with an LC_{50} of 151 µg/mL, followed by *C. camphora* leaf oil (LC_{50} = 574 µg/mL) and *C. tamala* root oil (LC_{50} = 1320 µg/mL). The nematicidal activity of *C. glaucescens* oil on *C. elegans* can be attributed to its major component, methyl (*E*)-cinnamate (LC_{50} = 138 µg/mL), which had previously shown nematicidal activity against the pine wood nematode, *Bursaphelenchus xylophilus* [48,49]. The other major component in *C. glaucescens* oil, 1,8-cineole, was inactive against *C. elegans*, as well as the root knot nematode, *Meloidogyne incognita* [50]. Notably, camphor, the major component in both *C. camphora* and *C. tamala* oils, was also inactive against *C. elegans*. Camphor was reported by Ntalli and co-workers [51] to be inactive against the root knot nematode, *M. incognita*, in contrast to an earlier report by Al-Banna *et al*. [52].

Plant-derived natural products have been used for thousands of years, both for their medicinal properties [53], as well as pesticidal utilities [54-56]. Essential oils are attractive as pesticidal agents because they are readily available, renewable, readily degraded, and non-polluting. Essential oils have been utilized as fungicides [57], herbicides [58], insecticides [59], and nematicides [60,61]. In this work, we have demonstrated the pesticidal potential of essential oils derived from *Cinnamomum camphora*, *C. glaucescens*, and *C. tamala*. In particular, *C. camphora* leaf essential oil was phytotoxic to lettuce, antifungal to *Aspergillus niger*, and insecticidal, particularly toward midge and butterfly larvae, fruit flies, and fire ants. *C. camphora* oil was also toxic to brine shrimp and human breast tumor cells. *C. glaucescens* fruit essential oil showed notable nematicidal activity, as well as termiticidal and mosquito larvicidal activity. The root essential oil of *C. tamala* was toxic to mosquito larvae and fire ants.

Experimental

Plant material: *Cinnamomum camphora* (#15), *C. tamala*, and *C. glaucescens* were collected on the 18th May, 2011, from Hetauda, Makwanpur (27.42° N, 85.03° E, 1550 m above sea level), Nepal. An additional sample of *C. camphora* (#72) was collected from Dhudikhel, Kavre (27.61°N 85.59°E, 1512 m above sea level) on 12th July, 2011. The plants were identified by Kiran Kumar Pokharel and voucher specimens (M001, M002, and M003, respectively) have been deposited in the Botany Department, Tribhuvan University, Post-Graduate Campus. Fresh leaves (100.0 g) of *C. camphora*, roots (100.0 g) of *C. tamala*, and berries (200.0 g) of *C. glaucescens*, were shredded and hydrodistilled for 4 h using a Clevenger apparatus to give essential oils: 1.3 g, 0.9 g, 1.0 g, and 1.5 g for *C. camphora* (#15), *C. camphora* (#72), *C. tamala*, and *C. glaucescens*, respectively.

Gas chromatographic–mass spectral analysis: The *Cinnamomum* 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 = 45-400 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 0.25 µm, length 30 m, and an 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 200°C and detector temperature 280°C. The GC oven temperature program was used as follows: 40°C initial temperature, hold for 10 min; increased at 3°C/min to 200°C; increased 2°/min to 220°C. A 1%, w/v, solution of the sample in CH_2Cl_2 was prepared and 1 µL was injected using a splitless injection technique.

Identification of the oil components was based on their retention indices determined by reference to a homologous series of *n*-alkanes, and by comparison of their mass spectral fragmentation patterns with those reported in the literature [62] and stored on the MS library [NIST database (G1036A, revision D.01.00)/ChemStation data system (G1701CA, version C.00.01.080)]. The percentages of each component are reported as raw percentages based on total ion current without standardization.

Allelopathic activity assays: An allelopathic bioassay based on lettuce (*Lactuca sativa* L.) and perennial rye grass (*Lolium perenne* L.) germination and subsequent radicle and hypocotyl growth [63] was measured to study the effects of the *Cinnamomum* essential oils and major essential oil components. Stock solutions of each essential oil or compound (2.0 g/L essential oil and 1.0 g/L Tween-80 in water) were prepared and used for the assays. Two-fold serial dilutions of stock test solutions were prepared to give test

concentrations of 4000, 2000, 1000, 500, and 250 μg/mL with the control being 1.0 g/L aqueous Tween-80. Seeds were placed in 6-well test plates (10 seeds per well), each well lined with 2 layers of Whatman No. 1 filter paper moistened with test solution, and the test plates were sealed with Parafilm®. The test plates were incubated at room temperature in the dark for 5 days, after which the number of germinated seeds was determined and the root (radicle) and shoot (hypocotyl) lengths were measured. Student's *t*-test [64] was used to compare radicle and hypocotyl test means with controls. Seed germination IC_{50} values were determined using the Reed-Muench method [65].

Antimicrobial screening: The essential oils and major components were screened for antimicrobial activity against Gram-positive bacteria, *Bacillus cereus* (ATCC No. 14579) and *Staphylococcus aureus* (ATCC No. 29213); Gram-negative bacteria, *Pseudomonas aeruginosa* (ATCC No. 27853) and *Escherichia coli* (ATCC No. 10798). Minimum inhibitory concentrations (MIC) were determined using the microbroth dilution technique [66]. Dilutions of the essential oil were prepared in cation-adjusted Mueller Hinton broth (CAMHB) beginning with 50 μL of 1%, w/w, solutions of essential oil in DMSO plus 50 μL CAMHB. The extract solutions were serially diluted (1:1) in CAMHB in 96-well plates. Organisms at a concentration of approximately 1.5×10^8 colony forming units (CFU)/mL were added to each well. Plates were incubated at 37°C for 24 h; the final minimum inhibitory concentration (MIC) was determined as the lowest concentration without turbidity. Geneticin was used as a positive antibiotic control; DMSO was used as a negative control. Antifungal activity against *Aspergillus niger* (ATCC No. 16888) was determined as above using potato dextrose broth inoculated with *A. niger* hyphal culture diluted to a McFarland turbidity of 1.0. Amphotericin B was the positive control.

Cytotoxicity screening: Human MCF-7 breast adenocarcinoma cells (ATCC No. HTB-22) [67] were grown in a 3% CO_2 environment at 37°C in RPMI-1640 medium, supplemented with 10% fetal bovine serum, 100,000 units penicillin and 10.0 mg streptomycin per L of medium, 15 mM of Hepes, and buffered with 26.7 mM $NaHCO_3$, pH 7.35. Cells were plated into 96-well cell culture plates at 2.5×10^4 cells per well. The volume in each well was 100 μL. After 48 h, supernatant fluid was removed by suction and replaced with 100 μL growth medium containing 1.0 μL of DMSO solution of the essential oil (1%, w/w, in DMSO), giving a final concentration of 100 μg/mL for each well. Solutions were added to wells in 4 replicates. Medium controls and DMSO controls (10 μL DMSO/mL) were used. Tingenone [68] was used as a positive control. After the addition of compounds, plates were incubated for 48 h at 37°C in 5% CO_2; medium was then removed by suction, and 100 μL of fresh medium was added to each well. In order to establish percent kill rates, the MTT assay for cell viability was carried out [69]. After colorimetric readings were recorded (using a Molecular Devices SpectraMAX Plus microplate reader, 570 nm), average absorbances, standard deviations, and percent kill ratios ($\%kill_{cmpd}/\%kill_{DMSO}$) were calculated.

Brine shrimp lethality assay: The brine shrimp, *Artemia salina* (L.), lethality test was carried out using a modification of the procedure by McLaughlin [70]. *A. salina* eggs were hatched in a sea salt solution (Instant Ocean®, 38 g/L) with an incandescent light bulb as the heat source. After 48 h, the newly hatched nauplii were counted using a micropipette and transferred to 20 mL vials. Nine vials were prepared, each containing 10 *A. salina* nauplii in 10 mL of sea salt solution (same as the hatching solution). Three vials were labeled as controls with the first containing no DMSO, another with 10 μL, and the last with 100 μL DMSO. Three replicate vials contained 10 μL of 1% essential oil solution in DMSO, and the other 3 were prepared by adding 100 μL of 1% essential oil solution in DMSO. Surviving *A. salina* were counted after 24 h. LC_{50} values (Table 3) were determined using the Reed-Muench method [65].

Butterfly larvicidal activity assay: Cabbage white butterfly, *Pieris rapae* (L.), eggs were purchased from Carolina Biological Supply (Item # 144100). The egg strip was cut into smaller sections and the eggs placed onto organically grown collared green (*Brassica oleracea* L.) leaves. The eggs hatched in 48 h, after which they were placed on collard green leaves in a Petri dish. Three different essential oil concentrations were made in 1% tween water solutions (1000 μg/mL, 500 μg/mL, and 250 μg/mL). Leaf disks (6.5 cm) were prepared from collar leaves and dipped into the different concentrations of sample solution separately for 30 sec. Solvents were evaporated in a fume hood for 30 min. The control was made from 1% tween water solution only. The leaf disks were each placed in a Petri dish and 10 *P. rapae* larvae were transferred into each disk. The number of dead larvae was counted after 48 h.

Termiticidal assay: Worker termites, *Reticulitermes virginicus* (Banks), were purchased from Carolina Biological Supply (Item # 143736). The termiticidal assay was carried out using a 6-well culture plate, each well fitted with a filter paper disc. Solutions of the essential oils and major components were prepared in 1% aqueous Tween® 80 solution at 2000, 1000, and 500 μg/mL. Sample solutions (200 μL) of each concentration were sprayed into 3 of the wells. Water and 1% aqueous Tween® 80 were used as controls in the remaining wells. Six termites were placed into each well and termiticidal activity was determined after 24 h.

Mosquito larvicidal assay: Larvae of *Culex pipiens* L. were obtained from Carolina Biological Supply (Item # 144478). For the bioassay, 10 mL of sterile water was placed in five 20 mL vials. Into each vial, 10 mosquito larvae were transferred using a soft brush. Three vials were labeled as control with the first one containing 10 μL DMSO, the second containing 100 μL DMSO and the third containing only sterile water. Into the remaining 2 vials were added 10 μL of 1% solution of essential oil in DMSO and 100 μL of 1% essential oil/DMSO solution (*i.e.*, final concentrations of 10 and 100 μg/mL). Surviving mosquito larvae were counted after 24 h. The experiments were carried out at 23 ± 2ºC. An analogous assay was carried out using midge (*Chaoborus plumicornis* Fab.) larvae (glassworm), which were obtained from a local aquarium shop.

Fruit fly lethality assay: Wild type *Drosophila melanogaster* Meigen were obtained from Carolina Biological Supply and a breeding colony maintained using a *Drosophila* culture kit. *Drosophila* medium (2 mL) was placed into each of five 20 mL glass vials. Three vials were labeled as control, the first containing only *Drosophila* medium, the second with 20 μL DMSO, and the third with 150 μL of DMSO. Another 2 vials contained 20 μL of 1% essential oil solution in DMSO, and the other vial was prepared by adding 150 μL of 1% essential oil solution in DMSO. Ten individual fruit flies were transferred into each vial. The bioassay was carried out in triplicate. Surviving fruit flies were counted after 24 h.

Fire ant lethality assay: Worker red imported fire ants, probably *Solenopsis invicta* × *richteri* hybrid [71], were collected from the University of Alabama in Huntsville. Sample solutions of 1000 μg/mL, 500 μg/mL and 250 μg/mL were prepared in 1% aqueous Tween-80® solution. The control was 1% Tween solution. Each

assay was carried out using a 400 mL beaker, fitted with a filter paper disk on the bottom. The filter paper was sprayed with 600 µL of sample solution and 20 fire ant workers were transferred to the beaker, which was sealed with Parafilm®. The mortality of fire ants was recorded after 24 h. The bioassay was carried out at room temperature in triplicate. LC_{50} values were calculated using the Reed Muench method [65].

Nematicidal assay: A nematicidal assay using *Caenorhabditis elegans* (Maupas) was carried out using a modification of the procedure of Park and co-workers [72]. A 1% solution of each essential oil and major component in DMSO was prepared. Dilutions of the sample solution were prepared in sterile water solution beginning with 50 µL of the 1% essential oil solution plus 50 µL sterile water. The sample solution was serially diluted (1:1) with sterile water in a 96-well plate. Into each well, 10-30 *C. elegans* (mixtures of juvenile and adult nematodes, male: female: juvenile ~1:1:2) per 50 µL of sample solution. Sterile water and serially diluted DMSO were used as controls. The dead and living nematodes were counted after 24 h under a microscope. Dead nematodes were identified by their immobility, and straight body, even after transfer to clean water. LC_{50} values (Table 3) were determined using the Reed-Muench method [65].

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Essential Oil Characterization of Two Azorean *Cryptomeria japonica* Populations and Their Biological Evaluations

Cristina Moiteiro[a,*], Teresa Esteves[a], Luís Ramalho[b], Rosario Rojas[c], Sandra Alvarez[d], Susana Zacchino[d] and Helena Bragança[e]

[a]*Universidade de Lisboa, Faculdade de Ciências, Departamento de Química e Bioquímica; Centro de Química e Bioquímica, Campo Grande, 1749-016 Lisboa, Portugal*

[b]*Laboratório Nacional de Energia e Geologia, LBA-Laboratório de Biocombustíveis e Ambiente, Estrada do Paço do Lumiar, 22, 1648-039 Lisboa, Portugal*

[c]*Laboratorios de Investigación y Desarrollo, Facultad de Ciencias y Filosofía, Universidad Peruana Cayetano Heredia, Av. Honorio Delgado 430 Urb. Ingeniería S.M.P. Lima 31, Perú*

[d]*Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Área Farmacognosia, Suipacha 531, 200 Rosario, Argentina*

[e]*Instituto Nacional de Investigação Agrária e Veterinária I.P., Quinta do Marquês, 2780-159 Oeiras, Portugal*

cmmoiteiro@fc.ul.pt

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Essential oils from foliage, bark and heartwood of *Cryptomeria japonica* D. Don from Azores Archipelago (Portugal) were analyzed by GC and GC-MS. Two populations, of black and reddish heartwood color, were studied. The main compounds found in the foliage of both populations were α-pinene (9.6-29.5%), (+)-phyllocladene (3.5-26.5%), *ent*-kaur-16-ene (0.2-20.6%), sabinene (0.5-19.9%) and limonene (1.4-11.5%), with a large variation in individual compounds from each population. Heartwood oils were characterized by a high content of cubebol (2.8-39.9%) and *epi*-cubebol (4.1-26.9%) isomers, which were absent in the foliage. Elemol and eudesmol isomers were found in the foliage and heartwood oils, while (+)-phyllocladene was absent in heartwood. Black and reddish bark oils were composed of the diterpenes dehydroferruginol (1.9-5.1%) and ferruginol (2.6-11.5%), along with the sesquiterpenes δ-cadinene (10.4-15.9%), α-muurolene (3.3-5.4%), *epi*-zonarene (4.0-5.0%), cubenol (9.3-14.0%), τ-muurolol (4.8-10.7%), β-eudesmol (3.0-9.9%), γ–eudesmol (1.9-7.0%) and hedycariol (1.4-6.2%). Azorean *C. japonica* oils exhibited significant chemical differences compared with native plants from Asia.

The essential oils showed moderate antimicrobial activity against the pathogenic fungus *Cryptococcus neoformans* and human pathogenic bacteria (especially against multidrug-resistant *Mycobacterium tuberculosis*). The antimicrobial activity of the essential oils may be attributed to compounds such as *ent*-kaur-16-ene, (+)-phyllocladene, ferruginol and elemol, which are present in different proportions within the complex oil mixture. These results suggest a potential use for *C. japonica* oils obtained from wood industry leftovers.

Keywords: *Cryptomeria japonica*, Essential oils, GC-MS, Pathogenic fungi, *Mycobacterium tuberculosis*.

Cryptomeria japonica D. Don. (Cupressaceae), also known as Sugi or Japanese cedar, is an Asian conifer that was introduced into the Azores Islands in the mid-19th century. *C. japonica* progressively overcame endemic tree species, and nowadays is widely distributed in the islands, representing 57% of the total wood producing forest area, and with sawmills generating wastes having only minor and non-commercial applications [1]. Typically, *C. japonica* heartwood has a red color, but, in some cases, a black/dark color can also occur, reducing its commercial value [2,3]. During the last years, the incidence of reddish heartwood color of *C. japonica* trees in the Azores islands has been declining, leading to a rise of economically lower valued black heartwood specimens. The regional Azores government, in 1998, initiated a Forest Breeding Program in order to evaluate the genetic basis of the Azores sugi population. This program intended to improve the genetic species of *Cryptomeria* and to increase the productivity and quality of wood [4].

In recent years, particular interest has been placed on the study of *C. japonica* essential oils (EOs) from Japan, Korea, Taiwan and China, with some significant activities being reported, such as antiulcer [5], antifungal [6,7], antimicrobial [8-10], cancer chemopreventive [11], neuropharmacological [12], antimosquito [13-15], antitermite [16,17] and insecticidal [18,19].

In a strategy of valorization of an unrecognized natural resource in the Azorean islands, the main purpose of this study was to find a promising potential use for the unutilized bark, foliage and wood leftovers of *C. japonica*, by obtaining and chemically identifying their EOs, as well as evaluating their antimicrobial activities against wood deteriorating fungi, human pathogenic fungi and bacteria. This is the first study reporting the composition of the EOs of the two different *C. japonica* populations, black and reddish heartwood trees, of Azores, Portugal (Faial island).

For these purposes, two tree sampling sites were chosen, taking into account sugi heartwood color, a physical property that could influence the composition of the EOs. The black heartwood population was assigned as A, and the reddish heartwood population as B. Table 1 shows the yields of obtained EOs from the different tissues of *C. japonica* analyzed.

Comparing oil yields, taking into account the heartwood color of each *C. japonica* specimen, the variation in the foliage of black heartwood trees was higher than in the oils of reddish heartwood foliage, based on fresh weight (A- 1.1-1.9% and B- 0.5-0.8%). For heartwood oils of both populations, the yields obtained showed little differences (A- 0.6-2.0% and B- 0.5-2.1%), with the same being

Table 1: Percentage limits of the components detected in EOs from the foliage, woods and barks of *C. japonica* from two populations of Faial Island (Azores).

		Relative content (%)					
Compound	**RI[a]**	**Foliage A***	**Foliage B***	**Heartwood A***	**Heartwood B***	**Bark A***	**Bark B***
Tricyclene	830	0.3 - 0.4	t - 0.4	-	-	-	-
α-Thujene	915	t - 1.2	0.3 - 1.2	-	-	-	-
α-Pinene	**931**	**9.6 - 29.5**	**13.5 - 23.8**	-	-	0.8	2.1
Camphene	943	1.8 - 2.8	0.7 - 2.6	-	-	-	-
Sabinene	**964**	**0.5 - 19.9**	**1.0 - 10.5**	-	-	-	-
β-Pinene	970	0.5 - 1.7	0.7 - 1.2	-	-	-	-
Myrcene	**978**	**2.5 - 3.9**	**2.4 - 3.1**	-	-	-	-
Δ^3-Carene	1007	0.5 - 1.9	t - 1.4	-	-	-	-
α–Terpinene	1011	t - 2.1	0.1 - 1.5	-	-	-	-
p-Cymene	1013	0.4 - 0.5	t - 0.4	-	-	-	-
Limonene	**1023**	**1.4 - 5.0**	**1.8 - 11.5**	-	-	T	0.6
γ-Terpinene	1049	t - 3.5	0.2 - 2.5	-	-	T	-
Terpinolene	1079	0.2 - 1.3	0.3 - 1.1	-	-	-	-
2-p-Menthen-1-ol	1106	t - 0.3	t - 0.2	-	-	-	-
Terpinen-4-ol	**1165**	**t - 8.9**	**0.4 - 4.6**	-	-	-	-
α-Terpineol	1174	t - 0.4	t - 0.3	-	-		0.4
Bornyl acetate	1273	1.1 - 2.6	0.5 - 1.8	-	-	-	-
α-Cubebene	1354	-	-	0.1 - 0.4	t - 0.4	-	-
α-Copaene	1383	-	-	T	t - 0.2	-	-
β-Elemene	1394	-	-	0.2 - 0.8	0.4 - 1.0	-	-
β-Caryophyllene	1426	t - 0.2	t - 0.2	t - 0,7	-	-	-
trans-*β*-Farnesene	1454	-	-	t - 0.5	0.4 - 0.8	-	-
α-Humulene	1461	-	-	0.4 - 0.7	t - 0.8	0.4	0.7
epi-Bicyclosesquiphellandrene	1469	-	-	-	-	1.3	3.4
γ-Muurolene	1471	-	-	0.8 - 1.4	t - 1.5	0.4	-
Germacrene-D	1480	0.2 - 0.4	0.2 - 0.8	T	-	-	-
β–Cubebene	1499	-	-	t – 2.0	t - 1.9	-	-
epi-Cubebol	1500	-	-	**4.1 - 26.9**	**12.3 - 21.2**	-	0.6
α–Muurolene	1503	0.1 - 1.5	0.3 - 0.5	**2.0 - 3.3**	**1.5 - 2.9**	**3.3**	**5.4**
γ-Cadinene	1516	0.2 - 1.2	0.2 - 0.3	t - 3.7	-	-	-
Cubebol	1520	-	-	**2.7 - 39.9**	**14.6 - 33.8**	-	0.5
epi-Zonarene	1522	-	-	-	-	4.0	5.0
δ–Cadinene	1524	0.8 - 6.5	0.8 - 1.4	**6.2 - 10.8**	**6.4 - 10.9**	**10.4**	**15.9**
Cadina-1,4-diene	1526	-	-	0.5 - 1.6	0.6 - 1.0	2.2	2.7
α-Calacorene	1532	-	-	0.43 - 0.6	0.4 - 0.9	1.1	1.9
Elemol	1542	**0.2 - 12.7**	**4.1 - 8.1**	**1.7 - 14.1**	**1.8 - 9.2**	**2.6**	**1.1**
Nerolidol	1551	-	-	t - 0.3	t - 0.3	0.5	1.4
Caryophyllene oxide	1580	0.2 - 3.0	0.3 - 0.7	t - 0.6	t - 0.5	-	-
Allo-Hedycariol	1580	-	-	0.7 - 2.9	0.7 - 2.5	2.3	1.7
Cubenol	1619	t - 0.7	0.3 - 1.2	**5.5 - 17.2**	**4.0 - 18.4**	**14.0**	**9.3**
γ-Eudesmol	1626	**0.2 - 9.1**	**2.8 - 7.6**	t - 2.9	t - 1.8	**7.0**	**1.9**
τ-Cadinol	1635	0.6 - 4.7	0.9 - 1.4	1.1 - 3.9	1.0 - 3.9	2.4	2.5
τ-Muurulol	1631	-	-	1.3 - 2.8	t - 6.9	**10.7**	**4.8**
β-Eudesmol	1646	**0.2 - 4.8**	**2.1 - 4.9**	**2.5 - 6.1**	**2.7 - 6.0**	**9.9**	**3.0**
α-Cadinol	1647		2.3 - 3.2	t - 5.2	t – 4.0	t	1.8
α-Eudesmol	1652	**4.4 - 7.1**	**1.6 - 5.9**	**0.6 - 10.7**	**2.2 - 5.8**	3.4	1.3
Hedycariol	1661	-	-	t - 5.5	1.1 - 3.3	**6.2**	**1.4**
Caladene	1665	-	t - 0.1	-	t - 0.3	1.2	1.4
Tremetone	1704	-	-	0.6 - 1.5	0.2 - 3.1	1.4	-
Pimara-8,15-diene	1938	0.5 - 2.1	t - 2.4	t - 0.4	t	-	-
Cupressene	1954	0.6 - 2.8	0.2 - 0.9	-	t	-	-
Sandaracopimaradiene	1967	0.5 - 1.3	0.3 - 1.4	-	-	-	-
Pimara-8(14),15-diene	1986	0.2 - 0.7	0.3 - 0.7	t - 0.4	t - 0.4	t	0.8
Isopimaradiene	2018	t - 0.3	0.2 - 0.3	-	-	-	-
(+)-Phyllocladene	2053	**7.9 - 24.1**	**3.5 - 26.5**	-	-	-	0.2
***ent*-Kaur-16-ene**	2078	**0.2 - 0.4**	**0.4 - 20.6**	t - 0.3	t - 0.2	-	0.3
8*β*–Hydroxysandaracopimarene	2149	0.6 - 3.6	1.2 - 3.3	0.2 - 0.9	0.2 - 0.9	-	-
Sandaracopimarinal	2193	t - 0.2	t	0.2 - 0.5	0.2 - 1.6	1.6	0.9
Kauran-16-ol	2223	-	t	0.1 - 0.5	t - 0.8	1.3	1.6
Sandaracopimararinol	2271	-	t	t - 0.5	t – 2.0	**2.2**	**0.5**
Dehydroferruginol	2296	-	t	t - 0.1	t - 0.5	**1.9**	**5.1**
Ferruginol	2305	-	t	t - 0.5	0.1 - 1.2	**2.6**	**11.5**
Identified components (%)		94.6 - 99.2	97.8 - 99.3	97.9 - 99.5	95.8 - 97.5	95.1	92.1
Nº Identified components		38	44	38	37	30	33
Grouped components variation (%)							
Monoterpene hydrocarbons		37.1 - 50.5	37.1 - 43.4	-	-	0.8	2.7
Oxygenated monoterpenes		2.0 - 10.7	1.4 - 5.6	-	-	-	0.4
Sesquiterpene hydrocarbons		1.4 - 9.7	1.5 - 3.2	12.3 - 21.7	12.0 - 32.7	24.3	36.5
Oxygenated sesquiterpenes		13.1 - 30.2	16.0 - 28.2	77.9 – 84.0	60.7 - 80.4	60.4	31.5
Diterpene hydrocarbons		12.5 - 28.2	19.1 - 32.9	t - 1.1	t - 0.4	-	1.3
Oxygenated diterpenes		0.6 - 3.6	1.2 - 3.3	0.8 - 2.2	0.5 - 6.4	9.6	19.6
Oil yields (%, w/w)		1.1 - 1.9	0.5 - 0.8	0.6 – 2.0	0.5 - 2.1	0.2	0.1

[a]Relative to C_8-C_{24} *n*-alkanes on a OV-101 column; t- trace (<0.1%); * Population A- Black color of the heartwood; Population B- reddish color of the heartwood.

observed for the barks (A- 0.2% and B- 0.1%). It appears that no significant relationship exists between the color of heartwood and oil yields. The sapwoods EO yield was extremely low (0.1%).

The 22 oil samples analyzed by GC were complex mixtures and, depending on the part of the plant, a range of 30 to 44 compounds were identified, totalizing a percentage variation of 94.6-99.5%. The compounds identified in the EOs from the different tissues analyzed, as well as theirs percentage limits, are shown in Table 1. It can be seen that, from each individual sample in the same population, a larger range of percentage limits of each component among the foliage and heartwoods were detected.

The foliage of both populations are characterized by high contents of monoterpene hydrocarbons (A- 37.1-50.5% and B- 37.1-43.4%), followed by oxygenated sesquiterpenes (A- 13.1-30.2% and B- 16.0-28.2%), diterpene hydrocarbons (A- 12.5-28.2% and B- 19.1-32.9%), and oxygenated monoterpenes (A- 2.0-10.7% and B- 1.4-5.6%), but a low content of sesquiterpene hydrocarbons (A- 1.4-9.7% and B- 1.5-3.2%) and oxygenated diterpenes (A- 0.7-3.6% and B- 1.2-3.3%). The main common constituents of the foliage of the black and reddish population were α-pinene (A- 9.6-29.5% and B- 13.5-23.8%), (+)-phyllocladene (A- 7.9-24.1% and B- 3.5-26.5%), *ent*-kaur-16-ene (A- 0.2-0.4% and B- 0.4-20.6%), sabinene (A- 0.5-19.9% and B- 1.0-10.5%), limonene (A- 1.4-5.0% and B- 1.8-11.5%), elemol (A- 0.2-12.7% and B- 4.1-8.1%), α-eudesmol (A- 4.4-7.1% and B- 1.6-5.9%), β-eudesmol (A- 0.1-4.8% and B- 2.1-4.9%) and γ-eudesmol (A- 0.2-9.1% and B- 2.8-7.6%). The isolation and characterization of the isomers (+)-phyllocladene and *ent*-kaur-16-ene allowed us to conclude that, among the EO components, *ent*-kaur-16-ene is clearly the unique compound that is present only in the reddish population, while its isomer, (+)-phyllocladene, exists in high amount in both populations. α-Pinene, *ent*-kaur-16-ene, sabinene, elemol and eudesmol isomers have also been identified in the leaves of *C. japonica* EOs from Taiwan [6,15,18,19] and Korea [8], although with different percentages than those found in Azorean oils. On the contrary, the main compounds identified in the EOs of the leaves of *C. japonica* from China were α-elemol (20.1%), kaur-16-ene (14.8%), β-phellandrene (6.0%) and β-elemene (5.9%) [20].

Heartwood oils were characterized by a high content of two isomers, cubebol (A- 2.7-39.9% and B- 14.6-33.8%) and *epi*-cubebol (A- 4.1-26.9% and B- 12.3-21.2%), which are absent in leaves. So, the oxygenated sesquiterpenes form, respectively, 77.9-84.0% and 60.7-80.4% of the black and reddish heartwood populations, followed by sesquiterpene hydrocarbons (A- 12.3-21.7% and B- 12.0-32.7%). These groups of terpenes are also representative of the foliage EOs, while the more volatile monoterpene hydrocarbons and oxygenated monoterpenes present in the leaves were lacking in the heartwood oils. Furthermore, cubenol (A- 5.5-17.2% and B- 4.0-18.4%), elemol (A- 1.7-14.1% and B- 1.8-9.2%), α-eudesmol (A- 0.6-10.7% and B- 2.2-5.8%), β-eudesmol (A- 2.5-6.1% and B- 2.7-6.0%), τ-cadinol (A- 1.1-4.0% and B- 1.0-3.9%), τ-muurulol (A- 1.3-2.8% and B- t-6.9%) and δ-cadinene (A- 6.2-10.8% and B- 6.4-10.9%) were also present in both heartwood populations. The main foliage constituent, (+)-phyllocladene, is not present in heartwood EOs and, consequently, diterpene fractions were largely decreased in heartwood EOs, for both populations.

Oxygenated diterpenoids, such as sandaracopimarinal, kauran-16-ol, sandaracopamarinol, dehydroferruginol and ferruginol, are characteristic of black and reddish bark oils, representing about 9.6% and 19.6%, respectively, of the total oil percentage. δ-Cadinene (A- 10.4% and B- 15.9%), α-muurolene (A- 3.3% and B- 5.4%), and *epi*-zonarene (A- 4.0% and B- 5.0%) are the main sesquiterpene hydrocarbons present in both bark populations, which represent 24.3% and 36.5% of the total bark EOs. Oxygenated sesquiterpenes (A- 60.4% and B- 31.5%) are the major group of constituents of black and reddish bark populations, due to a considerable presence of cubenol (A- 14.0% and B- 9.3%), τ-muurulol (A- 10.7% and B- 4.8%), β-eudesmol (A- 9.9% and B- 3.0%), γ-eudesmol (A- 7.0% and B- 1.9%) and hedycariol (A- 6.2% and B- 1.4%). So, sesquiterpenes, both oxygenated and hydrocarbons, are the main group constituents for both bark EOs, although black barks had higher amounts of oxygenated sesquiterpenes than reddish barks.

Comparing the results obtained from Azores *C. japonica* EOs with those reported for the same species from other countries, deep differences, not only in chemical constituents, but also in their percentages, were observed. According to the study of Cheng *et al.* [14], there is no apparent relationship between the age of *C. japonica* specimens and the chemical composition of the leaf EO from the same origin. Cheng also suggests that the discrepancy with other authors could be related with different seasons in which the samples were collected. Therefore, the differences observed in the Azorean EOs are probably due to specific microclimate and temperature of a Macaronesian region, in association with edaphic factors, and to the plant adaptation to this specific environment. Another important contribution to the differences observed in compound amounts among the several EOs analyzed could be the use of individual *C. japonica* specimens, instead of a homogenized mixture of foliage and woods. These results may provide useful information for further studies of EOs of *C. japonica* trees from other Archipelago islands.

C. japonica EOs from foliage, heartwoods and barks, together with the main compounds isolated from EOs, were tested for antimicrobial activity against human pathogenic bacteria and pathogenic and wood surface contaminant fungi. The antifungal evaluation is depicted in Table 2. The fungus *B. cinerea* was inhibited by all EOs at a MIC of 100 μg/mL with fungistatic activity; inversely, the isolated compounds, (+)-phyllocladene, *ent*-kaur-16-ene and a mixture of elemol, cubebol and *epi*-cubebol did not display any antifungal activity (MIC > 200 μg/mL). The fungi *F. circinatum* and *C. parasitica* showed resistance towards all the samples tested. The wood surface contaminants *A. niger* and *T. harzianum* displayed a different behavior towards the tested samples, with almost all of them showing a moderate activity against the last fungus and being, in general, inactive against *A. niger* (MIC ≥ 200 μg/mL). All the oil samples tested inhibited the growth of *C. cladosporioides* and *Cladosporium* sp, with MICs between 100-200 μg/mL. Among the pure compounds, only a mixture of cubebol, *epi*-cubebol and elemol, from the heartwood EO, exhibited antifungal activity against *Cladosporium* spp. This result is significant, since sapstain fungi are responsible for wood deterioration processes.

Additionally, none of the EOs inhibited any of the human pathogenic fungi up to 250 μg/mL. Regarding the activity of the main compounds isolated from EOs, only sandaracopimarinal and a fraction enriched in elemol showed moderate MICs of 62.5 and 125 μg/mL, respectively, against *C. neoformans* (Table 2). By taking into account this last result, we deepened the knowledge of the inhibition capacity of the oils and components by analyzing the percentage inhibition of *C. neoformans* by each sample at 250 μg/mL and by each two-fold dilution of this concentration up to 7.8 μg/mL (Table 3). It was shown that the EOs do not possess significant antifungal properties against *C. neoformans,* since the foliage EO inhibited only 15.6% of growth at 250 μg/mL (it is important to take into account that at least 50% inhibition is necessary to consider either an oil or a compound with antifungal properties). In contrast, some components of the oil showed interesting activities. Among them, (+)-phyllocladene and ferruginol, showed 50% inhibition at 250 μg/mL. In addition, sandaracopimarinal, which had a MIC_{100}= 62.5 μg/mL, showed 50% of inhibition at 31.25 μg/mL. Also, the enriched fraction in elemol, which had a MIC_{100}=125 μg/mL, showed a MIC_{50} = 62.5 μg/mL.

Furthermore, EOs of foliage and heartwood of *C. japonica* populations were tested *in vitro* against the sensitive strain $H_{37}Rv$

Table 2: Minimum inhibitory concentration (MIC in µg/mL) of *Cryptomeria japonica* EOs and of the main compounds isolated from them against pathogenic fungi and yeast.

	Ca	*Ct*	*Sc*	*Cn*	*A fl*	*A fu*	*A ni*	*Mg*	*Tr*	*Tm*	*Th*	*Bc*	*Cc*	*Csp*
EO Foliage	i	I	i	i	i	I	i	I	I	i	150	100*	200	200
EO Heartwood	i	I	i	i	i	I	i	I	I	i	200	100*	200*	I
EO Bark	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	200	n.t.	n.t.	n.t.	200	100*	200*	I
Sandaracopimarinol	i	I	i	i	i	I	i	I	I	i	n.t.	n.t.	n.t.	n.t.
Sandaracopimarinal	i	I	i	62.5	i	I	i	I	I	i	n.t.	n.t.	n.t.	n.t.
β-Eudesmol	i	I	i	i	i	I	i	I	I	i	n.t.	n.t.	n.t.	n.t.
Ferruginol	i	I	i	i	i	I	i	I	I	i	n.t.	n.t.	n.t.	n.t.
(+)-Phyllocladene	i	I	n.t.	i	i	I	i	I	I	i	I	I	I	I
ent-Kaur-16-ene	i	I	n.t.	i	i	I	i	I	I	i	>200	>200	>200	>200
Pt-Cj-Ele+Eud[a]	i	I	i	i	i	I	i	I	I	i	n.t.	n.t.	n.t.	n.t.
Mix-Elemol[a]	i	I	i	125	i	I	i	I	I	i	n.t.	n.t.	n.t.	n.t.
Cubebol+epi-Cubebol+Elemol	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	i	n.t.	n.t.	n.t.	I	I	I	100*
Amphotericin B	0.98	1.95	0.49	0.25	0.49	0.49	0.49	0.12	0.06	0.06	n.t.	n.t.	n.t.	n.t.
Terbinafine	1.95	1.95	3.90	0.49	0.98	0.98	1.96	0.04	0.01	0.03	n.t.	n.t.	n.t.	n.t.
Ketoconazole	0.49	0.98	0.49	0.25	0.12	0.49	0.25	0.06	0.03	0.03	n.t.	n.t.	n.t.	n.t.
Carbendazim	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	0.4*	n.t.	n.t.	n.t.	200*	200*	0.2*	0.2*

[a]These fractions are enriched in compounds; i = >250 µg/mL; n.t. = not tested; * = bacteriostatic / fungistatic activities; *Ca*: *C. albicans*; *Ct*: *C. tropicalis*; *Sc*: *S. cerevisiae*; *Cn*: *C. neoformans*; *An*: *A. niger*; *Afu*: *A. fumigatus*; *Afl*: *A. flavus*; *Mg*: *M. gypseum*; *Tr*: *T. rubrum*; *Tm*: *T. mentagrophytes*; *Th*: *T. harzianum*; *Bc*: *B. cinérea*; *Cc*: *C. cladosporioides*; *Csp*: *Cladosporium sp*.

Table 3: Antifungal activity (% inhibition = mean ± SD) of EOs and pure compounds against *Cryptococcus neoformans*.

	MIC (µg/mL)					
	250	**125**	**62.5**	**31.25**	**15.6**	**7.8**
EO Foliage	15.6 ± 2.2	9.9 ± 2.7	4.6 ± 0.5	0	0	0
EO Heartwood	0	0	0	0	0	0
(+)-Phyllocladene	58.7 ± 0.6	38.2 ± 7.5	12.3 ± 8.2	0	0	0
ent-kaur-16-ene	26.0 ± 2.02	24.8 ± 7.9	17.7± 2.2	12.1± 2.9	0	0
Sandaracopimarinol	28.4 ± 12.5	8.7 ± 1.1	4.0 ± 1.8	0	0	0
Sandaracopimarinal	100	100	100	54.3 ± 5.2	0	0
β-Eudesmol	41.7 ± 9.1	39.3 ± 10.4	33.8 ± 3.7	33.6 ± 6.2	34.3 ± 3.7	32.1 ± 2.8
Ferruginol	51.5 ± 13.1	48.7 ± 8.5	46.5 ± 3.8	44.6 ± 7.3	35.8 ± 2.6	31.7 ± 3.5
Elemol+Eudesmol	4.6 ± 10.8	1.4 ± 3.6	0	0	0	0
Mix-Elemol	100	100	50.5 ± 4.9	30.53 ± 4.49	18.5 ± 4.9	0
Amphotericin B	100	100	100	100	100	100
Ketoconazole	100	100	100	100	100	100

and a multidrug-resistant (MDR) strain of *Mycobacterium tuberculosis* (Table 4). EOs of the foliage (MIC 25 µg/mL) proved to be more active than those of the heartwood (MIC 50 µg/mL), especially against the MDR strain. The main compounds present in these EOs were isolated and tested in the antimycobacterial assay. None of them alone was active against the sensitive $H_{37}Rv$ and MDR strain (MIC≥ 25 µg/mL), except for (+)-phyllocladene and *ent*-kaur-16-ene, which showed a good activity (MIC 12.5 µg/mL), against the latter strain. These results lead us to think that the antimicrobial activity of the EOs of foliage may be attributed to the presence of active compounds such as *ent*-kaur-16-ene and (+)-phyllocladene, and probably also to the synergetic effects between them and the proportions in which they are present within a complex mixture. This complexity makes it often difficult to explain the aforementioned activities, as well as relating them to specific compounds or mixtures present in oils.

Table 4: *In vitro* activity of EOs and their main constituents against *M. tuberculosis* $H_{37}Rv$ and MDR strains.

Samples	**MIC (µg/mL)**	
	$H_{37}Rv$	MDR
EO Foliage A	50	25
EO Foliage B	50	25
EO Heartwood A	50	50
EO Heartwood B	200	50
(+)-Phyllocladene	>25	12.5
ent-Kaur-16-ene	>25	12.5
Elemol	>25	>25
Cubebol+*epi*-Cubebol	>25	25
Isoniazid	0.125	4
Rifampin	0.063	16

A- Black population; B- reddish population.

The use of the wastes produced by the *C. japonica* wood industry in Azores Archipelago, until now being only seen as disposable, can represent an extremely important commercial advantage for the islands economy. The amount of wood/foliage not used and lost in the fields is considerable, and the upscale of the oil production is relatively easy and inexpensive.

Experimental

Plant material: Foliage (needle-like juvenile foliage), bark, and heartwood from 5 individual plants of 2 *C. japonica* populations, black and reddish heartwood color specimens from Faial island, were collected in July 2007 and provided by the Azorean Direcção Regional dos Recursos Florestais (DRRF). The individual plant samples were collected from 2 locations in Faial: black populations, altitude 140 m; latitude N 38:32:52.883; longitude W 28:38:21.171; reddish populations, altitude 480 m; latitude N 38:34:25.88; longitude W 28:40:20.203. The foliage and woods were air dried during 4 days and 6 weeks, respectively, at room temperature.

Extraction procedure: The leaves were cut into small pieces and heartwoods were ground in a Fritsch-Germany mill through a 1-mm screen width. Leaves and woods were then hydrodistilled for 4 h using a Clevenger-type apparatus [21]. The yields of EOs obtained are given in Table 1.

Analysis of essential oils: The EOs were analyzed by gas chromatography with flame ionization detection (GC-FID) and gas chromatography-mass spectrometry (GC-MS). The GC-FID analysis was performed on a Hewlett Packard model 5890 series II gas chromatograph equipped with an Automatic sampler HP 6890 series injector and a flame ionization detector (FID), using 2 different fused-silica capillary columns: OV-101 (50 m x 0.25 mm, 0.25 µm film thickness) and Supelcowax 10 (30 m x 0.25 mm, i.d. 0.25 µm film thickness). The oven temperature was held at 70°C for 5 min and then programmed to 210°C at 2°C/min. Injector and detector temperatures were 260°C and 270°C, respectively. The carrier gas was helium at a constant pressure of 55 kPa for the OV-101 and 45 kPa for the Supelco wax10 columns. The intensity of each peak was integrated. Each sample was analyzed 3 times. The average peak areas of all GC signals were added together and the percentage of each component peak was calculated by comparing its average area to the total areas.

The GC-MS analysis was carried out on a Carlo Erba GC-MS system, model HRGC/MS, with a Kratos mass detector model MS25RF (sector instrument), fitted with a HP-5MS column (30 m x 0.25 mm, 0.25 μm film thickness). The oven was programmed initially from 70ºC with 2 min hold up time to the final temperature of 250ºC with a 5ºC/min ramp. The final temperature hold time was 5 min, injector temperature was 250°C and GC/MS interface temperature was 280ºC. The carrier gas was helium at a constant pressure of 90 kPa, with a split ratio 1:20, ionization energy 70 eV source at 200ºC with full scan (25–450 amu) and a scan time of 0.5 s. The GC identification of the EO components is given in Table 1 and was assigned by comparison of their retention indices (RI) relative to a C_8-C_{24} *n*-alkane series, and by analysis of standards in the same gas chromatographic conditions. The mass spectra of EO components were identified by comparing the obtained mass spectra of the analytes with those of authentic standards from the mass spectra of Wiley 6.0 and Mass Spectra Library (NIST 98) and with corresponding RI and mass spectral data of components from reference oils analyzed in our laboratory.

General methods: Medium pressure column chromatography (CC) was carried out on silica gel 60 (12 x 75 mm, 40-63 μm, Buchi, 044884) filled columns. Preparative TLC, silica gel 60 F_{254} (20x20 cm 0.25 mm thickness, Merck, Germany) or RP-18 preparative TLC (20 x 20 cm 0.25 mm thickness, F254, Merck, 1.05559) plates were used for isolation of compounds. 1D and 2D NMR, Brüker AMX 300 MHz NMR spectrometer. Optical rotation, $[\alpha]_D$, Perkin-Elmer 241 Polarimeter.

Isolation procedures: Samples of the oils (*ca*. 100 mg) from the foliage of both populations with the higher percentage of kaurene and phyllocladene diterpenoids, selected by GC analysis, were subjected to silica gel preparative TLC and eluted with *n*-hexane and *n*-hexane/ethyl acetate (3:1), successively. The fractions obtained were analyzed, and the pure compounds characterized by GC-FID, GC-MS, ^{1}H NMR, ^{13}C NMR, MS and $[\alpha]_D$ measurements, and by comparison with data reported in the literature [22-24]. In order to determine the absolute stereochemistry of phyllocladene and kaurene, both pure isolated compounds were compared with retention times obtained in a cyclodextrine chiral capillary GC column (program: 70ºC for 2 min to 175ºC at 5ºC/min and 175ºC for 35 min; ASTEC CHIRALDEX^{TM} G-T 30m x 0.25mm, 0.12 μm) for the authentic samples [22], as well as by comparing $[\alpha]_D$ values with those in the literature [22,23]. The two isomers were lastly assigned as (+)-phyllocladene and *ent*-kaur-16-ene.

A sample of the oil obtained from heartwood (1g) was fractionated by silica gel medium pressure CC, using *n*-hexane and *n*-hexane/diethyl ether mixtures as eluents. One of the fractions obtained (F54-66; 144.5 mg) was subjected to preparative RP-18 TLC and eluted successively with methanol/water (8:2), to obtain elemol and a mixture of the isomers *epi*-cubebol and cubebol, which were analyzed and characterized by ^{1}H NMR, ^{13}C NMR and GC-MS. NMR and MS data of elemol are in agreement with those of the literature [25]. Cubebol and epi-cubebol were also identified by comparison of the mass fragmentation patterns and ^{13}C NMR spectra described in the literature [26,27].

Bioassays: Fungal and bacterial strains. Pathogenic fungi used in this study: *Botrytis cinerea* CCMI 899, *Trichoderma harzianum* CCMI 711, *Cladosporium cladosporioides* CCMI 478, and *Cladosporium* sp. CCMI 700 were obtained from the Culture Collection of Industrial Microorganisms (CCMI) Laboratório de Microbiologia Industrial, ex-INETI, Lisbon, Portugal. *Fusarium circinatum* FC0004 and *Cryphonectria parasitica* CO722 were obtained from the Culture Collection of Instituto Nacional de Investigação Agrária e Veterinária I.P., Oeiras, Portugal.

Candida albicans ATCC 10231, *C. tropicalis* CCC 131 2000, *Saccharomyces cerevisiae* ATCC 9763, *Cryptococcus neoformans* ATCC 32264, *Aspergillus flavus* ATCC 9170, *A. fumigatus* ATCC 26934, *A. niger* ATCC 9029, *Trichophyton rubrum* CCC 110, *T. mentagrophytes* ATCC 9972 and *Microsporum gypseum* CCC 115 were obtained from the American Type Culture Collection (ATCC), Rockville, MD, USA, and CEREMIC (CCC), Centro de Referencia en Micología, Facultad de Ciencias Bioquímicas y Farmacéuticas, Rosario, Argentina. Inocula of cell or spore suspensions were adjusted to 1-5 x 10^3 cells/spores with colony forming units (CFU) /mL [28].

Antimicrobial activity screenings: The Minimum Inhibitory Concentration (MIC) yielding no visible growth of the active compounds, and of the oils, was determined by the broth dilution method, according to reported procedures [28,29], from 250 to 0.98 μg/mL, with a final DMSO concentration ≤ 1%. Minimal bactericidal/fungicidal activities were determined by sub-cultivation of the samples into normal culture media at appropriate temperature and incubation times. MICs were visually recorded at 48 h for yeasts, and, for the rest of the fungi, at a time according to the control fungal growth. Carbendazim®, Amphotericin B, Terbinafine and Ketoconazole were used as positive controls for fungi. Compounds with MICs > 250 μg/mL were considered inactive.

Inhibition percentage determination: The test was performed in 96-well microplates according to previous reports [30]. Tests were performed in duplicate. Reduction of growth for each compound concentration was calculated as follows: % of inhibition = 100 - (OD_{405} ETW - OD_{405} SCW)/(OD_{405} GCW - OD_{405} SCW), where ETWs = EOs or compounds test wells; GCW = growth control well; SCW = sterility control well and OD = optical density.

MIC_{50} was defined as the lowest concentration of a compound that showed 50% reduction of the growth control and was determined from the results obtained in the inhibition percentage determination.

Antimycobacterial activity* in vitro*: Bioassays were conducted on sensitive H_{37}Rv ATCC 27294 (American Type Culture Collection) and MDR (clinical isolate, strain 02TBDM039EP097) *Mycobacterium tuberculosis* strains. For the preparation of the inoculum, a suspension of *M. tuberculosis* was made by mixing growth from slants (20-30 days old) with 100 μL of Tween 80 in 0.2% bovine serum albumin (Sigma Chemical Co., St. Louis, MO). Turbidity of the suspension was then adjusted to a McFarland standard No. 1 (3 x 10^7 CFU/mL) by adding Tween 80 and bovine serum albumin. The bacterial suspension (300 μL) was further transferred to 7.2 mL of 7H9GC broth (4.7 g of Middlebrook), 7H9 broth base (Difco, Detroit, MI), 20 mL of 10% glycerol, 1 g of Bacto Casitone (Difco), 880 mL of distilled water and 100 mL of OADC (oleic acid, albumin, dextrose, catalase) (Remel, Lenexa, KS). For the bioassay, the compounds were resuspended in DMSO at a concentration of 1 mg/mL (stock solution). These stock solutions were further diluted with appropriate volumes of 7H9GC broth to yield final concentrations of 0.1 to 50 μg/mL. Final drug concentration ranges of standard antibiotics used as positive controls were 0.125 to 32 μg/mL for isoniazid and 0.063 to 16 μg/mL for rifampin. The standard drugs or compounds (100 μL) were mixed in the wells with 100 μL of bacterial inoculum, resulting in a final bacterial concentration of approximately 1.2 x 10^6 CFU/mL. The wells in column 11 served as inoculum-

only controls. Solvent (DMSO) was included in every experiment as a negative control. The plates were sealed in plastic bags and then incubated at 37°C for 5 days. On day 5, 50 µL of the tetrazolium-Tween 80 mixture {1.5 mL of tetrazolium [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Aldrich Chemical Co., Milwaukee, WI) at a dilution of 1 mg/mL in absolute ethanol and 1.5 mL of 10% Tween 80} was added to the wells, and the plate was incubated at 37°C for 24 h. After this incubation period, the growth of the microorganism was visualized by the change in color of the dye from yellow to purple. The tests were carried out in triplicate. MIC is defined as the lowest drug concentration that prevents the aforementioned change in color.

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Antioxidant, Antiproliferative and Antimicrobial Activities of the Volatile Oil from the Wild Pepper *Piper capense* Used in Cameroon as a Culinary Spice

Verlaine Woguem[a,b], Filippo Maggi[c,*], Hervet P. D. Fogang[a,b], Léon A. Tapondjou[a], Hilaire M. Womeni[b], Luana Quassinti[c], Massimo Bramucci[c], Luca A. Vitali[c], Dezemona Petrelli[d], Giulio Lupidi[c], Fabrizio Papa[e], Sauro Vittori[c] and Luciano Barboni[e]

[a]*Laboratory of Environmental and Applied Chemistry*, *Faculty of Science, University of Dschang*, *P.O. Box 183*, *Dschang*, *Cameroon*

[b]*Laboratory of Biochemistry of Medicinal Plants*, *Food Science and Nutrition*, *Faculty of Science*, *University of Dschang*, *P.O. Box 67*, *Dschang*, *Cameroon*

[c]*School of Pharmacy*, *University of Camerino*, *I-62032 Camerino*, *Italy*

[d]*School of Biosciences and Biotechnology*, *University of Camerino*, *I-62032 Camerino*, *Italy*

[e]*School of Science and Technology*, *Chemistry Division*, *University of Camerino*, *I-62032 Camerino*, *Italy*

filippo.maggi@unicam.it

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Wild pepper (*Piper capense* L.f., Piperaceae) is a spice traditionally used in western Cameroon to make soups called '*Nkui*' and '*Nah poh*'. In the present work, the essential oil hydrodistilled from fruits was analyzed by GC-FID and GC-MS, and for *in vitro* biological activities, namely cytotoxic, antioxidant and antimicrobial, by MTT, DPPH, ABTS and agar disc diffusion methods. The oil composition was dominated by monoterpene hydrocarbons (56.5%) responsible for the pepper odor, such as β-pinene (33.2%), sabinene (10.0%) and α-pinene (8.9%). The oil induced a concentration-dependent inhibitory effect on human tumor cells MDA-MB 231 (breast adenocarcinoma), A375 (malignant melanoma) and HCT116 (colon carcinoma), showing IC_{50} values of 26.3, 76.0 and 22.7 μg/ml, respectively. The oil showed total antioxidant activity with a Trolox equivalent antioxidant concentration (TEAC) value of 140 μmol/g. The essential oil of *P. capense* proved to be an effective scavenger of the $ABTS^+$ radical, with an activity only about 30 times lower than that of Trolox. Moderate activity was observed against the Gram-positive species *Staphylococcos aureus* and *Enterococcus faecalis*, and the yeast *Candida albicans*. The notable inhibition of some human tumor cells is worthy of further investigation to discover the possible mechanisms of action responsible for the observed cytotoxic effect of this essential oil.

Keywords: *Piper capense*, Essential oil, Cytotoxic activity, Antioxidant, Antimicrobial.

Species belonging to the genus *Piper* L. (Piperaceae) are distributed largely in tropical and sub-tropical regions of the world. They have high commercial, economic and medicinal value since they constitute an important source of secondary metabolites. Notably, they produce aromatic fruits which are mainly used as spices. Especially in Cameroon, two traditional soups made by people in the western region, called '*Nkui*' and '*Nah poh*', contain the fruits of wild pepper (*P. capense* L.f.), which is one of three *Piper* species recognized to be indigenous to Cameroon [1]. The plant is an under storey shrub common in gallery forest, with V-shaped branchless and pointed ovate leaves bearing seven basal nerves. The fruits ripen around January and March [1]. They are believed to be the cause of sweating and sleepiness [2]. The leaves are used in S. Tomé and Principe, on the western equatorial coast of Central Africa, as a stomachic and carminative in cases of indigestion, flatulence and colic. Furthermore, a handful of leaves is included in Cameroonian recipes to treat epilepsy in Fongo-Tongo village [3]. The roots are traditionally used in South Africa to treat wounds, vaginal discharge, infertility, sore throat and tongue sores. The same parts in Kenya are boiled and used against malaria [4].

Main secondary metabolites isolated from the plant are lignans [5,6], terpenoids [2,7], amides [8], amide alkaloids and flavones [9]. As far as we know, few investigations have been made on the biological activity of volatiles obtained from this spice. The essential oils obtained from leaves and fruits collected in the western province of Cameroon were reported to show negligible antifungal activity [10]. Other papers described the toxicity of the essential oil against the insect infecting maize grains, *Sitophilus zeamais* [11], and the larvicidal activity against the malaria vector *Anopheles gambiae* [12].

As part of our continuous search for bioactivity of essential oils from Cameroonian spices [13,14], we evaluated the antioxidant, cytotoxic, and antimicrobial capacities of *P. capense* essential oil. Chemical investigation has been undertaken to establish the composition-activity relationship.

Sixty-two components were identified in the oil (Table 1), corresponding to 89.5% of the total composition. The oil was mainly constituted by monoterpenes (64.7%), among which the hydrocarbons were predominant (56.5%). Main representatives of this chemical class were β-pinene (33.2%), sabinene (10.0%) and α-pinene (8.9%), accounting together for 52.1% of the whole oil. This high concentration of hydrocarbons is essential to give the pepper odor [15]. The second fraction in terms of abundance was represented by sesquiterpenes (24.1%), among which hydrocarbons were again the most abundant (17.8%). Main representatives of this group were (*E*)-caryophyllene (6.3%) and germacrene D (3.8%). The chemical composition of the oil obtained from dry fruits was quite consistent with that reported in previous studies [10,11].

Table 1: Chemical composition of the essential oil hydrodistilled from fruits of *Piper capense*.

N.	Constituent[a]	RF[b]	Calc. LRI[c]	Lit. LRI[d]		Essential oil		ID[f]
				ADAMS	NIST08	%[e]	mg/g	
1	α-Thujene	1.1	927	930	927	0.6±0.0	14.5±0.3	RI,MS
2	α-Pinene	1.1	932	939	932	8.9±0.0	234.8±4.1	Std
3	Camphene	1.1	947	954	947	0.6±0.0	16.2±0.4	Std
4	Sabinene	1.1	973	975	973	10.0±0.8	264.9±24.5	RI,MS
5	β-pinene	1.1	975	979	965	33.2±0.7	876.9±5.9	Std
6	Myrcene	1.1	993	990	993	0.9±0.0	23.0±0.5	Std
7	α-Phellandrene	1.1	1005	1002	1005	Tr±0.0	0.7±0.0	RI,MS
8	δ-3-Carene	1.1	1011	1011	1011	0.5±0.0	12.3±0.3	Std
9	α-Terpinene	1.1	1019	1017	1019	Tr[g]±0.0	0.5±0.1	RI,MS
10	*p*-Cymene	1.1	1028	1024	1028	0.4±0.0	11.4±0.3	Std
11	Limonene	1.1	1031	1029	1033	1.8±0.0	48.1±0.9	Std
12	1,8-Cineole	1.5	1034	1031	1034	0.4±0.0	10.2±0.7	Std
13	(*Z*)-β-Ocimene	1.1	1045	1034	1043	Tr±0.0	0.8±0.0	RI,MS
14	(*E*)-β-Ocimene	1.1	1055	1050	1052	Tr±0.0	0.9±0.0	RI,MS
15	γ-Terpinene	1.1	1063	1059	1063	Tr±0.0	1.1±0.2	Std
16	*cis*-Sabinene hydrate	1.5	1071	1070	1068	0.4±0.0	11.8±0.3	RI,MS
17	Terpinolene	1.1	1089	1088	1089	Tr±0.0	0.9±0.1	Std
18	*trans*-Sabinene hydrate	1.5	1098	1098	1098	Tr±0.0	0.8±0.0	RI,MS
19	Linalool	1.5	1103	1096	1103	1.3±0.0	34.2±0.9	Std
20	*cis*-*p*-Menth-2-en-1-ol	1.5	1125	1121	1126	0.1±0.0	3.1±0.5	RI,MS
21	*trans*-Pinocarveol	1.5	1140	1139	1141	0.6±0.0	15.7±0.7	Std
22	Camphor	1.5	1146	1146		0.2±0.0	4.5±0.8	Std
23	Pinocarvone	1.5	1165	1164	1165	0.1±0.0	3.2±0.3	RI,MS
24	Borneol	1.5	1167	1169	1167	0.6±0.0	14.9±0.5	Std
25	Terpinen-4-ol	1.5	1179	1177	1179	1.2±0.0	31.6±0.5	Std
26	α-Terpineol	1.5	1192	1188	1192	0.6±0.0	16.6±0.4	Std
27	Myrtenal	1.5	1195	1195	1195	0.3±0.0	8.3±0.3	Std
28	Myrtenol	1.5	1197	1195	1196	0.4±0.0	10.2±0.9	RI,MS
29	Isobornyl acetate	1.5	1286	1285		2.0±0.0	52.9±1.3	Std
30	α-Cubebene	1.1	1348	1348		0.4±0.1	10.3±2.1	RI,MS
31	Cyclosativene	1.1	1360	1371	1360	0.2±0.0	6.0±0.1	RI,MS
32	α-Copaene	1.1	1372	1376		0.6±0.0	16.0±0.2	RI,MS
33	β-Bourbonene	1.1	1380	1388		0.1±0.0	1.6±0.2	RI,MS
34	β-Cubebene	1.1	1387	1388	1387	1.2±0.0	32.7±0.3	RI,MS
35	β-Elemene	1.1	1389	1390		1.0±0.0	26.0±0.2	RI,MS
36	(*E*)-Caryophyllene	1.1	1413	1419		6.3±0.1	166.8±1.6	Std
37	β-Copaene	1.1	1424	1432		0.1±0.0	3.9±0.3	RI,MS
38	γ-Elemene	1.1	1431	1436		0.3±0.0	6.7±0.3	RI,MS
39	6,9-Guaiadiene	1.1	1440	1444		0.2±0.0	5.4±0.2	RI,MS
40	α-Humulene	1.1	1448	1454	1448	1.1±0.0	29.4±0.5	Std
41	(*E*)-β-Farnesene	1.1	1459	1456	1459	0.1±0.0	1.5±0.3	RI,MS
42	Germacrene D	1.1	1476	1485		3.8±0.0	99.7±0.6	RI,MS
43	β-selinene	1.1	1480	1490	1480	0.3±0.0	7.4±0.1	RI,MS
44	*trans*-Muurola-4(14),5-diene	1.1	1485	1493		0.1±0.0	3.2±0.0	RI,MS
45	α-Muurolene	1.1	1496	1500		0.6±0.0	16.1±0.0	RI,MS
46	*n*-Pentadecane	1.4	1500	1500	1500	0.2±0.0	5.9±0.1	Std
47	β-Bisabolene	1.1	1506	1505		Tr	0.7±0.0	RI,MS
48	*trans*-Calamenene	1.1	1519	1522		Tr±0.0	0.5±0.0	RI,MS
49	δ-Cadinene	1.1	1523	1523		0.8±0.0	21.8±0.5	RI,MS
50	*trans*-Cadina-1,4-diene	1.1	1528	1534		Tr±0.0	0.4±0.0	RI,MS
51	Germacrene B	1.1	1551	1561	1552	0.6±0.0	16.7±0.4	RI,MS
52	Elemicin	1.4	1561	1557	1561	Tr±0.0	0.8±0.0	RI,MS
53	(*E*)-Nerolidol	1.3	1564	1563	1564	1.5±0.0	40.3±0.6	RI,MS
54	Spathulenol	1.3	1573	1578		Tr±0.0	0.8±0.0	RI,MS
55	Caryophyllene oxide	1.3	1576	1583	1576	2.8±0.0	74.2±0.8	Std
56	Salvial-4(14)-en-1-one	1.3	1588	1594		0.2±0.0	5.1±0.5	RI,MS
57	Humulene epoxide II	1.3	1602	1608	1602	0.3±0.0	8.2±0.3	RI,MS
58	1,10-di-*epi*-Cubenol	1.3	1626	1619	1623	0.4±0.0	11.6±0.6	RI,MS
59	Caryophylla-4(12),8(13)-dien-5-ol[h]	1.3	1632	1640	1635	0.1±0.1	2.9±0.6	RI,MS
60	Cubenol	1.3	1638	1646		0.3±0.0	7.4±0.4	RI,MS
61	α-Muurolol	1.3	1644	1646	1644	0.4±0.0	10.0±0.3	RI,MS
62	Eudesma-4(15),7-dien-1-β-ol	1.3	1682	1688		0.3±0.0	9.2±1.9	RI,MS
	Total identified (%)					89.5		
	Oil yield (%)					1.9		
	Grouped compounds							
	Monoterpene hydrocarbons					56.5	1495.5	
	Oxygenated monoterpenes					8.2	165.1	
	Sesquiterpene hydrocarbons					17.8	474.1	
	Oxygenated sesquiterpenes					6.3	169.7	
	Others					0.7	71.5	

[a] Compounds are listed in order of their elution from a HP-5MS column. Their nomenclature was in accordance with Adams [30]. [b] Response factor (RF) of FID detector for the main chemical groups occurring in the essential oil. [c] Linear retention index on HP-5MS column experimentally determined using homologous series of C_8-C_{30} alkanes. [d] Linear retention index taken from Adams [30] and/or NIST08 [31]. [e] Percentage values are means of three determinations. [f] Identification methods: Std, based on comparison with authentic compounds; MS, based on comparison with Wiley, ADAMS and NIST08 MS database; RI, based on comparison of RI with those reported in ADAMS and NIST08. [g] Tr, traces (mean value below 0.1%). [h] Correct isomer not identified.

In these studies the major volatiles of fruits from western Cameroon were again monoterpene hydrocarbons, namely β-pinene, sabinene and α-pinene (46.8, 17.4 and 14.4%, and 59.3, 14.7 and 10.5%, respectively), and sesquiterpene hydrocarbons, namely (*E*)-caryophyllene and germacrene D (4.0 and 5.2%, and 3.4 and 2.5%, respectively), but percentages were slightly different with respect to those detected by us. The aerial parts of *P. capense* growing in S. Tomé and Principe [2] were also characterized by monoterpenes such as β-pinene (32.5%) and α-pinene (8.6%), and sesquiterpenes such as (*E*)-caryophyllene (12.6%), along with a low content of sabinene (0.7%), and benzenepropanoic acid ethyl ester (7.8%), which was not detected in our study. Finally, the essential oil obtained from the whole plant collected in Kenya [12] showed a different composition consisting mainly of sesquiterpene hydrocarbons (43.9%) such as δ-cadinene (16.8%), β-bisabolene (5.6%) and bicyclogermacrene (3.3%), while monoterpene hydrocarbons, including β-pinene (7.2%) and α-phellandrene (4.8%), were less abundant (30.6%).

The antioxidant activity of the essential oil from *P. capense* was evaluated using DPPH and ABTS methods. From results reported in Table 2 we observed that the oil showed lower antioxidant activity in the DPPH assay, while worthy of mention was the activity against the $ABTS^+$ radical. On the other hand, no reducing capacity power (FRAP assay) of the oil was pointed out. Proton radical scavenging activity is an important attribute of antioxidants and $ABTS^+$, a protonated radical, has a characteristic maximum absorbance at 734 nm, which decreases with the scavenging of the proton radicals [16]. As shown, the essential oil from *P. capense* was an effective scavenger of the $ABTS^+$ radical with an activity about 30 times lower than that of Trolox and the scavenging activity was much higher than that against the $DPPH^•$ radical. Factors like radical stereoselectivity or sample solubility in different testing systems have been reported to affect the capacity of samples to react and quench different radicals [17]. Wang and co-workers [18] found that some compounds having $ABTS^+$ scavenging activity did not show inhibition against the DPPH radical. Low values of DPPH scavenging activity were also reported for different kinds of essential oils with a typical monoterpene hydrocarbon pattern [19-21]. In addition, the discrepancy observed in the results of $ABTS^+$ and $DPPH^•$ scavenging ability might be due to their different mechanisms of reaction: $ABTS^+$ assay is based on hydrogen transfer reaction, while DPPH assay is based on electron transfer [22]. Evaluation of the scavenging activity of pure compounds used as standards, conducted in our laboratories, showed no activity for *α*-pinene, *β*-pinene, and (*E*)-caryophyllene, and very low activity for limonene. Therefore, the antioxidant effectiveness of the oil, as already demonstrated [19], may be due to the synergism of different constituents.

Table 2: *In vitro* antioxidant activity of the essential oil from *Piper capense*.

Essential oil	(IC_{50} µg/ml)[a]		
	ABTS	DPPH	FRAP
P. capense	300 (± 6)	>>1000	N.A.[b]
Reference			
Trolox	10.1(±0.6)	3.9 (± 0.4)	

[a] IC_{50} = The concentration of compound that affords a 50% reduction). [b] N.A., not active.

To investigate the cytotoxic activity of *P. capense* essential oil, we evaluated its effect on the human tumor cell lines MDA-MB 231 (human breast adenocarcinoma), A375 (human malignant melanoma) and HCT116 (human colon carcinoma) by MTT assay. As shown in Table 3, the essential oil induced a concentration-dependent inhibitory effect on all cancer cell lines tested in the dilution range 0.78-200 µg/mL.

Table 3: *In vitro* cytotoxic activity of the essential oil from *Piper capense*.

Essential oil	Cell line (IC_{50} µg/mL)		
	MDA-MB 231[a]	A375[b]	HCT116[c]
P. capense	26.3	76.0	22.7
95% C.I.	23.2 - 29.9	65.7 - 83.6	20.7 - 25.0
Major constituents			
β-Pinene	78.5	> 200	59.2
95% C.I.	73.2 - 84.4		54.5 – 64.0
(*E*)-Caryophyllene	45.3	63.3	55.7
95% C.I.	37.3 - 52.7	54.8 - 77.8	50.3 - 62.7
Reference			
Cisplatin	2.4	0.16	2.8
95% C.I.	2.0 - 2.7	0.12 - 0.22	2.6 – 3.0

IC_{50} = Concentration of compound that affords a 50% reduction in cell growth (after 72 h of incubation). [a] Human breast adenocarcinoma cell line. [b] Human malignant melanoma cell line. [c] Human colon carcinoma cell line.

The IC_{50} values of the oil were 26.3, 76.0 and 22.7 µg/mL on MDA-MB 231, A375 and HCT116 cell lines, respectively. The cytotoxic activity of *P. capense* essential oil may be attributed to specific components of the oil. We tested the cytotoxic activity of β-pinene and (*E*)-caryophyllene on the same cell lines used to analyze *P. capense* essential oil (Table 3). In a previous study, β-pinene proved to be less active than α-pinene against the same cell lines [13]. According to Bakkali and coworkers [23], sabinene has also a dose-dependent cytotoxic activity when tested either alone or added to the essential oil. The concentrations of β-pinene (33.2%), sabinene (10%) and α-pinene (8.9%) cannot fully justify the cytotoxic activity of *P. capense* essential oil, which means that some other compounds are active, probably sesquiterpenes such as (*E*)-caryophyllene (6.3%) and germacrene D (3.8%). (*E*)-caryophyllene showed cytotoxic activity against the tumor cell lines (Table 3), with IC_{50} values in the range 45.3-63.3 µg/mL. To our knowledge, also germacrene D was active against tumor cells such as human breast adenocarcinoma (MDA-MB 231 and MCF-7), human ductal carcinoma (Hs 578T), and human hepatocellular carcinoma (Hep G2) [24]. In addition, minor components could also contribute to the cytotoxic activity of the oil or be responsible for synergism along with other compounds [25,26].

Table 4: Activity of *P. capense* essential oil and some major constituents on bacterial and yeast species. Values are expressed as inhibition zones (mm,)[a].

Essential oil	*S. aureus*	*E. faecalis*	*E. coli*	*P. aeruginosa*	*C. albicans*
P. capense	10	10	6	6	8
Major constituents					
α-pinene	6	6	6	6	11
β-pinene	6	6	6	6	9
(*E*)-caryophyllene	8	10	8	6	6

[a] Sterile filter paper discs (6 mm in diameter) were placed on the surface of inoculated plates and spotted with 10 µL of a 1:1 dilution of essential oil/reference compound in DMSO.

Results of antimicrobial activity tests are summarized in Table 4. The essential oil showed a measurable, albeit low, activity against the two Gram-positive bacterial species *S. aureus* and *E. faecalis* and the yeast *C. albicans*. Activity on Staphylococci has already been reported for the methanolic extract from barks [27], while no data were available for the antibacterial activity of the essential oil from barks of this species. The moderate activity against *C. albicans* has been previously observed, but was measured by a microdilution method [10]. Given the differences in the methods, the results may be considered comparable and the activity presented herein as a sort of general positive control of the antimicrobial activity test. A remarkably strong activity was observed in a previous study using an acetone extract from roots, whose composition was, however, not determined [28]. Comparison between activities of the essential oil and three major constituents of the oil, namely α-pinene, β-pinene, and (*E*)-caryophyllene, showed

that the last may be responsible for the activity observed against all the bacterial species, except for *P. aeruginosa*, against which neither the oil nor the pure compounds were effective. In the *E. coli* culture we observed an inhibition by (*E*)-caryophyllene (zone diameter of 8 mm), not accompanied by an appreciable activity of the whole essential oil. This result may be due to the relative low concentration of (*E*)-caryophyllene within the oil. On the contrary, *C. albicans* was only susceptible to both forms of pinene. This may account for the measurable antifungal activity of the essential oil.

In conclusion, the high content of monoterpene hydrocarbons found in *P. capense* essential oil furnishes a typical pepper note and supports the use of the plant as an odorous spice in African traditional cuisine. The oil was an effective scavenger of the $ABTS^+$ radical with an activity only about 30 times lower than that of Trolox and so could be considered as a good food preservative. The essential oil showed notable inhibition activity against some human tumor cells, being worthy of further investigation to discover the possible mechanisms of action responsible for the observed cytotoxic effect. Finally, the results of antimicrobial activity against the bacterial Gram-positive and yeast species may sufficiently support the described use of some parts of this plant in the treatment of some infection related conditions (e.g. wounds, vaginal discharge, and sore throat).

Experimental

Plant material: Dry fruits of *P. capense* were bought in a market of Dschang (Cameroon, Menoua Division, Western Region). Identification was made by Mr Nana Victor, taxonomist at the Cameroon National Herbarium (Yaoundé), where a voucher specimen was deposited (N. 6018/HNC/SRF).

Extraction of the essential oil: The dry fruits (80 g) of *P. capense* were ground and subjected to hydrodistillation in a Clevenger-type apparatus using 750 mL of deionized water for 3 h yielding 1.98%, w/w, of a strong smelling. pale yellow oil. The oil collected was dried over anhydrous sodium sulfate, and then stored in the refrigerator (+4°C) until used.

GC-FID and GC-MS analyses: For gas chromatographic separations, an Agilent 4890D instrument coupled to an ionization flame detector (FID) was used. Volatile components were separated on a HP-5 capillary column (5% phenylmethylpolysiloxane, 25 m, 0.32 mm i.d.; 0.17 µm film thickness) (J and W Scientific, Folsom, CA), with the following temperature program: 5 min at 60°C, subsequently 4°C/min up to 220°C, then 11°C/min up to 280°C, held for 15 min, for a total run of 65 min. Injector and detector temperatures were 280°C. Helium was used as the carrier gas, at a flow rate of 1.4 mL/min; injection volume: 1 µL; split ratio, 1:34. A mixture of aliphatic hydrocarbons (C_8-C_{30}) (Sigma, Milan, Italy) in *n*-hexane was directly injected into the GC injector under the above temperature program, in order to calculate the retention index of each compound. Oil samples were diluted 1:100 in *n*-hexane and injected in a volume of 1 µL. Analysis was repeated 3 times. Data were collected by using HP3398A GC Chemstation software (Hewlett Packard, Rev. A.01.01). The relative amounts of essential oil components, expressed as percentages and mg/g e.o., were obtained according to a reported procedure [13].

GC-MS analysis was performed on an Agilent 6890N gas chromatograph coupled to a 5973N mass spectrometer using a HP-5MS (5% phenylmethylpolysiloxane, 30 m, 0.25 mm i.d., 0.1 µm film thickness) (J & W Scientific, Folsom) capillary column. The temperature programme was the same as above. Injector and detector temperatures were 280°C. Helium was used as carrier gas, at a flow rate of 1 mL/min. Split ratio: 1:50; acquisition mass range: 29-400 *m/z*. All mass spectra were acquired in electron-impact (EI) mode with an ionization voltage of 70 eV. Oil samples were diluted 1:100 in *n*-hexane and the volume injected was 2 µL. Data were analyzed by using MSD ChemStation software (Agilent, Version G1701DA D.01.00). Whenever possible, volatile components were identified by co-injection with authentic standards and isolated compounds. Otherwise, the peak assignment was carried out by the interactive combination of chromatographic linear retention indices that were consistent with those reported in the literature [29,30], and MS data consisting of computer matching with WILEY275, NIST 08 ADAMS, and a home-made library (based on the analyses of reference oils and commercially available standards).

Evaluation of antioxidant activity

DPPH free-radical scavenging activity: The DPPH free-radical scavenging assay was carried out on a microplate according to a previously described procedure [31]. Extract concentration providing 50% inhibition (IC_{50}) was calculated from the linear regression algorithm of the graph plotting inhibition percentage against extract concentration. Experiments were conducted in triplicate.

ABTS assay: Radical scavenging capacity was measured by a modified method originally described by Re and coworkers [32] for application to a 96-well microplate assay [33]. Trolox was used as positive control. Each experiment was repeated 3 times.

Ferric reducing antioxidant power (FRAP) assay: Determination of antioxidant activity by ferric reducing antioxidant power (FRAP) assay was carried out according to Müller and coworkers [34]. The ability of the essential oil to scavenge the different radicals in the assays was compared with that of Trolox, used as positive control, and the activity of the oil was expressed as tocopherol equivalent antioxidant capacity (µmol TE/g product). Tests were repeated 3 times.

MTT cytotoxicity assay: Human colon carcinoma cell line HCT116 was cultured in RPMI1640 medium with 2 mM L-glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin, and supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS) (PAA Laboratories GmbH, Austria). Human breast adenocarcinoma cell line MDA-MB 231, and human malignant melanoma cell line A375 were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 2 mM L-glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin, and supplemented with 10% HI-FBS. Cells were cultured in a humidified atmosphere at 37°C in the presence of 5% CO_2. The MTT assay was used as a relative measure of cell viability. Cell-viability assays were carried out according to a previously reported procedure [35]. Cytotoxicity was expressed as the concentration of compound inhibiting cell growth by 50% (IC_{50}). The IC_{50} values were determined with the GraphPad Prism 4 computer program (GraphPad Software, S. Diego, CA, USA).

Antimicrobial activity: Microorganisms included in this study were: *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212, and *Candida albicans* ATCC 24433. Antimicrobial activity of the essential oil of *P. capense* was assessed by the disc diffusion test, as reported by the European Committee for Antimicrobial Susceptibility testing [36], with previously described minor modifications due to the nature of the substance tested [37]. Ten µL of each reference compound (α-pinene, β-pinene, (*E*)-caryophyllene) per paper disc was used in the control experiments; the known antimicrobials ciprofloxacin (5 µg

disc) and fluconazole (25 µg disc) were used as a reference against bacteria and fungi, respectively. Each test was repeated at least twice.

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Boldine and Related Aporphines: From Antioxidant to Antiproliferative Properties

Darina Muthna*, Jana Cmielova, Pavel Tomsik and Martina Rezacova

Department of Medical Biochemistry, Faculty of Medicine in Hradec Kralove, Charles University in Prague, Simkova 870, Hradec Kralove 500 38, Czech Republic

muthnad@lfhk.cuni.cz

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Plant and folk medicine represent nowadays a source of either new therapeutic substances or substrates for drug synthesis. One such promising group for possible further exploitation is the family of aporphine alkaloids containing boldine and related compounds. In this mini-review we focus on boldine and its newly described effects, which predominantly arise from its antioxidant properties. Moreover, we try to compare its antiproliferative properties with other better known members of the aporphine group.

Keywords: Boldine, Aporphine alkaloids, Antioxidant, Antiproliferative properties.

A wide range of compounds of natural origin that have been exploited in folk medicine for a long time are currently being intensively studied. Because of their multiple benefits, these compounds represent a possible source of new therapeutic substances for the treatment of various diseases. One such promising group is the family of aporphine alkaloids including boldine, neolitsine, dicentrine, glaucine, cassythine, actinodaphnine, and nantenine [1-3]. Because of their chemical structures they are potent antioxidants. Oxidative stress results in direct or indirect reactive oxygen species (ROS) mediated damage of nucleic acids, proteins, and lipids, and has been implicated in carcinogenesis, neurodegeneration, atherosclerosis, diabetes and aging [4]. Thus the regulation of redox status remains a promising therapeutic approach [5]. In this review we focus on boldine and its newly described effects, which predominantly arise from its antioxidant properties. Moreover, we try to compare its described *in vitro* antiproliferative properties with other better known members of the aporphine group.

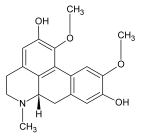

Figure 1: Boldine - (6a*S*)-1,10-Dimethoxy-6-methyl-5,6,6a,7-tetrahydro-4H-dibenzo[de,g]quinoline-2,9-diol

Antioxidant activities of boldine: Main sources of boldine are the leaves and bark of the Chilean tree *Peumus boldus* (common name, boldo) from the Monimiaceae family, but it can also be isolated from plants of the related Lauraceae family, among others [2,6,7]. Like other members of the aporphine group, boldine behaves as a potent antioxidant. Although boldine has various beneficial effects, the majority of them arise just from its antioxidant properties. Due to its free radical scavenging properties, boldine influences cell proliferation, survival, differentiation and metabolism [4]. Aporphines generally are easily oxidized to dehydro- and oxo-aporphines. Even in the absence of hydroxyl groups in non-phenolic aporphines, the antioxidant capacity is maintained by the stabilization of the benzylic C-6a radical with the nitrogen lone pair [8].

Generally, with respect to the phenolic structure, these phytochemicals can exert a biphasic effect. In cancer cells, they can scavenge the constitutively high amounts of H_2O_2 that stimulate proliferation. On the other hand, the same phytochemicals under certain experimental conditions can exhibit pro-oxidant activity and generate ROS in amounts beyond the threshold for its tolerance [9]. This is true also for boldine. At concentrations of 10 and 50 μM, it exhibits intense free radical scavenging properties; however, at a concentration of 100 μM, boldine potentiates lipoperoxidation induced by ischemia, indicating a pro-oxidant effect in rat hippocampal slices [10]. Similar concentration-dependent effects can be observed on regulators of adipogenesis and adiponectin levels. Boldine protects adiponectin from both oxidative stress and inflammatory cytokines at lower concentrations (10–25 μM), giving better protection than higher concentrations (more than 50 μM) [11]. In diabetic conditions, boldine restores NO bioavailability and thus attenuates oxidative stress and endothelial dysfunction [12].
Another property of boldine and related alkaloids which seems to be closely related to its free radical scavenging effects is its antinociceptive activity. Boldine inhibited the rapid pain response and exerted a central antinociceptive effect [2]. Moreover, boldine as an antioxidant, strongly inhibits the synthesis of prostaglandin, which results in its anti-inflammatory and antipyretic effects [13].

Enzyme inhibitory activities of boldine: Besides exhibiting significant antioxidant properties, boldine exerts inhibitory activity on some enzymes. Boldine inhibits acetylcholinesterase activity with an IC_{50} value of 8.5±1.4 μM. For comparison, the IC_{50} value of the standard acetylcholinesterase substrate inhibitor, huperzine A, is 1.8 μM [6]. The inhibition of acetylcholinesterase and the consequent increase in colon motility are the reasons why the infusion of *P. boldus* is used in folk medicine to treat digestive and hepatic problems [14]. However, boldine itself possess an ileum relaxant property [15]. In addition, boldine exhibits other properties with respect to which a connection with antioxidant capacity has not yet been proven. Boldine might be able to interact with the promoter of the peroxisome proliferator-activated receptor (PPAR) gene directly and/or modulate its responsive genes and thus have strong beneficial effects on obesity-related diseases. This effect seems to be independent of its antioxidant property [11].

Hoet *et al*. [16] focused on boldine as well as other aporphine alkaloids within the context of DNA. These alkaloids are non-planar molecules and thus lack the structure of conventional DNA intercalators. Nevertheless, boldine intercalates weakly into DNA and may also be an adaptive intercalator such as dicentrine, undergoing a conformational change to a planar conformation upon binding to DNA [17]. Despite this weak intercalation, no effect on topoisomerase I and II activity was observed after boldine treatment. Its cytotoxicity is, therefore, probably independent of interactions with DNA or topoisomerases.

Nevertheless, boldine is a popular compound in the synthesis of derivatives with improved inhibitory activities. Boldine methine can inhibit angiotensin-II-induced leukocyte-endothelial cell interactions *in vivo*. The effects are mediated partly through the inhibition of the generation of ROS, the down-regulation of P-selectin expression on the endothelial cell, the inhibition of CXC chemokine and PAF release, and the blockade of PAF receptor. In contrast, boldine did not markedly affect the inflammatory activity elicited by angiotensin-II [18]. Other activities of boldine are of an anti-α-glucosidase, anti-leishmanial and anti-fungal nature [6].

Antiproliferative activities of boldine: Whereas the antioxidant activities of boldine are well documented [19], relatively little is known about its antiproliferative properties. A considerable difference is observed in the effects of boldine alone compared with boldo extract, which is a mosaic of active components. In infusions of boldo leaves about 40 compounds were detected. Thus, the medicinal properties reported for such infusions should be attributed not only to the presence of catechin and boldine, but also to other compounds [20]. That is, that the various effects of the decoction or extract from boldo leaves were not confirmed or observed after simple treatment with boldine, catechin, quercetin or rutin, even at concentrations exceeding those in the extract [21].

The anti-proliferative effect of *P. boldus* water extract is strongly cell dependent. The extract at a concentration as high as 3.5 mg of *P. boldus* extract/mL did not cause any appreciable decrease in Caco-2 cell viability. For HeLa cells, a *P. boldus* water extract concentration of 0.66 mg/mL was able to reduce cell viability by 50% [14]. In addition, the methanolic extract (containing 1.05% of boldine) inhibited cell growth and induced apoptosis in the M14 cell line [21].

Despite its weak effect on cell viability, a strong modification in the protein profile of HeLa cells appears. Some of these proteins may correspond to the cadherins or to the connexins. This may help cell communication in the gap-junctions, as treatment with *P. boldus* results in the detachment of cells [14]. In the M14 human cancer cell line, *P. boldus* leaf extract at 5 and 10 mg/mL induced a reduction in Hsp70 protein expression. This decrease was in correlation with a high level of DNA fragmentation and a significant increase in caspase-3 enzyme activity. A parallel growth in ROS production could amplify the apoptosis cascades. *P. boldus* leaf extract at higher concentrations (20–40 mg/mL) causes necrosis associated with high lactate dehydrogenase release and a further increase in ROS production. These processes generate intolerable oxidative stress in cancer cells [21]. A very important fact is that the extract of leaves of *P. boldus* exhibited no effect on the viability of normal fibroblast cells [20]. Also Gerhardt *et al*. suggested that boldine is selectively toxic because it may not affect hippocampal cells to the extent that it affects tumor cells [22].

Boldine reduced cellular proliferation by inducing G2 arrest followed by a reduction in mitotic cells in U138-MG exposed to 80 μM for 72 hours. Boldine did not induce apoptosis in these cells. Neither activation of caspases 3 or 9 nor an increase in the cleavage of PARP or DNA fragmentation were observed after exposure for 24 hours. Only C6 cells were propidium iodide positive after 500 μM boldine treatment for 72 hours [22].

Further, the antiproliferative properties of boldine are improved by the synthesis of different derivatives. Two new compounds – 3-thiocarbamateboldine and 2,9-*O*,*O*-diacetyl-3-thiocarbamateboldine – were synthesized and their cytotoxicity evaluated by Thomet *et al* [23]. The derivatization of boldine to a diphenylphosphinyl derivative results in its enhanced lipophilicity as well as an increase in its intercalating behavior and thus in significant cytotoxic activity in breast cancer cells lines (MDA-MB-231 and MCF-7). Boldine itself has no cytotoxic capacity at an indicated concentration of 100 μM for 72 hours [24]. Finally, Thomet *et al*. used a boldine derivative as ligand to synthetized boldiplatin. This compound has a comparable inhibitory concentration to oxaliplatin and, moreover, exerts greater selectivity against cancer tumor cell lines [25].

Antiproliferative activities of related alkaloids: It was reported that aporphine alkaloids, especially those containing a 1,2-methylenedioxy group, are effective against cancer cell lines [26]. On the other hand, nantenine, with a 9,10-methylendioxy, and apomorphine, with no methylendioxy group, also have proven antiproliferative properties [27,28]. Thus which structural part is responsible for the antiproliferative activity remains an open question.

All the discussed alkaloids can be isolated from various plant species; only representatives of these plants are named. Further information about sources of aporphine alkaloids are reviewed by Guinaudeau, Leboeuf and Cave [29,30].

Nantenine

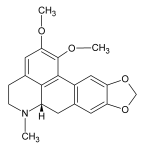

Figure 2: Nantenine- (6a*S*)-1,2-Dimethoxy-6-methyl-5,6,6a,7-tetrahydro-4H-benzo[de][1,3]benzodioxolo[5,6-g]quinoline

Nantenine can be isolated from *Nandina domestica* (Berberidacae) and *Uvaria chamae* (Annonaceae). Nantenine exerts a cytotoxic effect on both HCT-116 and Caco-2 cell lines with a potency comparable with that of the clinically available anticancer therapeutic drug etoposide [27], and a cytotoxic effect in mouse L929 transformed cells [28]. Like boldine, nantenine also seems to be selectively cytotoxic. At a concentration corresponding to the IC_{50} against L929 cells, it was nontoxic to mouse thymocytes [28]. The C1 alkoxy derivative and the C1 benzoate derivative of nantenine exhibit marginally higher selective cytotoxicity to normal cells. The C1 phenolic group on the aporphine scaffold is detrimental to the cytotoxic activity [27].

Dicentrine

Dicentrine, isolated from *Lindera megaphylla* (Lauraceae), is an adaptative intercalator, a molecule that can adopt a relatively flat conformation. It is a strong topoisomerase II inhibitor with an IC_{50} of 27 μM, but is not a topoisomerase II poison [17]. Dicentrine may be compared with classical anti-cancer drugs such as anthracyclines. Its DNA intercalation, together with its inhibition of topoisomerases I and II, results in the formation of DNA strand breaks, which is followed by the activation of multiple signaling routes such as BRCA1-mediated DNA damage response, p53 signaling, G1/S and G2/M cell cycle regulation, and

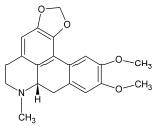

Figure 3: Dicentrine - (7a*S*)-10,11-Dimethoxy-7-methyl-6,7,7a,8-tetrahydro-5H-[1,3]benzodioxolo[6,5,4-de]benzo[g]quinoline

aryl hydrocarbon receptor pathways. CDKN1A appeared as one of the differentially expressed genes upon dicentrine treatment. Moreover, dicentrine is selectively cytotoxic to EGFR-expressing cells; the overexpression of EGFR is associated with different types of cancer [3]. Dicentrine significantly inhibited cell growth and biosynthesis of the macromolecules DNA and RNA, and decreased the colony formation. Its cytotoxicity was confirmed in multiple human, as well as mouse tumor cell lines by an MTT and *in vitro* tumor growing assay [31,32]. Also, its derivative, (−)-nordicentrine, showed cytotoxicity against various human cancer cell lines [33].

Anonaine

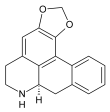

Figure 4: Anonaine - (7a*R*)-6,7,7a,8-Tetrahydro-5H-[1,3]benzodioxolo[6,5,4-de]benzo[g]quinoline

Anonaine from *Michelia alba* (Magnoliaceae) induces dose-dependent DNA damage that is correlated with increased intracellular nitric oxide, ROS, glutathione depletion, disruptive mitochondrial transmembrane potential, activation of caspase 3, 7, 8 and 9, and PARP cleavage. (−)-Anonaine up-regulated the expression of Bax and p53 proteins in HeLa cancer cells. It is worth noting that the DNA damage did not occur in (−)-anonaine-treated non-cancer cell lines, either MDCK or Vero cells [34].

Apomorphine

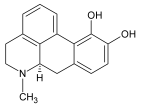

Figure 5: Apomorphene - (6a*R*)-6-Methyl-5,6,6a,7-tetrahydro-4H-dibenzo[de,g]quinoline-10,11-diol

Apomorphine displays scavenger properties at low concentrations [35]. After treatment with low concentrations, an antiapoptotic response (increased bcl-xL, bcl-2*;* decreased bax) and increased cell viability were manifested [36]. At high concentrations of apomorphine a proapoptotic response, including the up-regulation of caspases-3 and 10, fas and fas-ligand, NF-κB p105 subunit, and tumor suppressor protein p53 mRNAs was observed [36]. It causes cell death at higher concentrations. Typical necrotic DNA degradation was found in C6 cells treated with 400 μM of apomorphine for 6 hours (loss of membrane integrity; degeneration of cytoplasmic organelles, especially mitochondria; and DNA fragmentation) [37].

Roemerine

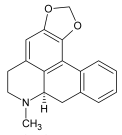

Figure 6: Roemerine - (7a*R*)-7-Methyl-6,7,7a,8-tetrahydro-5H-[1,3]benzodioxolo[6,5,4-de]benzo[g]quinoline

Roemerine, isolated from *Annona senegalensis* (Annonaceae), has no cytotoxic properties alone, but is able to enhance the cytotoxic response mediated by vinblastine with multidrug-resistant KB-V1 cells. This effect seems to be achieved via its interaction with P-glycoprotein [38]. Some cytotoxicity of roemerine was proven against K562S cells [39].

Duguetine

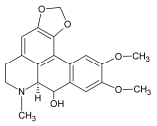

Figure 7: Duguetine - (7aS)-10,11-Dimethoxy-7-methyl-6,7,7a,8-tetrahydro-5H-[1,3]benzodioxolo[6,5,4-de]benzo[g]quinoline-8-ol

Dugeutine, isolated from *Duguetia furfuracea* (Annonaceae), proved to have *in vitro* antitumor activity comparable with that of doxorubicin [26].

Conclusion: After the ascendancy of synthetic chemistry over natural product drug discovery and development during the latter part of the 20th century [40], nowadays the interest returns to the plant and folk medicine as a source of either new therapeutic substances or chemical scaffolds for drug synthesis. One such promising group is the family of aporphine alkaloids comprising boldine and its derivatives that is used in folk medicine to treat headache, earache, rheumatism, dyspepsia, menstrual pain, urinary tract inflammation and others [19]. Ample evidence demonstrates the ability of boldine to act as a potent free radical-scavenger and antioxidant molecule. However, recent studies show that boldine and other aporphine alkaloids also exhibit antitumor properties which are not related to oxidative stress management. Boldine can be isolated quite easily from various plants, and, due to its beneficial properties, could represent another new phytotherapeutic drug arising from folk medicine.

Abbreviations: ROS reactive oxygen species, PPAR peroxisome proliferator-activated receptor, PAF platelet-activating factor, PARP poly ADP ribose polymerase

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New Therapeutic Potentials of Milk Thistle (*Silybum marianum*)

Nataša Milić[a], Nataša Milošević[a*], Ljiljana Suvajdžić[a], Marija Žarkov[b] and Ludovico Abenavoli[c]

[a]*Department of Pharmacy, Faculty of Medicine, University of Novi Sad, Novi Sad, Serbia*
[b]*Department of Neurology, Faculty of Medicine, University of Novi Sad, Novi Sad, Serbia*
[c]*Department of Health Sciences, University Magna Graecia, Catanzaro, Italy*

milosevic_natasa@ymail.com

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Silymarin is a bioflavonoid complex extract derived from dry seeds of Milk thistle [(*Silybum marianum*(L.) Gaernt. (Fam. Asteraceae/Compositaceae)] whose hepatoprotective effect has clinically been proved. Low toxicity, favorable pharmacokinetics, powerful antioxidant, detoxifying, preventive, protective and regenerative effects and side effects similar to placebo make silymarin extremely attractive and safe for therapeutic use. The medicinal properties of silymarin and its main component silibinin have been studied in the treatment of Alzheimer's disease, Parkinson's disease, sepsis, burns, osteoporosis, diabetes, cholestasis and hypercholesterolemia. Owing to its apoptotic effect, without cytotoxic effects, silymarin possesses potential applications in the treatment of various cancers. Silymarin is being examined as a neuro-, nephro- and cardio-protective in the damage of different etiologies due to its strong antioxidant potentials. Furthermore, it has fetoprotective (against the influence of alcohol) and prolactin effects and is safe to be used during pregnancy and lactation. Finally, the cosmetics industry is examining the antioxidant and UV-protective effects of silymarin. Further clinical studies and scientific evidence that silymarin and silibinin are effective in the therapy of various pathologies are indispensable in order to confirm their different flavonolignan pharmacological effects.

Keywords: Milk thistle, Silymarin, Silibinin, Therapeutic, Protective.

Milk thistle [*Silybum marianum* (L.) Gaernt., family Asteraceae/Compositaceae] is probably the oldest and the best-studied plant in the treatment of liver diseases. The first records of milk thistle healing effects are recorded in the Old Testament [1]. Silymarin standardized dry extract is a bioflavonoid complex consisting of at least seven flavonolignans (Figure 1). Silibinin is the major component of this complex extract (about 60-70%) and is biologically the most active constituent of silymarin, followed by silicristin (20%), silidianin (10%), and isosilybin (5%). Silibinin is a mixture of two stereoisomers, silibinin A and silibinin B in the equimolar ratio 1:1 [2].

Modern Western medicine has confirmed hepatoprotective properties of silymarin by conducting animal studies and clinical trials [1]. Silymarin has antioxidant, anti-inflammatory, antifibrotic, detoxifying and regenerative properties. It stimulates protein synthesis and liver regeneration. Silymarin has numerous hepatoprotective effects: it prevents lipid peroxidation by scavenging free radicals and increases the levels of reduced glutathione (GSH). It regulates membrane permeability and membrane stability in damage caused by xenobiotics, regulates nuclear expression and inhibits the transformation of hepatocytes in myofibroblasts (with cirrhosis) [3, 4]. New *in vivo* studies have proved silymarin to be a strong hepatoprotective in hepatic fibrosis induced by carbon tetrachloride (CCl_4). After silymarin had been administered to the rats treated previously with CCl_4, the levels of glutamic oxaloacetic transaminase and glutamic pyruvic transaminases were normalized and the secretion of the connective tissue growth factor was decreased, indicating that silymarin ameliorates the destructive changes in the liver after hepatic fibrosis was provoked [5]. An insight has recently been published into the possible mechanism of silymarin defense capacity, as an antioxidant against various hepatotoxicants. Orally administered silymarin to the experimental mice in only three doses of 100 or 200 mg/kg increased the amount of metabolites generated from homocysteine in the transsulfuration pathway (cystathionine, cysteine, and glutathione), elevated the activity of cystathionine β-synthase, while down-regulated cysteine dioxygenase. This resulted in the augmented oxygen radical scavenging capacity of the liver cytosol and reduced lipid peroxidation. For the first time, it was demonstrated that the antioxidant capacity of silymarin was connected to the hepatic glutathione production in the liver by cysteine synthesis increment and inhibited degradation to taurin [6]. Silymarin inhibits the absorption of α-faloidine and *α*-amanitin toxins (from the mushroom *Amanita phalloides*) as it prevents their binding to the cell surface and limits the transportation systems on the cell membranes. Silymarin/silibinin (SIL) stimulates the regeneration of hepatocytes by nuclear A polymerase activation, increasing protein synthesis and inhibiting the expression of the adhesion molecules (E-selection) [7]. The anti-inflammatory effect of silymarin prevents the activation of the intrahepatic nuclear factor kappa B (NF-kB), reducing the levels of tumor necrosis factor alpha (TNF-α), interelukin-2 (IL-2), interferon gamma (IFN-γ) and the inducible nitric oxide synthase (iNOS) [8]. It was shown that silymarin was hepatoprotective [9], and it was used in the treatment of nonalcoholic fatty liver [4], alcoholic liver cirrhosis [10], and acute and chronic hepatitis [11].

SIL possesses some other potential remedying properties and the new therapeutic effects of milk thistle extract have been considered and proved in various preclinical and clinical studies which are presented in this paper.

Pharmacokinetics of SIL: Silymarin/silibinin is rapidly, but poorly absorbed due to its high liposolubility and poor water solubility. After an oral administration of silymarin (equivalent to 120 mg silibinin), the peak plasma concentration was reached within 1-2 h, but the maximum concentration was only 1.1 to 1.3 mg/mL [12]. The increased bioavailability of silymarin was achieved by complexation with phosphatidylcholine, β-dextran or a suitable

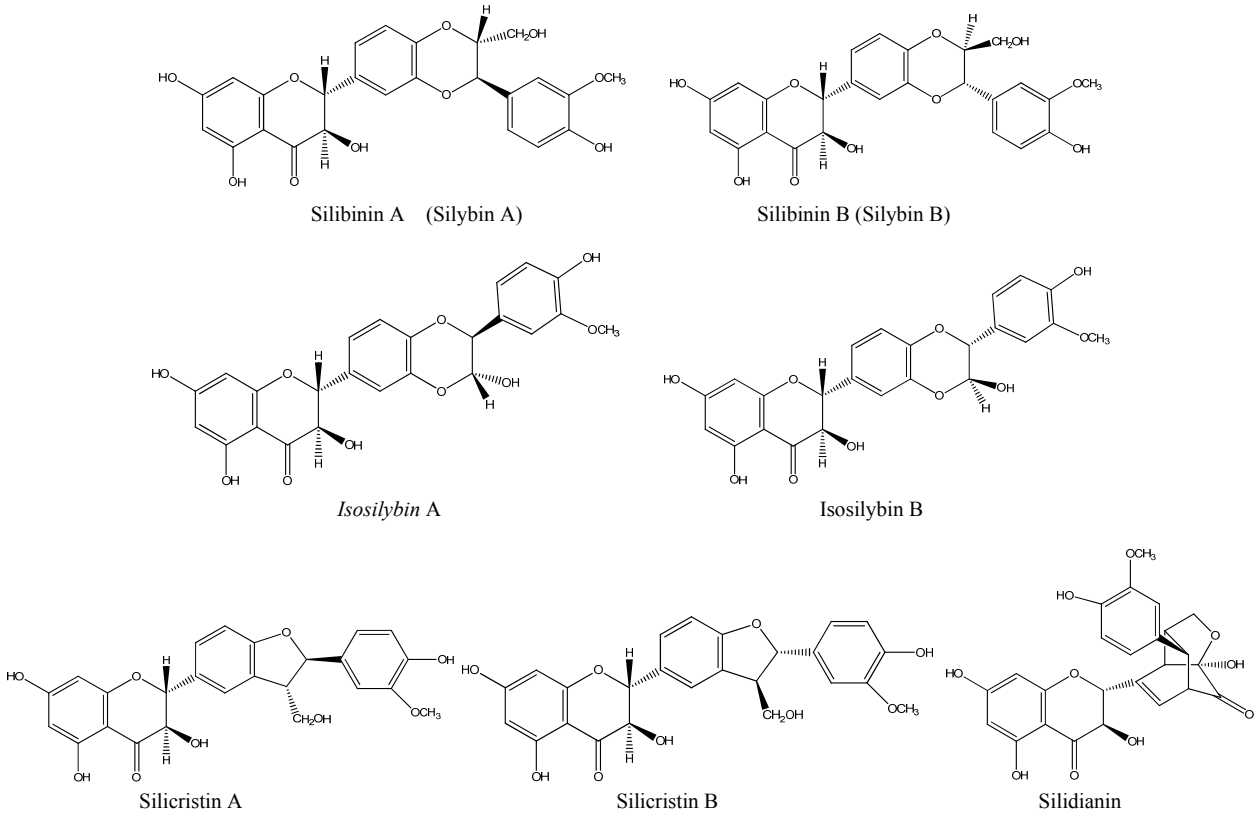

Figure 1. Flavonoligans of Milk thistle extracts.

material for capsulation. Silibinin in plasma is rapidly distributed in liver, lungs, skin, prostate, and pancreas, and the maximum level is achieved within just one hour after oral administration. About 70% of silibinin binds to plasma proteins, and the concentration in the bile is 100 times higher than in plasma. In humans, higher doses of silibinin are subjected to I and II metabolism phases, whereby the liver microsomes produce one dominant demethylated metabolite in the first phase, while three mono-hydroxyl metabolites and one dihydroxyl metabolite are generated in small amounts. Silibinin monoglucuronide, silibinin diglucuronide, silibinin monosulfate and silibinin glucuronide sulfate are produced in the second phase, and then *O*-demethyl silibinin glucuronide and silibinin triglucuronide. The halftime elimination of silymarin (silibinin equivalent to 25%) is 6.32 h and it is mainly excreted via bile. Only 1 to 7% of silymarin is excreted into the urine [13-15]. However, the urinary excretion of 31 detected metabolites was followed by clearance for 24 h while substantial amounts of glucuronides, sulfo-glucuronides and diglucuronides were excreted within 48 h. A non-marginal entero-hepatic recirculation, which preferentially occurs for silymarin sulfate–glucuronides and diglucuronides, may be the explanation for a delayed clearance of the conjugated metabolites. Although, polyphenols are drastically modified by the colon microbiota, no microbially derived compounds were detected after the oral administration of silymarin [16].

Silymarin side effects: German Commission E approved silymarin for various liver conditions and stated that it had no side effects at the recommended doses. It is safe for use in pregnancy (560 mg/day), in children (20-50 mg intravenously) and in elders over 75 years (420 mg/day). The toxic effects of the milk thistle extracts have not been recorded at a dose of 1200 mg/day, although it was administered at doses of 160-600 mg/day divided in three equal doses in most of the clinical studies. However, mild allergies were recorded at doses greater than 1500 mg a day. A few patients reported heartburn, diarrhea, bloating, flatulence and dyspepsia. It is proved that silymarin is well-tolerated and safe for use up to 41 months [17].

Silymarin interaction with drugs: Silymarin inhibited the enzymes of phase I metabolism: CYP2E1, CYP2D6, CYP2C19, CYP1A2 and CYP2A6 and the enzymes of the phase II metabolism such as uridine diphosphoglucuronyltransferase (UGT) 1A1 isozymes, and due to this ability it could cause interactions with the drugs that are metabolized with the same enzymes. It was also detected that silymarin, in a dose-dependent manner, inhibited P-glycoprotein, which is responsible for the excretion of the xenobiotics from the liver into the hepatobiliary duct and could cause the drug accumulation that is excreted via those routes. However, only the interactions of silymarin with metronidazole (increases the clearance of metronidazole by about 30%), pyrazinamide (prevents its excretion) [13] and warfarin (increases the risk of bleeding) have been detected so far [18].

Effect on Alzheimer's disease and dementia: Alzheimer's disease (AD) is characterized by progressive cognitive impairment and protein plaque deposits. The extracellular aggregation of amyloid β (Aβ-peptide), a protein plaque component, found in the brain cells of the patients with AD, presents the major histopathological change in this disease. It is assumed that Aβ-peptide generates H_2O_2, which is instantly converted into a highly reactive hydroxyl radical which causes an oxidative stress. In the *in vitro* study on human neuroblastoma SH-SZ5Y, it was proved that silibinin, in a dose-dependent manner, inhibited the aggregation of Aβ-peptide, decreased Aβ-peptide cytotoxicity, reduced hydrogen peroxide production and cell injury. Silibinin is a powerful anti-oxidative agent which increases GSH and superoxide dismutase (SOD) levels

and inhibits lipid peroxidation. The study confirmed the anti-oxidative effects of silibinin and assumed that the decrease of H_2O_2 production, induced by Aβ aggregation, was the main route for its neuroprotective activity [19].

Pretreatment with silibinin in a dose-dependent manner improved the spatial memory and energy metabolism in the brain, reduced cholinergic dysfunction and prevented the accumulation of lipid peroxides in mice after streptozotocin (STZ) injection had been administered. Several studies reported that the impaired brain energy metabolism was the key event in AD pathophysiology. It was demonstrated that dysregulated Ca^{2+} intracellular homeostasis and increased free radical formation disturbed the mitochondrial function and caused the changes in brain energy metabolism. The pretreatment with silibinin eliminated the disruption of the metabolism energy (the defects in the electron-transport chain of mitochondria and the reduced production of ATP in the mice brains that STZ caused), reduced the levels of reactive oxygen species (ROS) and malonyldialdehyde (MDA), and increased GSH and Ca^{2+} levels in the synaptic space. Loss or down-regulation of the neuronal nicotinic acetylcholine receptors (nAChRs) was also observed in AD. Silibinin reduced the anti-acetylcholinesterase action, restored the function of acetylcholinesterase and mRNA expression in a dose-dependent manner in the brain of the mice that were treated with STZ. In addition, it prevented the decreased expression of the nAChRs (particularly the subtypes which are predominant in the brain and cause the impairment of the cognitive functions) [20].

The cognitive deficit was observed in patients with diabetes and this disease was often cited as a risk factor for the development of vascular dementia and Alzheimer's disease. It is supposed that the stimulation of ubiquitous and inducible heme oxygenase (HO)-1, caused by silibinin, was accountable for the silibinin antioxidant and anti-inflammatory activity as the HO isoforms catalyzed the conversion of heme to carbon monoxide and bilirubin, and its increased expression was the response to the oxidative stress in the CNS. The level of HO-1 was reduced in the forebrain and cerebellum of the diabetic mice, while it was unexpectedly increased in the brainstem; treatment with silibinin caused a considerable induction of HO-1 in the forebrain and cerebellum, but it provoked inhibition in the brainstem. Silibinin decreased isoprostanes and 8-OH deoxyguanosine (markers of lipid peroxidation and DNA damage, respectively) in the hippocampus and hypothalamus of the treated diabetic animals. Silibinin protected DNA from the oxidative stress and therefore, it could be considered as an important phytotherapeutic agent in the treatment strategy of the accompanying diabetic side effects on the CNS [21].

Silibinin's favorable effects on the cognitive function was confirmed by *in vivo* studies on mice whose memory loss was caused by methamphetamine (METH), which reduced the ability to recognize a new object and the five-fold choice. METH provokes hallucinations and delusions in humans, and METH chronic use causes cognitive deficits after withdrawal. Silibinin neutralized the METH effects and reduced the time necessary to recognize the new items (impairment in the novel object recognition test). Silibinin administered to the healthy mice did not cause any changes in the serotonin and dopamine levels, but annulled the effects that METH had on these systems if it had been given as a pretreatment. It is believed that silibinin inhibited monoamine oxygenase (MAO) and reduced the dopamine and serotonin metabolisms. The silibinin antioxidant effect reduced the peroxynitrile concentration which caused the dopamine and serotonin concentrations to be maintained in the brain. Silibinin anti-inflammatory effects protected microglial activation in the midbrain, striatum, thalamus, orbitofrontal and insular cortices provoked by METH. It additionally gave protection from the neurotoxicity caused by lipopolysaccharides that initiated a pro-inflammatory cascade and released the cytokines. Therefore, silibinin can have a potentially important role in the therapy of drug addicts [22].

Effect on Parkinson's disease: The possible causes of the degeneration of the dopaminergic neurons in Parkinson's disease (PD) are: reduced GSH levels, DNA damage, iron deposition and primarily, the oxidative stress that impairs the oxidative phosphorylation and energy metabolism that lead to dopaminergic neuron death. Silymarin postponed the oxidative damage of the neurons (reducing the synthesis of ROS and maintaining SOD levels) that had been developed in many neurodegenerative diseases by inhibiting MAO in glial cells, by stimulating peroxidation product secretion and, probably, by stimulating the synthesis of ribosomal RNA as it bonds via the estradiol receptors [23].

Anticarcinogenic effects: The anti-tumor effect of silymarin was detected in tumors induced in the epidermis of mice in 1994 [24]; this led to a number of studies. Silymarin is classified in the group of effective chemoprotective and chemopreventive agents in the treatment of various cancer conditions. In 1999, it was reported that silibinin was probably responsible for its anti-proliferative effect since it inhibited DNA synthesis in prostate carcinoma LNCaP and DU145 cells, breast carcinoma MCF-7 cells and cervical carcinoma A431 cells [25].

Effect on skin cancer: Silymarin demonstrated the capacity to reduce the expression of the TNF-α endogenous promoter in SENCAR mice with chemically induced skin cancer. Orally administered silymarin not only inhibited the tumor growth, but it further decreased the volume of the existing tumors (80-97%) with no signs of toxicity to the animals. Silymarin inhibited all the proteins of the mitogen-activated protein kinase (MAPK) family: ERK1/2, JNK and p38 and induced apoptosis; it was irrelevant whether silymarin had been administered orally or topically [26-28]. It can be applied in a benign condition such as premalignant keratosis. It was found that silymarin possessed a protective effect in SKH-1 mice with UVB radiation-induced skin tumor initiation, tumor growth and complete carcinogenesis. Silymarin significantly affected the inhibition of UVB-induced sunburns and the formation of apoptotic cells, skin oedema, catalase activity reduction, induction of expression and activity of cyclooxygenase (COX) and ornithine decarboxylase (ODC), when applied either topically or orally before or after exposure to UVB. Regulating the p53-p21 cascade that protected the DNA from damage, silymarin reduced a number of the thymidine dimer-positive cells and slowed the kinetics of the DNA damage by stimulating its reparation after the UV exposure. Silymarin induced the level of Cip1/p21 protein in the epidermis of the mice during the acute radiation with UVB rays, thus inhibiting the cell proliferation and expression of the genes responsible for the cell growth in a cascade way [29-31]. In addition, in novel studies, it was found that the main constituent of silymarin, silibinin, saved the cells from apoptosis through inhibition of IGF-1R activation followed by repression of ERK1/2 and JNK phosphorylation [32] and induced autophagy protection of human carcinoma A431 cells from UVB-induced apoptosis [33] in UVB-irradiated A431 cells. Furthermore, silibinin contributed to the reduction of A, E and D1 cyclins, increased the protein levels of CDKIs, Cip1/p21 and Kip1/p27 in the tumor cells, inhibited the activity of cyclin-dependent kinase (CDK) and thus decreased the proliferation and growth of the tumor cells. Therefore, it is expected that the silibinin non-toxic photo-protective effects will be

considered as a potentially useful chemo-preventive agent for the treatment of skin cancer [34].

Effect on hepatocellular carcinoma: The positive effects of silymarin in the treatment of hepatocellular carcinoma were reported in several *in vivo* and *in vitro* studies. In the study conducted on Wistar rats with induced hepatocellular carcinoma, treatment with silymarin (1000 ppm orally, 16 weeks before or 5 weeks after tumor induction) regulated the increased levels of aminotransferases (AST and ALT), phosphatases (ACP and ALP), lactate dehydrogenase, gamma-glutamyl transferase and 5'-nucleotidase, tumor marker alpha-fetoprotein (AFP) and carcinoembryonic antigen (CEA). The reduction in MDA-DNA formation was recorded in the animals with tumors that had received silymarin compared with the control group [35]. The research on H4IIE hepatocytes, where the tumor had been induced with a high ethanol concentration, confirmed the hepatoprotective effects of SIL: silibinin inhibited CYP2E1 (but not alcohol dehydrogenase) and ethanol metabolism, reduced the ROS level, while it also reduced the proliferation and progression of the cell cultures in the rats suffering from hepatocellular carcinoma at a dose of 10 μM [36], which was consistent with the findings that silibinin inhibited cell proliferation, matrix metalloproteinase 2 enzymatic activity, NO production and ERK 1/2 phosphorylation in a dose-dependent manner in the HepG-2 cell line [37]. Furthermore, recent studies confirmed that pretreatment with silymarin down-regulated the cytokines-gene expression in the human hepatocellular carcinoma cell lines susceptible to amphotericin B-induced oxidative stress [38].

Effect on breast cancer: Silymarin inhibited both anchorage-dependent and anchorage-independent cell growth in dose- and time-dependent manners in the treatment of human breast carcinoma. Its effects on the cell growth and proliferation were probably connected with an induction of up to 19-fold in the protein expression of CDK inhibitor Cip1/p21G1 which caused the arrest in the cell cycle progression [39]. In addition, silibinin had a synergistic effect with anticancer chemotherapeutics such as doxorubicin, cisplatin and carboplatin in the human breast cancer cells. Silibinin, in a dose-dependent manner, inhibited the expression of matrix metalloproteinase 9 (MMP-9), preventing the transcription of this protein by blocking the pathway that went through MAPK and by suppressing the AP-1 proteins with oligonucleotides containing the sequence for the AP-1 DNA at the binding site of the MMP-9 , preventing the expression of MMP-9 in the *in vitro* cultures of the human breast cancer cells [40, 41]. The results were even more impressive with the use of silibinin-loaded lipid nanoparticles containing D-α-tocopheryl-polyethyleneglycol 1000 succinate (TPGS) [42]. It was recently found that silibinin was a Wnt/β-catenin signaling inhibitor by suppressing Wnt co-receptor LRP6 expression at the transcription level, and that the anti-cancer activity of silibinin was associated with its inhibitory effect on Wnt/LRP6 signaling [43].

Effect on ovarian cancer: Silipide (IDB 1016 - silibinin complex with phosphatidylcholine), administered orally to hairless mice which had been embedded with the xenograft of human ovarian cancer cells, contributed to a significant reduction in the tumor mass by 78% [44]. It was found that silibinin enabled the overcoming of resistance to paclitaxel. It reduced the invasiveness of the paclitaxel-resistant tumor cells in another *in vitro* study on paclitaxel-sensitive and paclitaxel-resistant cells [45]. If these effects are proved in *in vivo* conditions, silibinin in combination with paclitaxel could become a part of the strategy in the treatment of tumors in patients resistant to paclitaxel.

Effect on cervical cancer: Silymarin has a possible application in chemo-prevention and in the treatment of cervical carcinoma. Silymarin caused the apoptosis of HeLa cells of human cervical cancer at low doses (80 μmol/L) and cell necrosis at high doses (160 μmol/L). The apoptosis caused by silymarin via MAPK activation was due to chromatin condensation and nuclear fragmentation of the apoptotic cells [46]. It was found that silibinin inhibited hypoxia-induced HIF-1α accumulation and HIF-1 transcriptional activity in human cervical (HeLa) and hepatoma (Hep3B) cells, activated Akt, and reduced hypoxia-induced vascular endothelial growth factor (VEGF) released by HeLa and Hep3B cells, and finally, inhibited cell proliferation [47].

Effect on bladder cancer: Silibinin inhibited cell growth and caused cell cycle arrest in the G1 and G2/M phases of the highly invasive TCC-SUP cells (apoptosis), and the G1 phase of the cell cycle in T-24 of human bladder carcinoma cells (without apoptosis), respectively. Silibinin acted in the T-24 cells through erbB1-mediated mitogenic signalization, inhibiting the cell growth without causing apoptosis [48]. It was found that orally administered silymarin, at a dose of 1000 ppm for 8 weeks during the initiation of cancer, or 24 weeks after the initiation, caused statistically significant reduction in the number of pre-neoplastic lesions and the rate of the disease progression of bladder cancer in the *in vivo* study on ICR mice with induced bladder cancer [49]. Furthermore, silibinin inhibited the tumor RT4 xenograft growth by decreasing the tumor volume and weight, reduced cell proliferation and increased apoptosis in the tumors in the *in vivo* studies. The assumed mechanism of action was decrement of the surviving protein expression and its nuclear localization [50].

Effect on lung cancer: Silibinin slowed the growth of A549 human lung cancer cells and enhanced the effect of doxorubicin modulation of the NF-kB route thus inhibiting the resistance to doxorubicin, significantly reduced the levels of iCOX-2 and diminished the adverse effects of the chemotherapeutics [51]. In addition, silibinin reversed the resistance to etoposide and to doxorubicin in human small-cell lung carcinoma and acted synergistically with the chemotherapeutics [52]. Oral pretreatment with silibinin statistically reduced the number of tumors and large tumors (> 1.5 mm) in relation to the control group in the study on A/J mice, where lung cancer had been provoked by urethane. This study evaluated the density of blood vessels where it was found that silibinin decreased the number of highly vascularized tumors and the density of the blood vessels in the vascularized tumors. Finally, silibinin decreased the levels of iNOS and COX-2 enzymes whose expression increased in the process of angiogenesis responsible for the tumor development [53]. The pretreatment with silibinin reduced the phosphorylation of STAT1 and STAT3 induced by cytokines responsible for the proliferation of the A549 human lung cancer cells under the *in vitro* conditions. Silibinin inhibited the AP-1 transcription factor of DNA, blocked the MAPK cascade and inhibited iNOS at the concentration of 200 mmol/L, indicating that it could be a potential chemo-preventive agent for lung cancer treatment [54]. Silibinin-meglumine, a water-soluble form, inhibited the growth of non-small-cell lung carcinoma mouse xenografts as efficiently as the "gold standard" gefitinib and reversed the resistance of gefitinib-unresponsive tumors [55].

Effect on prostate cancer: SIL possessed anti-carcinogenic activity both with hormone-dependent and hormone-independent prostate cancer and its component isosilybin B was demonstrated as the most effective suppressor of the topo IIa gene promoter activity. The inhibiting effect on the prostate cancer cells in the LNCaP, PC3 and DU145 cultures that SIL exhibited was not registered in the

normal prostate epithelial cells [56]. Silibinin induced increased expression of cytokeratins and chromogranin A and modulated the phosphorylation status of retinoblastoma (Rb) and Rb-related proteins in human prostate carcinoma LNCaP cells. Furthermore, it caused a considerable reduction in the prostate specific antigen (PSA) in the LNCaP cells, which was important for cell growth inhibition. SIL down-regulated epidermal growth factor receptor (EGFR) signaling in PCA, which consequently led to the cascade that inhibited CDK and cancer cell cycle arrest. Silibinin/silymarin inhibited tumor growth factor (TGF-α) mRNA expression and decreased secreted and cellular levels of TGF-α in both LNCaP and DU145 cells. It not only reduced NF-κB signaling activity in DU145 prostate cancer cells, but also increased the sensitivity of the cells to TNFα-induced apoptosis that was commonly inhibited [57]. Additionally, the silipide/silibinin effect in prostate cancer was accomplished by reducing the level of insulin-like growth factor-1 (IGF-1) and/or elevating the level of insulin-like growth factor-binding protein-3 (IGFBP-3) in TRAMP mice [58]. A dose-dependent decrease was also observed in cyclin B1, cyclin E, and cyclin A protein levels by silibinin, which suggested that oral silibinin blocked PCA growth and progression via modulation of the tumor IGF-IGFBP-3 axis and cell cycle regulation [59].

Effect on oral cancer: Silymarin was proved to decrease the viability of human pharynx squamous carcinoma (FaDu) cells in an *in vitro* study. Apoptosis of the FaDu cells was preceded by 10-fold diminished Akt phosphorylation and five to six-fold upregulated expression of the phosphatase and tensin homolog. This caused the inhibition of Bcl-2 expression and elevation of caspases activity which led to the apoptosis of the FaDu cells [60].

Effect on gastric cancer: Silibinin dose-dependently inhibited the growth of human gastric carcinoma SGC-7901 cells. Namely, it provoked G2 phase arrest in the cell-cycle progression of the SGC-7901 cell line. SGC-7901 cells growth inhibition was observed after silibinin significantly decreased the expression of p34cdc2 levels and induced p53 and p21 expression. A final common pathway, involving the activation of caspase, is usual for most chemotherapeutic agents. Surprisingly, silibinin caused apoptotic death of SGC-7901 cells in a caspase-independent manner [61].

Effect on colon cancer: Silibinin, in a dose-dependent manner, induced cell cycle arrest in the G2/M phase of the FET and GEO cell lines of human colon cancer and in the poorly differentiated HCT116 cells in the G1 phase. As with prostate cancer, silibinin inhibited CDK and the activity of D1, and E cyclins in colon carcinoma, but did not reduce COX-2 expression and activity. It also inhibited autocrine TGF-α secretion, and its binding to the EGFR and the EGFR expression [62]. Significant reduction in the frequency of colonic ACF (aberrant crypt foci) in a dose-dependent manner in F344 rats was achieved after dietary administration of silymarin (100, 500 and 1,000 ppm), either during or after 4 weeks of exposure to the carcinogens [63]. Orally administered silibinin, before or during the tumor initiation, caused statistically significant reduction in the number of formed tumors, while it prevented the development of macroscopically visible tumors (only polyps were developed) during the entire period of the study on Wistar rats with induced colon cancer, using 1.2-dimethylhydrazine (DMH). Silibinin modulated the enzyme activity of an intestinal micro flora, reduced oxidative stress in the colon and prevented the retoxification of DMH (it inhibited cytochrome P450 activity, the creation of the DMH carcinogenic metabolites, and also induced the enzyme induction of second phase metabolism which facilitates DMH excretion). Furthermore, silibinin facilitated the establishment of the antioxidant enzyme functions [64]. Another *in vivo* study reported that silibinin had an anti-proliferative effect against human colorectal carcinoma HT29 xenograft and an antiangiogenic effect, which is very important in preventing metastasis. The silibinin antiproliferative activity was achieved via down-regulation of ERK1/2 and Akt phosphorylation, as well as the cyclin D1 expression. The silibinin antiangiogenic activity *in vivo* was connected with the inhibition of iNOS, COX-1, COX-2, hypoxia-inducing factor-1α (HIF-1α) and VEGF [65]. Therefore, silibinin could be considered as a promising chemo-preventive agent for the treatment of colon cancer.

Effect on pancreatic cancer: Dietary silibinin suppressed the growth of human pancreatic carcinoma BxPC-3 and PANC-1 tumor xenografts (representing early and late clinical stages of the disease, respectively), inhibited the cell proliferation and induced the apoptosis in BxPC-3 tumors in *in vitro* and *in vivo* studies. It caused a strong G1 arrest in BxPC-3 cells in a dose-and/or time-dependent manner and a moderate arrest in PANC-1 cells. The authors assumed that silibinin inhibited the G1/S cell cycle progression via down-regulation of cyclin D1 and CDK4/2 or by up-regulation of CDKIs [66].

Effect on leukemia: Silibinin, in a dose-dependent manner, inhibited the proliferation of the human promyeolitic leukemia HL-60 cell line and induced cell differentiation, which was even more pronounced when combined with 1,25-(OH)2D3 vitamin via both protein kinase C α and β level elevation and enhanced the activity [67]. Silymarin strongly inhibited Akt protein levels in K562 leukemia cells, caused caspases activation and PARP cleavage, which inhibited the growth of the human leukemia cells and induced their apoptosis [68]. This was confirmed for both silibinin A and silibinin B on the same cell culture, which showed that the diastereoisomers were more potent than the silibinin racemic mixture [69].

Nephroprotective effect: Silibinin-hemisuccinate (also silicristin and silidianin) stimulated the proliferation of Vero cells similar to fibroblasts (African green monkey, *C. aethiops*) by about 23% more than the control group, increased the synthesis of DNA molecules in the cells, protein synthesis, and reduced the toxic effects of paracetamol. In addition, silibinin had a positive effect on cells chemically damaged by cisplatin and vincristine, but only when it had been administered before these drugs [70]. Silymarin treatment of diabetic nephropathy patients at the final phase caused the normalization of immunoregulatory defects in *in vitro* studies [71]. Silymarin, in a dose-dependent manner, acted preventively in ischemia and reperfusion after renal injury and decreased the morphological changes that included the dilation and vacuolization of tubules, pelvic inflammation, interstitial inflammation, perirenal fat infiltration, and glomerular and tubular necrosis caused by the nephrotoxic drugs: cisplatin, doxorubicin, aminoglycosides, vincristine, cyclosporine and acetaminophen in *in vitro* studies [72].

Neuroprotective effect: Silymarin had a neuroprotective effect in rats with brain damage caused by acetaminophen in an *in vivo* study. In the group of rats treated only with silymarin, increased GSH and ascorbic acid levels and slightly increased SOD levels were observed in relation to the control, while the MDA levels were significantly reduced in the group that had received silymarin three days before paracetamol was administered. It is supposed that silymarin binds nonenzymatic ROS into CNS, or it increases the protein synthesis involved in the antioxidant activity, and, therefore, the authors recommended the use of silymarin in the treatment of neurodegenerative and neurotoxic disorders [73]. Silibinin, administered intragastrically 30 min before a permanent middle

cerebral artery occlusion, significantly alleviated neurological deficit, reduced an infarct volume and suppressed a brain oedema, which was assumed to have been accomplished by up-regulation of pAkt, pmTOR, HIF-1α, Bcl-2 and by down-regulation of Bax, and NF-κB in the ischemic brain tissue after a stroke [74]. The pretreatment with silymarin had a statistically significant neuroprotective effect in the *in vivo* studies on the rats with the neurotoxicity caused by Na-fluoride since it increased the activity of the antioxidant systems (SOD, CAT and GSH) [75].

Cardioprotective effect: Fat infiltration in the myocardium of obese db/db mice was induced to correspond to myocardium steatosis of obese and diabetic patients or to patients with non-alcoholic steatosis. Silibinin decreased the ALT levels in the serum three times in comparison with the untreated group and reduced the insulin and glucose levels in the serum during the fasting period causing the elimination of the generated insulin resistance. The treatment with silibinin significantly decreased myocardial injury and mitigated morphological abnormalities of the majority of the myocardiocytes, reduced the damage caused by oxidative stress and maintained the GSH level. Silibinin completely eliminated the increased TNF-α expression and decreased the expression of genes for IL-6, which was observed in the obese mice [76]. Silibinin attenuated the phenylephrine-induced hypertrophic response in H9c2 cardiac cells, up-regulation of atrial natriuretic peptide and the increase of cellular protein levels by blocking ERK1/2 and Akt signaling pathways. Pretreatment of the H9c2 cells with silibinin also protected them from H_2O_2-induced cellular stress [77].

Hypocholesterolemic and hypolipidemic effects: Silymarin had the same effect on serum cholesterol as probucol (an antioxidant hypocholesterolemic drug) in rats on a high-cholesterol diet. Silymarin increased the HDL level mildly, reduced the cholesterol content in the liver and stimulated LDL cholesterol excretion from the liver, which contributed to the anti-atherogenic effect [78]. The results were confirmed in a study in which silymarin dose–dependently reduced total cholesterol, LDL and triglyceride levels in rabbits on a high cholesterol diet and thus inhibited the formation of atherosclerotic plaques [79]. In a four-month randomized double blind study silymarin, administered three times a day (200 mg tablet) with a standard therapy in non-insulin dependent hyperlipidemic patients caused a statistically significant reduction in total cholesterol, LDL and triglyceride levels compared with the control group which received only a standard therapy with placebo [80]. Silibinin-cyclodextrin significantly decreased triglyceride levels in relation to the placebo group in a double-blind six-month-study with patients with poorly controlled non-insulin dependent diabetes [81]. The treatment with silymarin, during one month, significantly reduced total cholesterol, LDL and VLDL levels, and increased HDL levels slightly (silymarin showed the same effect as lovastatin) in the randomized clinical study with 57 patients with dyslipidemia [82].

Effect on diabetes: In a study on Wistar rats, silibinin showed a significant hypoglycemic effect in the liver, inhibiting gluconeogenesis during fasting and glycolysis in satiety, respectively, opening the possibility of the clinical application of silibinin in the treatment of diabetes [8]. The blood glucose level of patients with diabetes mellitus II, in the group who received silymarin, was statistically significantly lower after four months of administration in relation to the placebo group, where the increased glucose level was recorded in a double-blind placebo-controlled clinical study. The patients treated with silymarin, compared with the control group, had a slight, but not statistically significant weight loss and reduction in blood pressure [80].

A long-term treatment with silymarin reduced lipoperoxidation and insulin resistance in the patients with alcohol liver cirrhosis who had similar disease histories and pathological characteristics, like patients with alcoholic liver disease and NASH. Namely, the significant decrease in fasting blood glucose levels, insulin level decrement (40%), reduction of exogenous insulin needs, decrease of mean daily blood glucose levels, reducing daily glycosuria and HbA1c levels, were recorded in the group that received silymarin together with the standard therapy after only 4 months of administration [83]. In addition, the rise in glucose and fatty acid levels in the diabetics led to ROS increment accompanied by insulin resistance, dysfunction of the pancreatic β-cells and insulin secretion – the effects that may be significantly mitigated due to the silymarin antioxidant action [84].

Effect on sepsis: The endotoxins (lipopolysaccharides) that activate macrophages and produce cytokines and lead to oxidative stress are considered to have the key role in sepsis development and multiple body function disorders. Pretreatment with silymarin had the same effect as pretreatment with antioxidant *N*-acetylcysteine (NAC) in a study on rats with induced septic lung and brain damage. The survival rate in the group treated with NAC was the same as in the group treated with silymarin after 72 h. Both silymarin and NAC effectively eliminated the increase of TNF-α, IL-1 and IL-6, LDH and MDA caused by sepsis. Silymarin inhibited the oxidative injury caused by the lungs and brain sepsis owing to its ability to balance the antioxidative status and to regulate the inflammatory mediators [85].

Effects on osteoporosis and osteoarthritis: Seidlova-Wuttke *et al.* were the first to observe that silymarin had a raloxiphen-like effect on bone structure [86]. In another study it was demonstrated that silymarin did not affect the luteinizing hormone (LH) level, or cholesterol, LDL and HDL levels in rats after ovariectomy, but it increased the uterine weight, height and hypertrophy of endometrial luminal epithelium compared with the control group, probably due to partial binding to ERα receptors in the uterus. However, silymarin augmented the density of the trabecular part of the long bones, did not affect the osteocalcin levels, but reduced the alkaline phosphatase levels and increased the calcium and phosphorus levels in the serum and stimulated the secretion of parathyroid hormone 5.5 times more than that with ethinyl estradiol. The potential use of silymarin against osteoporosis development was confirmed in this study, assuming that its effect could not be achieved only through the estrogen receptor, but some other mechanisms of action should be included [87].

The destructive changes of moniodoacetate-provoked osteoarthritis in rats were ameliorated with both celecoxib and silymarin. Celecoxib (100 mg/kg) and silymarin (50 mg/kg) applied separately or in parallel (celecoxib in a dose of 100 mg/kg and silymarin in a dose of 25 mg/kg) lowered the osteoarthritis MDA and NO levels. A combination therapy, containing both celecoxib and silymarin, resulted in IL-1β reduction in the serum more significantly than the application of each drug separately. Silymarin potentiated the effect of celecoxib, and applied together, they also amended the histopatological findings (fibrillated surface, presence of osteoclasts and connective tissue) in the rats with induced osteoarthritis. The authors proposed further testing of celecoxib and silymarin as a combined osteoarthritis therapy in humans [88].

Preventive effect for fetal alcohol syndrome: Research on the influence of silymarin on fetal alcohol syndrome was made on Fisher female rats with confirmed pregnancies. The hatchlings from the ethanolic group had significantly less mass, less total brain mass

and less mass of the corpus callosum than the control and in the group treated with silymarin, while the female group, along with ethanol was fed with silymarin during the pregnancy, gave birth to offspring of a nearly identical body mass as the control group [89]. In another study, exposure to alcohol during fetal development influenced the spatial orientation of the adult rats, and the spatial memory impairment was more pronounced in the female rats, but was significantly lower in the group pretreated with silymarin. It is assumed that silymarin scavenged the free radicals produced by ethanol oxidation thus preventing the potential negative effects on the migration of the cells, then, it acted on the hippocampus stimulating the DNA and proteins synthesis, maintaining the number of cerebral Purkinje cells, or it acted through the NMDA receptors [90].

Prolactive effect: Female rats were treated either with a standardized extract (silymarin BIO-C ® = Pilùtatte ® - Sil ®) or with metoclopramide intraperitoneally (the control). Sil ® caused a statistically significant increase in body weight and serum prolactin levels in the female rats compared with the control. After interruption of the treatment with Sil ® after 66 days, the prolactin level was still elevated. Bromocriptine, an agonist of D2-dopamine receptors, dose-dependently, significantly reduced the prolactin levels in the serum induced by Sil ® treatment. It is clear that silymarin increased the circulating prolactin levels in the female rats and that the dopamine D2 receptors were probably at least partially involved in this effect [91].

Immunomodulatory effect: Silymarin modulated CD4+ splenocytes proliferation in mice activated αCD3 mAb (anti-mouse CD3 monoclonal antibody) in the *in vitro* study, but did not have a direct mitogenic activity and cytotoxic effects. Silymarin, at the concentration of 50 μM, significantly inhibited the CD3-induced NF-kB nuclear translocation and production of IL-2 and IFN-γ in the activated splenocytes after 72 h in relation to the untreated cells and showed immunomodulatory potential [92]. Silibinin exerted anti-inflammatory and anti-fibrotic effects on the CD14+ cells by NF-kB mediated inhibition of TNF-α, IL-10, TNF-β1, PGE_2 and NO production [93]. The down-regulation of NF-kB, induced by pretreatment with silibinin, prevented mouse-ovalbumin induced allergic airway inflammation in the *in vivo* study [94]. Silibinin inhibited the leukotriene formation significantly by Kupffer cells *in vitro*, inhibiting the 5-lipo-oxygenase pathway [95], which is consistent with the studies indicating that sylimarin and its constituent silibinin inhibited arachidonic acid metabolism *in vitro* [96, 97].

Effect on burns: The burns caused by hot water in Wistar rats caused severe oxidative stress and tissue damage. The dermal or dermal/oral application of silymarin restored the increased LDH and MDA levels and decreased the GSH levels to the values of the control group. When the burns were treated with silymarin, the higher levels of TNF-α were reduced compared with the group without the silymarin treatment. Myeloperoxidase activity, which indicated the increased infiltration of neutrophils in the damaged tissue, was significantly increased 48 h after the burns had been caused, but was fully restored to the level of the control group after the silymarin treatment. Silymarin eliminated the increased thromboplastin activity observed in the burns and reduced the oxidative damage of the epidermis and dermis caused by heat [98].

Effect on cholestasis: It was shown that silibinin had a therapeutic application in cholestasis because it affected the transport of tauro- and glyco-conjugated bile salts through the canalicular membrane in isolated hepatocytes of Wistar rats. Silibinin possessed an anti-cholecystic effect on estradiol-17ß-D-glucuronide and taurolitocholates, and induced cholestasis by increasing the level of cytosolic Ca^{2+} through cAMP, which opened the possibility for further research of the silibinin activity in the prevention of gallstone formation [99].

SIL effect in cosmetics: Cosmetic products containing silymarin to treat rosacea and to maintain the skin moist and lips smooth are already on the market. As the aging processes are accompanied by oxidative stress, SIL is expected to be used in cosmetic preparations against wrinkles and aging skin. The ability of silymarin to protect the skin from epidermal hyperplasia and DNA epidermal cell damage caused by UVB radiation makes SIL appropriate and benefitting to be used in cosmetics preparations with a UV protection factor in sunscreens [100].

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Biomedical Properties of Edible Seaweed in Cancer Therapy and Chemoprevention Trials: A Review

Farideh Namvar[a,e*], Paridah Md. Tahir[a], Rosfarizan Mohamad[a,b], Mahnaz Mahdavi[c], Parvin Abedi[d], Tahereh Fathi Najafi[c], Heshu Sulaiman Rahman[f] and Mohammad Jawaid[a]

[a]*Institute of Tropical Forestry and Forest Products (INTROP), Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia*

[b]*Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia*

[c]*Shiraz Branch, Islamic Azad University, Shiraz, Iran*

[d]*Reproductive Health Center, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran*

[e]*Department of Medicine, Applied Biology Research Center, Mashhad Branch, Islamic Azad University, Mashhad Iran*

[f]*Department of Microbiology and Pathology, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia*

farideh.namvar@gmail.com

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This review article summarizes *in vitro* and *in vivo* experiments on seaweed anticancer activity and seaweed chemical components. Seaweed use in cancer therapy, chemopreventive randomized control trials (RCTs) and quasi-experiments are discussed. The literature reviewed in this article was obtained from various scientific sources and encompasses publications from 2000-2012. Seaweed therapeutic effects were deemed scientifically plausible and may be partially explained by the *in vivo* and *in vitro* pharmacological studies described. Although the mechanisms of action remain unclear, seaweed's anticancer properties may be attributable to its major biologically active metabolites. Much of the seaweed research outlined in this paper can serve as a foundation for explaining seaweed anticancer bioactivity. This review will open doors for developing strategies to treat malignancies using seaweed natural products.

Keywords: Anticancer, Apoptosis, Cancer Therapy, Chemoprevention, Cytotoxicity, Macro algae, Seaweeds.

The number of patients diagnosed with cancer has been increasing each year, and cancer remains a major cause of death. According to the WHO, cancer accounts for approximately 13.3% of deaths worldwide. Rates of occurrence are expected to rise by 50% worldwide over the next 20 years [1]. Individuals diagnosed with cancer struggle with managing symptoms and coping with treatment side effects. Many clinicians and patients turn to alternative or traditional medicine to avoid the unpleasant or dangerous side effects inherent to modern cancer therapies. Recently, chemopreventive strategies using natural products and dietary supplements have become attractive options as potential means of controlling cancer incidence [2]. Gathering information on and analyzing purported traditional medicine practices is an important element of anticancer drug development involving natural products. For thousands of years, humans have utilized certain marine algae for multiple purposes [3]. Seaweed medical use dates to at least 5,000 years ago in ancient China, where the emperor ShenNung listed seaweed as a medicine in his classic pharmacopeia, the Pen Ts'ao [4]. Seaweeds have been used in traditional Chinese medicine to treat goiter, scrofula, swelling and pain of testes, and edema [5]. Additionally, some seaweed species have also been used in modern Chinese medicine to treat arteriosclerosis, skin diseases, high blood pressure, hepatosplenomegaly, neurosis, angina pectoris, acute esophagitis, and chronic bronchitis [6]. Marine algae belong to one of two groups: microalgae or macroalgae (seaweeds). Macroalgae are plant-like organisms. They are classified according to their pigmentation into green (chlorophytes), red (rhodophytes) and brown (phaeophytes) macroalgae.

Known for their richness in polysaccharides, minerals and certain vitamins, seaweeds also contain other bioactive substances, such as proteins, lipids and polyphenols. These chemicals possess potential foruse in treating cancer [7], oxidative stress [3,8], inflammation [9], allergy [10], diabetes [11], thrombosis [12], obesity [13], lipidemia [14], hypertensive [15,16] and other degenerative diseases. This review provides an overview of seaweed *in vitro* and *in vivo* investigations, and it summarizes our own research on seaweed components and their anticancer activity. Additionally, seaweed RCTs and quasi-experiments directed toward cancer therapy and prevention are discussed.

The chemical and nutritional contents of seaweeds vary with age, organism, species, and habitat [17]. Seasonal, environmental, and physiological variations also affect the contents [18]. Cultivated seaweed composition is affected by the time of harvest and the processing methods (e.g., drying, cutting, and milling) [19,20]. Typical air-dried seaweed contains3–47% carbohydrate, 33–75% protein, 1.5–4% lipid, and 10–35% ash [21,22].

Seaweed contains considerably more protein than high-protein legumes, such as soybeans, and the protein content is generally higher in red and green algae (10–47% DM) than in brown algae (3–16%DM) [23]. The quality of a food protein depends on the variety and amounts of essential amino acids that that particular protein contains. Macroalgal proteins contain all essential amino acids;however, variations in specific concentrations occur between phyla [24]. The organoleptic characteristics of algae arise primarily from their free amino acid profile, which in turn depends on the

environment where the algae grow [25]. Some contain important light-harvesting, protein-pigment complexes called phycobiliproteins. When present, these proteins generally constitute 1–10% of the dry algal biomass. Phycobiliproteins are oligomeric proteins containing two chromophore-bearing polypeptides. This broad class of proteins encompasses three protein subclasses that absorb various red and blue wavelengths – phycoerythrins (PEs), phycocyanins and allophycocyanins. PEs are contained in certain red algae, and phycocyaninscan be found in certain red and blue-green algae [8]. Phycobiliproteins also fluoresce *in vitro*. This property has enabled them to be used in biotechnological applications. For example, the biomedical diagnostic community employs phycobiliproteins, primarily in immunochemical methods. Moreover, in some countries, phycobiliproteins are used as natural colorants for food and cosmetics (e.g., chewing gum, dairy products, jellies, ice sherbets, lipsticks and eyeliners) [26]. The most commonly used phycobiliprotein is phycoerythrin, which is present in fluorescent immunoassays, fluorescent immunohistochemistry and other methodologies [8].

Recent studies have shown that the antioxidative capacity of phycobiliproteins can be exploited to preventor treat several chronic diseases [27]. Phycobiliproteins also exhibit anti-inflammatory, neuroprotective, hypocholesterolemic, hepatoprotective, antiviral, antitumor, antiarteriosclerotic, serum lipid-reducing and lipase inhibition activities. Lectins are glycoproteins that have health applications and uses in clinical diagnosis. Within organisms that possess them, lectins function in aggregation and recognition roles [28]. They have been isolated from primitive marine organisms, including fishes, sponges and sea invertebrates [28]. Recently, a new lectin structural family was identified in *Bryothamnion triquetrum*, a red marine alga. The lectin contained 91 amino acids and two disulfide bonds. The amino acid sequence of the *B. triquetrum* lectin was not similar to any known animal or plant lectin structure [3]. Finally, mitogenic and antineoplastic isoagglutinin glycoproteins have been discovered in the red alga *Solieria robusta* [8].

A normal total lipid content in a macroalgae is between 1.5 and 5%; therefore, its contribution as an energy source appears to be low [29]. Generally, seaweed fatty acids are even-numbered, linear chains that possess one or more double bonds [30]. Algae contain higher proportions of saturated and unsaturated fatty acids than terrestrial plants [18]. PUFAs account for almost half of the seaweed lipids. Much of the seaweed PUFAsoccur in the form of omega-3 (n-3) and omega-6 (n-6) fatty acids, such as eicosapentanoic acid (EPA) and arachidonic acid (AA) [31]. In addition to fatty acids, the unsaponifiable fraction of macroalgal lipids contains carotenoids (e.g., fucoxanthin in brown seaweeds; carotene, lutein and violaxanthin in red and green seaweeds), tocopherols, sterols (e.g., fucosterol in brown seaweeds) and terpenoids [32].

Algal polysaccharides differ from terrestrial plant polysaccharides, and polysaccharide type varies greatly between algal species (Table 1). Although their carbohydrate content is relatively high (33–75% DM), algae are not energy-rich foods because the digestibility of these carbohydrates is low [33]. Most algal polysaccharides simply pass through the human gastrointestinal tract as dietary fiber [34]. “Dietary fibers” are a wide range of complex materials, and numerous definitions have been proposed for this term. Dietary fibers are classified as soluble (SDF) or insoluble (IDF) according to their water solubility. The total dietary fiber content of seaweeds ranges from 29.3–62.3 g/100 g [35], which is higher than the fiber content of most fruits and vegetables. The typical Asian individual’s algal intake of 8 g/daysatisfies up to 12.5% of the daily recommended dietary fiber requirement [31].

Table 1: Carbohydrate content of seaweeds

Division	IDF	SDF
Phaeophyta (Brown A.)	-Structural cellulose -Insoluble alginate	-Alginate (10-40%) -Fucans (3.6 to 20%)/ -Fucoidans -Ascophyllans -Glycuronogalactofucans -Neutral glucans (up to 30%) - Laminarans -Mannitol
Rhodophyta (Red A.)	- Cellulose - Residual polysaccharides	-Solublexylans -Sulfated galactans - Porphyrans -Agar (mixture of sulfated galactans, D-galactose and 3,6-anhydro-l-lactose) - Carrageenan (linear sulfated galactans)
Chlorophyta (Green A.)	- Glucoxylan - Glucuromannan, Amorphous cellulose uronic acids. Rhamnose, xylose, galactose, arabinose	-Ulvans (approximately 8–29% DM)

IDF: insoluble dietary fiber;SDF: soluble dietary fiber

The health benefits of algal fiber consumption are well documented [2]. Polysaccharide composition is a principal characteristic used in classifying algae. Algae contain three types of polysaccharides: structural cell wall polysaccharides, intercellular mucilage polysaccharides, and storage polysaccharides. Storage polysaccharides, such as carrageenans and alginates, are common to seaweeds and are the most commercially exploited seaweed components. Storage polysaccharides exhibit textural and stabilizing properties that are useful in several food applications [31].

Found extensively in the cell walls of macroalgae, fucoidans are a class of sulfated, fucose-rich polymers. Fucoidans are a major therapeutic component of brown algae. Depending on the specific seaweed species, these polymers constitute up to 25–30% of algal dry weight [36]. Fucoidans display numerous bioactive properties, including anticoagulant, antiviral, antibacterial [37], antithrombotic, anti-inflammatory, antitumor and antioxidant activities [38]. Fucoidans can be easily extracted using either hot water or acidic solutions. In addition, the therapeutic potential of fucoidans increases with the degree of sulfation [39].

Fucoidans vary extensively in their polysaccharide structures.Every fucoidan-producing species possesses multiple types of fucoidan that differ in theirmonosaccharide proportions. In addition,brown algae synthesizespecies-specific fucoidan sugar compositions. Fucoidan structure and biological significance has been studied [38,40,41]. Fucoidans of various molecular weights and of different origins (and also other seaweed contents) have exhibited a marked anticancer effect *in vitro* and *in vivo*, whether they are administered orally or injected directly into the blood or peritoneal cavity (Table 2).

A significant reduction in the cancer development was observed in mice and rats after cancer cells had been implanted [41,49]. Similar effects were observed in several other cancer animal models, including leukemia and breast cancer [50].The phenomenon was attributed to the stimulation of both innate and specific immunity. Isolated fucoidans from brown algae have also been shown to possess broad immunomodulating effects. In human studies, fucoidan has been shown to stimulate the enzyme granzyme A, which is believed to induce cytokine production in a variety of cells [51]. This observation may partially explain the mechanism of

fucoidan immunomodulation [49,52]. In another human study involving fucoidan ingestion, low serum levels of fucoidans coincided with the presence of stromal cell-derived factor 1 and increased chemokine receptor CXCR4-expressing hematopoietic stem cells and chemokine IFN-γ [52].

Table 2: Bioactivity of seaweed components.

Characteristic	Bioactivity	Study design	Ref.
Total polysaccharide	Antitumor	*In vitro* (HepG2, A549 and MGC-803 cells)	[42]
Carrageenan	Antitumor immunomodulation	*In vivo* (Mice)	[43]
Agar	Antitumor	*In vivo* (inhibited the transplantation of Ehrlich ascites carcinoma in mice)	[43]
Laminaran	Stimulates immune systems; B and helper T cell	*In vivo*	[44]
Porphyran	Potentially apoptotic	*In vitro*	[45]
Carotenoids	Cancer prevention	*In vitro - In vivo*	[46]
fucoxanthin	Apoptotic	*In vitro* (human promyelocytic leukemia cells)	[47]
Peptides	Antitumor	*In vitro - In vivo*	[48]

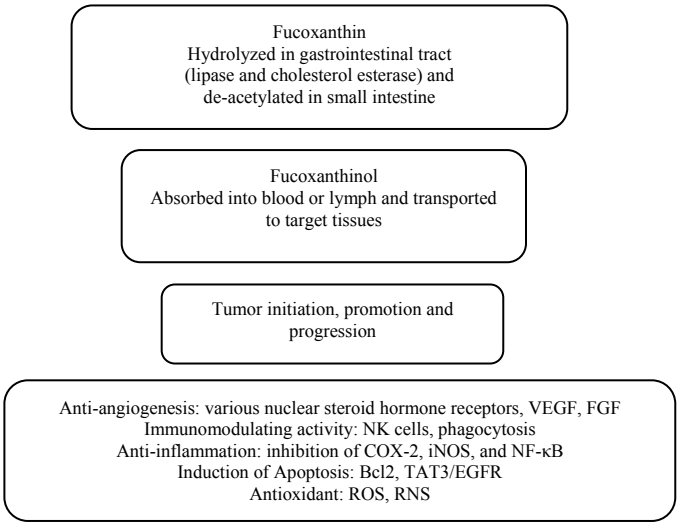

Figure 1: Flowchart representing the mechanism of action of fucoxanthin antitumor initiation, promotion and progression. VEGF: vascular endothelial growth factor; FGF: fibroblast growth factor; HPA:hypothalamic-pituitary-adrenal; NK: natural killer cell; COX-2: cyclooxygenase-2; iNOS: inducible nitric oxide synthase; NFκB: nuclear factor kappa B; cFLIP: cellular form of FLICE inhibitory protein; Bcl2: B-cell lymphoma; ROS: reactive oxygen species; and RNS: reactive nitrogen species.

Large, controlled studies have been proposed to evaluate fucoidans therapeutic effects for inoperable, advanced or recurrent colorectal cancer [49]. Strong archival support documentsthe promising bioactivities of fucoidan. Researchers hope to use fucoidans for treating thrombosis and some forms of cancer [37]. Wijesekara *et al.* [53] reported that algal sulfated polysaccharides possess potential for new pharmaceutical and food anticancer products.

Fucoxanthin is an orange carotenoid found in edible brown seaweeds. This carotenoid exhibits anticarcinogenic effects, apoptotic induction of cancer cells, anti-inflammatory effects, and radical-scavenging activity [23,54] (Figure 1). Additionally, fucoxanthin significantly inhibits numerous enzymes, e.g., matrix metalloproteases, hyaluronidases and elastases [55]. This inhibitory activity limits tissue breakdown in inflammatory settings caused by injury and disease; moreover, the activity even inhibits metastasis [56].

Laminaran (or laminarin) is a water-soluble,approximately 5000 Da polysaccharide containing 20-25 glucose units [57] and is the second most numerous storage glucan in brown algae. Most laminarans are dietary fibers because they form complex structures that are stabilized by inter-chain hydrogen bonds and are resistant to hydrolysis in the upper gastrointestinal tract (GIT). Laminaran modulates intestinal metabolism by affecting mucus composition, intestinal pH and short-chain fatty acid production. In addition to their role as dietary fibers, laminarans function as prebiotics and possess antibacterial and antitumor activities [58]. Environmental factors such as water temperature, salinity, waves, sea currents and depth affect the structure and biological activities of laminaran [57]. The antimetastatic effects of laminaran sulfate have been investigated [57,59]. Laminaran sulfate inhibited heparanase activity in mouse B16-BL6 melanoma cells and rat 13762 MAT mammary adenocarcinoma cells. Consequently, the tumor cells' ability to degrade heparan sulfate in their extracellular matrixes was reduced, and an antimetastatic effect was, therefore, produced [59].

At the effective concentrations, laminaran sulfate exerted a small effect on tumor cell proliferation and primary tumor growth*in vivo*. These results further illustrate heparanase involvement in tumor metastasis, and the study supports potential clinical applications of laminaran sulfate in cancer treatment [59].

Alginic acid, or alginate, is the common name given to a family of linear polysaccharides produced by brown seaweed [60]. Alginates are available commercially in both acid and salt (sodium and calcium) forms. The acid form is a linear polyuronic acid referred to as alginic acid. The salt form is an important cell wall component in all brown seaweed, constituting up to 40 - 47 % of dry algal biomass. Alginate is widely used in the food and pharmaceutical industries because of its ability to chelate metal ions and to form highly viscous solutions [57]. Alginate possesses diverse bioactivities: anticoagulative [61], antitumor [62], antiviral, anti-hypertension and antioxidant [53]. Alginate protects against carcinogens by clearing them from the digestive system and protecting stomach and intestinal surface membranes from the effects of carcinogens[50]. Intestinal enzymes cannot digest alginic acid. Consequently, heavy metals are gelated or rendered insoluble by alginic acid in the intestines and cannot be absorbed into the body [58]. In several countries, the use of alginic acid and its derivatives for gastritis, gastro duodenal ulcer and anti-ulcer treatment is patent protected. Clinical trials have shown that sodium alginate promotes the regeneration of stomach mucous membranes, suppresses inflammation, and eradicates mucous membrane colonies of *Helicobacter pylori* [63]. Additionally, alginate promotes the restoration of intestinal biogenesis [8]. Other studies show effects on fecal microbial fauna, changes in compound and acid concentrations, and prebiotic properties that can promote health, particularly in regard to cancer prevention [64,65].

Carrageenans are a family of linear, hydrophilic, sulfated galactans that are obtained from different Rhodophyta species [13]. Carrageenans are traditionally split into six categories based on their chemical composition and commercial use [71]. Because of their sulfate group concentration, all carrageenan fractions are soluble in water and insoluble in organic solvents, oil and fats [13]. Carrageenans are classic agents for inducing inflammation and inflammatory pain in experiments [72]. From a human health perspective, carrageenans exhibit several promising pharmaceutical properties [62], including antitumor [73], immunomodulatory [74], antihyperlipidemic [13,75,76], anticoagulative [77] and antiviral activities [78]. Recently, researchers confirmed that carrageenan is an extremely potent infection inhibitor of a broad range of human genital papillomaviruses (HPVs); therefore, carrageenan-based sexual lubricant gels may offer protection against HPV transmission [13,52,58,79].

At concentrations approximately 1000 times higher than those required to inhibit papilloma viruses, carrageenans inhibited HIV [78,80]. Carrageenans are approved and widely used as a food additive [71,81]. In the food industry, they are employed as a thickener, emulsifier, and stabilizer [10,82].The food products that contain carrageenan include chocolate milk, ice cream, whipped cream, dietetic foods, low-fat processed meats, infant formula, beer, and salad dressings. Carrageenans' unique ability to combine with milk proteins improves the texture of many food products [6,13,76]. The safety of carrageenans as food additives have been investigated by many researchers. In a review of 45 studies using animal models (rats, mice, rabbits and Guinea pigs), low molecular weight (degraded) carrageenans were associated with the induction and proliferation of intestinal neoplasms and ulcerations [76]. These results warn of potential problems associated with human carrageenan consumption, such as inflammatory bowel disease and gastrointestinal malignancies [13,36,83]. One research group performed a time-trend analysis to see if carrageenan use correlated with the incidence of mammary carcinoma in twentieth century United States. This analysis revealed that the increasing consumption of several alginate gums correlated positively with increased incidences of breast carcinoma [81].

Mannitol is an important sugar alcohol produced by photosynthesis [84]. This sugar alcohol occurs naturally in many brown algal species, accounting for 20–30% of the dry weight of some *Laminaria* species [60,85]. Mannitol is only 70% as sweet as sugar, but has a low glycemic index rating because it cannot be metabolized by intestinal cells [86]. Mannitol is commonly used in chewing gum and mints. The mannitol content of a seaweed varies widely with season and, particularly, environment. The applications of this sugar alcohol are extremely diverse. Because of its hydrating and antioxidant properties, mannitol is used in numerous cosmetic and pharmaceutical products [60,70,86]. In addition, mannitol is used in the paint and varnish industry, leather and paper manufacture, the plastics industry and explosives. A variety of foods contain mannitol. It can replace sucrose in candy and chocolate-flavored coatings. Sugar-free chocolates are popular among people with diabetes, a growing problem in modern society. Mannitol is used as a flavor enhancer because of its sweet taste and pleasantly cool feel in the mouth. Mannitol is non-hygroscopic and chemically inert; therefore, it can be used to maintain the proper moisture level in foods and increase shelf-life and stability[86]. Mannitol is non-carcinogenic. Finally,it can be used in pediatric and geriatric food products because it does not contribute to tooth decay [58,87].

Ulvans are highly charged, sulfated polyelectrolytes with average molecular weights from 189 to 8,200kDa [88]. These cell wall polysaccharides comprise 8 to 29% of dry algal matter [58,66]. Ulvans' unique physicochemical properties make them attractive candidates for novel food, pharmaceutical, chemical aqua cultural, and agricultural bioactive polymers. Two major types have been identified: water soluble ulvans and insoluble, cellulose-like ulvans. Numerous researchers have demonstrated ulvans' antitumor, immunomodulatory [89], antiviral [90], anticoagulative [91] and antioxidant activities [92,93]. Ulvans were able to affect the adhesion and proliferation of normal and tumor human colonic cells. Ulvans were also able to affect the expression of transforming growth factors (TGF) and surface glycosyl markers related to cellular differentiation [89,94]. Moreover, ulvans induced mucin secretion in rat colon and, therefore, increased protection of the colonic mucosa. Because of their ion-exchange properties, ulvans are used as bioindicators for monitoring heavy metal pollution in coastal waters [95]. Lahaye and Robic extensively reviewed the available ulvan literature, emphasizing its structure and properties [94].

Species of *Porphyra* contain a sulfated, complex galactan called porphyran [66,67]. This has been used as a gelling agent, a nutritional supplement and an antioxidant [66,68]. Porphyran is a quality dietary fiber that chemically resembles agar. A 20% nori (*Porphyra*) powder mixed with a basic diet and given orally to rats prevented, 1,2-dimethylhydrazine-induced intestinal carcinogenesis. Porphyran has exhibited appreciable antitumor activity against Meth-A fibrosarcoma [69,70]. In addition, porphyran was demonstrated to significantly lower an artificially enhanced level of hypertension and bloodcholesterol in rats [77]. An agar-type polysaccharide obtained from a cold water extract of a *Gracilaria* species displayed antioxidant and antitumor activities [43]. Agaro-oligosaccharides have also been shown to suppress the formation of a pro-inflammatory cytokine and an enzyme associated with nitric oxide production[96]. Agarose can be separated from agar in 42% yield; agar content varies seasonally from 26 to 42% in *Gelidium* species.

Algal oligosaccharides are carbohydrate molecules with low degrees of polymerization [77]. They are produced naturally and by chemical, physical or biochemical degradations of algal polysaccharides. Numerous oligosaccharides have been shown to possess antioxidant, immune stimulative and antitumor properties [22]. Additionally, a diet containing oligosaccharides benefits health because oligosaccharides are prebiotics. These digestion-resistant oligomers function as food sources for and promote the growth of beneficial bacteria [22]. For example, xylo- and fructo-oligosaccharides remain intact until the large intestine, where aerobic bacteria, such as bifidobacteria and lactobacilli, preferentially consume these sugars [97]. Moreover, non-digestible oligosaccharides have exhibited other biological activity, such as immunostimulative, antioxidative, antiangiogenic and antithrombotic properties [22]. In their review [77], Mussatto and Mancil showed that transgalactosylated disaccharide intake reduces fecal pH and ammonia, *p*-cresol and in dole concentrations. They also assert an increase in bifidobacteria and lactobacilli populations and a decrease in Bacteroidaceae populations. Because these changes in fecal physiological parameters are believed to reduce the risk of cancer, macroalgal non-digestible oligosaccharides are considered potential anticarcinogenic food ingredients [66].

Generally, algae contain a variety and high concentrations of minerals (8–40%) [21]. Their high mineral contentsresult from the ability of their cell surface polysaccharides to retain various inorganic marine substances [31]. However, actual mineral content varies between species and because of oceanic residence time, the geographic harvesting site, wave exposure, seasonal changes, environmental factors, physiological factors, processing, and the mineralization methods used [21,23]. Physiologically important minerals accumulate in seaweeds at considerably higher levels than in edible terrestrial plants and animals [31]. In fact, edible seaweed value in human nutrition is based on its high content of several essential minerals – As, Na, Mg, P, K, I, Fe, and Zn. Algal Na and K levels are particularly high, although their Na/K ratios are usually below 1:5 [98]. Because seaweeds also contain large amounts of trace elements that are limited in terrestrial vegetables, algal-based supplements could provide adults with the daily requirements of these minerals [21]. All seaweeds contain relatively large amounts of sulfates (1.3–5.9%), which are typical components of their polysaccharides. Fucans in brown algae and galactans in red algaebind sulfates; such sulfated mucilages are not found in terrestrial plants [99]. Sulfate levels relate to specific aspects of ionic regulation and salt concentrations in the algal aqueous environments [100]. Most algae contain higher Mg levels (500–1,000 mg/100 g) than terrestrial plants and animals [21]. Algae also

contain higher Fe levels than terrestrial organisms, but the concentration varies considerably depending on the species and harvesting season. A study on iron bioavailability revealed that iron absorption and retention was lower in seaweed-fed animals than a control. The researchers attributed the cause to the polyphenolic compounds or peptides from the partially digested algal protein in the diet [101]. Calcium is a major element in algae and is present at concentrations of 470–1,400 mg/100 g of DM[21]. P is found at levels of 200–800 mg/100 g of DM. Because of its high Ca/P ratio (3:5), algae could compensate for the calcium scarcity in several foods, such as cereals and meats [16,21]. Moreover, seaweed consumption may prove useful to individuals at risk of calcium deficiency, namely expectant mothers, adolescents and the elderly. Algal iodine concentrations, which vary greatly among species [101,102], also differ according to growth conditions, habitat, and pre-harvest conditions[103]. Brown seaweeds accumulate many elements. They are a good source of magnesium, copper, iron, iodine and other nutritionally rare elements. In the developed world, Japan has one of the lowest age-adjusted breast cancer mortality rates [1,104]. The incidence of breast cancer in Japanese immigrants to the United States and in their successive generations, has gradually reached the rates of white United States women,which suggests a dietary link [105]. High iodine intake may be a key protective factor against breast cancer development in Japanese women. One correlation study in Spain found a significant positive association between regions where iodine intake was low and breast cancer mortality rates high [106]. The use of iodine in treating breast cancer warrants study. Traditional eastern Asian medicine has long used iodine-rich seaweeds as a cancer treatment to ‘‘soften’’ tumors and ‘‘reduce’’ nodulation [107]. Recent *in vivo* animal studies suggest iodine possesses an antitumor effect [108]. In dimethylbenz [a] anthracene-induced mammary carcinoma in rats, iodine supplementation suppressed the disease’s development [106]. This suppressive activity was enhanced when the iodine treatment was combined with progesterone treatment. The suppressed tumors possessed significantly higher mean iodine content than the nonsuppressed tumors. Progesterone enhanced the iodine uptake [109]. An anticarcinogenic role for iodine in experimental animals was suggested by the work of Funahashi and co-workers. They observed that Lugol’s iodine or iodine-rich Wakame seaweed administration to rats treated with the carcinogen 7,12-dimethylbenzanthracene suppressed mammary tumor development[35]. Those researchers additionally demonstrated that seaweed induced a greater degree of apoptosis in human breast cancer cells than fluorouracil, a chemotherapeutic agent used to treat breast cancer. This finding led the authors to speculate that ‘seaweed may be applicable for prevention of breast cancer’[109].

A major nutritive characteristic of edible algae is their high vitamin content, primarily with regard to water-soluble vitamins C and B and fat-soluble vitamins A and E. The vitamin C levels in green and brown algae range between 50 and 300 mg/100 g of DM, whereasthe values for red algae range from 10 to 80 mg/100 g of DM [21]. Certain dried green and red algae, e.g. *Spirulina*, contain extensive amounts of vitamin B12. However, other edible algae contain little or none [110]. Daily ingestion of only 1 g of *Spirulina* supplies the daily requirements of B12. Because of their vitamin B12 content, algae have been recommended as dietary supplements for vegetarians, among whom megaloblastic anemia is common. Seaweed folate content (as folic acid or vitamin B9) ranges from 61.4 to 161.6 μg/100 g of DM [21]. Red and brown algae contain high levels of folic acid and folate derivatives, including 5-methyltetrahydrofolate, 5-formyltetrahydrofolate and tetrahydrofolate. On a DM basis, thiamine (vitamin B1) content is high compared with that of terrestrial vegetables [111]. Brown seaweeds contain higher vitamin E levels (2.3–41.2 mg/100 g of DM) than green and red seaweeds (0.8 mg/100 g of DM) [112]. Brown algae contain α, β, and γ-tocopherols; green and red algae contain only α-tocopherol [113].

Phenolic compounds in seaweed possess a wide range of bioactivity: antimicrobial, antioxidant, antiviral and antitumor. These molecules are assumed to function in algae as chemical defenses against grazers, pathogens and epiphytes [114,115]; phenolic compounds in algae are also assumed to provide photoprotection [116]. Algal polyphenols, also called phlorotannins, are composed of phloroglucinol units (1,3,5-trihydroxybenzene). They constitute a heterogeneous group of molecules that vary greatly in their structure and degree of polymerization. Phlorotannins at high levels are found primarily in Fucales (20–30% dw). However, their extractable polyphenol levels (less than 0.4% algal DM) are lower than other phytochemicals. Phlorotannin concentrations are genetically variable and depend on the season of harvesting, habitat, and other extrinsic environmental factors such as salinity, nutrient and light availability, ultraviolet radiation, and herbivore grazing. Furthermore, phenolic content varies within an individual alga. Brown seaweeds display the highest phlorotannin concentrations [117]. Although phlorotannins have different structures and molecular weights, their chemical properties are similar [118]. Phlorotannins are ecologically important. They protect brown algae from ultraviolet irradiation and may be a chemical defense against herbivores. Like gallotannins or condensed tannins in terrestrial plants, phlorotannins are capable of precipitating proteins or metal ions. Polymeric phlorotannins inhibit enzymes such as phospholipase A, lipoxygenase,cyclooxygenase-1, hyaluronidase [118], and tyrosinase. Several polyphenolic compounds and complex mixtures have been isolated from brown algal species to explore the potential phlorotannin role in preventing degenerative diseases linked to oxidative stress [119]. Nutritionists agree that cancer incidence and neurodegenerative disease probability can be diminished by diets rich in fruits, grains and vegetables. The damage of DNA by reactive oxygen species is widely believed to be a significant contributor to age-related cancer development [63]. Until recently, algae were largely ignored as sources of antioxidant. Studies over the last several years have revealed that several seaweed components have appreciable antioxidant capability [20,120]. Tierney *et al.* thoroughly reviewed the available literature on macroalgal antioxidant activities[121].Numerous researchers have studied seaweed health benefits *in vitro*[122] and *in vivo*.

A few human studies have assessed the impact of seaweed consumption on the risk for future disease (Table 3). Such data should be interpreted with caution because they indicate associative relationships but not necessarily causal relationships between seaweed intake and health outcomes. Different seaweeds appear to have different effects on disease risk. Yang reported that premenopausal women who ate the greatest amounts of seaweed were 56% less likely to be diagnosed with breast cancer than premenopausal women who ate the least amounts of seaweed [123]. An analysis of postmenopausal women revealed an even more dramatic risk reduction (68%) when comparing the highest and lowest quintiles of seaweed intake [123]. Preliminary research has demonstrated that selected seaweed phytochemicals (i.e., alginic acid, fucoidan, fucoxanthin and laminarin) interfere with cancer cell proliferation *in vitro* 124]. Human studies offer additional clues regarding possible mechanisms. Insulin-like growth factor 1 (IGF-1) is an anabolic hormone important for growth and development.

Table 3: Summary of recent observational studies on seaweed intake and health.

Disease/ Health condition	Type of study	Study design	Ref.
Breast cancer	Case-control study	362 cases	[123]
Cardiovasculardisease	Prospective study	40547 Japanese men and women	[135]
Allergicrhino sinusitis	Cross-sectional study	1002 Pregnant Japanese women	[136]
Serum IGF-1 Concentrations	Double-blinded, randomized, placebo-controlled crossover clinical trial.	30 Healthy Postmenopausal American Women	[125]
Obesity	3760 Japanese women aged 18-20 yr.	Cross-sectional study	[137]
Diabetes, Type II	Retrospective study	3,405 Korean individuals, aged 20 -65 yr.	[138]
post-load plasma glucose and insulin concentrations	Double-blind, randomized, placebo-controlled crossover study	Twenty-three participants (11 men, 12 women) aged 19–59 yr.	[129]
Serum totalcholesterol	Retrospective study	> 7000 people	[139]
estrogen and phytoestrogen metabolism	Double-blind trial	15 Healthy postmenopausal women	[126]
Metabolic syndrome	Randomizeddouble-blinded placebo-controlled trial	13 Men (mean age 47.4 ± 9.9 yr) and 14 women (average age 45.6 ±12.2 yr) with at least one symptom of the metabolic syndrome	[140]
Immunomodulatory effects	open-label combined Phase I and II study	10 Healthy individuals aged between18 and 65 yr	[52]

High-circulating serum concentrations of IGF-1 in adulthood are associated with an increased risk of postmenopausal breast cancer. Breast cancer incidence is typically lower in Asian countries where soy is commonly consumed. Notably, in trials with American women, soy supplements significantly increasedIGF-1. However, seaweed is also consumed regularly in Asian countries with low breast cancer risk. Thirty healthy postmenopausal women participated in a 14-week double-blinded, randomized, placebo-controlled, crossover clinical trial. Participants consumed 5 g/day of either placebo or seaweed in capsules for 7 weeks. During the 7th week, a high-soy protein isolate powder was added. Soy significantly increased serum IGF-1 concentrations compared with the placebo. Seaweed reduced this soy increase by approximately 40%. The researchers concluded that concurrent seaweed and soy consumption may be important for moderating the effects of soy on IGF-1 serum concentrations, hence providing a possible mechanism for seaweed anticancer effects [125].

In another study, fifteen healthy postmenopausal women participated in a double-blind trial of seaweed supplementation with a soy challenge. Seaweed positively altered estrogen and phytoestrogen metabolism[126].These observations may explain why the rates of estrogen-dependent cancers are higher in Western and lower in Eastern countries[127]. Results from our lab have shown that polyphenol-rich red seaweed is tumor-suppressive. The mechanism occurred via apoptosis induction, down regulating the endogenous estrogen biosynthesis, and improvement ofthe antioxidative status of the rats [128]. Because cholesterol is a precursor to sex hormone biosynthesis, kelp consumption may alter circulating sex hormone levels and menstrual cycling patterns. In particular, dietary kelp may be beneficial to women at high risk for estrogen-dependent diseases.

Additionally, brown seaweed was administered to three premenopausal women with either abnormal menstrual cycling patterns or menstruation-related disease histories. Seaweed intake was associated with significant increases in menstrual cycle lengths. Moreover, hormone measurements for one woman revealed significant anti-estrogenic and progestagenic effects following kelp administration. These pilot data suggest that dietary kelp may lengthen the menstrual cycle and exert anti-estrogenic effects in premenopausal women. Furthermore, these studies suggest that, in addition to soy, seaweed consumption may be responsible for the reduced risk of estrogen-related cancers observed in Japanese populations [127]. In another study, seaweed consumption was associated with a 12.1% reduction in the insulin incremental area. Glucose and insulin responses were similar between men and women. No adverse effects were associated with seaweed capsule consumption. These data suggest that brown seaweed may alter the insulin homeostasis in response to carbohydrate ingestion [129]. High levels of circulating insulin and an inadequate intake of iodine, a trace mineral found in seaweeds, are thought to contribute to breast cancer incidence. However, not every type of seaweed afforded the same level of protection. These results highlight the variability in the bioactive components of seaweed. Even within a single species, research has suggested that significant seasonal variations in nutritional content exist, which is likely to impact the biological effects of edible components outlined above.

Historically, seaweed has been consumed around the world. However, seaweed has been consumed in appreciable amounts only in certain areas. Although chemical analyses suggest numerous nutritional benefits to consuming seaweed, more evidence is needed linking seaweed dietary intake to health. Epidemiological studies suggest dietary factors could be critical to understanding cancer rates. Seaweed whole extracts or purified components have exhibited high antitumor activity *in vitro*. *In vivo* research with different animal models have demonstrated that fresh seaweed or seaweed extracts (as part of a regular diet, drinking water and/or as an injection) both inhibit and treat cancer.

Although little is known about relative cancer risk and seaweed intake in humans, a small body of *in vivo* and *in vitro* research suggests seaweed may be useful in cancer prevention and treatment. Seaweeds have been used to treat tumors in traditional Chinese and Japanese folk medicine. Populations that consume seaweed regularly, as in Japan, have dramatically lower rates of hormone-sensitive cancers, such as breast and prostate cancers [130]. Epidemiological studies performed in Japan before Westernized diets were common reported that Japanese women had lower rates of premenopausal and postmenopausal breast cancer [131]. In addition, after developing breast cancer, Japanese women were more likely to survive five years or more than United States women with breast cancer [132].

No clinical studies of cancer and seaweed have been reported. However, in a large prospective dietary study in Japan, investigators reported that, after 9 years of follow-up, miso soup (made from a concentrated hot water extract of seaweed plus a tablespoon or less of soybean paste and usually a few vegetables) was the food most closely associated with low breast cancer risk out of the foods investigated [133]. Women who ate three or more bowls of miso soup daily had approximately half the rate of breast cancer as those who ate two or fewer bowls. In two other epidemiological studies of diet and breast cancer in Japan, daily miso soup consumption was associated with 15% lower rate of breast cancer; women who drank miso soup at least five times /week had 13% lower rates [134].

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Methods for Extraction and Determination of Phenolic Acids in Medicinal Plants: A Review

Agnieszka Arceusz, Marek Wesolowski* and Pawel Konieczynski

Department of Analytical Chemistry, Medical University of Gdansk, 80-416 Gdansk, Poland

marwes@gumed.edu.pl

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Phenolic acids constitute a group of potentially immunostimulating compounds. They occur in all medicinal plants and are widely used in phytotherapy and foods of plant origin. In recent years, phenolic acids have attracted much interest owing to their biological functions. This paper reviews the extraction and determination methods of phenolic acids in medicinal plants over the last 10 years. Although Soxhlet extraction and ultrasonic assisted extraction (UAE) are commonly used for the extraction of phenolic acids from plant materials, alternative techniques such as supercritical fluid extraction (SFE), and accelerated solvent extraction (ASE) can also be used. After extraction, phenolic acids are determined usually by liquid chromatography (LC) owing to the recent developments in this technique, especially when it is coupled with mass spectrometry (MS). Also detection systems are discussed, including UV-Vis, diode array, electrochemical and fluorimetric. Other popular techniques for the analysis of this group of secondary metabolites are gas chromatography coupled with mass spectrometry (GC-MS) and capillary electrophoresis (CE).

Keywords: Phenolic acids, Medicinal plants, Extraction, Chromatographic techniques, Capillary electrophoresis, Secondary metabolites.

Introduction

Vascular plants synthesise many organic compounds, often called secondary metabolites [1]. These low-molecular-weight compounds determine the basic life processes of plants. It has been estimated that about 8000 compounds naturally occurring in plants are phenols. Their characteristic structural feature is an aromatic ring with at least one hydroxyl substituent. Many phenolic compounds occur constitutively, but some stress factors contribute to increases in or *de novo* synthesis of phenolics, such as infection, plant tissue damage, UV radiation and elevated temperature [2].

The popularity and consumption of medicinal plants have grown significantly in recent years. This phenomenon has led to a faster and better evaluation of the quality of plant products. One of the tools playing a crucial role as an element controlling the quality of plant material used in medicine is the so-called fingerprint analysis [3-5].

Determination of phenolic compounds can be very helpful in estimation of pharmacological activity of medicinal plants [6]. Within this huge group of compounds, a significant role is played by phenolic acids, aromatic secondary metabolites widespread in the plant kingdom. These compounds contain both hydroxyl and carboxyl groups. Phenolic acids include hydroxyl derivatives of benzoic and cinnamic acids (Table 1). Much attention has been focused on gallic, vanillic, salicylic, caffeic and *p*-coumaric acids, which are active constituents of many plants [7,8]. Examples include thyme, whose phenolic acids comprise gallic, caffeic and rosmarinic acids, also sage containing ferulic, gallic, rosmarinic, vanillic, and caffeic acids, as well as rosemary, characterised by a high content of vanillic, caffeic and rosmarinic acids [7].

Source and roles played by phenolic acids in plants

Phenolic acids are secondary metabolites and constitute an important group of hydrophilic compounds in plant tissues [9,10]. They seldom occur in the free form and, therefore, they appear in low concentrations. In plants, phenolic compounds can appear mainly in their bound forms, for example as glycosides (phenolic glycosides) and in esters as depsides and depsidones [11]. In addition, in plant tissues, compounds of phenolic acids with other natural constituents have been identified, for instance, with flavonoids, fatty acids, sterols and cell wall polymers.

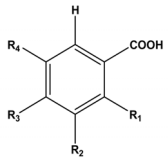
The contents of phenolic acids in plant raw materials, as well as of other phenolic compounds, depend on many factors. The biggest impacts come from climatic conditions, cultivation, fertilization and time of harvest [2,12,13]. Also important are storage conditions of the plant materials and the methods of preparation [10]. It is known that plants from the *Lamiaceae* family contain especially large amounts of phenolic acids [14]. These compounds can also be found in large amounts in fruits and vegetables. The main source of phenolic acids is herbal infusions; they also occur in black and green tea, and coffee [15]. In food products, phenolic acids may influence the color, flavor, fragrance and oxidation stability of food. In this case, their level depends, among other factors, on the type of technological process applied to a given plant material [10].

Source and roles of phenolic acids in human

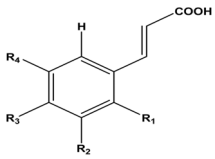
Phenolic acids constitute an important group of compounds, characterised by a wide spectrum of pharmacological activity. They are responsible for free radical scavenging, metallic ions chelation, and changing enzymatic activity. Moreover, these compounds exhibit antiviral activity, for instance rosmarinic acid, anti-inflammatory activity, for example a mixture of esters of benzoic and cinnamic acids; diuretic, anti-allergic, as well as cholagogic and choleretic activities [15-17]. Phenolic acids participate in regeneration and adaptation processes in humans and are used in prevention against many diseases [18,19]. It was proved that they prevent coronary disease, inflammation, type-2 diabetes, as well as aiding in the treatment of cancer. Several of these compounds, for instance ferulic and caffeic acids, are called cancer growth inhibitors. Furthermore, recent studies have also shown pro-coagulant activity of phenolic acids isolated from *Blumea riparia* DC [20] and possible degradation pathway of salvianolic acid B in water solution and simulated gastric and intestinal fluids [21].

Table 1: Structural formulas of phenolic acids.

Hydroxybenzoic acids	R_1	R_2	R_3	R_4
Benzoic acid	H	H	H	H
p-Hydroxybenzoic acid	H	H	OH	H
Vanillic acid	H	OCH_3	OH	H
Gallic acid	H	OH	OH	OH
Syringic acid	H	OCH_3	H	OCH_3
Protocatechic acid	H	OH	OH	H
Gentisic acid	OH	H	H	OH
Veratric acid	H	OCH_3	OCH_3	H
Salicylic acid	OH	H	H	H



Hydroxycinnamic acids	R_1	R_2	R_3	R_4
Cinnamic acid	H	H	H	H
o-Coumaric acid	OH	H	H	H
m-Coumaric acid	H	OH	H	H
p-Coumaric acid	H	H	OH	H
Ferulic acid	H	H	OH	OCH_3
Caffeic acid	H	OH	OH	H



The availability of phenolic acids in humans is mostly influenced by their chemical form, as well as the morphological part of the plant [22]. More hydrophilic compounds are characterised by a higher bioavailability and are more readily absorbed in the upper part of the digestive system. On the other hand, substances in their bound form are absorbed after enzymatic hydrolysis, which is mediated by intestinal microflora.

The biological significance of phenolic acids calls for the need of elaboration of appropriate analytical methods enabling their monitoring in drugs and foods of plant origin, as well as in plant raw materials used for their production. Bearing all this in mind, the aim of this work is to present an up-to-date review of the most often used methods for extraction and determination of phenolic acids in plant materials.

Extraction methods

One of the main sources of errors committed during analytical procedure is the stage of sample preparation. As this stage determines the time of analysis, it is crucial to shorten the procedure and to enhance its accuracy and selectivity while identifying the majority of chemical compounds [23]. In order to separate the analytes from solid samples, the most often used solvents are those with low evaporation heats and low boiling points. The solvents also have to be non-toxic, non-flammable and chemically neutral, and they should not influence negatively the instrumentation and stability of the analysed substances. Solvents such as methanol, ethanol, acetone, ethyl acetate and their mixtures with water are commonly used [6,10,24,25].

Phenols and phenolic acids also exist as insoluble bound complexes, which are coupled to cell wall polymers through ester and glycosidic links and they are not extractable by organic solvents [11,26]. Bound phenolic acids are typically liberated using base hydrolysis, acid hydrolysis, or both before extraction [27-29]. Many extraction procedures incorporate the use of an antioxidant as stabilizer; compounds that have been used for this purpose include butylated hydroxyanisole, tert-butylhydroquinone and ascorbic acid.

Soxhlet extraction

Extraction is a first stage of the process leading to isolation of secondary metabolites from plant materials. A classical method used for this purpose is Soxhlet extraction. This method ensures multiple digestion of a plant material and continuous extraction, while a fresh portion of a solvent is delivered. Thanks to this, the largest difference between the concentrations of analyte in the cell solution and in the solvent is maintained throughout, which leads to total extraction of a plant material.

Multiple extraction with the use of a Soxhlet apparatus was applied for extraction of phenolic acids from plant material (*Sambucus nigra* L., *Polygonum aviculare*). Methanol was a solvent and the digestion process lasted 15 h. Application of this modification of extraction in the case of wild common lilac allowed a high extraction yield of *p*-hydroxybenzoic, vanillic and ferulic acids [30]. Soxhlet extraction was also used for qualitative and quantitative analysis of phenolic compounds occurring in *Salvia halophila* and *S. virgata* [31]. To optimize the extraction conditions, *n*-hexane, ethyl acetate, methanol and 50% aqueous methanol solution were used during 8 h. It was found that the highest extraction yield of total phenolics was obtained when 50% methanol was used, and the lowest by using *n*-hexane. Koşar *et al*. [32] tested the same solvents when they investigated extraction of *S. halophila*.

Soxhlet extraction was also used by Karasová and Lehotay [33], who reported on the isolation of benzoic acid derivatives from *Melissa officinalis*. Different extraction times were tested (1 h, 4 h, 8 h), as well as the solvent, which was a mixture of methanol with water at different volume ratios (60:40 and 80:20). Eventually, for separation of the derivatives, the 80:20 mixture of methanol and water was applied, and the extraction was performed during 1 h. It has been concluded that the time of extraction had no influence on the results. The only exception was gallic acid, because, after extending the extraction time, the lowest concentrations of this compound were obtained.

The main disadvantages of Soxhlet extraction are that it is a time-consuming process and uses costly solvents, which must be of appropriate quality. For these reasons, in recent years other methods have been preferred that are less time-consuming and require smaller volumes of solvents [34]. Among these methods are ultrasound-assisted extraction (UAE), supercritical fluid extraction (SFE), and accelerated solvent extraction (ASE). These methods can be successfully used for extraction of phenolic compounds from plant materials. A comparison of these methods is presented in Table 2.

Ultrasound-assisted extraction (UAE)

Ultrasound-assisted extraction (UAE) is based on the action of ultrasonic vibrations directed toward an extracted sample, which enhance efficacy of penetration of a sample by solvent. This method

Table 2: Comparison of extraction techniques.

Parameter	Soxhlet	UAE	MAE	SFE	ASE
Costs	low	low	average	high	high
Time of extraction	6-48 h	< 30 min	< 30 min	< 60 min	< 30 min
Solvent volume to be used [mL]	200-600	< 50	< 40	<10	< 100

is characterized by high speed, simplicity, and usually takes several minutes. Apart from the type of a solvent, sample size, pH of extract, temperature and pressure, factors such as particle size, duration of sonification and its amplitude, also have an impact on the efficacy of the extraction process [35,36]. Regardless of these factors, the method is considered as the simplest one possible to perform in a laboratory [37,38]. One of its advantages is the possibility to carry out the extraction of several samples simultaneously within a relatively short time. However, it is necessary to decant the extract or to filter it through appropriate paper filters. On the other hand, an opaque solution after extraction could get stuck on an HPLC column. In practice, during the UAE process disruption of plant cell walls takes place, this enabling the effective extraction of metabolites contained in the cells. This method was applied by Pawar *et al.* [39], who analysed different species of ginger. The UAE process was conducted for 30 min, using methanol as a solvent.

Ultrasound-assisted extraction is not the sole process used for extraction. Sometimes it is preceded by another procedure in order to prepare adequately the sample, for example to moisten it with a solvent. Such a procedure has been proposed by You *et al*. [29], who extracted the fruits of blackberry with an 80:20 (v/v) methanol/water mixture, with addition of acetic acid, during 16 h in a dark place. Then, sonication was performed and the analysed samples were centrifuged twice. Benetis *et al*. [40] have also used ultrasound for the analysis of phenolic compounds in *Achillea millefolium* L. Preliminary studies conducted with the use of ethanol/water solutions of concentrations from 40% to 96% (v/v), and applying different extraction times of 5, 10, 20, 30 and 60 min, have shown that the best results were obtained with the 70% ethanol solution. An extraction profile in relation to ultrasonication time has shown that by extending the time of extraction the yield increased, but after 60 min a decrease in yield was noticed. Finally, for further experiments, a 70% ethanolic solution was chosen as solvent and a 30-min ultrasonication used, with a 0.25g sample and 25 mL of solvent.

Among numerous references describing application of UAE to sample preparation, there is one, which compares the efficacy of this method with the classical liquid-liquid extraction [41]. For both processes, the following solvents were used: a 60% methanolic solution, a 60% acetone solution, water, and a 60:30 (v/v) mixture of water and ethyl acetate. The analysed material was aromatic plants, including *Rosmarinus officinalis* and *Origanum majorana.* Classical digestion was performed in a water bath at 90°C for 2 h, whereas ultrasonication was performed for 1 h at 25° and 60°C. Both processes were carried out in triplicate using each of the above mentioned solvents. The methanolic solvent appeared to be the best, and the temperature of 60°C. Under these conditions, the highest yield of extraction was achieved.

Supercritical fluid extraction (SFE)

This type of extraction often precedes analysis by HPLC [42]. Supercritical fluid extraction is a modern technique, which has many advantages over the classical extraction methods [38,43]. Among these are low temperatures, which is a positive feature in the case of analysis of thermally labile compounds. Other advantages are high selectivity, significant reduction in solvent volumes used for extraction, low mass of sample for extraction (around several mg), short extraction time, possibility of automation, as well as off-line and on-line coupling with a majority of chromatographic techniques (GC, HPLC). The solvent used in SFE is a fluid in a supercritical state, usually carbon dioxide, owing to its low price and low toxicity. However, in the case of phenolic compounds, it is not the best solvent, since its polarity is low in comparison with that of phenolics [44]. In spite of this, Castro-Vargas *et al*. [45] used carbon dioxide for isolation of phenolic fractions from the seeds of *Psidium guajava* L., family *Myrtaceae*.

Many parameters influence the efficacy of extraction by supercritical fluids. They can be divided into two groups [46]. The first encompasses parameters related to conducting the extraction, such as pressure, temperature, time, sample weight, and flow intensity. The other group involves parameters connected with the matrix of a sample, such as its form, homogeneity, solubility and desorption ability of analytes. Apart from this, the SFE process depends on the pH. Its variations can influence the extraction yield and the speed of extraction of the analyte from an aqueous phase.

SFE is commonly applied in the pharmaceutical industry, as well as in the food and cosmetic areas [38]. Supercritical fluid extraction serves for the isolation of biologically active compounds from plant materials, mainly for those which cannot be separated by the use of simple solvent extraction.

Accelerated solvent extraction (ASE)

The accelerated solvent extraction method is a relatively new technique developed and distributed mainly by Dionex (Dionex Corporation, Sunnyvale, CA). In the literature, there are alternative names for this technique, such as pressurized fluid extraction (PFE) and pressurized liquid extraction (PLE).

In the ASE technique the same solvents are used as those in classical methods, but a higher pressure (about 3.3-20.3 MPa) and elevated temperature, about 40-200°C, are applied [38]. A sample extracted by this technique is placed in an extraction vessel made of stainless steel. The time of analysis is short, within the range of 5-15 min [47].

Basic advantages of ASE are: the possibility to extract samples with high humidity, short extraction time, better penetration of a sample by solvent, good extraction kinetics, automation of the process and relatively easy use of instrumentation [38]. Also, lesser volumes of solvents are needed. For example, during ASE extraction lasting about 20 min, only 10-50 mL of solvent were used, whereas in classical methods, such as Soxhlet extraction, extraction time is usually from 10 to 48 h, and the volume of solvent exceeds 400 mL [47].

Shake extraction

Beside the above-mentioned methods of sample extraction used for the analysis of plant material, extraction with various shaking devices has been performed. The process of shaking is aimed at enlarging the surface area where the solvent interacts with plant material, and in this way enhancing efficacy of the whole process and shortening its time. In this method, a sample is suspended in a certain volume of solvent and then shaken at a set stirrer speed. The method was applied by Li *et al*. [48] for the analysis of phenolics in *Phalaris canariensis* L. The

procedure was as follows. To the analysed sample, an 85:15 (v/v) mixture of 95% ethanol and 1 M hydrochloric acid was added, and the suspension was shaken on an orbital (rotary) shaker for 1 h at 30°C. After centrifugation of the extract, the plant material was re-extracted under the same conditions, and finally the combined filtrates were evaporated under reduced pressure. For further analysis, the dry residue was used after dissolving it in a 50% methanolic solution.

Fernand *et al*. [49] have also applied the shaking process for the extraction of a plant material. An ethanolic solution was used as the extractant, whereas the dry residue, after evaporation of the solvent, was dissolved in a 1:1 (v/v) mixture of ethanol and water. In the next step, solid-phase extraction was used to remove from the extract constituents interfering with phenolic components.

Santos-Gomes *et al.* [50] conducted their investigation in a similar way. However, instead of an ethanolic solution they used acetone and a shortened extraction time. A triple extraction was applied and simultaneous reduction of the solvent's volume: 50 mL over 15 min, 50 mL over 10 min and 25 mL over 5 min. Then, the residue left after concentration of the extracts under reduced pressure was dissolved in a methanolic solution, which was analysed by HPLC. Extraction by shaking was also applied for the analysis of rosmarinic acid in the leaves of lemon balm [51]. Aqueous methanol solutions were used as solvents, with methanol/water ratios of 40, 60 and 80% (v/v). Also, the impact of extraction time (30, 60 and 90 min) and temperature (25, 40 and 55ºC) on the process was studied. The optimum extraction conditions were obtained when the process was conducted during 60 min with use of 60:40 (v/v) methanol/water solution.

Solid-liquid extraction carried out in a separation funnel is also considered as one of the shaking methods, but extraction yields are more variable because shaking speed and strength cannot be controlled with great accuracy. In the case of analysis using gas chromatography (GC), the most often applied extraction processes are: solid-phase micro-extraction (SPME), headspace single-drop micro-extraction (HSDME) and microwave-assisted *extraction* (MAE) [52,53]. The last is commonly used for isolation of active substances from plant material [54]. The parameters most affecting its efficacy, are the following: the type and volume of solvent, radiation potential, and extraction time and temperature of the process [53,55-58]. Usually organic solvents are used, and their volume depends on the kind and mass of a sample [53,58]. In comparison with Soxhlet extraction (Table 2), lesser amounts of solvent are needed, and the extraction time is shorter. The root of *Salvia miltiorrhizae* was extracted by the MAE technique in order to determine selected phenolic acids [59], as well as dry roots of *Eucommia ulmodies*, in which chlorogenic acid, among others, was determined by using a methanol and water mixture and conducting the extraction at 40ºC [60]. A slightly lower temperature, 30ºC, was used for determining phenolic compounds in the herbs of *Hypericum perforatum* and *Thymus vulgaris*. HCl solution was used as the extractant [61].

Methods for the determination of phenolic acids

For separation, purification and identification of phenolic compounds in plant materials numerous chromatographic methods have been applied [10]. Moreover, chromatographic techniques are also used for investigation of interactions of phenolic compounds with other food constituents.

High-performance liquid chromatography (HPLC)

Quantitative analysis of phenolic compounds in plant material depends on the chemical nature of the constituents, the method of extraction, particle size, time and conditions of storage of the plant material prior to analysis, as well as on the determination method and the presence of interfering agents, such as fats, terpenes and chlorophyll [10]. Usually, chromatographic methods are applied, of which high-performance liquid chromatography [62,63] is the most common. In this method, different types of columns, mobile phases, column temperature and, to a smaller extent, the flow rate of the mobile phase, have been tested. Water, methanol and acetonitrile are the most common constituents of the mobile phase. Sometimes it is necessary to add modifiers, which facilitate the resolution of the components. The most popular modifiers are formic acid, ammonium acetate and acetic acid, the presence of which prevents tailing in the chromatograms. As far as the time of analysis is concerned, it is not a fixed parameter, because by modification of the flow rate of the mobile phase it is possible to extend or to shorten it. In the HPLC technique it is possible to use detection systems such as a UV-Vis spectrometer with either single wavelength or diode-array capability, chemiluminescence detector (CL), coulometric electrode array system (CEAD) and mass spectrometer (MS).

Reversed-phase, high-performance, liquid chromatography with UV-Vis detection was used by Waksmundzka-Hajnos *et al*. [30] for the analysis of the inflorescence of *Sambucus nigra* L. and the foliage of *Polygonum aviculare* L. Isocratic elution was applied with the use of two mobile phases. The first, used for *S. nigra*, was a 22:78 (v/v) mixture of methanol and orthophosphoric acid, and the other, applied for *P. aviculare* L., was a methanol/water (25:75 v/v) mixture, with addition of 1% of acetic acid. Phenolic compounds were detected at 520 nm.

Santos-Gomes *et al*. [50] applied HPLC for the analysis of phenolics in several plant species of *Lamiaceae*. The mobile phase was a mixture of acetonitrile, water and acetic acid (15:84:0.85 v/v) as solvent A and methanol as solvent B. Gradient elution was applied and the flow rate was set at 0.8 mL/min. The analytical wavelength was 280 nm, and identification of particular phenolic compounds was performed based on comparison of the retention times of the compounds with those of the standards.

Benetis *et al*. [40] have used HPLC with UV-Vis detection for the analysis of phenolic compounds in *Achillea millefolium* L. Gradient elution was applied with a mobile phase of acetonitrile/water, and trifluoroacetic acid (TFA) as a modifier to prevent ionization of phenolic groups. Before performing the analysis, two types of columns were tested: Xterra RP18 (Waters) and Ascentis RP-Amide Supelco; the latter column was chosen. The phenolic compounds were analysed at 25°C, and a 10 µL sample was injected into the column with a flow rate of 1.5 mL/min and detection at 360 nm.

Liquid chromatography with UV detection was also applied for the determination of phenolic acids in *Echinacea purpurea* [64], the leaves of lemon balm [65], aqueous extracts of *Hypericum perforatum* [66], and in 32 medicinal plants growing in Poland [67]. In the first case, the derivatives of caffeic acid were determined at 330 nm. In the second case, caffeic, ferulic and *p*-coumaric acids were determined at 325 nm, gallic, vanillic and syringic acids at 280 nm, and in the third case, all phenolic acids at 210 nm.

Liquid chromatography with diode array detection (DAD), in comparison with single wavelength UV/Vis detection, enables registration of the absorption spectrum of a compound over a wide range of wavelengths and determination of the absorbance maxima. This type of detection was applied for establishing the chemical composition of extracts of *Salvia halophila* [32], for identification and quantification of *p*-hydroxybenzoic acid derivatives in lemon balm (*Melissa officinalis*) [33], phenolic acids in the roots of *Salvia*

Table 3: Comparison of LC-MS with GC-MS for the analysis of phenolic compounds in medicinal plants [77].

Parameters	LC-MS	GC-MS
Time of sample preparation	20 min	180 min
Time of analysis	60 min	50 min
Range of linearity	limited	good
Selectivity	good	high
Limit of detection	5-15 ng/mL	10-80 ng/mL
Identification	possibility of calculation of empiric equation	mass spectral library for large group of compounds; fragmentation enables evaluation of molecular structure
Ruggedness of the system	satisfactory	very good

miltiorrhiza [68], in preparations of Chinese plants [69], and in plant-derived foods [27]. Another example of UV detection of polyphenolic compounds in HPLC was the use of a photodiode array detector by scanning between 200 and 400 nm, with a resolution of 1.2 nm [28].
In recent years, a growing interest has been gained in HPLC with chemiluminescent detection, the advantage of which is high sensitivity and selectivity. However, this method also has some drawbacks. First is its limited application, due to the small number of HPLC-CL chemiluminescent reactions. There are several chemical agents enabling derivatization, and in spite of the fact that they broaden the identification range for constituents not connected with chemiluminescent reactions, their excess or lack of integration can interfere with identification of analytes. Another disadvantage appears when the sample constituents are detected at a slow flow rate, and the mobile phase is incompatible with the chemiluminescent reactions; this precluding identification of these constituents. Another drawback of the method is that the intensity of chemiluminescence depends on such environmental factors as the kind of solvent, pH and ionic strength [70]. Despite those disadvantages, Cui *et al*. [70] decided to use this method in their research. They performed both isocratic and gradient elution at 25°C and a flow rate of 1.0 mL/min. The mobile phase was chosen out of three mixtures: acetonitrile-orthophosphoric acid-water, methanol-acetic acid-water, and phosphate-acetonitrile-methanol. It was found that when the concentration of acetic acid was 1.5%, the intensity of chemiluminescence was highest. Similar effect was obtained with a 35% methanolic solution and, accordingly, the 35:65 (v/v) of methanol-1.5% acetic acid mixture was selected as the mobile phase for the analysis. After completion of these measurements, it was found that peaks in the chromatograms registered using CL detection were higher than those obtained by the DAD detection system.

A less commonly used type of detection system is coulometric electrode array detector (CEAD). This detector was applied for determining ferulic and *p*-coumaric acids in the seeds of common flax and in the roots of nettle [71]. HPLC-CEAD is characterised by high sensitivity and precision, based on redox activity of the analyte [72].

In some laboratories, HPLC coupled with mass spectrometry (MS) is also used [73]. Guo *et al. [*74] have applied the LC-MS technique for the simultaneous determination of six phenolic acids in rat plasma after intravenous administration of a traditional Chinese medicinal preparation *Guanxinning* in a form of a lyophilised powder containing *Salvia miltiorrhiza* Bge. The same type of detection was used for determining caffeic acid in products from *Echinacea* sp. [45], chlorogenic acid in the leaves of lemon balm [65], and caffeic and protocatechuic acids in the roots of sage [75]. Furthermore, LC-MS and NMR techniques were also used for identification and structure establishment of eighteen phenolic acids and mono- and diglycosidic flavonoids [76].

Gas chromatography (GC)

Gas chromatography is one of the less common techniques used for the determination of phenolic acids. Using this technique, it is possible to analyse only several small molecule phenolic acids (below 600 D) [66,77]. Moreover, GC requires high temperatures, which can lead to sample decomposition [78].

For the analysis of phenolic acids in plant materials, GC coupled with mass spectrometry (GC-MS) is very often used. In comparison with LC-MS, this method allows better selectivity, precision and accuracy, especially when small amounts of compounds have to be analysed in plant material [77].

GC-MS was applied by Fiamegos *et al*. [79], who determined the contents of phenolic acids in medicinal plant raw materials and their infusions. They used GC-MS instrumentation with SIM (*selective ion monitoring*). Owing to this modification of the method, the currents were registered only for selected ions with characteristic masses, typical of the studied analyte. The method with SIM is useful for determining constituents occurring at low concentrations in complex mixtures.

Proestos *et al*. [41] have also applied GC-MS for the determination of phenolic acids in aromatic plants, such as nettle and common rue. They used capillary gas chromatography, silylation as a derivatization procedure, and *N,O-bis*(*trimethylsilyl*)-*trifluoro-acetamide* and *trimethylchlorosilane as* silylating *agents. A*long with capillary gas chromatography (CGC-MS), they also used HPLC with UV detection at 280 nm.

A comparison of GC-MS with LC-MS (Table 3) has shown that the first method requires a time-consuming sample preparation step prior to analysis, but the time of analysis is shorter, and the linearity and selectivity of the method is much better than that in the case of LC/MS [77]. The sample preparation step includes dynamic sonication-assisted extraction of herbal samples, followed by the extracts being further treated by liquid-liquid extraction and derivatization. In GC-MS the compounds are identified by comparison of their spectra with those available in the mass spectra library.

It is also worth mentioning that, among the chromatographic methods, high-speed, counter-current chromatography (HSCCC) [80] was used by Yang *et al.* [81] for isolation and purification of phenolic acids originating from a Chinese medicinal plant, *Smilax china*.

Capillary electrophoresis (CE)

Recently, capillary electrophoresis (CE) has achieved growing significance in the analysis of phenolic compounds [82]. This method allows the separation and determination of polar substances of both ionic and non-ionic character, as well as non-polar, non-ionic substances. Advantages of CE are a small volume of electrolytes, short analysis time, high resolution potential and small sample weight [83-86]. In order to achieve proper resolution by this method, it is necessary to optimize such parameters as the type of buffer, its pH and concentration, the type of capillary and its volume, temperature of electrophoresis, voltage and the way of injection of a sample [87].

Each of these parameters will depend on CE methodology and on the chemical properties of the studied phenolic acid and its matrix.

Before analysis by capillary electrophoresis, the active compounds are extracted into solution, usually by supported liquid *extraction* (SLE) [37,44,88-92], supercritical fluid *extraction* (SFE) [93,94], and liquid-liquid extraction (LLE) [9,25]. The less common methods are microwave-assisted solvent *extraction* (MAE), taking only several minutes [84], and Soxhlet extraction [95].

Apart from CE, another popular technique applied for the determination of phenolic compounds, is *capillary* zone electrophoresis (CZE) [96,97]. This method was used, among others, for the determination of rosmarinic acid in commercial sage tea-bags [85]. Of the tested solvents, such as methanol, acetone and acetonitrile, the best was methanol. As the extraction technique, ultrasonication was used, and for detection, UV spectrometry at 210 nm. Resolution of the compounds was performed in a quartz capillary, 50 μm in diameter, and a solution of borate as a separating buffer was used. Beside CE and CZE, sometimes micellar electrokinetic chromatography (MEKC) is also used. By using this technique, phenolic acids were determined in the roots and pods of *Echinacea purpurea* [9], as well as in the herb of *Artemisia capillaris* [98]. C*apillary* electrochromatography (CEC) was applied for the analysis of extracts obtained from the flowers of chamomile [82].

As detection methods in capillary electrophoresis and its modifications, optical methods are used (fluorimetric, phosphorimetric, chemiluminescent, UV-Vis, IR and Raman spectrometries, and refractometry), as well as electrochemical methods (conductometric, potentiometric, amperometric and voltamperometric), and other, such as mass spectrometry and radiometry. The most frequently used detectors are those based on UV, MS and amperometric methods. They assure high sensitivity and selectivity.

CE with UV detection is commonly used for the analysis of phenolic acids in plant materials, and the most often applied electrolyte is a borate buffer of pH 9.2 [99]. This buffer was used for the determination of phenolic acids in *Strobili lupuli* [91], *Cortex fraxini* [92], and the fruits of sea buckthorn berries [100]. Less frequently, a MOPSO buffer solution is used; this was applied together with Tris solution and boric acid, pH 8.3, for the determination of chlorogenic acid in *Hypericum perforatum* [101]. Also a phosphate buffer with acetonitrile, pH 2.8, was used for the determination of caffeic and chlorogenic acids in *Matricaria chamomilla* L. [82].

MS detection is useful for identification of chemical compounds in mixtures as it can elucidate chemical and structural information about molecules from their molecular weight and distinctive fragmentation patterns [102]. In the CE method coupled with MS, resolution of the constituents can be regulated by changing the pH. This technique is useful when other analytical techniques do not provide reliable results. Moreover, the CE-MS combination opens more analytical options, such as increase in sensitivity and specificity of the method. Application of this hyphenated method does not require using complicated sample preparation procedures and in comparison with the chromatographic methods, it is a good alternative for simultaneous analysis of phenolic compounds, because it provides rapid and efficient separations and uses reduced sample and solvent consumption [103].

Concluding remarks

Phenolic acids are secondary metabolites widespread in the plant kingdom. Because of their wide spectrum of biological activity, isolation, quantification and structure establishing of these compounds is still a crucial matter. On the one hand, there are methods of isolation of phenolic acids from medicinal and dietary plants, on the other, analytical techniques for quantitation of these secondary metabolites. Literature screening has shown that although Soxhlet and ultrasound-assisted (UAE) extractions are commonly used for isolation of polyphenols from plant matrices, alternative techniques of extraction, such as supercritical fluid (SFE) and accelerated solvent (ASE) are more and more often used. Non-questionable advantages of Soxhlet extraction are its low cost and good recovery, but there are some disadvantages, such as large volume of solvent, long time of extraction and tedious handling procedure. Recently introduced advanced techniques of extraction, such as SFE and ASE, are not as profitable as initially expected. Supercritical fluid extraction takes place under subcritical conditions, and this time-consuming and expensive procedure is limited to compounds of low or medium polarity. On the other hand, accelerated solvent extraction is performed with high extraction temperatures that may lead to degradation of thermolabile compounds. Furthermore, this technique is considered as a potential alternative to SFE for extraction of polar compounds.

Quantitation of phenolic acids is usually performed by liquid chromatography (LC) owing to the recent developments in this technique, especially when it is coupled with mass spectrometry (MS). Other advanced techniques used for quantitation of the secondary metabolites are gas chromatography coupled with mass spectrometry (GC-MS) and capillary electrophoresis (CE). All the above techniques have some limitations. For example, LC with DAD and MS provided short time analysis with less sample preparation, while GC method, which is used very often with MS detection, required a relatively high temperature, which can lead to sample decomposition. Moreover, GC can be used only for several small molecule phenolic acids (below 600 D). Recently, CE has achieved growing significance in quantitation of phenolic acids. This technique provides short analysis time and small volume of electrolytes, and the most common detectors are UV, MS and amperometric.

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