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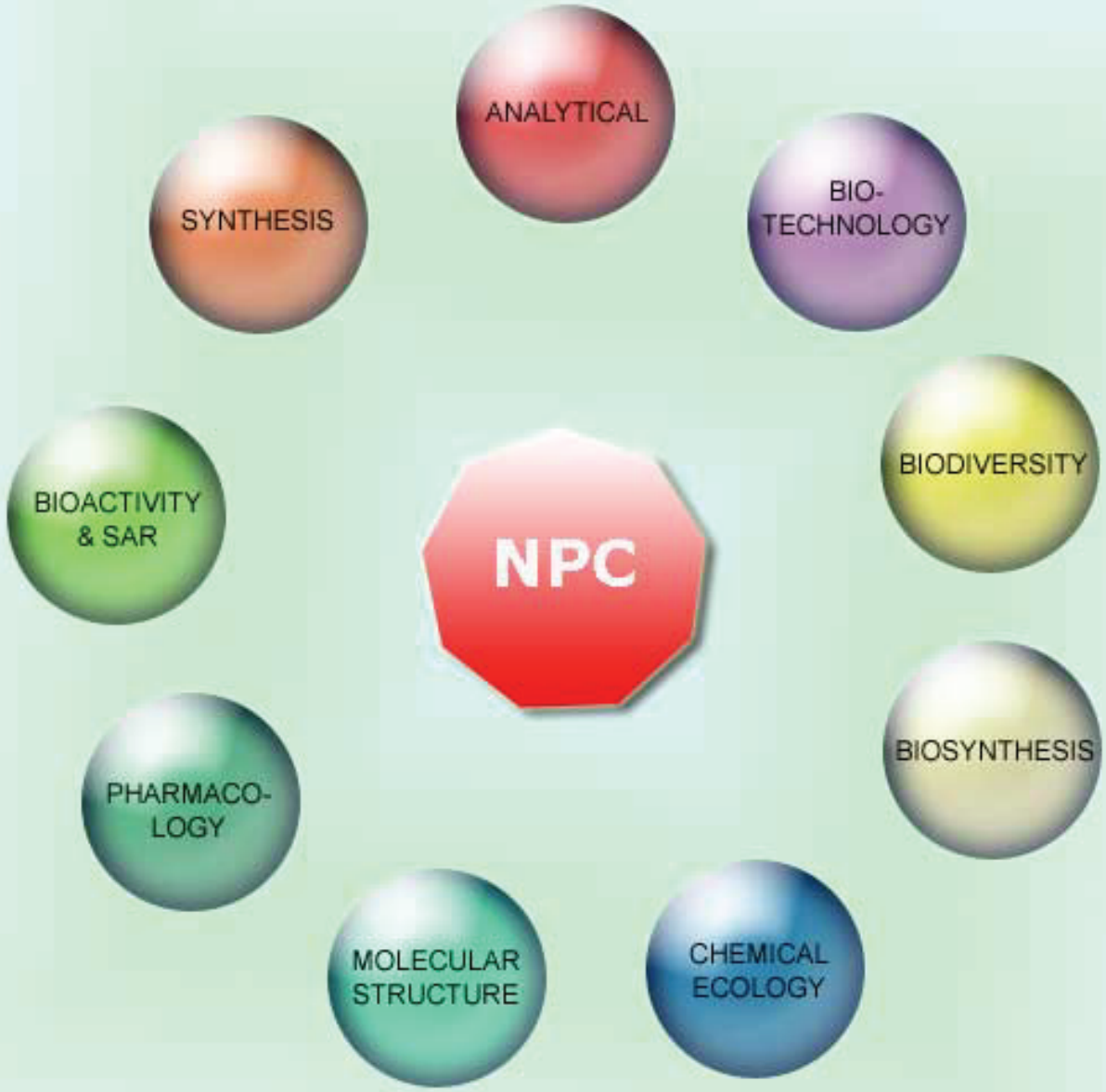

NPC-CMAPSEEC Issue

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Editorial

NPC- CMAPSEEC: Special Issue

I am very grateful to Prof. Vassya Bankova, Chairman of 9th Conference of the Association of Medicinal and Aromatic Plants of South-Eastern Europe (9th CMAPSEEC), Centre of Phytochemistry, Bulgarian Academy of Sciences, Sofia, Bulgaria, Prof. Zora Dajic, President of Association of Medicinal and Aromatic Plants of South-Eastern European Countries (AMAPSEEC), as Honorary Chairperson 9th CMAPSEEC, and Organizing Committee, for arranging this issue, originating from the CMAPSEEC-2016 which was held in Plovdiv, Bulgaria, from May 26–29, 2016, and attended by a large number of participants. The first part of February 2017 edition is devoted to selected manuscripts (16) presented at CMAPSEEC-2016. I am very grateful to Prof. Vassya Bankova for extending an invitation to participate in this scientific meeting as well as for organizing this issue. The editors join me in thanking Prof. Bankova, the authors and the reviewers for their efforts that have made this issue possible, and to the production department for putting it into print.

Pawan K. Agrawal
Editor-in-Chief

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Introduction to the Special Issue on the 9th Conference on Medicinal and Aromatic Plants of the South-Eastern European Countries (9th CMAPSEEC, 2016)

This special issue contains a selection of papers originally presented at the 9th Conference on Medicinal and Aromatic Plants of the South-Eastern European Countries (9th CMAPSEEC), which was held in the beautiful city of Plovdiv, Bulgaria, on May 26 – 29, 2016. It was organized by the Association of Medicinal and Aromatic Plants of the South-Eastern European Countries, together with the Bulgarian Phytochemical Society and the Institute of Organic Chemistry with the Centre of Phytochemistry of the Bulgarian Academy of Science, and with the President of AMAPSEEC, Prof. Zora Dajic, as Honorary Chairperson.

The 9th CMAPSEEC continued the practice of the AMAPSEEC conference series of presenting the most recent advances and state of the art research in the field of medicinal and aromatic plants in South-Eastern Europe, a region with rich biodiversity and a long standing tradition of use of plants for medication. As usual, the Conference gathered scientists, professionals and representatives of companies working in the exciting field of medicinal and aromatic plants not only from South-Eastern Europe but also from many countries all over the world. The conference provided a great opportunity to share research results, new approaches and ideas, and views and visions for the development of the application of medicinal and aromatic plants for the benefit of society. It became a celebration of human curiosity and endeavour to explore Nature. Coverage of the Conference included: Medicinal and aromatic plants (MAP) diversity at all levels and tools for its evaluation; Pharmacology and biological effects of active MAP compounds; and MAP cultivation, breeding and biotechnology. The 9th CMAPSEEC was very successful: it attracted over 150 participants from 26 countries, with 10 prominent scientists as invited lecturers, 31 short lectures and over 160 poster presentations.

This issue of *Natural Product Communications* presents some of the highlights of the conference. Edited by members of the conference organizing committee Prof. Milena Popova, Prof. Milen Georgiev, Prof. Vassya Bankova, and the Journal Editor in Chief, Dr. Pawan Agrawal, the articles selected here were submitted shortly after the conference and rigorously peer-reviewed and revised before being accepted for publication. The editors received a high number of articles from the conference participants, and all submissions received extensive feedback from the editors and anonymous peer-reviewers. Unfortunately, not all submissions were accepted, but the editors wish all those who submitted well with their future research and careers, and we look forward to reading their work with interest.

We would like to extend a special thanks to the authors and reviewers of the papers. Also, we are grateful to Dr. Pawan K. Agrawal, the Editor in Chief of *Natural Product Communications*, and the editorial team for their assistance in the preparation of this issue and for continued support and collaboration between the AMAPSEEC conference and *NPC*.

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Phytochemical Profile of *Inula britannica* from Bulgaria

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The flower heads of *Inula britannica* L. of Bulgarian origin afforded sesquiterpene lactones (gaillardin, britannin, 11,13-dihydroinuchinenolide B, ivalin, pulchellin C), triterpenoids (3-*O*-palmitates of 16β-hydroxylupeol, 16β-hydroxy-β-amyrin, and faradiol) and flavonoids (quercetin, luteolin, luteolin-7-*O*-glucoside). All compounds are known and they were identified by spectral methods. The observed differences in the chemical content of the chloroform and methanol extracts were also reflected in their free radical scavenging activity, evaluated by DPPH and ABTS assays. Intraspecific variability of *I. britannica* is discussed.

Keywords: *Inula britannica*, Asteraceae, Sesquiterpene lactones, Triterpenoids, Flavonoids, DPPH and ABTS assays.

The genus *Inula* (Asteraceae) consists of approximately 100 species, distributed mainly in Asia and Europe. Many studies have been carried within the genus due to the significant structural diversity and biological activity of sesquiterpene lactones, diterpenes, triterpenes, and flavonoids, isolated from a number of *Inula* species [1,2]. *I. britannica* is used in Chinese folk medicine to treat digestive disorders, bronchitis and inflammation, bacterial and viral infections, as well as some tumors [2b]. That is why most of the studies are on *I. britannica* of Chinese origin. The application in traditional medicine requires extensive phytochemical study and pharmacological evaluations of this species. It should be noted that sesquiterpene lactones were the largest group. More than 40 lactones of different skeletal types – germacranolides, eudesmanolides, guaianolides, secoeudesmanolides, seco-guaianolides, and lactone dimers, have been described so far. To the best of our knowledge, from the European taxa only Russian [3] and Serbian [4] have been investigated up to now.

In continuation of our studies on species of the Asteraceae family this work is a part of a phytochemical investigation of Bulgarian taxa from the genus *Inula*. The $CHCl_3$ extract of the flowers of *I. britannica* was fractioned by column chromatography on silica gel. IR control allowed selection of the fractions (characteristic absorption band at 1770-1740 cm^{-1}) for further determination of the lactone profile. Thus, the sesquiterpene lactones ivalin [5], britannin [3a,4], gaillardin [4,5a], 11,13-dihydroinuchinenolide B [4], and pulchellin C [3b,4] have been isolated and identified. All these compounds have been previously found in *I. britannica* of different origins. With exception of ivalin, the other compounds were reported for the Serbian taxon [4]. From the latter one, 1,10-seco-eudesmanolides – 14-(3-methylpentanoyl)-6-deoxybritannilactone, 14-(3-methylbuta-noyl)-6-deoxybritannilactone, and 14-(2-methyl-propanoyl)-6-deoxybritannin, as well as 4H-tomentosin have been isolated. Varieties of lactones with secoeudesmane and secoguaiane skeletons have been reported before in the Asian populations of *I. britannica* [1,2]. It should be noted that no one of these substances was detected in the studied Bulgarian sample.

It was found (IR control) that the less polar fractions did not contain sesquiterpene lactones, but the absorption band at 1720 cm^{-1} showed the presence of compounds bearing ester groups. Further on, prep. TLC yielded the known triterpenoids 3-*O*-palmitates of 16β-hydroxylupeol, 16β-hydroxy-β-amyrin, and faradiol. Only the first one has been found in a Turkish population of *I. britannica* so far [6a], while the other two compounds are isolated now for the first time from this species, but they have been detected earlier in *Achillea alexandri-regis* [6b] and *Calendula officinalis* [6c], respectively. β-Sitosterol and β-amyrin were also detected. More or less, different triterpenoids have been isolated from the genus *Inula*, but esters of long-chain fatty acids have been detected only in one Turkish population of *I. britannica* [6a]. It should be noted that the literature data for triterpenoids in *Inula* species are insufficient for discussion of their chemotaxonomic significance.

Besides the described above compounds, 3 flavonoids and 1,5-dicafeloylquinic acid were isolated from the MeOH extract. Luteolin, luteolin-7-*O*-glucoside and quercetin are known for *I. britannica*, while 1,5-dicafeloylquinic acid was detected in *I. viscosa* [2]. Being very common plant components and usually in high concentrations, at this point of the investigation, these compounds were not regarded as compounds of chemotaxonomic interest.

Further, TLC comparison of extracts from flowers and leaves showed that both plant organs accumulated the same sesquiterpene lactones. Regarding phenolic components, it was found that luteolin, luteolin-7-*O*-glucoside, and 1,5-dicafeloylquinic acid were present in both leaves and flowers, while quercetin was detected only in flowers. Finally, the observed differences in the chemical composition of the chloroform and methanol extracts were also reflected in their free radical scavenging activity, evaluated by DPPH and ABTS assays (Table 1). As can be seen, both methanol extracts (from flowers and leaves) possessed the highest antioxidant activity and maximal total phenolic and flavonoid contents. A good correlation between antiradical activity in the studied extracts and their total phenolic content was also observed ($R^2 = 0.9954$ and $R^2 = 0.9910$ for ABTS and DPPH, respectively).

Table 1: Total phenolic (TPC) and flavonoid (FC) contents in different *I. britannica* extracts and their radical scavenging activity.

Plant parts	Extract	TPC [mgGA/gDM]	FC [mgC/gDM]	ABTS [μMT/gDM]	DPPH [μMT/gDM]
Leaves	$CHCl_3$	0.4±0.02	0.3±0.02	1.6±0.1	0.7±0.1[a]
Flowers	$CHCl_3$	0.1±0.02	0.1±0.02	1.1±0.1	0.7±0.02[a]
Leaves	MeOH	2.3±0.1	0.6±0.01	15.5±0.2	13.7±0.7
Flowers	MeOH	7.9±0.4	0.9±0.02	44.4±0.4	37.6±0.6

[a]Values with the same letter are not significantly different, $p \leq 0.05$. Standard deviations were calculated on the base of three samples.

Diversity of lactones found in the so far studied taxa of *I. britannica* of Asian and European origin revealed significant intraspecific variability. Nevertheless, on the basis of the skeleton type of lactones it could be suggested that secoeudesmanolides and secoguaianolides characterized the Asian populations. On the other hand, Bulgarian and Russian (European) populations are free of these types of lactones. Further, secoeudesmanolides and one secoguaianolide have been isolated from a Serbian taxon, but bicyclic lactones are principal components. So, the existence of secoeudesmanolides and secoguaianolides in the European *I. britannica* cannot be ruled out. Thus, the domination of guaianolides and eudesmanolides or their seco-derivatives characterize European and Asian taxa, respectively. Further phytochemical investigation on *I. britannica* will clarify the lactone profile of European populations.

Experimental

Plant material: *I. britannica* was collected from a natural locality in the Southern Balkan Region in Bulgaria. A voucher specimen (SOM 172474) was deposited in the Herbarium of the Institute of Biodiversity and Ecosystem Research, Bulg. Acad. Sci.

Extraction and isolation: A portion (1 g) of air-dried flower heads and leaves of *I. britannica* were extracted with $CHCl_3$ and MeOH. The corresponding crude extracts were compared by TLC (Silica gel 60 and RP-18, F_{254} (Merck), $CHCl_3$-diethyl ether, 50:1; $CHCl_3$-acetone, 10:1 and 1:1; MeOH-H_2O, 1:1) and used for determination of the total phenolics, flavonoids and antioxidant capacity. Further, the $CHCl_3$ and MeOH extracts from flower heads (30 g) of the plant were worked up for isolation of the individual compounds. The $CHCl_3$ extract (1.5 g) was fractionated by column chromatography (CC) on silica gel using $CHCl_3$-acetone mixtures with increasing polarity to give 11 fractions (F_1-F_{11}). Further separation by CC and prep. TLC (silica gel, *n*-hexane-diethyl ether, 5:1) of Fr. F_2 (50mg) afforded 3-*O*-palmitates of 16β-hydroxylupeol (5.7 mg), 16β-hydroxy-β-amyrin (5.3 mg), and faradiol (5.0 mg). The presence of β-amyrin and β-sitosterol was proved by TLC of F_3 (silica gel, *n*-hexane-diethyl ether, 1:1) using these compounds as standards. Prep. TLC (silica gel, $CHCl_3$-acetone, 5:1) of F_7 (47 mg) afforded ivalin (7 mg) and britannin (14 mg). Gaillardin (33 mg) and pulchellin C (18 mg) were obtained from F_9 (65 mg) and F_{11} (92 mg), resp. after recrystallization ($CHCl_3$). Prep. TLC (silica gel, $CHCl_3$-acetone, 5:1) of F_{10} (18 mg) yielded 11,13-dihydroinuchinenolide B (7 mg). The MeOH extract (0.5 g) was separated into 2 fractions F-1 and F-2 by CC (Sephadex LH-20, MeOH). Prep. TLC (silica gel RP-18, MeOH-H_2O, 1:1) of F-2 (50 mg) yielded luteolin (9 mg), quercetin (1.8 mg), luteolin-7-*O*-glucoside (2.0 mg), and 1,5-dicafeoylquinic acid (6.4 mg). All isolated compounds were proved by comparison of their ^{1}H NMR spectral data with those in the literature.

Determination of the content of total phenolic compounds (TPC) and total flavonoids (FC): TPC and FC were determined by procedures described in ref. [7a,b] and expressed as mg gallic acid equivalents per g dry plant material [mgGA/gDM] and mg (+)-catechin equivalents per g dry plant material [mgC/gDM], respectively.

DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity and ABTS [2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)] assays: The DPPH and ABTS assays were performed according to the procedures described by Thaipong *et al.* [7c] and the antioxidant activity was expressed as μM Trolox equivalents per g dry plant material [μMT/gDM].

Statistical analysis: Correlation coefficients (R^2) for determination of the relationship between the radical scavenging activity and the TPC and FC were calculated using MS Excel software.

Acknowledgments - This work was supported by the framework of the Bulgarian-Swiss Research Programme under Grant No. IZEBZ0_142989; DO2-1153).

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Production of Δ^7-Sterols from *In Vitro* Root Cultures of Endangered *Gypsophila trichotoma*

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Species from the genus *Gypsophila* are known for their medicinal, industrial and decorative applications. *G. trichotoma* Wend. is an endangered plant species for the Bulgarian flora according to the Red Data Book. Δ^7-Sterols, which are unusual and rare in the plant kingdom, are present in the roots of this species. In previous studies different *in vitro* cultures were established from aerial parts of the species. The objective of this study was to explore the possibility for production of Δ^7-sterols from *in vitro* cultured roots of *G. trichotoma*. The root cultures were grown on six modified MS media and the quantity of sterols was analyzed. These findings will serve to solve the important matter of the role of nutrients on sterols biosynthesis.

Keywords: Sterols, *In vitro*, Prostate hyperplasia, *Gypsophila trichotoma*.

Gypsophila trichotoma Wend. (Caryophyllaceae) is a perennial herbaceous plant. It is distributed in southeast Europe, southwest Asia, Kazakhstan, West Mongolia, Russia, and Turkmenistan. The plant is an endangered species for the Bulgarian flora according to the Red Data Book and protected by the Law of Biodiversity. Some *G. trichotoma* populations grow in the territory of the Kaliakra Reserve (Tyulenovo area) and are thus managed by the government. The plants grow on salt-rich, clay-type soils on the rock of the seacoast at around 50 m.a.s.l. The population of the taxon has decreased as a result of the reduction of its distribution. *G. trichotoma* has been proved to have high anti-inflammatory, antiviral, antibacterial, antioxidant, and hepatoprotective properties [1]. Phytochemical examination revealed the presence of triterpene saponins, flavonoids, sterols, triterpenes and volatiles [2]. The plant was introduced for *in vitro* cultivation at the Department of Pharmacognosy of the Faculty of Pharmacy in Sofia [3].

Rare Δ^7-sterols (Figure 1), previously isolated from the roots of the plant [4], have been reported to possess antitumor activity. They also have a positive effect in cases of prostate hyperplasia [5].

In vitro plant cultures were established on standard liquid MS–Li and modified MS–Li growth media (Table 1). An optimized HPLC method for determining the content of sterols was used.

Analysis of the data (Table 2) showed that NaCl had a positive effect on steroidal glycoside accumulation, while Mg^{2+} and Ca^{2+} had a beneficial effect on steroid synthesis. These findings could be assigned to the natural metabolism of the plant, which is naturally growing on salt-rich soils. Sodium chloride, Mg^{2+} and Ca^{2+} should be present in considerable concentration in order for Δ^7-sterols to be accumulated in the roots. The optimal culture media should contain these inorganic ingredients in the mentioned concentration.

In all of the tested samples stigmast-7-ene-3-ol (t_R = 20.879 min) was detected. This fact could be assigned to its role as an initial precursor in the biosynthesis of the two other sterols – stigmast-7-ene-3-one and stigmast-7-ene-3-*O*-β-D-glucopyranoside.

Table 1: Composition of Murashige and Skoog–Linum modified media (MS–Li) for 1 L medium, pH 5.6 (before autoclaving).

Compound [mg]	MS-Li	MS-Li +Mg^{2+}	MS-Li -Mg^{2+}	MS-Li +Ca^{2+}	MS-Li -Ca^{2+}	MS-Li +NaCl
NH_4NO_3	1650	1650	1650	1650	1650	1650
KNO_3	1900	1900	1900	1900	1900	1900
$MgSO_4x7H_2O$	370	740	0	370	370	370
$CaCl_2x2H_2O$	440	440	440	880	0	440
KH_2PO_4	170	170	170	170	170	170
NaCl	-	-	-	-	-	1.8
H_3BO_3	6.20	6.20	6.20	6.20	6.20	6.20
$MnSO_4x4H_2O$	16.9	16.9	16.9	16.9	16.9	16.9
$ZnSO_4x4H_2O$	8.60	8.60	8.60	8.60	8.60	8.60
KJ	0.83	0.83	0.83	0.83	0.83	0.83
$Na_2MoO_4x2H_2O$	0.25	0.25	0.25	0.25	0.25	0.25
$CuSO_4x5H_2O$	0.025	0.025	0.025	0.025	0.025	0.025
$CoCl_2x6H_2O$	0.025	0.025	0.025	0.025	0.025	0.025
Na_2EDTA	37.3	37.3	37.3	37.3	37.3	37.3
FeSO4x7H2O	27.8	27.8	27.8	27.8	27.8	27.8
Nicotinic acid	0.25	0.25	0.25	0.25	0.25	0.25
Vitamin B_1	0.05	0.05	0.05	0.05	0.05	0.05
Vitamin B_6	0.25	0.25	0.25	0.25	0.25	0.25
Mio-inositol	100	100	100	100	100	100
Glycine	2	2	2	2	2	2
2-Naphthylacetic acid	200	200	200	200	200	200
Sucrose	30000	30000	30000	30000	30000	30000

Table 2: Content of sterols in *in vitro* cultivated samples.

Root Culture	Stigmast-7-ene-3-*O*-β-D-glc, % ± SD	Stigmast-7-ene-3-one, % ± SD	Stigmast-7-ene-3-ol, % ± SD
MS – Li	0	0.61 ± 0.01	2.11 ± 0.01
MS – Li +NaCl	1.65 ± 0.01	0	0.63 ± 0.04
MS – Li +Mg^{2+}	0	2.47 ± 0.02	2.73 ± 0.02
MS – Li -Mg^{2+}	0	0	0.59 ± 0.04
MS – Li +Ca^{2+}	0	1.33 ± 0.02	1.14 ± 0.03
MS – Li -Ca^{2+}	0	0	0.78 ± 0.02

Further optimization of the concentration of inorganic components in the nutrient media is needed in order to gain maximal amount of these rare compounds.

Experimental

Plants samples: Seeds of the native population of *G. trichotoma* were collected at the Black Sea Coast, Zelenka locality, near Balgarevo village, Bulgaria with permission from the Ministry of Environment and Water of the Republic of Bulgaria. Seeds were surface-sterilized with 95% EtOH for 60 sec, then in a 20% solution of commercial bleach (20 min), followed by 3 times rinsing with

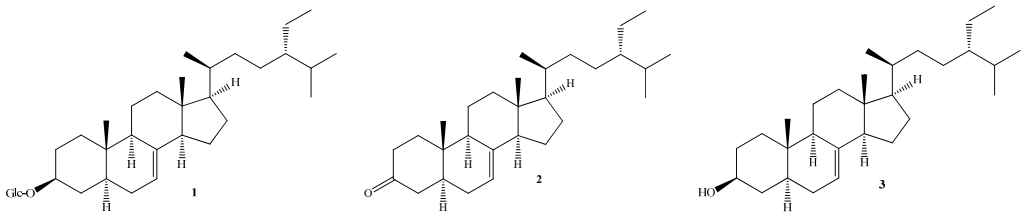

Figure 1: Sterols from roots of *G. trichotoma*: **1** – Sigmast-7-ene-3-*O*-β-D-glucopyranoside; **2** – Stigmast-7-ene-3-one; **3** – Stigmast-7-ene-3-ol.

sterile water. After sterilization the seeds were germinated aseptically. Variations in culture media were prepared as MS medium was supplemented with phytohormones and additional quantities of inorganic salts. The germinated seeds were transferred into flasks (300 mL) containing solid Murashige and Skoog (MS) plant growth media [6], and grown in an illuminated chamber (20°C) to produce shoots. After 4 weeks the roots were well developed. They were aseptically cut from the plants and transferred into modified liquid MS–Li media with addition of 2-naphthylacetic acid (Table 1) and grown at 20°C in the dark. The roots were aseptically sub-cultured every 15 days on fresh media, having the same composition.

Sample preparation: Air-dried powdered plant material (0.20 g) was extracted with methanol twice on a water bath under reflux for 30 min. The resulting extract was filtered and diluted to 10.0 mL in a volumetric flask with the same solvent. An aliquot of the sample solution (20 µL) was injected.

***Reference solution*:** Reference solutions were prepared by dissolving a requisite amount of sterols in methanol.

Instrumentation: Chromatographic analyses were performed on a chromatographic system Young Lin 9100 (Hogye-dong, Anyang, Korea), which consisted of a YL 9101 vacuum degasser, YL 9110 quaternary pump, YL 9131 column compartment, YL 9160 PDA detector, 7725i manual injector, equipped with a 4.6 x 250 mm column Luna® 5U C18 (2) 100 Å, Phenomenex® ODS with particle size 5 µm. YL-Clarity® software was used.

Chromatographic conditions: An isocratic mobile phase of methanol: acetonitrile (30:70 v/v) was used; 210 nm wavelength; column temperature 40°C; flow rate 1 mL/min. Stigmast-7-ene-3-*O*-β-D-glucopyranoside (t_R = 16.312 min); Stigmast-7-ene-3-one (t_R = 18.143 min); Stigmast-7-ene-3-ol (t_R = 20.879 min).

Standard and chemicals: Air-dried powdered roots (700 g) were exhaustively extracted with 80% methanol. After partial evaporation, the aqueous solution was extracted with CH_2Cl_2. The dichloromethane extract was further subjected to flash-chromatography over silica gel to gain the sterols. These were purified *via* recrystallization. Structural assessment of the glycoside was carried out by acid hydrolysis, followed by TLC with authentic reference substances and for all sterols – MS and ^1H and ^{13}C NMR spectroscopic experiments were performed, as previously described in [4].

The organic solvents (HPLC-gradient grade) and all other reagents were provided by Merck (Germany).

Statistical analysis: Each experiment was performed in triplicate. MedCalc 12.3 (MedCalc Software 2012) was used to perform the calculations. The Krushkal-Wallis one-way analysis of variance was conducted to define the statistical significance of sterol amount. The results were defined as mean ± SD. Probability value of $P \leq 0.05$ was used as significance criteria.

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Evaluation of Glaucine Content in Bulgarian Black Sea Coast Localities of *Glaucium flavum* Cranz. (Papaveraceae)

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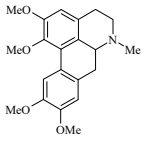
The content of the alkaloid glaucine was evaluated in *Glaucium flavum* plants from seven localities along the Bulgarian Black sea coast during two consecutive years, in order to select those with highest glaucine content. Some fluctuations of glaucine content were observed during the two years, and in most of the localities the alkaloid was lower in 2015. Pomorie and Ahtopol maintained high percentages of glaucine in the dry plant material in the two investigated years, being 2.3% for Pomorie in 2014 and for Ahtopol in 2015. The lowest percentages of glaucine were recorded in the plant material from Shkorpilovtsi (0.9% and 0.6%, respectively in 2014 and 2015). Fluctuations in glaucine content were probably due to some abiotic factors as light, temperature, precipitation, soil substrate, salinity, etc.

Keywords: Yellow hornpoppy, Glaucine, Alkaloids, Bulgarian localities.

Glaucium flavum Crantz. (yellow hornpoppy) is a medicinal plant from Papaveraceae family, which spreads along seacoast sands, shingle and rocky places [1a], sometimes it grows inland in river valleys with sandy-pebble beds [1b]. Species' natural area of distribution is West and South Europe, Caucasus [1a], and Scandinavian Peninsula [1c]. In Bulgaria the yellow hornpoppy is mainly distributed along the Black Sea coast.

G. flavum is a source of isoquinoline and morphinane alkaloids and the principal of them are glaucine, isocorydine, corydine, protopine, isoboldine, corunnine, chelidonine, sanguinarine, cataline, 7-oxoglausine, thaliporphine [2]. The major aporphine alkaloid glaucine (**1**) is used for preparation of medicines with antitussive activity, which efficacy is comparable to codeine, but glaucine does not cause the typical side effects of codeine such as addiction, constipation and respiratory depression [3].

Plant resources are limited and the plant harvest is forbidden on the whole territory of Bulgaria on the strength of annual ordinance of the Ministry of Environment and Water, in compliance with the Medicinal Plants Act (2000). Previous studies revealed differences in the alkaloid content of several Bulgarian populations [2c], however, data actualization is needed, and moreover some localities were no more confirmed during the last years. Our previous research concerning four Bulgarian localities in 2013, also ascertained differences in their glaucine content [4].



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The present study aimed to determine and evaluate in two consecutive years the glaucine content in *G. flavum* plants growing in different localities along the Bulgarian Black sea coast, and to specify the localities with the highest glaucine content. Seeds from them could be further used for *in vitro* cultures initiation and multiplication of high yielded glaucine plants.

The values of glaucine content of the plant material collected from five of the investigated localities during 2014 were comparable, being about 2%, (Table 1). The other two localities, near Skorpilovtsi and Durankulak, distinguished as their glaucine contents were twice and more lower than the average for that year. The values of glaucine quantity of 2015 could be divided into three groups regarding glaucine content in the dry plant material: lowest

Table 1: Localities of *Glaucium flavum* along the Bulgarian Black Seacoast, and glaucine content in the crude alkaloid mixture (CAM) and in the dry plant material.

Locality, GPS coordinates, altitude	Glaucine in the dry plant material (%)		Glaucine in CAM (%)		CAM in the dry plant material (%)		Sample dry weight (g)	
	2014	2015	2014	2015	2014	2015	2014	2015
Durankulak N 43.69110, E 28.56325, 1 m	1.0±0.1	1.4±0.04	43.6±2.9	72.5±2.1	2.3	2.0	15.1	18.0
Shabla N 43.54282, E 28.60612, 3 m	2.1±0.1	1.5±0.03	76.0±3.1	68.1±1.4	2.8	2.2	18.0	18.0
Skorpilovtsi N 42.95834, E 27.89775, 3 m	0.9±0.1	0.6±0.1	47.8±2.8	36.5±4.8	1.9	1.7	18.5	18.0
Pomorie N 42.58634, E 27.63191, 6m	2.3±0.1	2.0±0.04	74.2±1.7	59.4±1.3	3.0	3.4	11.0	18.0
Varvara N 42.13460, E 27.89718, 0 m	2.0±0.04	0.8±0.02	68.4±1.1	47.2±1.4	2.9	1.7	16.7	18.0
Ahtopol N 42.10403, E 27.93655, 4 m	2.0±0.04	2.3±0.1	68.7±1.2	58.1±2.3	3.0	4.0	15.0	8.4
Sinemorets N 42.06564, E 27.97379, 6 m	2.1±0.1	1.6±0.1	60.6±4.1	50.5±1.5	3.4	3.2	12.5	18.0

(Skorpilovtsi and Varvara), intermediate (Durankulak, Shabla and Sinemorets), and highest (Pomorie and Ahtopol) (Table 1).

Some fluctuations have been observed comparing glaucine content in the plants from the investigated localities during the two years. In most of them the alkaloid was lower in 2015, as well as the crude alkaloid mixture (CAM). An exception was the locality of Ahtopol. The lowest percentages of glaucine were recorded in the plant material from Shkorpilovtsi (0.9% and 0.6%, respectively in 2014 and 2015). These results complemented and confirmed our first data concerning Shkorpilovtsi locality [4]. In this locality glaucine is not the prevailing alkaloid, its quantity is almost twice less of the main alkaloid isocorydine [5]. The dry plant material from Pomorie and Ahtopol maintained high percentages of glaucine in the two investigated years, being 2.3% for Pomorie in 2014 and for Ahtopol in 2015. These values were much higher than previously reported data for the two localities [2c].

Variations in concentration of glaucine in *G. flavum* were observed also among four Israeli populations (from 36% to lack of glaucine) [6]. Fluctuations of the alkaloids composition and content were observed in other species of the family, as well. In *Papaver somniferum*, for example, morphine content in different countries varied between 3 and 30% [7a]. Variations in alkaloid content and accumulation could be caused by a complex of ecophysiological factors: light intensity and duration [7a], temperature and its interaction with light [7b], water supply, salt stress [6], macro- and micronutrients [7c], pH of the soil, fertilizer supply, altitude etc., but their impact is depending on the species and the chemotypes. In *G. flavum* alkaloids' content was found to depend on Ni and K concentrations in plants [7c]. Alkaloids synthesis and accumulation were improved at Ni and K suboptimal concentrations, otherwise they inhibited these processes. In the case of *P. somniferum*, the total alkaloids accumulation increased under tropical conditions (short day with high light intensity) and in all ecotypes morphine content became higher [7b].

In conclusion, glaucine amount in the crude alkaloid mixtures of the investigated *Glaucium flavum* localities varied during the two examined years, although the ratio of the alkaloids remained similar. Fluctuations in glaucine content values were probably due to some abiotic factors as light, temperature, precipitation, soil substrate, salinity, etc., following the localities microclimatic conditions. More investigations are required to find out the factors that influence glaucine biosynthesis, accumulation and proportion in the total alkaloid content in plants.

Experimental

Plant material: The plant material of yellow hornpoppy was collected during the mass blooming stage in July 2014 and in July 2015 from seven localities of the species scattered along the entire Bulgarian Black Seacoast - Durankulak, Shabla, Shkorpilovtsi, Varvara, Ahtopol, and Sinemorets villages (Table 1). Locality plant samples were gathered consisting of the aboveground part.

Crude alkaloid mixture isolation: The air-dried and ground plant material (Table 1) was exhaustively extracted in Soxhlet apparatus with 200 ml 96% ethanol. Concentrated ethanol extracts were acidified with 3% HCl and left in dark for 24 hours. The acidified and filtered solution was subjected to three times petroleum ether extraction. Thus purified acidic solution was alkalized with NH_4OH (pH 9-10) and then extracted five times with CH_2Cl_2. The combined CH_2Cl_2 extracts were dried over anhydrous Na_2SO_4, filtered and then evaporated under reduced pressure to give crude mixtures of alkaloids.

Glaucine determination: 15 mg of each crude alkaloid mixture were dissolved in 0,7 ml $CHCl_3$ and 0.1 ml MeOH. 3 µl from sample were applied three times to DC Alufolien Kieselgel 60 F_{254} (Merck) and the sheets were developed with solvent system petroleum:$CHCl_3$:Me_2CO:MeOH (4:4:1:1). The spots were visualized by spraying with Dragendorff's reagent. Glaucine percentages in crude alkaloid mixtures were quantified using QuantiScan ® densitometry program (Biosoft, Cambridge, UK) as mean values.

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Crataegus orientalis Leaves and Berries: Phenolic Profiles, Antioxidant and Anti-inflammatory Activity

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The present study was designed to define the phenolic content, antioxidant and anti-inflammatory activity of *Crateagus orientalis* Pall. ex M. Bieb., traditionally used by local people in southern parts of F.Y.R. Macedonia. The presence and content of 7 phenolics in ethanolic extracts of leaves and berries were studied using HPLC-DAD, where the most dominant compounds were hyperoside, isoquercitrin and chlorogenic acid. The leaf extract was more effective as a DPPH radical scavenger (IC_{50} = 29.7 μg/g) than the berry extract, as well as in the relative reducing power on Fe^{3+}. Anti-inflammatory potential was studied by means of cyclooxygenase-1 (COX-1) and 12-lipoxygenase (12-LOX) inhibitory activity; both extracts evinced activity. Furthermore, *C. orientalis* leaf extract showed a concentration dependent inhibition of COX-1 pathway products 12-HHT and TXB_2, reaching IC_{50} values below the lowest applied concentration (68.9% and 55.2% of 12-HHT and TXB_2 production inhibition, respectively, at concentration of 0.4 mg/mL). Although inhibitors such as acetylsalicylic acid and quercetin showed higher activity, this study demonstrates that the investigated extracts are potential anti-inflammatory agents.

Keywords: Hawthorn, Phenolics, Hyperoside, Radical scavenging, Anti-inflammatory, Reducing power.

Medicinal use of extracts prepared from the leaves, flowers, and fruits of hawthorn (*Crataegus* spp., Rosaceae) dates back to ancient times. The plant is still a popular herbal medicine, widely used in traditional as well as in official medicine for preventing and treating cardiovascular diseases including angina, hypertension, arrhythmias, and congestive heart failure [1,2]. Hydroalcoholis extracts of *Crataegus* species have been found to possess hypocholesterolemic effect [3] and to inhibit angiotensin-converting enzyme [4]. The main secondary metabolites of hawthorn are flavonoids, proanthocyanidins, anthocyanins, organic and phenolic acids. Terpenes, lignans, phenylpropanoids, hydroxycinnamic acids and even essential oils have been reported [2]. Flavonoids and oligomeric proanthocyanidins are considered to be most important for pharmacological activity [3]. Different *Crataegus* species have been used as herbal medicines in many countries, including species that are official in the European Pharmacopoeia 7.0 [5], such as *C. monogyna* and *C. oxyacantha*. On the other hand, the usage of *C. orientalis* has been reported only in a few Middle East ethnobotanical surveys [6,7]. A recent ethnobotanical study locates the usage of *C. orientalis* in southern parts of F.Y.R. Macedonia (unpublished data). Thus, studies on *C. orientalis* are scarce, compared to other *Crayaegus* species.

The flavonoid content of leaves, flowers and unripe fruits of *C. orientalis* were investigated by Melikoglu *et al*. [8]; apigenin, apigenin-7-glucoside, hyperoside, vitexin and vitexin-4'-rhamnoside were isolated from the leaves, apigenin, quercetin, hyperoside, vitexin 4'-rhamnoside and rutin from the flowers, and apigenin, quercetin, hyperoside and rutin from the fruits. Arslana *et al*. [9] indicated that the ethanol extract of *C. orientalis* leaves suppress the formation of thrombosis in the carrageenan-induced mice tail thrombosis model and that it could be a good candidate for the development of a new antithrombotic medicine. Furthermore, Bor *et al*. [10] showed that the ethanol extract of *C. orientalis* leaves exhibits remarkable antinociceptive, antiinflammatory, and antioxidant activities.

To the best of our knowledge, the chemical composition of *C. orientalis* originating from the Balkan Peninsula has not been investigated till now and since the biological potency of this species is not investigated sufficiently, the aim of our study was to investigate the polyphenolic profile, and the antioxidant and anti-inflammatory activity of leaves and fruits of *C. orientalis* native to the southern parts of F.Y.R. Macedonia.

The total phenolic content of extracts of *C. orientalis* leaves and berries varied from 77.4 to 94.2 mg GAE/g of dry extract (Table 1); the leaves contained higher phenolic content than the berries. Our results pointed out greater phenolics accumulation in Balkan growing *C. orientalis* compared with several hawthorn species from Turkey where the phenolic content of methanol extracts of berries ranged from 35.7 to 55.2 mg GAE/g dry weight [11]. On the other hand, a high value for total phenolic content (343.5 mg GAE/g) in the ethyl acetate extract of *C. monogyna* leaves was reported by Öztürk *et al*. [12]. Park *et al*. [13] studied the ethyl acetate fraction of hawthorn berries, which were also characterized by high phenolic and flavonoid contents (140.2 tannic mg/g and 56.5 catechin mg/g, respectively). Literature data show significant differences in total phenolic content of hawthorn, probably due to several factors such as natural habitat, genotype, growth stage, extraction procedure and method for determination of total phenolics.

Previous studies have shown that the polyphenolic profile of hawthorn fruits and leaves were different and depended on the growth stage [14]. Few reports indicated that the flavonoids were the major markers for the interspecific distinction between

Table 1: The content of polyphenols (mg/g DW), total phenolic content (mgGAE/g DW) and free radical scavenging activity (μg/g DW) of methanol extracts of hawthorn (*C. orientalis*) leaves and berries.

#	Compound	Leaves	Berries	p [a]
1	Chlorogenic acid	11.8 ± 0.5	0.9 ± 0.0	0.000002
2	Epicatechin	7.2 ± 0.4	n.d.	0.000006
3	Vitexin	2.7 ± 0.0	0.5 ± 0.0	0.000000
4	Rutin	2.5 ± 0.0	0.6 ± 0.0	0.000000
5	Hyperoside	12.9 ± 0.1	1.4 ± 0.0	0.000000
6	Isoquercitrin	10.4 ± 0.1	0.9 ± 0.0	0.000000
7	Quercetin	0.5 ± 0.0	0.1 ± 0.0	0.000000
	Total phenolic	94.2 ± 0.1	77.6 ± 0.8	0.000003
	IC_{50}	29.7 ± 0.1	111.9 ± 0.2	0.000001

[a] *p*-value represent the probability that the null hypothesis is true.
n.d. – not detected.

Crataegus species [15]. Our study presents the phenolic composition of the methanol extracts of *C. orientalis* leaves and fruits analyzed by HPLC-DAD at 280 nm and 360.

Identification of seven compounds (chlorogenic acid, epicatechin, vitexin, rutin, hyperoside, isoquercitrin and quercetin) was made by comparison of their retention times and UV spectra with pure standards.

The results of quantitative analyses are shown in Table 1. The main compounds of the tested extracts were two *O*-glycosylflavones, hyperoside and isoquercitrin. Higher content of these flavonoids (12.9 mg/g DW and 10.4 mg/g DW, respectively) was found in the methanol extract of *C. orientalis* leaves. Leaves were also characterized by high amounts of chlorogenic acid and epicatechin, unlike in the berries. HPLC analysis of *C. orientalis* showed similarity with previously published results for *C. monogyna* collected in Serbia [16] and hawthorn species collected in Turkey and China [11,17]. Hyperoside was found to be the main flavonoid in the leaves and flowers of *C. tanacetifolia*, *C. orientalis*, *C. stevenii* and *C. microphylla* [18]. Liu *et al*. [17] also showed that hyperoside and isoquercitrin are the most abundant flavonol glycosides in the extracts of Chinese hawthorn berries. Polyphenols are known to be potent antioxidants and radical scavengers [19,20]. Some *Crataegus* constituents are good antioxidants, among them hyperoside, quercetin, epicatechin, and chlorogenic acid. They are also good antilipoperoxidants [21,22]. DPPH scavenging activity of the investigated methanol extracts are presented in Table 1. The leaf extract was a more effective DPPH radical scavenger (IC_{50} = 29.7 μg/g) than that of the berries (IC_{50} = 111.9 μg/g). This strong antioxidant activity is associated with high total phenolic content and structural characteristics of the most abundant compounds hyperoside and isoquercitrin. The berries extract exhibited lower antioxidant activity probably due to considerably lower amounts of hyperoside, isoquercitrin, chlorogenic acid, and epicatechin. Remarkable antioxidant activity of a *C. orientalis* ethanolic extract was also reported by Bor *et al*. [10] using *in vivo* tests. Reducing power on Fe^{3+} is one of the most frequently used tests for evaluation of the antioxidant potential of phytochemicals. Figure 1 shows the reducing power activity of methanol extracts of *C. orientalis* leaves and berries as a function of their concentrations. Trolox was used as a positive control. The reducing ability was found to be dose–dependent. The relative reducing power of hawthorn extracts on Fe^{3+} was higher for leaves of *C. orientalis* than for berries. Like the radical scavenging activity, the reducing power is also influenced by phenolic composition of these extracts.

Anti-inflammatory potential of methanol extracts of *C. orientalis* leaves and berries was determined using the intact cell system (platelets) as a source of cyclooxygenase-1 (COX-1) and 12-lipoxygenase (12-LOX) enzymes and a highly sensitive and specific LC-MS/MS technique for detection of the main arachidonic acid

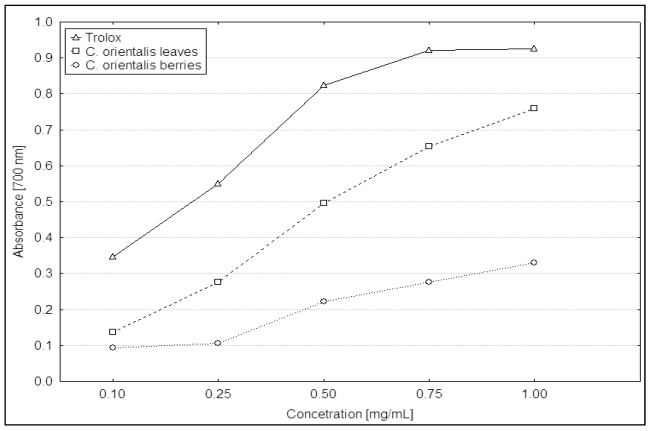

Figure 1: Reducing power activity of methanol extracts of hawthorn leaves and berries.

metabolites formed by them. Explicitly, 12-HHT (12(*S*)-hydroxy(5*Z*,8*E*,10*E*)-heptadecatrienoic acid), TXB_2 (thromboxane B2) and PGE_2 (prostaglandin E_2) are inflammation mediators derived from arachidonic acid metabolism, which is catalyzed by COX-1 enzyme, while 12-HETE (12(*S*)-hydroxy-(5*Z*,8*Z*,10*E*,14*Z*)-eicosatetraenoic acid) is a product of a 12-LOX pathway inflammatory response. The results of the inhibition potential of *C. orientalis* extracts, as well as of the known antiinflammatory agents aspirin and quercetin are shown in Table 2.

Table 2: IC_{50} values for COX-1 and 12-LOX assay of examined *C. orientalis* extracts.

		COX-1 pathway						12-LOX pathway	
		12-HHT[a]		TXB_2		PGE_2		12-HETE	
Ext.	*L*	< 0.4	c	< 0.4	c	na[b]	d	2.8 ± 0.1	d
(mg/mL)	*B*	2.0 ± 0.2	d	2.0 ± 0.2	d	1.6 ± 0.2	c	0.5 ± 0.1	b
Std.	*A*	5.0 ± 0.4	a	5.0 ± 0.1	a	5.6 ± 0.5	a	na	e
(mg/mL)	*Q*	22.4 ± 2.1	b	53.7 ± 2.5	b	12.8 ± 0.3	b	7.4 ± 0.6	a

[a]Values are means ± SD of three measurements. Means with different letters (a-f) differ significantly ($p < 0.05$). [b]na, 50% inhibition not achieved.
Samples tested: L – leaves; B – berries; A – aspirin; Q – quercetin.

C. orientalis leaves extract showed concentration-dependent inhibition of COX-1 pathway products 12-HHT and TXB_2, reaching IC_{50} values below the lowest applied concentration (68.9% and 55.2% of 12-HHT and TXB_2 production inhibition, respectively, at a concentration of 0.4 mg/mL). Lower, but also significant inhibitory activity towards production of the same metabolites was also achieved by *C. orientalis* berries extract. Previously, it has been reported that *C. oxycantha* tincture and its isolated flavonoids inhibit the formation of potent inflammatory and platelet aggregation mediator TXA_2 an unstable metabolite which is hydrolyzed within about 30 seconds to TXB_2 [23]. Taking into account the TXB_2 inhibition potential of *C. orientalis* extracts determined in our study, some attention should be drawn to *Crataegus* species as a possible inhibitor of TXA_2 synthase. Since TXA_2 synthase is an enyzme that follows COX-1 in the formation of TXA_2, a possible target for moderating inflammation by *Crataegus* species could be this point of arachidonic acid metabolism. Inhibition of PGE_2 production by *C. orientalis* was not concentration-dependent. In contrast, both examined extracts showed potential of 12-LOX inhibition. This finding could lead to meaningful studies of cytotoxic activity, because it was found that 12-HETE is also involved in the progression of various cancers [24]. Generally, the obtained results are in accordance with previously reported *in vivo* anti-inflammatory activity of *C. orientalis* collected in Turkey [8]. Overall, better activity of *C. orientalis* leaf extracts could be, at least partially, ascribed to higher content of phenolics than in berries. Particularly, the synthesis of compounds in the COX-1 and 12-LOX pathways, which go through radical reactions [25] might be terminated by phenolics. The

determined COX-1 and 12-LOX pathway inhibition potential of *C. orientalis* extracts was significantly lower than the activity of a well-known potent inhibitor of COX-1, aspirin and 12-LOX, quercetin. On the other hand, the achieved range of extract activities is indisputably comparable with that of some species traditionally used and well-known as anti-inflammatory agents [26].

In the present study, *C. orientalis* leaves and berries were investigated for their chemical composition, antioxidant and anti-inflammatory activity. This species showed notable phenolic content with hyperoside, isoquercitrin and chlorogenic acid as dominant compounds. Both extracts showed DPPH radical scavenging activity where leaves were more effective due to a high total phenolic content. Relative reducing power on Fe^{3+} was also higher for leaves of *C. orientalis*. Anti-inflammatory potential was shown by inhibition of cyclooxygenase-1 (COX-1) and 12-lipoxygenase (12-LOX) enzymes. Thus, *C. orientalis* leaves and berries may be considered as a significant therapeutic source.

Experimental

Plant material: Plant material was collected at the mountain Galičica (altitude 950 m) in southern F.Y.R. Macedonia. Leaves were collected in June 2010, while fruits were collected in October 2010. Voucher specimens (IPLB 345L and IPLB 346F) have been deposited in the herbarium of the Institute for Medicinal Plants Research 'Dr Josif Pančić', Belgrade, Serbia.

Extraction procedure: Collected leaves and berries of *C. orientalis* were air dried, ground and extracted in a Soxhlet apparatus for 72 h with 96% ethanol. The amount for extraction was: leaves 28.2 g and berries 38.1 g. Extracts were evaporated under vacuum at 40°C and were used for further analysis. The yields of the extracts were as follow: leaves 8.5 g, berries 15.5 g. Prior to HPLC analysis, the dry extracts of leaves (20 mg) and berries (50 mg) were dissolved in methanol (1 mL) and filtered through 0.45 μm membrane filters.

Total phenolic content: The content of total phenolics was determined according to the Folin-Ciocalteu colorimetric method, with some modification [27]. In brief, a methanol solution of extract (100 μL) was oxidized with Folin-Chiocalteu reagent (500 μL, previously diluted 10-fold with distilled water). The reaction was neutralized with saturated sodium carbonate (400 μL, 75 g/L). After 2 h of incubation at room temperature, absorbance was measured by an UV-Visible spectrophotometer (Agilent 8453) at 765 nm. Quantification was performed based on the standard curve of gallic acid and results were expressed as mg of gallic acid equivalents (GAE) per g dry weight of extract (DW).

Quantification of polyphenols: Phenolic acids and flavonoids were quantified by high-performance liquid chromatography using an Agilent 1100 chromatograph equipped with a DAD detector. HPLC analysis was performed with an Agilent Zorbax SB-C18 analytical column (250 mm x 4.6 mm, 5 μm particle size). The mobile phase consisted of solvent A (1%, v/v, orthophosphoric acid in water) and solvent B (acetonitrile), using gradient elution as follows: 10% B 0 min, 10-25% B 0-30 min, 25-55% B 30-40 min, 55-100% B 40-50 min, 100% B 50-55 min. Detection wavelengths were set at 280 and 360 nm, and the solvent flow rate was 0.8 mL/min. The amounts of the compounds were calculated using calibration curves of standards. The results were expressed as mg per g dry weight of extract.

Reducing power: The reducing power of the tested extracts was evaluated according to the method described by Li *et al*. [28], with several modifications. The methanol solution of extracts (200 μL, 0.1-1 mg/mL) was mixed with phosphate buffer (500 μL, 0.2 mM, pH=7) and potassium ferricyanide (400 μL, 10 mg/mL). After incubation at 50°C for 20 min, trichloroacetic acid (500 μL, 100 mg/mL) was added and the mixture was centrifuged at 2000 × *g* for 10 min. The supernatant (500 μL) was mixed with distilled water (500 μL) and $FeCl_3$ (100 μL, 1 mg/mL), and the mixture was left to stand for 10 min at room temperature. Finally the absorbance of solution was measured by an UV-Visible spectrophotometer (Agilent 8453) at 700 nm. Trolox was used as a positive control. The increased absorbance indicated increased reducing power.

Free radical scavenging activity: The free radical scavenging activity of the fractions was analyzed using the DPPH assay following a method described by Brand-Williams *et al*. [29]. The reaction mixture (1 mL) contained 500 μL of daily prepared DPPH solution (150 μM) and 500 μL of various concentrations (0.03, 0.06, 0.125, 0.25 and 0.5 mg/mL) of the tested extracts dissolved in methanol. After vortex mixing, the solutions were kept in the dark for 20 min at room temperature. Thereafter, the absorbance was measured at 517 nm. Trolox and ascorbic acid were used as positive controls. The percent inhibition was calculated against the control solution containing methanol instead of test solution.

Anti-inflammatory activity: *Ex vivo* COX-1 and 12-LOX assay was undertaken according to the method previously described [30]. An aliquot of human platelet concentrate, viable, but outdated for medical treatment, which contained 4 × 108 cells was suspended in buffer (0.137 mol/L NaCl, 2.7 mmol/L KCl, 2.0 mmol/L KH_2PO_4, 5.0 mmol/L Na_2HPO_4 and 5.0 mmol/L glucose, pH 7.2) to obtain a final volume of 2 mL. This mixture was slowly stirred at 37°C for 5 min. Subsequently, 0.1 mL of either extracts or standard compound solutions in DMSO (concentration ranging from 10.0 to 300.0, 0.156 to 5.0 and 0.01 to 0.6 mg/mL for extracts, quercetin and aspirin, respectively) and 0.1 mL of calcimycin (125 μmol/L in DMSO) were added and incubated for 2 min at 37°C, with moderate shaking. The exact amount of extract in control and calcimycin in blank probe were substituted with solvent (DMSO). Thereafter, 0.3 mL of $CaCl_2$ aqueous solution (16.7 mmol/L), substituted with water in a blank probe, was added and the mixture was incubated for a further 5 min at 37°C with shaking. Acidification with cold 1% aqueous formic acid (5.8 mL) to pH 3 terminated the reaction. If gel formation occurred, vortexing was applied before mixing with the acid. Internal standard PGB_2 (50 μL of 6 μg/mL solution in DMSO) was added and the mixture extracted with chloroform and methanol (1:1, 8.0 mL) with vigorous vortexing for 15 min. After centrifugation at 7012 × *g* for 15 min at 4°C, the organic layer was separated, evaporated to dryness, dissolved in methanol (0.5 mL), filtered and used for further LC-MS/MS analysis, previously described by Beara *et al*. [26]. The percent of COX-1 and 12-LOX inhibition achieved by different concentrations of extract was calculated by the following equation: $I(\%)=100\times(R_0-R)/R_0$, where R_0 and R were response ratios (metabolite peak area/internal standard peak area) in the control reaction and in the examined samples, respectively. Both R and R_0 were corrected for the value of the blank probe. Corresponding inhibition–concentration curves were drawn using Origin 8.0 software and IC_{50} values (concentration of extract that inhibited COX-1 and 12-LOX metabolites formation and cell growth by 50%) were determined.

Statistical analysis: For each assay and extract composition determinations, all the results were expressed as mean±standard deviation of 3 different measurements. A comparison of the group means and the significance between the groups were verified by one-way ANOVA followed by *post-hoc* Duncan's multiple range test. Level of statistical significance was set at $P<0.05$.

Compounds names: 12-HETE: 12(*S*)-hydroxy-(5*Z*,8*Z*,10*E*,14*Z*)-eicosatetraenoic acid; 12-HHT: 12(*S*)-hydroxy(5*Z*,8*E*,10*E*)-heptadecatrienoic acid; 12-LOX: 12-lipoxygenase; COX-1: cyclooxygenase-1; PGB_2: prostaglandin B_2;PGE_2: prostaglandin E_2; TXA_2: thromboxane A_2;TXB_2: thromboxane B_2;.

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Analysis of Antioxidant Polyphenols in Loquat Leaves using HPLC-based Activity Profiling

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Leaves of *Eriobotrya japonica* (loquat) have been used in Traditional Chinese Medicine with beneficial effects in numerous diseases. Extracts from loquat leaves are rich in antioxidants, containing among others: triterpenes, sesquiterpenes, flavonoids, tannins, and megastigmane glycosides. However, there is no conclusive study revealing which of these compounds are the main bioactive principles. The goal of this study was to pinpoint compounds responsible for strong antioxidant activity. *Eriobotryae folium* was extracted and fractionated between solvents of increasing polarity. All extracts and fractions were screened for total polyphenols and tannins, and antioxidant activity was checked by DPPH, phosphomolybdenum and linoleic acid tests. The ethyl acetate fraction demonstrated the highest antioxidant activity and contained the largest amount of polyphenols. Applying HPLC-based activity profiling to localize antioxidants revealed that cinchonain IIb, as well as flavonoid glycosides such as hyperoside, isoquercitrin, kaempferol glycosides, quercetin-rhamnoside, as well as two tentatively identified protocatechuic acid derivatives are the main substances responsible for the strong antioxidant activity of the ethyl acetate fraction.

Keywords: *Eriobotrya japonica*, Cinchonain, Antioxidant, Polyphenols.

Eriobotrya japonica Lindl. (Rosaceae), also known as 'loquat', has been widely used in Traditional Chinese Medicine with beneficial effects in numerous diseases, such as asthma, gastroenteric disorders, diabetes mellitus, chronic bronchitis and pulmonary inflammatory diseases [1]. Triterpenes, sesquiterpenes, flavonoids, tannins and megastigmane glycosides have been found in the leaves of *E. japonica* and some of these have demonstrated antitumor, antiviral, hypoglycemic and anti-inflammatory properties [1-3]. These beneficial effects could be partly attributed to their antioxidant and free radical scavenging activities [4-6]. Despite loquat having been proved to be one of the richest sources of antioxidants [7], there is still a lack of knowledge about the substances responsible for its strong antioxidant activity. The goal of this study was to pinpoint these compounds. In the first step, raw material from China was extracted using ultrasound-assisted extraction and fractionated between solvents of increasing polarity. All extracts and fractions were screened for total polyphenols and tannins. Antioxidant activity was checked by DPPH, phosphomolybdenum and linoleic acid tests (Table 1).

The ethyl acetate fraction demonstrated the highest ability to scavenge the 2,2'-diphenylpicrylhydrazyl radical, as well as the highest capacity to reduce metal ions, and the ability to prevent the oxidation of linoleic acid (Table 1). The reducing power of each fraction, expressed as the percentage of ascorbic acid equivalent activity, was weak at 37°C for all fractions. What is interesting, despite the *n*-butanol fraction having significantly weaker capacity to reduce metal ions at 37°C in the phosphomolybdenum assay, at 90°C it was equally as strong as the ethyl acetate fraction. This may be caused by a significantly greater content of thermolabile antioxidants such as polyphenols and tannins in this fraction [8]. We assessed the inhibition of peroxidation of linoleic acid by detection of final peroxidation products, such as malonyl dialdehyde and other molecules that react with thiobarbituric acid. The ethyl acetate and butanol fractions showed the highest inhibition, lowered TBARS by 69.5% and 66.5%, respectively, at 500 µg/mL concentration. These fractions revealed strong inhibition, even at very small concentration (10 µg/mL), by 48.1% and 48.0%, respectively. Spearman's rank correlation showed strong correlation between DPPH assay (EC_{50}) and the content of polyphenols (-0.942) and tannins (-0.828) (Table 2).

Table 1: Phenolic and tannin content of each fraction and their antioxidant activity.

Fraction	DPPH EC_{50} ug/mL	Reducing power AAE (%) 37°C	Reducing power AAE (%) 90°C	LA-Peroxidat-ion % inhibition	TPC Total Polyphenols (GAE) mg/g fraction	Tannins content (GAE) mg/g fraction
Acetone	39.4	1.7	37.3	23.7	78.4	25
Dichloromethane	60.2	1.9	43.5	24.8	22.4	6
Diethyl ether	47.3	2.4	18.7	20.9	39.4	14
Ethyl acetate	22.3	4.5	57.4	69.5	177.4	54
n-Butanol	35.4	3.1	57.5	66.5	89.4	32
Water	98.1	1.1	46.5	15.3	35.4	24

Taking into account the results above, we selected the ethyl acetate fraction for further study. HPLC-based activity profiling was applied to localize compounds responsible for the strongest antioxidant activity. With this approach, sub-mg amounts of a fraction were efficiently separated by analytical scale HPLC and automatically fractionated into micro-fractions in 96-well plates.

After drying, the plates were retested for antioxidant activity by DPPH assay. Micro-fractionation was carried out with a short C18

Table 2: Spearman Rank Order Correlation. Marked correlations are significant at $p<0.05$.

Variable	DPPH EC50 ug/mL	Reducing power AAE (%) 37°C	Reducing power AAE (%) 90°C	LA-Peroxid. % inhibition	TPC Total Polyphenols (GAE) mg/g fraction	Tannins content (GAE) mg/g fraction
DPPH EC50	1.00	-0.83	-0.43	-0.83	-0.94	-0.83
AAE 37°C	-0.83	1.00	-0.43	-0.83	0.71	0.54
AAE 90°C	-0.43	-0.43	1.00	0.60	0.48	0.66
LA-Peroxid.	-0.83	-0.83	0.60	1.00	0.66	0.60
TPC	-0.94	0.71	0.45	0.66	1.00	0.94
Tannins	-0.83	0.54	0.66	0.60	0.94	1.00

analytical column selected after preliminary experiments for its reasonable performance, fast separation and low solvent consumption. The chemical composition of the ethyl acetate fraction was evaluated by HPLC-DAD-MS^3. We identified 18 constituents: one phenolic acid (**3**), two flavonolignans (**4, 8**), nine flavonoids (**5-7**, **9-14**), one sesquiterpene glycoside (**17**), one triterpene acid (**18**) and tentatively determined two protocatechuic acid glycosides (**1**, **2**) and two kaempferol glycosides (**15**, **16**) (Table 3). All identified compounds were previously detected in this plant [1, 9-14]. Figure 1 shows a chromatogram of the ethyl acetate fraction acquired at 220-450 nm, and Table 3 presents the UV and mass spectral data of the detected compounds. Although most of the eluting microfractions have some anti-radical activity, there are only a few clearly outstanding peaks. Compounds **1**, **2**, **8**, **10-12**, and **14** are especially interesting due to their strong capacity to scavenge DPPH radicals. The strong antioxidant activity of flavonoid glycosides is widely known [15], but using HPLC-based activity profiling we can see which of them play the most important roles in the antioxidant properties of the ethyl acetate fraction. The major highly active compound was **8**, identified as cinchonain IIb. Despite a small amount of it in this fraction, it markedly contributed as the second strongest DPPH scavenging peak. Earlier study confirmed strong DPPH free radical scavenging activity of cinchonain IIb, where it achieved an EC_{50} of 5.05 µmol/L compared with 30.1 µmol/L for ascorbic acid [16]. In the ethyl acetate fraction there are also substances with very strong antioxidant activity (compounds **1** and **2**), not fully characterized so far.

However, based on their MS-MS spectra and molecular formula prediction by HR-TOF-MS we identified compound 1 tentatively as a protocatechuic acid hexoside. The negative ionization parent ion at *m/z* 315 after the loss of the hexosyl molecule would yield a fragment at *m/z* 153, corresponding to protocatechuic acid - H^+ and the fragment at *m/z* 109 would be protocatechuic acid after loss of a carboxyl moiety [17]. The same fragmentation pattern was observed for compound **2** (*m/z* 505), which leaves an unidentified moiety responsible for the ion at *m/z* 190, but the respective fragment ion was not detected in the MS, so it could be a result of a neutral loss. We hypothesize that it could be another derivative of a protocatechuic glycoside. The molecular formula predicted by the software is quite unlikely for the obtained mass spectrum, though.

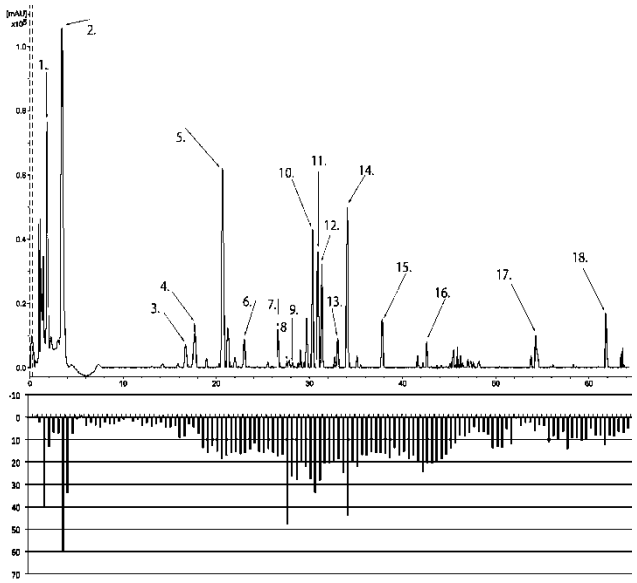

Figure 1: HPLC-DAD chromatogram of ethyl acetate fraction of *Eriobotrya japonica* leaves acquired at 220-450 nm joined with antioxidant activity of subfraction (% reduction of DPPH). For illustration purposes, a baseline correction in the Bruker Data Analysis ver. 4.2 was used.

In conclusion, we can confirm that HPLC-based activity profiling is a good method to localize the compounds with highest antioxidant activity. For the ethyl acetate fraction of *E. japonica* leaves it allowed detection and direct assessment of strong antioxidant activity of a few polyphenols, but also pointed to a very strong activity and abundance of two putative dihydroxybenzoic derivatives that are worth investigating further aiming at their isolation, full identification and detailed bioactivity testing.

Table 3: UV, MS^n and HR-MS data of compounds detected in ethyl acetate fraction.

	Compound	Retention time [min]	UV [nm]	$[M+H]^+$ *m/z*	MS^2	$[M-H]^-$ *m/z*	MS^2	qTOF m/z $[M-H]^-$	Error (ppm)	formula**
1	*Protocatechuic acid *O*-hexoside	1.9	285				153, 109	315.0732	-3.1	$C_{13}H_{16}O_9$
2	*putative Protocatechuic acid –*O*-hexoside derivative	3.5	260, 294	507	155	505	315,153, 109	504.9900	-0.7	$C_{20}H_{10}O_{16}$/ $C_{20}H_{26}O_{15}$
3	Chlorogenic acid	16.7	220, 324	355	338	353	337	353.0897	-5.5	$C_{16}H_{18}O_9$
4	Cinchonain Id 7-*O*-β-glucopyranoside	17.7	241, 295	615	308	613	306	613.1621	-9.5	$C_{30}H_{30}O_{14}$
5	(-)Epicatechin	20.7	239, 280	291		289	245,205	289.0726	-2.9	$C_{15}H_{14}O_6$
6	Kaempferol- 3-*O*-α-L-(2",4"-di-*E*-feruloyl)-rhamnoside	23.1		785	435	783	437	783.1951	-2.5	$C_{41}H_{36}O_{16}$
7	Procyanidin C1	26.7	239, 279	867		865	739, 695, 577, 425, 287	865.2356	-23.5	$C_{45}H_{38}O_{18}$
8	Cinchonain IIb	27.9	281	741	589	739	587	739.1668	-2.5	$C_{39}H_{32}O_{15}$
9	Rutin	28.9	220, 283	611	453	609	451	609.1475	-2.3	$C_{27}H_{30}O_{16}$
10	Hyperoside	30.4	255, 352	465	303	463	301	463.0891	-2.0	$C_{21}H_{20}O_{12}$
11	Isoquercitrin	30.9	256, 352	465	303	463	301	463.0885	-0.8	$C_{21}H_{20}O_{12}$
12	*Kaempferol pentosyl-hexoside	31.4	266, 344	597	465	595	463	593.1518	-1.1	$C_{27}H_{29}O_{15}$
13	Kaempferol-3-glucoside	32.7		449	287	447	285	447.0950	-3.9	$C_{21}H_{20}O_{11}$
14	Quercetin rhamnoside	34.1	257, 345	449	303	447	301	447.0944	-2.6	$C_{21}H_{20}O_{11}$
15	*Kaempferol glycoside	37.8	220, 264	433	287	431	285	431.0999	-3.6	$C_{21}H_{20}O_{10}$
16	unknown compound	42.6	220	711	485	709	591	709.3805	-0.9	$C_{37}H_{57}O_{13}$
17	Nerolidol-3-*O*-α-L-rhamnopyranosyl-(1-4)-α-L-rhamnopyranosyl-(1-2)-[α-L-(4-*t*-feruloyl)- rhamnopyranosyl-(1-6)]-β-D-glucopyranoside	54.3	221, 321	999	823	997	821	997.4646	0.4	$C_{49}H_{74}O_{21}$
18	3-*O*-*p*-Coumaroyl-tormentic acid	61.8	221, 311	635		633		no data		

* tentatively identified

**prediction of molecular formula based on SmartFormula package of Bruker Compass software.

Experimental

***Plant material*:** The leaves of *Eriobotrya japonica* (Rosaceae), were obtained from a vendor of traditional Chinese medicines, Beijing Tong-Ren-Tang (China). A voucher sample is deposited in the herbarium of Botanical Garden of Medicinal Plants (specimen database entry "Rosac_Eriobotrya_japonica_CN_Nawrot2015-1"). The dried leaves (70.0 g) were extracted with acetone/water (70:30, v/v, 5 x 500 mL) at 30°C, in an ultrasonic bath (Polsonic, Poland). Acetone and water were evaporated under reduced pressure. The dry extract (4.0 g) was suspended in water (100 mL) and partitioned between dichloromethane (5 x100 mL), diethyl ether (4 x100 mL), ethyl acetate (5 x100 mL) and finally *n*-butanol (5 x100 mL), affording 0.86, 0.34, 0.11, and 0.11 g of each dried fraction, respectively and 0.55 g of water residue fraction.

Reagents: 2,2-Diphenyl-1-picrylhydrazyl (DPPH), thiobarbituric acid (TBA), and hide powder were purchased from Sigma-Aldrich (Steinheim, Germany). Linoleic and gallic acid were purchased from Fluka AG, and trichloroacetic acid from Ubichem UK. All other reagents and solvents were obtained from Avantor-POCh, (Gliwice, Poland).

DPPH scavenging assay: The ability to scavenge the DPPH free radical was monitored according to a modified method of [17]. Briefly, DPPH solution (0.3 mM) was prepared in methanol. The extract and fractions were dissolved in a mixture of methanol and water (9:1, v/v) to obtain stock solution (1 mg/mL). Then each stock solution was diluted to obtain final concentrations of 1-250 µg/mL in the assay mixture. DPPH solution (125 µL) and 125 µL of the test extract and fractions at different concentrations were added to a 96-well plate. The absorbance at 517 nm was measured 30 min after mixing using a microplate reader (µQUANT, BioTek, USA). Vitamin C was the positive control. The percentage of scavenged DPPH was then calculated according to Eq1:

$$\%DPPH=((Abt-Abr)/Ab0)\times100$$

where Abt is the absorbance of DPPH solution with the test extracts, Ab0 is the absorbance of DPPH solution with a mixture of methanol and water (9:1, v/v) and Abr is the absorbance of the test extract solution with the addition of methanol. The antiradical activity of extracts was expressed as an EC_{50} value.

Phosphomolybdenum reduction assay: The antioxidant capacity of the extract and fractions was assessed as described by Prieto *et al.* [18], with our modifications [19]. Extract and fractions were dissolved in a mixture of methanol and water (9:1 v/v) to obtain stock solution (5 mg/mL). Then each stock solution was diluted to obtain final concentrations of 10-500 µg/mL in the assay mixture. The extract and fractions were combined with the reagent solution containing ammonium molybdate (4 mM), sodium phosphate (28 mM) and sulfuric acid (600 mM). The reaction mixture was incubated in a water bath at either 37°C or 90°C for 90 min. The absorbance of the colored complex was measured at 695 nm. The antioxidant activity was compared with that of ascorbic acid in the same concentration range.

Inhibition of linoleic acid peroxidation: The procedure of Wozniak *et al*. [19], using Fenton reaction induced lipid peroxidation, has been adapted for this assay. The extract and fractions dissolved in water, achieved a concentration range of 10-500 µg/mL in the assay mixture. Each fraction (150 µL) was mixed with 500 µL phosphate buffer (0.1M, pH 7.4), and 550 µL linoleic acid emulsion (linoleic acid mixed with Tween 80, 3:1, w/w); next 1.12 g emulsion was mixed with 50 mL 0.1 M phosphate buffer (pH 7.4)), and 150 µL 10 mM ascorbic acid. The peroxidation was started with the addition of 150 µL 10 mM $FeSO_4$. The reaction mixture was incubated for 90 min. at 37°C. Thereafter, 1.5 mL of 10% ice cold trichloroacetic acid was added and 1.5 mL of 1% thiobarbituric acid in 50 mM NaOH. The samples were heated in a water bath at 90°C for 10 min. After cooling the samples, 2 mL of *n*-BuOH was added and mixed well. The absorbance was read at 532 nm after transferring 300 µL of BuOH phase from samples to the 96-well plate. The percentage of linoleic acid peroxidation inhibition was calculated as in [19], using appropriate controls. Quercetin was used as a positive control.

Total polyphenols and tannins: Total phenolic content was determined with the Folin-Ciocalteu reagent according to a procedure described previously [20]. Tannin compounds were measured by parallel experiments with extracts vortexed for 1 h with 10 mg mL^{-1} using hide powder. The results were expressed as gallic acid equivalents according to the standard gallic acid calibration curve. Total tannins were calculated by subtraction of polyphenols non-absorbed by hide powder from the total phenol content.

HPLC apparatus: HPLC analyses were performed using an Ultimate 3000RS series system (Thermo Dionex, Idstein, Germany) equipped with a low-pressure quaternary gradient pump for a working pressure of up to 103MPa with vacuum degasser, an auto-sampler, a column compartment, and a diode array detector. For mass spectrometry, either a high resolution Time-of-flight mass spectrometer (Bruker qTOF Compact, Bruker Daltonik, Bremen, Germany) or an ion trap (Bruker Amazon SL) equipped with ESI interface was used. The system was controlled by Bruker HyStar. Microfractioning approach was adapted from Hamburger *et al.* [21]. Collecting of fractions was made with a Gilson 203 fraction collector.

HPLC-DAD conditions for micro-fractionation: Micro-fractionation was carried out using a Kinetex C18 analytical column (50 mm×3.0 mm×1.7 µm) (Phenomenex Torrance, CA, USA). Column temperature was maintained at 25°C. Elution was conducted using mobile phase A {water:formic acid (100:0.1, v/v)} and mobile phase B {acetonitrile:formic acid (100:0.1, v/v)} with a gradient as follows: 0–35 min 0–21% B, 35–40 min 21–30% B, 40-52 min 30–35% B, 52-62 min 35–100% B, and 62-65 min 100% B; the flow rate was 0.250 mL/min. A microfraction was collected every 30 sec. The fractions were dried under a gentle stream of nitrogen and tested for bioactivity. Volumes of 5, 10 or 20 µL of ethyl acetate fraction (3 mg/mL concentration) were introduced by the autosampler to the column. Each of the injections was performed in duplicate. UV spectra were recorded in the range of 200–450nm.

LC-MS analysis: The eluate leaving the DAD detector was introduced into the mass spectrometer without splitting. For MS^1 and HR-MS we used the qTOF mass spectrometer, whereas for MS^3 the ion trap detector was used. The parameters for ESI source were: nebulizer pressure 40 psi; dry gas flow 7 L/min; dry temperature 200°C and capillary voltage 2.2 kV. Analysis was carried out using scanning from *m/z* 50 to 2200. Compounds were analyzed in negative and positive ion mode and processed using Bruker Compass software.

Statistical analysis: Each of the antioxidant tests and analysis of total polyphenols and tannins was made in 6 repetitions. Spearman's rank correlation and EC_{50} were calculated using Statistica 12 (Statsoft, Poland).

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LC/DAD/MS^n and ICP-AES Assay and Correlations between Phenolic Compounds and Toxic Metals in Endemic *Thymus alsarensis* from the Thallium Enriched Allchar Locality

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Samples of *Thymus alsarensis* Ronniger, an endemic species for the Allchar locality, were evaluated for their polyphenolic composition and heavy metals. Allchar district is an abandoned antimony-arsenic-thallium deposit in the north-west of Kožuf Mountain, R. Macedonia, with a unique mineral composition affecting the mineral composition of the flora. A systematic method for phenolic compounds characterization was developed using mass spectrometry coupled to HPLC/DAD. Analyses were focused on the polyphenolic compounds to establish a possible correlation to the region specific heavy metals As and Tl in the different organs of *T. alsarensis*. Twenty-seven polyphenols: phenolic acid derivatives and flavonoid glycosides of luteolin, apigenin, quercetin, and kaempferol were detected; contents were higher in the leaves and flowers compared with stems and roots. Quinic acid (**1**), prolithospermic acid (**6**), salvianolic acid B (**7**), salvianolic acid A (**8**), monomethyl lithospermate (**9**), luteolin dihexoside (**12**), luteolin pentosyl-hexoside (**14**), luteolin acetyl pentosyl-hexoside (**16**), luteolin acetyl hexoside (**17**), luteolin dipentoside (**21**), luteolin pentoside (**24**), luteolin acetyl dipentoside (**25**), kaempferol pentosyl-hexoside (**19**) and kaempferol acetyl pentosyl-hexoside (**22**) were detected in *T. alsarensis* for the first time.

To assay the content of As and Tl, root, stem, leaf and flower samples were analyzed by inductively coupled plasma atomic emission spectrometry (ICP-AES). Significant accumulation of As and Tl was observed with As content from 0.25 to 140 mg/kg and Tl from 0.10 to 496 mg/kg. The content of As was much higher in the roots, while the content of Tl was significantly higher in the roots, flowers and leaves in all *T. alsarensis* specimens. Comparison of the results obtained for total polyphenols and for As and Tl content does not suggest any correlation (positive or negative) between the total phenolic content and the content of Tl and As. On the other hand, it is evident that the soil rich with specific heavy metals (Tl and As) affects the type of polyphenolic compounds produced in different organs, compared with other *Thymus* species growing on soil that is not contaminated.

Keywords: *Thymus alsarensis* Ronniger, Allchar, Polyphenols, Heavy metals, Correlation.

Allchar district is an abandoned antimony-arsenic-thallium deposit in the north-west of Kožuf Mountain, Republic of Macedonia, with a complex and unique mineral composition that affects the flora in the region. The genus *Thymus* is one of the most polymorphic genera of the Lamiaceae family. Species of this genus are characterised by emphasized polymorphism and the presence of a number of subspecies, varieties and forms. *Thymus* flora is very rich and diverse in the territory of the Republic of Macedonia [1]. *T. alsarensis* Ronninger of the genus *Thymus* L., Sect. Marginati (A. Kerner) A. Kerner, Subsect.Verticillati (Klok. et Shost.) Menitsky is an endemic aromatic and medicinal plant present in the Allchar locality [2].

Wild thyme is used as an expectorant, antispasmolitic, antiseptic and anthelmintic [3]. Infusion and decoction of aerial parts of *Thymus* species are used to produce tonics, carminatives, digestive aids, antispasmodics, anti-inflammatories and expectorants [4]. Furthermore, such preparations are used for the treatment of the common cold in Macedonian traditional medicine. Previous investigations on Macedonian *Thymus* samples have attempted to characterise the composition of essential oil as well as the content of flavonoids [5a-5c] and some trace elements [6] in these herbs.

Certain plants can accumulate essential and nonessential heavy metals in their roots and shoots to levels far exceeding those present in the soil. Metal accumulating plant species are invariably restricted to metalliferous soils found in different regions around the world [7a]. The mechanisms of metal accumulation, which involve extracellular and intracellularmetal chelation, precipitation and translocation in the vascular system, are poorly understood [7b]. Only a restricted number of plants from the local flora are able to grow in metalliferous soils, and it probably affected their metabolic pathway which is in correlation with polyphenolic compounds production. In the present work, a thorough study has been made to determine the polyphenolic profiles and content of *T. alsarensis* from the Macedonian flora, using HPLC coupled to UV-Vis diode array detection and tandem mass spectrometry with an electrospray ionization source (LC/DAD/ESI-MS^n). The systematic analysis for identification and quantification of all present phenolic compounds including phenolic acids, flavonols, flavones and flavanones has been carried out in order to establish a possible correlation between polyphenolic compounds and specific heavy metals (As and Tl) in the different organs of the endemic *T. alsarensis*.

Qualitative analysis: In total, 27 phenolic compounds were identified and classified into four groups: phenolic acid derivatives (9), flavones (11), flavonols (5) and flavanones (2). All retention and spectral data for the detected compounds are given in Table 1.

Phenolic acids: Compound **2** had a $[M–H]^-$ ion at *m/z* 341, which fragmented in MS^2 to *m/z* 179 and 161, characteristic for caffeic acid. The loss of 162 amu indicates the presence of a sugar moiety.

Table 1: Retention times, UV-Vis, and mass spectral data of phenolic compounds present in *Thymus alsarensis*

	Compounds	t_R (min)	UV(nm)	$[M–H]^-$ (*m/z*)	$-MS^2[M–H]^-$ (*m/z*)
	Phenolic acids				
1	Quinic acid	7.9	238, 284	191	173, 111
2	Caffeic acid hexoside	8.5	238, 288sh, 328	341	179, 163
3	Salviaflaside derivative	20.2	238, 290sh, 330	701	521, 359
4	Rosmarinic acid	22.6	238, 288sh, 328	359	197, 179, 161
5	Hydroxy jasmonic acid *O*-hexoside	22.8	252, 300sh, 332	387	369, 207, 163
6	Prolithospermic acid	22.9		357	313, 269
7	Salvianolic acid B	23.8	238, 296sh, 330	717	519, 475
8	Salvianolic acid A	28.0	240, 298sh, 328	493	359, 161
9	Monomethyl lithospermate	34.8	290, 326	551	519, 359
	Flavone				
10	Apigenin *C*-hexoside-*C*-hexoside	22.9	238, 272, 336	593	473, 353
18	Apigenin 7-*O*-glucoside	29.6	286, 332	431	269
12	Luteolin dihexoside	24.7	254, 286sh, 308, 334	609	447, 285
14	Luteolin pentosyl-hexoside	25.5	234, 256, 328	579	447, 285
15	Luteolin 7-*O*-glucoside	26.4	234, 256sh, 328	447	285
16	Luteolin acetyl pentosyl-hexoside	29.1	234, 292sh, 326	621	579, 561, 285
17	Luteolin acetyl hexoside	29.2	266, 344	489	285
20	Luteolin 7-*O*-rutinoside	30.7	240, 288, 328	593	285
21	Luteolin dipentoside	31.0	292, 334	549	417, 285
24	Luteolin pentoside	32.6	232, 288	417	371, 285
25	Luteolin acetyl dipentoside	36.4	256, 350	591	549, 531, 285
	Flavonols				
11	Quercetin 3-*O*-glucoside	24.5	236, 282, 348	463	301
23	Quercetin 3-*O*-rutinoside	31.9	236, 282, 348	609	301
19	Kaempferol pentosyl-hexoside	29.9	288, 334	579	417, 285
22	Kaempferol acetyl pentosyl-hexoside	31.3	256, 350	621	417, 285
27	Kaempferol	43.3	256, 352	285	241, 216
	Flavanone				
13	Hesperetin glucoside	25.0	290	463	301
26	Naringenin	38.9	290	271	177, 151

Compound **4** produced two fragments $[M–H-198]^-$ (*m/z* 161, 100%) and $[M–H-162]^-$ (*m/z* 197) corresponding to rosmarinic acid [8a].

Compound **3** had a deprotonated molecular ion at *m/z* 717. The MS^2 spectra of the $[M–H]^-$ ion showed fragment ions at *m/z* 519 $[M–H-198]^-$ and 359 $[M–H-198-162]^-$, indicating a loss of a sugar moiety. This compound was tentatively identified as a salviaflaside (rosmarinic acid 3'-glucoside) derivative. The MS of compounds **7** and **8**, salvianolic acid B and A, respectively, had characteristic fragmentation pattern indicating the presence of caffeoyl moieties. Their identification was tentative and supported by literature data [8b]. Compound **9** had a molecular ion at *m/z* 551, and MS^2 fragment ions at *m/z* 519 and 359, which also indicate the presence of rosmarinic acid in the structure. It was identified as monomethyl lithospermate. Compound **5** had a deprotonated molecular ion at *m/z* 387, and MS^2 fragments at *m/z* 367, 207, and 163, which correspond to a loss of a hydroxyl group and sugar moiety. This compound was identified as hydroxyjasmonic acid *O*-hexoside. In the literature there are data about the presence of 5′-hydroxyjasmonic acid 5′-*O*-hexoside in *Thymus* species [9]. Compound **6**, prolithospermic acid, had a deprotonated molecular ion at *m/z* 357 and characteristic MS^2 fragments at *m/z* 313 and 269. This compound was not previously found in *Thymus* species, but lithospermic acid has been reported in samples of wild thyme [10a].

Rosmarinic acid is common in *Thymus* species [10a], whereas compounds such as salviaflaside, salvianolic acid A and B are more characteristic for species of the genus *Salvia* [10b].

Flavones: To the best of our knowledge, flavones are the dominant group of flavonoids present in *Thymus* species, followed by flavanones and flavonols. From the MS data, eleven flavones were detected. The $[Y_0]^-$ ions at *m/z* 269 and 285 indicated the presence of apigenin (**10** and **18**) and luteolin (**12**, **14**-**17**, **20**, **21**, **24** and **25**) derivatives. Apigenin 7-*O*-glucoside (**18**), luteolin 7-*O*-rutinoside (**20**) were previously found in wild thyme species [10a], whereas luteolin *O*-glucoside (**15**) is described as either 7-*O*-glucoside in wild thyme species [10a] or 5-*O*-glucoside in *Thymus x citriodiuc* [9]. Compound **10** had a deprotonated molecular ion at *m/z* 593, and MS^2 fragments at 473 and 353. Both subsequent losses of 120 amu indicate *C*-glucosylation [10c]. So, compound **10** was tentatively identified as apigenin *C*-hexosid-*C*-hexoside, which was previously reported in *T. vulgaris* [11].

The most abundant peak for the luteolin derivatives (with $[M–H]^-$ of the aglycon *m/z* 285) was produced after consecutive loss/es of acetyl (42 amu), hexose (162 amu), pentose (132 amu) and their combinations. According to their characteristic fragmentation patterns, these compounds were tentatively identified as luteolin dihexoside (**12**) ([M–H-162-162]), luteolin pentosyl-hexoside (**14**) ([M–H-132-162]), luteolin acetyl pentosyl-hexoside (**16**) ([M–H-42-132-162]), luteolin acetyl hexoside (**17**) ([M–H-42-162]), luteolin dipentoside (**21**) ([M–H-132-132]), luteolin pentoside (**24**) ([M–H-132]), and luteolin acetyl dipentoside (**25**) ([M–H-42-132-132]). These compounds have not previously been reported in *Thymus* species.

Flavonols: Five flavonols were identified according to the UV–Vis absorption maxima and MS data (Table 1). The $[Y_0]^-$ ions at *m/z* 301 and 285 indicated two aglycones with the corresponding molecular ions: quercetin (aglycone of **11** and **23**) and kaempferol (**19**, **22** and **27**). Compound **23** was determined to be quercetin 3-*O*-rutinoside by comparison with a reference standard. Quercetin 3-*O*-glucoside (**11**), quercetin 3-*O*-rutinoside (**23**) and kaempferol (**27**) were previously identified in *T. vulgaris* [11].

Compounds **19** and **22** had deprotonated molecular ions at *m/z* 579 and 621, respectively. In the MS^2 spectra of both compounds peaks at *m/z* 417 and 285 were detected. The losses of 162 and 204 amu, for compounds **19** and **22**, respectively, correspond to hexose $[M–H-162]^-$ and acetyl hexose $[M–H-162-42]^-$, and the loss of 132 amu indicates the presence of pentose. Then, from the fragmentation pattern it can be concluded that compounds **19** and **22** can be identified as kaempferol pentosyl-hexoside and kaempferol acetyl pentosyl-hexoside, respectively. These two compounds have not previously been identified in extracts of *Thymus* species.

Flavanones: From the literature it can be found that flavanones, especially naringenin, eriodictiol and hesperetin, are present in different forms (as aglycones, glucosides and ruthinosides) in

Table 2: Total content of phenolic acid derivatives (PA) (mg/g), flavonoids (F) (mg/g), total phenolic compounds (mg/g), content of As (mg/kg) and Tl (mg/kg) in different organs (flower, leaf, stem and root) determined in 14 samples of *Thymus alsarensis*,

	Flower					Leaf					Stem					Root				
Sample	**PA**	**F**	**Total**	**As**	**Tl**	**PA**	**F**	**Total**	**As**	**Tl**	**PA**	**F**	**Total**	**As**	**Tl**	**PA**	**F**	**Total**	**As**	**Tl**
1	--	--	**--**	0.90	19.4	5.49	18.8	24.3	5.36	20.0	1.08	2.03	3.11	2.46	13.4	10.5	11.9	22.4	15.1	21.6
2	33.9	19.0	52.9	2.69	49.8	17.8	22.3	40.1	10.5	29.2	2.98	3.15	6.13	3.29	11.3	3.26	4.48	7.74	21.1	34.6
3	81.6	233	314	20.3	545	8.34	39.1	47.4	102	309	8.50	2.90	11.4	92.6	169	1.25	0.78	2.03	623	905
4	20.8	123	144	<0.5	0.24	13.1	211	224	0.50	<0.20	3.92	4.51	8.43	0.50	0.21	4.32	1.21	5.53	<0.5	1.28
5	13.1	8.59	21.7	<0.5	461	11.3	25.1	36.4	4.05	371	8.69	2.01	10.7	4.48	166	0.53	0.23	0.76	40.0	424
6	4.98	29.0	34.0	0.75	140	15.5	26.4	41.9	4.70	101	0.33	3.89	4.22	0.95	80.9	4.67	2.64	7.31	26.7	174
7	12.1	10.1	22.2	0.97	20.0	9.29	19.9	29.2	2.28	9.08	5.77	4.32	10.1	3.11	3.14	2.91	2.10	5.01	3.94	6.04
8	4.50	18.1	22.5	6.80	11.8	9.00	19.8	28.8	16.9	15.9	2.80	10.0	12.8	10.5	20.9	1.80	5	6.8	51.8	146
9	22.5	7.03	29.5	0.62	0.34	4.38	16.2	20.6	<0.5	<0.20	4.18	4.38	8.56	<0.5	<0.20	1.77	2.16	3.93	<0.5	0.44
10	16.6	7.3	23.9	26.8	426	19.8	31.9	51.7	44.0	523	6.44	4.76	11.2	29.5	352	3.29	1.81	5.1	86.1	273
11	3.44	8.32	11.8	1.24	<0.20	4.90	64.2	69.1	1.68	<0.20	1.23	1.77	3.00	0.59	<0.20	3.95	1.18	5.13	1.95	<0.20
12	10.6	9.79	20.4	<0.5	63.2	23.9	48.8	72.7	0.91	18.5	2.07	7.02	9.09	<0.5	10.8	--	--	--	<0.5	44.5
13	26.6	21.9	48.5	<0.5	1.77	5.99	51.2	57.2	0.93	1.38	3.31	2.29	5.60	<0.5	<0.20	1.53	1.16	2.69	0.83	<0.20
14	3.45	4.70	8.15	<0.5	104	13.7	21.7	35.4	1.65	63.5	--	--	**--**	<0.5	21.7	4.47	1.24	5.71	6.86	37.5
min	**3.44**	**4.7**	**8.15**	**0.62**	**0.24**	**4.38**	**16.2**	**20.6**	**0.5**	**1.38**	**0.33**	**1.77**	**3.00**	**0.50**	**0.21**	**0.53**	**0.23**	**0.76**	**0.83**	**0.44**
max	**81.6**	**233**	**315**	**26.8**	**545**	**23.9**	**211**	**224**	**102**	**523**	**8.69**	**10.0**	**12.8**	**92.6**	**352**	**10.5**	**11.9**	**22.4**	**623**	**905**

*min and max – minimum and maximum values

Thymus species [9, 10a]. In the extracts of *T. alsarensis* only two flavanones were detected: hesperetin *O*-glucoside ([M–H]$^-$ at *m/z* 463) and naringenin ([M–H]$^-$ at *m/z* 271).

Quantitative analysis: The quantification of all polyphenolic compounds was achieved using standard solutions of caffeic acid, quercetin 3-*O*-rutinoside (rutin), and apigenin as representative of their own group of polyphenols (Table S1-S4, Supplementary data). Total phenolic content was determined as a sum of phenolic acids and flavonoids (flavones, flavanones and flavonols).

The total phenolic content was the highest in flower, followed by leaf, root and steam extracts. It was in the range from 8.15 to 314 mg/g dry herb for flowers, 20.6 to 224 mg/g dry herb for leaves, 3.00-12.8 mg/g dry herb for stems and from 0.76 to 22.4 mg/g dry herb for roots (Table 2). The total amount of phenolic acid derivatives in *T. alsarensis* extracts ranged from 3.44 to 81.6; from 4.38 to 23.9; from 0.33 to 8.69; from 0.53 to 10.5 mg/g dry herb for flowers, leaves, stems and roots, respectively.

Salvianolic acid A (**8**) was found in all studied samples, and its content was around 85% of total phenolic acid content (Table S1-S4, Supplementary data). Compound **2** (caffeic acid glucoside) was the next most abundant phenolic acid compound in leaves, stems and root samples, whereas in the flowers the second dominant phenolic acid was hydroxyjasmonic acid *O*-hexoside (**5**), followed by monomethyl lithospermate (**9**).

Total content of flavonoids in the extracts of *T. alsarensis* ranged from 4.70 to 232; from 16.2 to 211; from 1.77 to 7.02; from 0.23 to 11.9 mg/g dry herb for flowers, leaves, stems and roots, respectively (Table 2). Flavones were the dominant group and their content counts for 80, 63, 41 and 77% of total flavonoid content for flower, leaf, stem and root extracts, respectively. Luteolin 7-*O*-glucoside (**15**) was present in all analyzed samples and its content contributes around 36 and 62% to the total flavone content for flower and leaf samples and 52% for stem and root samples. The contribution of flavonols to total flavonoid content was 20% for flowers and leaves and 57 and 3% for stem and root samples, respectively. Quercetin 3-*O*-glucoside (**11**) was present in all analyzed samples and its content to total flavonol content contributed to around 86% for flower, leaf and root samples, and 64% for stems.

ICP-AES assays: Of the 14 sites where *T. alsarensis* samples were collected (Table 3), 5 (No. 3, 5, 6, 10 and 14) around the Allchar mine are in an area known to possess the highest content of Tl and As in the soil [12a]. Comparison of samples collected near or around the Allchar mine (n = 5) with samples from the rest of the area (n = 9) revealed significant differences in the content of As and Tl.

It was evident that the samples of *T. alsarensis* were able to accumulate heavy metals, especially As and Tl, and distribute them in all parts of the plant. The content of As was significantly higher in the roots than in the stems and leaves and not detected in most of the flower samples (Table 2). The content of Tl was significantly higher in all samples of *T. alsarensis* collected from the Allchar locality (3, 5, 6, 10 and 14). The highest As accumulation was found in the roots, ranging from 0.83 to 623 mg/kg. For the above ground parts of this species, the highest values of As were found in the leaves, ranging from 0.5 to 102 mg/kg, followed by stems ranging from 0.5 to 92.6 mg/kg. The lowest content of As was observed in the flowers (ranged from 0.62 to 26.8 mg/kg). Comparison of the content of Tl in different parts of the plant indicated that the higher content of Tl was observed in the roots (147 mg/kg), flowers (131 mg/kg) and leaves (104 mg/kg), and slightly lower in stems (60.7 mg/kg).

Comparison of the results obtained for total polyphenols and for As and Tl content does not suggest any correlation (positive or negative) between the total phenolic content and the content of Tl and As. On the other hand, from the comparison on the identified compounds with those found in literature [13], it is evident that the soil rich with specific heavy metals (Tl and As) affects the type of polyphenolic compounds produced in different organs, compared with those growing on soil which is not contaminated. The compounds reported here for the studied extracts of *T. alsarensis* such as: quinic acid (**1**), prolithospermic acid (**6**), salvianolic acid B (**7**), salvianolic acid A (**8**), monomethyl lithospermate (**9**), luteolin dihexoside (**12**), luteolin pentosyl-hexoside (**14**), luteolin acetyl pentosyl-hexoside (**16**), luteolin acetyl hexoside (**17**), luteolin dipentoside (**21**), luteolin pentoside (**24**), luteolin acetyl dipentoside (**25**), kaempferol pentosyl-hexoside (**19**) and kaempferol acetyl pentosyl-hexoside (**22**) have not been reported previously for any *Thymus* species. This specific polyphenolic pattern could be attributed to the specific environment in the Allchar region characterized with soil rich in arsenic and thallium minerals that is also reflected in the mineral composition of the plants growing there.

Experimental

Plant material: Plant samples were collected at 14 locations within the abandoned mine described above (Table 3) during the summer of 2011. Specimens of *T. alsarensis* (Figure S1) were sampled according to their abundance and biomass (10–20 replicates from each sampling site). Entire plant samples were washed carefully with double distilled water to remove soil particles, left to dry and then separated into roots, stems, leaves and flowers. Plant material was dried and homogenized to a constant weight at room temperature for 7–14 days.

Table 3: Collection data for *Thymus alsarensis*.

Sample	N	E	Altitude/m	Location
1	41.16459	21.94564	740	R*
2	41.15731	21.94211	772	R
3	41.15699	21.9448	753	A
4	41.13479	21.94189	1035	R
5	41.15734	21.94783	875	A
6	41.16172	21.94494	736	A
7	41.15731	21.94211	772	R
8	41.17578	21.94872	735	R
9	41.13708	21.94469	986	R
10	41.14761	21.94656	895	A
11	41.14612	21.94504	934	R
12	41.15044	21.95099	795	R
13	41.15356	21.9569	966	R
14	41.15959	21.94511	757	A

*A- place near to Allchar rich with As and Tl; R- the other region

Sample preparation: For HPLC/MS analysis 0.1 g of powdered material (*n*=3) was processed. The extraction procedure was performed with 5 mL 70% methanol, using US bath for 30 min and centrifuged for 15 min at 3000 rpm. For ICP/AES analysis 0.5 g powdered material (*n*=3) was placed in a Teflon digestion vessel with 5 mL HNO_3 (69%, m/v) and 2 mL H_2O_2 (30%, m/v). Vessels were closed, caps tightened and placed in the rotor of a Mars microwave digestion system. Plant samples were digested at 180°C. After cooling, digested samples were quantitatively transferred into 25 mL calibrated flasks.

Instrumentation: Chromatographic separations were carried out on a 150 mm × 4.6 mm, 5 µm Zorbax (Eclipse) XDB C18 column (Agilent, Germany). The mobile phase consisted of 1% formic acid in water (v/v) (A) and 1% formic acid in methanol (v/v) (B). A gradient program was used as follows: 0-5 min 20% B, 10-30 min 40% B; 45 min 50% B, and 55-60 min 100% B. The flow rate was 0.25 mL min^{-1} and the injection volume was 10 µL. Spectral data from all peaks were accumulated in the range: λ = 190-600 nm and the chromatograms were recorded at λ=280, 300, 320 and 350 nm. The mass detector was an ion-trap mass spectrometer equipped with an electrospray ionization (ESI) system. Nitrogen was used as a nebulizing gas at a pressure of 65 psi and the flow was adjusted to 12 L/min. The heated capillary and the voltage were maintained at 325°C and 4 kV, respectively. MS data were acquired in the negative ionization mode. The full scan covered the mass range of *m/z* 100–1200. LC–DAD was used for separation and quantification. Peak assignment of the various classes of polyphenols in the chromatograms was based on comparison of their retention behavior and UV–Vis spectra with those of the authentic compounds and literature data. The conjugated forms of the polyphenolic compounds were further characterized by electrospray ionization mass spectrometric detection. Quantification was performed by HPLC/DAD using five-point regression curves ($R^2 \geq 0.999$) of authentic standards. Flavonols were determined at 350 nm using quercetin 3-*O*-rutinoside (rutin), phenolic acid derivatives at 320 nm using caffeic acid as external standard, and flavones and flavanones at 300 nm using apigenin. The contents of As and Tl were analyzed by atomic emission spectrometry with inductively coupled plasma, ICP-AES (Varian, 715-ES, USA) using ultrasonic nebulizer CETAC (ICP/U-5000AT^+) for better sensitivity [19].

Supplementary data: Total content of phenolic acids and flavonoids (in mg/g) for all compounds detected in the flower, leaf, stem and root extracts of the 14 samples of *T. alsarensis* are given in Table S1-S4, and photographs of *T. alsarensis* in Figure S1.

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Ultrasound and Microwave-Assisted Extraction of Elecampane (*Inula helenium*) Roots

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The aim of the current research was to perform two "green chemistry" extractions (ultrasound and microwave irradiation) with methanol, 70% (v/v) ethanol and water for extraction of biologically active substances from elecampane (*Inula helenium* L.) roots and to compare their contents in all extracts. The presence of carbohydrates (sugars, fructooligosaccharides and inulin), total phenols and flavonoids were established. *In vitro* antioxidant potential was also evaluated by four assays (DPPH, ABTS, FRAP and CUPRAC). Water extracts obtained by ultrasound-assisted extraction (UAE) showed the highest value of inulin (38 g/100 g dry weight plant material). The highest antioxidant activity was possessed by the 70% (v/v) ethanol extracts obtained by UAE: DPPH – 107.2 mM TE/g dw, ABTS – 86.0 mM TE/g dw, FRAP – 67.0 mM TE/g dw and CUPRAC -173.0 mM TE/g dw, respectively. The reason for this probably depended on the highest content of total phenols in the 70% UAE ethanol extract {7.9 mg GAE/g dw, phenolic acids (chlorogenic, caffeic, *p*-coumaric, sinapic and ferulic acids)}, especially chlorogenic acid (1.84 mg/g) and flavonoids (quercetin, kaempferol and catechin; 26.4 mg QE/g dw). UAE was evaluated as a promising approach for the simultaneous extraction of bioactive compounds (dietary fibers and antioxidants) from elecampane roots in comparison with microwave irradiation.

Keywords: *Inula helenium* L. roots, Elecampane, Inulin, Phenolic acids, Flavonoids, Antioxidant activity.

Elecampane (*Inula helenium* L.) is an important medicinal plant of the Compositae family, widely distributed in Europe, Asia and Africa [1-3]. Its roots are listed in several European pharmacopeias (e.g, PF X, Ned 5, BHP) as a diuretic, diaphoretic, expectorant and anthelminthic remedy [2,4-6]. Elecampane roots are applied in folk medicine as infusions and tinctures for curing asthma, bronchitis, lung disorders, tuberculosis, indigestion, chronic enterogastritis [5,6], and for wound healing [4,7], treatment of emesis, diarrhea and threatened abortion [2]. Its pharmacological properties include also anti-inflammatory, antioxidant, anticoagulant, anti-tumor, antimicrobial and insecticidal activities [5,8,9]. Previous research explained most of these biological activities by the presence of alantolactone, isoalantolactone, thymol derivatives, polysaccharides and phenolic compounds, especially chlorogenic and caffeic acids [3,6,7]. The extraction and identification of some phytochemical compounds from elecampane still remain a challenge.

Nowadays, the "green" methods for extraction of phytochemicals from medicinal plants gain more attention because of reducing time, energy and expense [3,10,11]. The new approach for chemical constituent isolation is the application of ultrasound and microwave irradiation for acceleration of the extraction process of the polysaccharides and other phytochemicals from elecampane [10-14]. Previous studies demonstrated the efficiency of alantolactone and isolanatolactone extraction from elecampane roots by UAE [10-12] and microwave assisted extraction (MAE) [13]. Compared with heat reflux extraction, UAE and MAE were more efficient and timesaving for the isolation of alantolactone and isoalantolactone from *elecampane* [11-13]. In addition, UAE was applied for extraction of total phenols [2], phenolic acids, especially chlorogenic and caffeic acid [3], flavonoids [11] and inulin from *Inula helenium* roots [14-16].

Until now, no relevant studies of the phytochemical profile of *Inula helenium* roots grown in Bulgaria were available. Earlier, Petkova *et al*. [16] reported the isolation of high molecular weight inulin (DP 30-33) from elecampane roots by UAE. However, the influence of microwave irradiation on the extraction process of sugars and inulin from elecampane roots was not studied. To the best of our knowledge the influence of extraction solvents with different polarity, together with the extraction techniques based on the principles of 'green' chemistry for the isolation of phytochemicals from elecampane roots was not investigated in detail.

Therefore, the aim of the current research was to perform two "green chemistry" extraction approaches (UAE and MAE) with methanol, 70% (v/v) ethanol and water for extraction of biologically active substances from elecampane roots and to compare their content in all extracts. The main interest was to evaluate elecampane root as a natural source of pharmaceuticals (inulin and polyphenols) with improved health benefits.

The screening of carbohydrates in the different extracts of elecampane roots was qualitatively made by TLC analysis (Table 1). TLC chromatograms showed that large numbers of carbohydrates were successively extracted by UAE and MAE with short extraction times. The presence of fructose (R_f = 0.50), sucrose (R_f = 0.44), fructooligosaccharides (FOSs), including 1-kestose (R_f = 0.37), nystose R_f = (0.32) and 7-8 FOSs oligomers, equivalent to Frutafit® CLR DP=7-9 (3 mg/mL) were established in the elecampane extracts (10 µL). High-molecular weight inulin that coincided with the used standard chicory inulin (DP=22) (3 mg/mL) was found only in water and 70% (v/v) ethanol extracts, because of its solubility.

The data from spectrophotometric and HPLC-RID analyses are summarized in Table 1.

Table 1: Carbohydrate composition and extraction yields (%) in different extracts of roots of *Inula helenium*, g/100 g dw (mean ± SD, n=4).

Carbohydrate	UAE MeOH	UAE 70%EtOH	UAE H_2O	MAE MeOH	MAE 70%EtOH	MAE H_2O
Fructose	2.3±0.2	2.5±0.2	2.5±0.2	2.2±0.1	2.4±0.2	2.5±0.3
Glucose	0.3±0.1	0.2±0.1	0.3±0.1	0.3±0.1	0.2±0.1	0.2±0.1
Sucrose	0.3±0.1	0.8±0.1	0.8±0.2	0.3±0.1	0.8±0.1	0.8±0.1
1-Kestose	0.4±0.1	0.5±0.1	0.5±0.1	0.4±0.1	0.5±0.1	0.5±0.1
Nystose	0.3±0.1	0.4±0.1	0.4±0.1	0.3±0.1	0.4±0.1	0.3±0.1
Inulin	**2.5±0.1**	**21.2±0.3**	**38.0±1.1**	**1.8±0.2**	**19.3±0.4**	**29.4±0.4**
Total fructans	**5.8±0.3**	**25.5±0.6**	**42.0±1.3**	**5.0±0.1**	**23.7±0.8**	**33.5±1.1**
Yield of extract	30	41	58	28	35	48

dw – dry weight, SD – standard deviation, MeOH – methanol, EtOH – ethanol

For the first time the carbohydrate content of methanol and 70% (v/v) ethanol extracts of elecampane roots were evaluated. The main detected compounds were inulin, nystose, 1-kestose, sucrose, fructose and glucose (Table 1). The low molecular weight carbohydrates were easily extracted by methanol, 70% (v/v) ethanol and distilled H_2O. No significant differences in their content were observed in the resulting extracts. However, the highest fructan content was found in both UAE and MAE water extracts, probably due to the better water solubility of inulin. In general UAE accelerated the total fructans extraction – 42 g/100 g dw. The UAE extract demonstrated the highest inulin quantity - 38 g/100 g dw. The lowest levels of fructans after MAE could be explained by the shorter extraction time – only 10 min. Moreover, UAE and MAE demonstrated a reduced time for fructans extraction. Better results were obtained by UAE. The sugar, inulin and total fructans content in water extracts was comparable with or higher than previously reported values for the same plants [17-19]. In the current study, the total fructan and inulin content obtained by UAE (42 g/100 g dw) was close to our earlier reported data by conventional extraction [19], as the analyzed roots had the same batch number. Therefore, UAE shortened the extraction time from six hours to 40 min and improved the efficiency (yield - 58 % by water extraction).

In addition the obtained elecampane extracts contained phenolic compounds. The amount of total phenolic and flavonoid contents varied with the different solvents used for UAE and MAE (Table 2). The highest values for total phenols were found in 70% ethanol extracts of *I. helenium* roots after UAE – 7.87 mg GAE/g dw. Our results were significantly higher than the previously reported data [2,20]. However, the polyphenol content in 70% (v/v) ethanol extracts was higher than that in the UAE 30% ethanol extracts (6.13 mg/g dw) [2] and conventional extracts of elecampane roots [19] – 3.5 mg/g dw. The values for the total phenolic content in UAE methanol extracts (5.84 mg GAE/g dw) were higher than their content in 100% [21] and 80% methanol - 3.65 mg/100 g dw [20]. The total flavonoids content was also the highest in the 70% (v/v) ethanol elecampane extract using UAE– 23.9 mg/g dw (Table 2).

Table 2: Total phenolic and total flavonoid contents and *in vitro* antioxidant activity (expressed as mM Trolox/g dw plant material) of elecampane root extracts.

	UAE MeOH	UAE 70%EtOH	UAE H_2O	MAE MeOH	MAE 70%EtOH	MAE H_2O
TPC	5.8±0.2	7.9±0.1	4.3±0.1	5.3±0.1	5.6±0.2	3.2±0.1
TF	9.4±0.2	23.9±0.1	18.5±0.2	8.2±0.1	6.2±0.2	6.3±0.1
DPPH	84.9±0.2	107.2±0.1	nf	90.3±0.1	27.5±0.1	nf
ABTS	56.1±0.2	86.0±0.2	nf	50.2 ±0.2	60.8±0.2	nf
FRAP	40.8±0.2	67.0±0.2	32.8±0.2	35.6±0.1	48.2±0.1	58.0±0.2
CUPRAC	117.1± 0.3	173.0±0.1	60.8±0.2	120.1±0.2	113.4±0.2	79.8±0.2

nf – not found; MeOH – methanol, EtOH – ethanol, TPC - Total phenolic content expressed as mg GAE /g dw; TF - total flavonoids expressed as mg QE/g dw,

These data confirmed the efficiency of the extraction process of flavonoids by UAE [11, 12]. However, our results from the current research were higher than the previously reported values for the total flavonoids from the elecampane root obtained by 60% ethanol UAE in the same solid to solvent ratio (17.4±0.9) mg/g [11]. Moreover, our results for total flavonoids in the 70% ethanol (MAE) extracts were consistent with those with 50% ethanol, with a solid to liquid ratio of 1:15 and MAE - 18.3 mg/g [13].

Scanty data for the antioxidant activity of foreign elecampane have been reported [20-22]. Our last study of the antioxidant capacity of 95% ethanol and subsequent water extracts of elecampane root (with the same batch number as these in this study) was evaluated by DPPH, ABTS, FRAP and CUPRAC [19]. To the best of our knowledge the antioxidant potential of UAE and MAE from elecampane roots still remained unevaluated. The current study showed that the highest antioxidant activity was possessed by 70% (v/v) ethanol extracts prepared by UAE (DPPH – 107.2 mM TE/g dw, ABTS – 86.0 mM TE/g dw, FRAP – 67.0 mM TE/g dw and CUPRAC -173.0 mM/g dw). Our results were significantly higher than those of the 80% methanol UAE extracts [20] and 95% ethanol and subsequent water extraction of elecampane roots after conventional extraction [19]. In general, promising antioxidant capacity was possessed by the methanol and 70% ethanol extracts obtained by UAE and MAE. The antioxidant activity positively correlated with the highest values of total phenolic content and total flavonoids and was in accordance with Spiridon *et al.* [21]. Obviously, the total phenolic content measured by the Folin-Ciocalteu method could not give detailed information about the individual constituents of the studied extracts. Therefore, an HPLC UV-VIS method was used for identification of phenolic acids and flavonoids in elecampane roots (Table 3). The identified compounds were flavonols, benzoic acid derivatives and cinnamic acid derivatives. The differences in their phytochemical content were probably due to the extraction methods used. The presence of 11 phenolic acids and 6 flavonoids was detected. Predominant flavonoids in the elecampane root extracts were quercetin (**12**), kaempferol (**13**) and myricetin (**14**) (Table 3). The highest content of these compounds was found in the 70% (v/v) ethanol UAE extract. Chlorogenic acid (**3**) was the dominant phenolic acid in 70 % ethanol and methanol UAE extracts – 1.84 mg/g and 1.34 mg/g dw, respectively.

Table 3: Quantitative analysis of major phenolic compounds in extracts of *Inula helenium* roots (mg/g dw plant material).

No	Compound	UAE MeOH	UAE 70%EtOH	UAE H_2O	MAE MeOH	MAE 70%EtOH	MAE H_2O
	Phenolic acid						
1	Gallic	nf	nf	0.2*	nf	0.1*	0.1*
2	2-Hydroxy benzoic	nf	nf	0.1*	nf	nf	nf
3	Chlorogenic	1.3±0.1	1.8*	0.1*	0.2*	1.0*	0.4*
4	Neochlorogenic	0.2±0.1	0.6*	0.2*	0.1*	0.2*	0.2*
5	Caffeic	nf	0.4*	0.1*	0.1*	0.2*	0.1*
6	*p*-Coumaric	0.1*	0.6±0.1	nf	nf	0.1*	0.1*
7	Sinapic	0.2±0.1	0.3±0.1	nf	trace	0.1*	nf
8	Ferulic	0.6±0.1	1.0±0.1	nf	nf	nf	nf
9	3,4-Dihydroxybenzoic	nf	nf	nf	nf	trace	0.1*
10	Vanillic	nf	nf	nf	trace	0.4±0.1	trace
11	Cinnamic	nf	nf	nf	trace	nf	nf
	Flavonoids						
12	Quercetin	nf	0.6*	0.2*	0.2±0.1	0.3*	0.2*
13	Kaempferol	nf	0.3*	0.1*	0.1*	0.2±0.1	nf
14	Myricetin	nf	0.7±0.1	0.2*	trace	0.3±0.1	0.1*
15	Catechin	nf	10.5±0.1	1.0±0.1	2.2±0.1	6.5±0.1	0.6*
16	Epichatechin	nf	nf	nf	nf	nf	0.1*
17	Quercetin-3-*O*-β-glucopyranoside	0.1*	nf	nf	nf	nf	nf

nf – not found, MeOH – methanol, EtOH – ethanol *SD < ±0.1, trace <0.1 mg/g dw

Neochlorogenic (**4**), caffeic (**5**), *p*-coumaric (**6**), sinapic (**7**) and ferulic (**8**) acids were also found in 70% (v/v) ethanol UAE extracts. This is the first report of the detection of sinapic and vanillic acids**,** as well as myricetin (**14**) and catechin (**15**) in elecampane root extracts. Syringic, cinnamic and rosmarinic acids were not found. Gallic acid (**1**) and 2-hydroxy benzoic acid (**2**) were mainly found in water extracts. Two early reports evaluated UAE with 95% ethanol and methanol for the extraction of chlorogenic and caffeic

acids [2,3]. These two phenolic acids possess a wide range of pharmacological activities [2,20,21]. Wojdylo *et al*. [20] found only caffeic, neochlorogenic, and ferulic acids in 80 % methanol UAE extracts [20]. However, our study showed the presence of only 5 phenolic acids in the UAE methanol extract and the absence of caffeic acid. This was detected only in water-containing solvents. Our findings for the presence of hydroxybenzoic, chlorogenic, neochlorogenic, *p*-coumaric, ferulic, and caffeic acids, and quercetin, epicatechin and quercetin-3-*O*-β-glucopyranoside in methanol elecampane extracts coincided with the previous TLC report [9], HPLC [3,20] and GC-MS analysis [21]. Moreover, the efficiency of UAE for extraction of phenolic acids was confirmed [3]. However, our results for chlorogenic acid were higher than other reports for UAE (40 kHz and 100 W; 30 min, solid-liquid ratio 1:20) [2,11]. Surprisingly, we did not detect caffeic acid in the methanol UAE.

The current research revealed the efficiency of UAE for extraction of phytochemicals from *I. helenium* roots, especially inulin and polyphenolics. The resulting phytochemical profile of 70% (v/v) ethanol extracts demonstrates high biological activity with promising future application in food and cosmetics. Bulgarian elecampane roots were evaluated as a source of phenolic acids (mainly chlorogenic, neochlorogenic, caffeic, *p*-coumaric, sinapic and ferulic), different flavonoids (quercetin, kaempherol, myricetin and catechin) with high antioxidant activities, and also inulin with potential immunostimulating and other health beneficial properties.

Experimental

Materials: All chemicals were of analytical grade (Sigma–Aldrich, Germany). FOSs Frutafit®CLR (DP 7-9) and inulin Frutafit®TEX (DP 22) were supplied by Sensus (Netherlands). Dried elecampane roots were purchased from a local drugstore (voucher specimen Alin L 52), finely ground and passed through a 0.5 mm sieve. The moisture content, analyzed by AOAC 945.32 [23], was 8.7%.

Preparation of plant extracts: Methanol, 70% (v/v) ethanol and distilled H_2O in a solid to liquid ratio of 1:20 were used for the extraction of phytochemicals from elecampane roots. UAE was performed in an ultrasonic bath (VWR, Malaysia, 45 kHz and 30 W) for 15 min, at 45ºC [24]. MAE was performed in a microwave oven {CROWN (700 W, 2450 MHz)} for 5 min at an average power of 541 W. The extraction process was repeated twice. The extracts were filtered, combined and used for further analyses. The results were calculated on the dry weight (dw) of plant material.

TLC analysis of sugars and fructans: Each extract (10 µL) was applied to a 10×20 cm silica gel 60 F_{254} plate (Merck, Germany) with a calibrated glass capillary (BLAUBRAND®, Germany). Thin-layer chromatography (TLC) was performed using the double-ascending method with a mobile phase of *n*-BuOH:i-Pro:H_2O:CH_3COOH (7:5:4:2) (v/v/v/v) that was developed over 4 and 8 cm, respectively. The TLC plates were dipped in diphenylamine-aniline-H_3PO_4–acetone (1:1:5:50) (w/v/v/v), heated at 120°C for 5 min [25] and scanned by HP Scanjet G2710 Photo Scanner [19]. Glucose, fructose, sucrose, 1-kestose, nystose (3 mg/mL) were used as standards (10 µL).

Fructans assay: The total fructans content in elecampane root extracts was defined spectrophotometrically at a wavelength of 480 nm; results are expressed as fructose equivalents [19].

HPLC-RID carbohydrate analysis: Extracts were evaporated to dryness. Each extract (10 mg) was dissolved in 2 mL distilled H_2O, filtered through a 0.45 µm PTFE filter (Isolab**,** Germany) and then 20 µL was injected. Separations were performed on an HPLC (Shimadzu) with an analytical column {Shodex® Sugar SP0810 (300 mm × 8.0 mm i.d.)} at 85°C, with a mobile phase of distilled H_2O at a flow rate 1.0 mL/min. Peaks were identified by retention times with standards of inulin, nystose, 1-kestose, sucrose, glucose and fructose. The analyte concentrations were calculated as peak areas with reference to the calibration curves, prepared at 5 concentration levels (0.5, 1, 2.5, 5 and 10 mg/mL). The calibration plots showed excellent linearity (r^2= 0.997) [26].

Determination of total phenolic and flavonoids contents: Total phenolic content was determined with Folin–Ciocalteu's reagent [27]. Gallic acid was used as a calibration standard and the results were expressed as mg gallic acid equivalents (GAE) per g (dw) plant material. Total flavonoids were determined with $Al(NO_3)_3$ reagent at 415 nm [28]. The results are presented as mg equivalents quercetin (QE) per g dw plant material.

In vitro antioxidant activity: The DPPH assay was performed as described [28]. The ABTS method [29] was used with some modifications [30]. FRAP assay was performed according to Benzie and Strain [31]. CUPRAC assay was according to Apak *et al*. [32]. The results are expressed as mM Trolox® equivalents (TE) per g dry weight (dw) plant material [30].

HPLC analysis of phenolic compounds: The qualitative analyses of phenolic components were performed on an Agilent 1220 HPLC system (Agilent Technology, USA), equipped with an Agilent TC-C18 column (5 µm, 4.6 × 250 mm) at 25ºC and UV–VIS detector at 280 nm. Separation of phenolic compounds was performed with mobile phases 0.5% acetic acid (A) and 100% acetonitrile (B) at a flow rate of 0.8 mL/min [33]. Phenolic acids (gallic, 2-hydroxy benzoic, neochlorogenic, caffeic, *p*-coumaric, sinapic, ferulic, 3,4-dihydroxybenzoic, vanillic, and cinnamic, and flavonoids (quercetin, kaempferol, myricetin, catechin, epichatechin, quercetin-3-*O*-β-glucopyranoside) (Sigma) were used for calibration of standard curves. The phenol concentrations were calculated as peak area with reference to the calibration curves, prepared at 5 concentration levels (5 µg/mL, 20 µg/mL, 50 µg/mL, 75 µg/mL and 100 µg/mL).

Statistical analysis: The data, expressed as mean ± SD, were statistically analyzed using MS-Excel software.

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ADME/Tox Properties and Biochemical Interactions of Silybin Congeners: *In silico* Study

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Silymarin, the active constituent of *Silybum marianum* (milk thistle), and its main component, silybin, are products with well-known hepatoprotective, cytoprotective, antioxidant, and chemopreventative properties. Despite substantial *in vitro* and *in vivo* investigations of these flavonolignans, their mechanisms of action and potential toxic effects are not fully defined. In this study we explored important ADME/Tox properties and biochemical interactions of selected flavonolignans using *in silico* methods. A quantitative structure–activity relationship (QSAR) model based on data from a parallel artificial membrane permeability assay (PAMPA) was used to estimate bioavailability after oral administration. Toxic effects and metabolic transformations were predicted using the knowledge-based expert systems Derek Nexus and Meteor Nexus (Lhasa Ltd). Potential estrogenic activity of the studied silybin congeners was outlined. To address further the stereospecificity of this effect the stereoisomeric forms of silybin were docked into the ligand-binding domain of the human estrogen receptor alpha (ERα) (MOE software, CCG). According to our results both stereoisomers can be accommodated into the ERα active site, but different poses and interactions were observed for silybin A and silybin B.

Keywords: Silymarin, Silybins, ADME/Tox properties, Estrogen receptor.

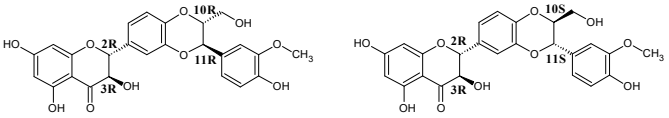
Silybum marianum (L.) Gaertn. (milk thistle) is an ancient medicinal plant that has been used for almost 2000 years for treatment of liver and gallbladder disorders of different etiologies [1,2]. The active component of this herb, silymarin, is a mixture of phenolic compounds, mainly silybin A, silybin B, but also other flavonolignans such as isosilybin A, isosilybin B, silychristin and silydianin, which are located predominantly in the fruit and seeds. The main component of silymarin is silybin, which is a quasi equimolar mixture of two diastereomers A and B (Figure 1) [3].

Today silymarin is best known for its antioxidant and chemoprotective effects on the liver [4], and is often either prescribed or self-prescribed as a complementary hepatoprotective medicine [5]. It has also gained attention due to its hypocholesterolemic, cardioprotective, neuroactive and neuroprotective properties [4]. Although silymarin is reported as nontoxic in human studies, little is known about its mechanism of action and biochemical interactions [6]. Recent works have explored inhibition and modulation of some drug transporters [7] and nuclear receptors [8] by silybin congeners as well as their biotransformation products [9]. For example, an *in vitro* study focusing on interactions of flavonolignans with the aryl hydrocarbon receptor (AhR) and estrogen receptor (ER) demonstrated that silymarin has partial estrogenic activity, with silybin B being probably responsible for it [8]. It was outlined that stereochemistry plays an important role for the investigated biological activities and there is a need for studies on the pure forms of the compounds that are otherwise therapeutically used as mixtures [10]. Another important prerequisite for broader and safer therapeutic use of flavonolignans is the better understanding of their metabolism, pharmacokinetics and potential toxic effects.

The pharmaceutical industry has used *in silico* methods for decades to search, optimize and evaluate drugs [9]. In recent years the *in silico* ADME/Tox (Absorption, Distribution, Metabolism, Excretion, and Toxicity) prediction is receiving particular attention due to the increased evidence that these pharmacokinetic properties should be considered earlier in the drug discovery process [11].

In the present study we aimed at exploring important ADME/Tox properties and biochemical interactions of selected flavonolignans (Figures 1 and 2) using *in silico* methods. For estimation of bioavailability after oral administration (gastrointestinal absorption) an in house developed quantitative structure–activity relationship (QSAR) model utilizing data from a parallel artificial membrane permeability assay (PAMPA) was used. Predictions of toxicity and metabolism were performed using knowledge-based expert systems, and molecular modelling studies were applied for investigation of the interactions of the stereoisomeric forms of silybin with the ligand-binding domain (LBD) of the human estrogen receptor alpha (ERα).

In silico estimation of gastrointestinal absorption was performed using a QSAR model for prediction of PAMPA permeability. PAMPA is a high throughput *in vitro* assay that evaluates transcellular permeation of small drug-like molecules [12]. PAMPA is used in pharmaceutical research to screen for human intestinal absorption since PAMPA permeability has been shown to correlate with both Caco-2 cell permeability and human intestinal absorption [13].



Silybin A Silybin B

Figure 1: Silybin A (2*R*, 3*R*, 10*R*, 11*R*) and silybin B (2*R*, 3*R*, 10*S*, 11*S*).

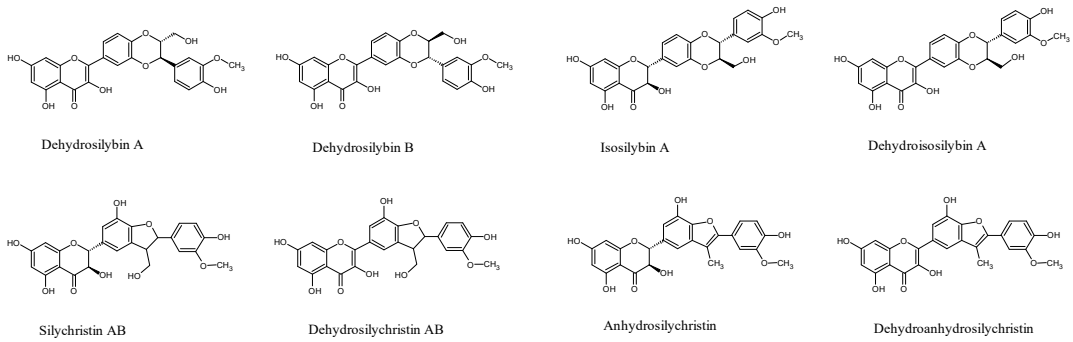


Figure 2: Chemical structures of the silybin congeners investigated in the present study.

In our study we used a highly predictive QSAR model derived from a data set of nearly 200 diverse drugs with PAMPA permeability coefficients measured at pH 6.5 and 7.4 (Equation 1: n – number of compounds used to derive the model; r^2 – adjusted multiple linear regression correlation coefficient, SEE – standard error of estimate; F – Fisher ratio, LOO q^2 – leave-one-out cross validation correlation coefficient; external validation q^2 – predicted correlation coefficient obtained on a test set of 50 compounds and a training set of 196 compounds [14]). The predicted values of PAMPA permeability (logPm), as well as the calculated values of the descriptors (logD – distribution coefficient at pH = 7.4; TPSA – topological polar surface area; MW – molecular weight) are shown in Table 1. According to the *in silico* prediction of PAMPA permeability the studied silybin congeners may be considered as moderate to highly permeable in the gastrointestinal tract.

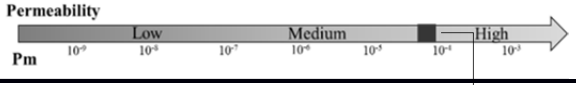
Equation 1: QSAR model for prediction of PAMPA permeability.

$$\mathbf{logPm} = -2.945(\pm0.228) + 0.600(\pm0.046)\ \text{logD} - 7.655(\pm0.811)\ \text{TPSA/MW}$$

$$n = 196,\ r^2 = 0.734,\ SEE = 1.108,\ F = 338.9$$

$$LOO\ q^2 = 0.729,\ external\ validation\ q^2 = 0.696\ (196/50)$$

Table 1: Calculated values of the descriptors and predicted values of logPm of the studied silybin congeners.



Compounds	logD at pH=7.4	TPSA / MW	Predicted logPm (cm/s)
1. Silybin A	1.77	0.322	-4.345
2. Silybin B	1.77	0.322	-4.345
3. Dehydrosilybin A	1.03	0.331	-4.862
4. Dehydrosilybin B	1.03	0.331	-4.862
5. Isosilybin A	1.82	0.322	-4.315
6. Dehydroisosilybin A	1.08	0.331	-4.832
7. Silychristin AB	1.7	0.345	-4.562
8. Dehydrosilychristin AB	1.4	0.354	-4.815
9. Anhydrosilychristin	2.77	0.323	-3.753
10. Dehydroanhydrosilychristin	2.07	0.333	-4.249

In silico toxicity and metabolism predictions were performed using the knowledge-based expert systems Derek Nexus and Meteor Nexus [15]. Derek Nexus generates a prediction by comparing the structural features of the target compound with a toxicophore encoded as structural pattern(s) in its knowledge base. The final predictions are derived from a reasoning scheme which takes into account the presence of a toxicophore (structural alert) in the query structure [16]. Based on the alerts detected in the structures of silybin congeners, three potential toxic effects in mammals (chromosome damage *in vitro*, ERα modulation and skin sensitization) are outlined as plausible (Table 2), but no stereospecific effects are specified.

Meteor Nexus predicts the metabolic fate of a chemical from its structure [17]. The likelihood of the predicted biotransformation is assessed by a Site of Metabolism Scoring (SOM) method using experimental data for compounds that match the same biotransformation, have similar molecular weights and are chemically similar around the site of metabolism to the query compound. The two most probable metabolic transformations for the studied compounds are presented: glucuronidation (Figure 3A) and oxidative *O*-demethylation (Figure 3B).

Table 2: Structural alerts and related toxicological effects of silybin congeners defined as 'plausible'.

Structural alerts	Toxicological effect	Compounds containing structural alert*
Flavonol	Mutagenicity *in vitro* in bacterium	3, 4, 6, 8
Flavonoid	Chromosome damage *in vitro* in mammal	3, 4, 6, 8
2-(Para-hydroxyphenyl)-benzoxazole or derivative	Estrogen receptor modulation in mammal	8, 9, 10
2-Phenyltetralin derivative	Estrogen receptor modulation in mammal	1, 2, 3, 4, 5, 6
Hydroxy-napthalene or derivative	Estrogen receptor modulation in mammal	3, 4, 6, 8, 10
Resorcinol or precursor	Skin sensitization in mammal	3, 4, 6, 8, 10
1,2-Dihydroxybenzene derivative	Skin sensitization in mammal	1, 2, 3, 4, 5, 6, 7, 8, 9, 10

* The numbers of the compounds are according to the numeration in Table 1.

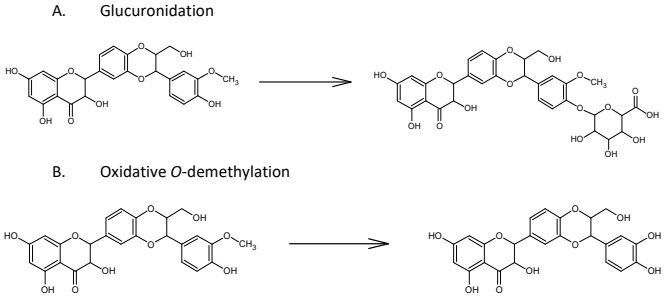


Figure 3: Metabolic transformations of silybin with highest scores predicted in Meteor Nexus.

In order to investigate the interactions of stereoisomeric forms of silybin with the LBD of the ERα a docking study of each of the isomers, silybin A and silybin B, was further performed. It is known that agonists and antagonists stabilize differently the helix 12 (H12) in the LBD C-terminus which plays a crucial role in determining ERα interactions with coactivators and corepressors [18]. The antagonist LBD conformation (with H12 position in green, Figure 4) was selected for docking of silybins as the agonist conformation (H12 position in magenta, Figure 4) was not large enough to accommodate the silybins (poses were generated with the compounds in unrealistic folded conformations, data not shown).

This selection was additionally justified by the analysis of ERα X-ray complexes of partial agonists in the Protein Data Bank (PDB) [19] that showed some partial agonists bound in the antagonist conformations of ERα. Our docking results demonstrate

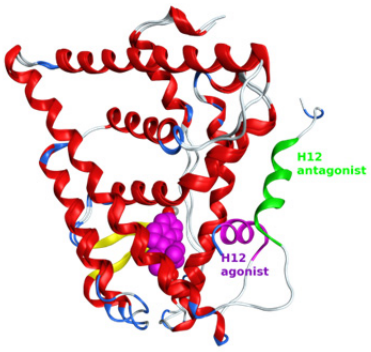


Figure 4: The ligand binding domain of ERα with the full agonist estradiol in the active site shown in space-filled rendering and colored in magenta. Positions of the activation helix H12: agonist conformation (magenta); antagonist conformation (green).

that both silybin A and silybin B can be accommodated into the ERα active site, but the stereoisomers showed different poses and interactions in the receptor active site. The results obtained from docking with MOE software [20] reveal no specific interactions of silybin A with amino acids in the active site of ERα (Figure 5). In contrast, silybin B forms hydrogen bond (HB) interactions with Leu525, located next to His524 (a residue that the agonist estradiol interacts with, Figure 6) and Asp 351 (Figure 5).

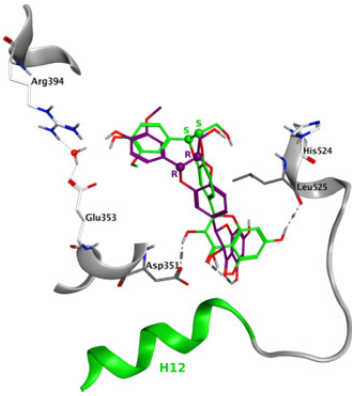


Figure 5: Poses of silybin A (purple) and silybin B (green) and HB interactions of silybin B in the ERα active site. The *R*- and *S*-stereo C-atoms of the compounds are shown as balls; the O-atom of the water molecule in the active site is shown as a red ball.

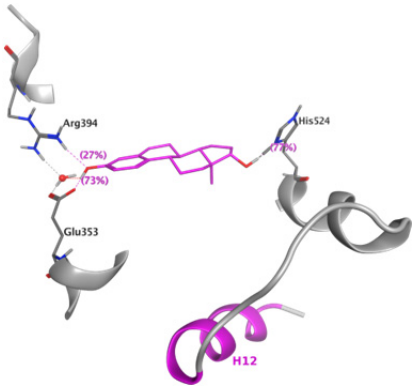


Figure 6: HB interactions of the full agonist estradiol into the LBD active site of ERα. The possible HBs with residues Glu353 and Arg394 are shown in magenta dot lines. HBs scores are expressed as percentages according to probability criteria derived from a large training set (Glu353 more preferred compared with Arg394). The active water molecule (red ball) and the helix H12 (magenta ribbon) are also shown.

Unlike the agonist estradiol and the antagonist 4-hydroxytamoxifen (Figure 7), silybin B does not interact directly with Glu353 and Arg394, but remains close to them and the active water molecule (distances not shown). Similarly to the antagonist 4-hydroxytamoxifen, silybin B interacts with Asp351 (Figure 7), but this interaction is through HB, and not through an ionic interaction.

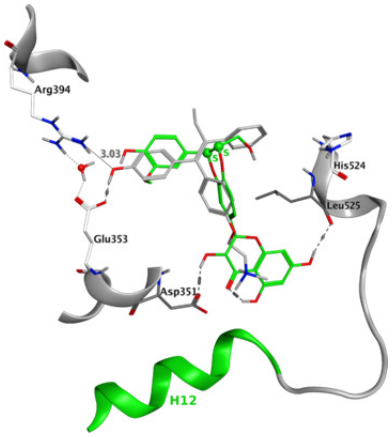


Figure 7: Poses of 4-hydroxytamoxifen (atom type colored) and silybin B (green) and their HB interactions in the ERα active site.

Using GOLD software [21, 22], stereospecific poses of silybin A and silybin B in the active site of ERα were also generated, but inverted orientations were observed with the 10*R*/11*R* and 10*S*/11*S* atoms of the isomers oriented towards H12, and not towards the inner side of the protein active site (Figure 8). In this region, the difference in the orientations of the two ligands is the most noticeable. Both silybins perform HB interactions with Glu353, but only silybin B additionally interacts with Thr347.

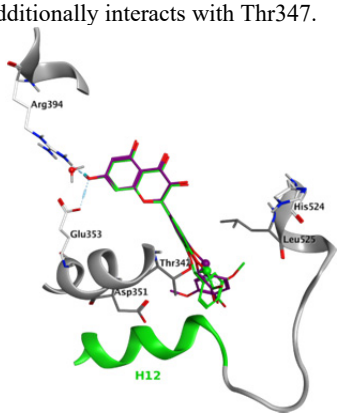


Figure 8: Poses of silybin A (purple) and silybin B (green) and HB interactions of silybin B in the ERα active site generated with GOLD software. The active water molecule (red ball) and the helix H12 (green ribbon) are also shown.

Our docking results clearly show stereospecific interactions of silybin A and silybin B in the ERα active site, independently of the orientation of the stereoatoms 10*R*/11*R* and 10*S*/11*S* in the active site (towards H12 or opposite to it). The recorded specific interactions of silybin B in the best docking poses reproduce some of the interactions observed for the agonists and some of the interactions of the antagonists thus implying the behavior of silybin B as a partial agonist. Our docking results are consistent with the *in vitro* study according to which silybin B and not silybin A is probably responsible for the partial ERα-mediated activity of silymarin [8]. They reveal possible orientations and interactions of these silybins in the receptor active site that, in combination with permeability, toxicity and metabolism predictions, can be useful for the rational modification and design of new natural product derivatives with potential positive effects for the human health.

Experimental

In silico prediction of the gastrointestinal absorption: The predictions were performed using a QSAR model implemented as a workflow in the KNIME platform that is freely accessible to execute in a web browser within the COSMOSTOX webportal (https://knimewebportal.cosmostox.eu/). The descriptors were

calculated with ACD/Percepta (logD) (http://www.acdlabs.com/) and CDK KNIME nodes (TPSA/MW) (http://www.knime.org/).

In silico predictions of toxicity and metabolism: The predictions were performed using Derek Nexus v.5.0.1 and Meteor Nexus v.3.0.0 knowledge-based expert systems (Lhasa Ltd.). Derek Nexus provides a level of likelihood for each prediction (certain, probable, plausible, equivocal, doubted, improbable, impossible, open and contradicted). In the present study the reasoning levels of the reported prediction are estimated as "plausible" by the expert system that means "the weight of evidence supports the proposition" [23]. The prediction performed by Meteor Nexus are assessed by SOM Scoring (with Molecular Mass Variance) and the two best scored metabolic transformations of the studied compounds are reported.

Molecular modelling studies: MOE 2015.10 software was used for docking studies, analysis and comparison of protein-ligand interactions and identification of important protein residues. The ERα ligand binding domain (X-ray structure of the ERα with 4-hydroxytamoxifen, PDB ID 3ERT) was initially prepared using the MOE tool "Protonate3D". The physiologically relevant parameters were set during the minimization: temperature = 310 K; pH = 7.4; ion concentration = 0.152 mol/L. The ligands were docked into the binding site of the prepared protein structure using the "Receptor + Solvent" protocol and the binding pocket of the receptor was specified by using the atoms of the co-crystallized ligand (4-hydroxytamoxifen). A rescoring with London dG scoring function was applied to rank the poses of the docked ligands without subsequent refinement and second rescoring. The best scored poses of each ligand with a negative value of the scoring function were kept. The ERα-ligand complexes were analysed using the MOE tool "Ligand Interactions".

GOLD v. 5.1 (Cambridge Crystallographic Data Centre Ltd.) software was used for additional docking studies. The ligands were docked into the LBD of the protein and the active site was specified by using the atoms of the co-crystallized ligand (4-hydroxy-tamoxifen). The docking was performed with an active water molecule in the binding site whose hydrogen positions were allowed to vary during the docking in order to maximise the hydrogen bonding score both from interactions with the protein and the ligand. The docking poses for each of the studied ligands were ranked according to the GoldScore scoring function and the best scored poses were selected. The visualization of the poses and the protein-ligand interactions were performed in MOE software.

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Comparative Study of Naphthoquinone Contents of Selected Greek Endemic Boraginaceae Plants - Antimicrobial Activities

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The cyclohexane (Ch) extracts of the roots of five Greek endemic Boraginaceae plants, *Onosma kaheirei* Teppner*, O. graeca* Boiss., *O. erecta* Sibth. & Sm.*, Alkanna sfikasiana* Kit Tan, Vold and Strid and *Cynoglossum columnae* Ten, were investigated for the presence of alkannin/shikonin-related compounds. All species,s except *C. columnae* and *O. erecta*, were found to contain this type of compounds. Seven compounds were obtained after several chromatographic separations from the Ch extracts of the investigated plants: deoxyalkannin (**1**), 2′′-(*S*)-*α*-methylbutyrylalkannin (**2**), isobutyrylalkannin (**3**), propionylalkannin (**4**), acetylalkannin (**5**), β-hydroxyisovalerylalkannin (**6**), and β,β-dimethylacrylalkannin (**7**). All structures were identified by 1D ^{1}H-/^{13}C- and 2D NMR spectroscopy, assisted also by ESI-MS. The extracts and the isolated compounds exhibiting an interesting antimicrobial profile when evaluated for their antimicrobial activity against six Gram-positive and -negative bacteria and three human pathogenic fungi.

Keywords: Boraginaceae, *Onosma*, *Alkanna*, *Cynoglossum*, Isohexenylnaphthazarins, Alkannins/Shikonins.

Boraginaceae is a family of herbs, shrubs and trees with a cosmopolitan distribution. The family comprises ca. 130 genera and 2300 species, occurring mainly in Europe (especially in the Mediterranean region) and Asia [1a, 1b]. Naphthoquinones are lipophilic red pigments that occur typically in the external layer of the roots of Boraginaceae as derivatives of the enantiomeric compounds alkannin and shikonin (A/S). They are responsible for the multiple pharmacological activities, which range from wound healing to anti-inflammatory, antimicrobial, antitumor, antithrombotic and antiviral properties [2a-2c].

In the framework of our research on the Boraginaceae family [1b, 3a-3c] we report herein the comparative analysis of the naphthoquinone contents of five Greek endemic plants, three of which belong to the genus *Onosma* (*O. kaheirei* Teppner*, O. graeca* Boiss. and *O. erecta* Sibth. & Sm.), while the two others are: *Alkanna sfikasiana* Kit Tan, Vold and Strid and *Cynoglossum columnae* Ten. To the best of our knowledge, there are no previous phytochemical reports in the literature on their naphthoquinone content, except for *O. kaheirei* that has been previously published by our team [1b]. In a previous report on Greek *Alkanna* species [4] only alkannin derivatives were found, while from international bibliographic data *Onosma* species are characterized by shikonin derivatives [5]. *C. columnae*, *O. kaheirei* and *O. erecta* are annual Boraginaceae species of the Mediterranean region [6].

Qualitative phytochemical analysis of cyclohexane (Ch) extracts of the above plants resulted to the isolation of seven naphthoquinones (Table 1), while the same (Ch) extracts of *O. erecta* and *C. columnae* were shown to be free of these compounds. Our results concerning the existence of alkannins in the studied Greek plants are in accordance with the bibliography, as it confirmed that all Boraginaceous species that are growing in Europe contain mainly alkannin derivatives, whereas those growing in Asia contain mainly shikonin derivatives [4].

C. columnae seeds from Greece were cultivated in Poland and the roots were treated as wild plants. Previous work on root samples of *Alkanna* species cultivated from seeds collected from wild populations in Greece showed no difference in A/S compounds compared with those obtained from the wild ones [4]. So, it can be safely claimed that wild *C. columnae* is free of A/S pigments.

Table 1: Isolated naphthoquinones

Naphthoquinones	*O. kaheirei*	*O. graeca*	*O. erecta*	*A. sfikasiana*	*C. columnae*
Deoxyalkannin (1)		√			
2′′-(*S*)-α-methylbutyrylalkannin (2)				√	
Isobutyrylalkannin (3)	√			√	
Propionylalkannin (4)		√			
Acetylalkannin (5)		√			
β-Hydroxyisovalerylalkannin (6)		√			
Mixture (2′′-(*S*)-α-methylbutyrylalkannin , Isobutyrylalkannin, β,β-Dimethylacrylalkannin (7)		√			

In addition, the plant extracts, as well as the isolated A/S pigments, were evaluated for their *in vitro* antimicrobial activity against two Gram-positive and four Gram-negative bacteria, as well as against a panel of three human pathogenic fungi (Table 2).

According to our results the Ch extracts showed a very interesting broad antimicrobial profile against all the assayed microorganisms and it appeared that the extracts of *O. graeca* and *A. sfikasiana* exhibited the strongest activities, probably due to their high content of naphthoquinones. Among the tested naphthoquinones, β-hydroxyisovalerylalkannin (**6**), appeared as the most active one with MIC values of $2.0 \bullet 10^{-3}$- $4.90 \bullet 10^{-3}$ mg/mL against all microorganisms, while all the isolated compounds followed a ranking for their antimicrobial activities of : **6** > **7**+**2**+**3** > **3** > **4** > **2** > **5** > **1**, which is in accordance with previous reported studies [2c].

Experimental

General: Specific rotation $[\alpha]_D$ values were measured using a Perkin Elmer 341 polarimeter. ^{1}H NMR, 2D NMR (400 MHz) and ^{13}C NMR (50 MHz) were recorded on Bruker DRX 400, Bruker

Advance III and Bruker AC200 spectrometers, respectively, using $CDCl_3$ as solvent. High resolution mass spectra (HRESI+) were recorded on a Thermo Scientific LTQ Orbitrap Discovery mass spectrometer, using the infusion method. The stationary phases used for column chromatography were silica gel 60H (< 45 μm, Merck) and flash silica gel (0.040-0.063 mm, Merck). TLC plates (Kieselgel 60 F_{254}) were purchased from Merck Chemical Co. Zones on TLC plates were detected under UV light (254 and 366 nm) and after spraying with a solution of vanillin, followed by heating at 105°C for 5 min. HPLC grade solvents were used.

Plant material: The roots of *O. erecta* (Crete, Chania)*, O. graeca* and *A. sfikasiana* (Parnon Mountain, Peloponnese) were collected and identified by Dr E. Kalpoutzakis (Dept. of Pharmacy, NKUA) and deposited at the Herbarium of the Dept. of Pharmacy. The aerial parts of *O. kaheirei* were collected and identified by Dr I Bazos (Dept. of Biology, NKUA), from Ymittos Mount, Attica. The cultivation of *C. columnae* has been previously described [3b].

Extraction and isolation: Roots were ground to powder and successively extracted with Ch. A portion of the Ch extract (0.66 g) of *A. sfikasiana* was subjected to column chromatography (CC), eluting with Ch/EtOAc mixtures of increasing polarity, to afford **3** (164.7 mg) and **2** (14.1 mg). The Ch extract (0.4 g) of *O. graeca* was also fractionated by CC eluting with Ch/CH_2Cl_2 mixtures of increasing polarity, affording **1** (6.0 mg), **4** (2.5 mg), **5** (20.8 mg) and an inseparable mixture of **2**, **3** and **7** (29.3 mg). Extraction and isolation of compound **3** from *O. kaheirei* is fully described by Orfanou *et al.* [1b].

Finally, the Ch extracts of *O. erecta* and *C. columnae* did not appear to contain alkannin/shikonin type secondary metabolites. Compounds **1**-**7** were deep red semi-solids; their ^{1}H- (400 MHz, $CDCl_3$) and ^{13}C-NMR (50 MHz, $CDCl_3$) spectra were in accordance with the literature [3a, 7].

Antimicrobial bioassay: Nine microorganisms, 2 Gram-positive bacteria: *Staphylococcus aureus* (ATCC 25923) and *S. epidermidis* (ATCC 12228), four Gram-negative: *Escherichia coli* (ATCC 25922), *Enterobacter cloacae* (ATCC 13047), *Klebsiella pneumoniae* (ATCC 13883) and *Pseudomonas aeruginosa* (ATCC 227853) and three yeasts *Candida albicans* (ATCC 10231), *C. tropicalis* (ATCC 13801) and *C. glabrata* (ATCC 28838) were assayed, using standard antibiotics.

Table 2: Antimicrobial activity of the Ch extracts and isolated naphthoquinones (MIC values in mg/mL)

Samples	*S. aureus*	*S. epidermidis*	*P. aeruginosa*	*K. pneumoniae*	*E. cloacae*	*E. coli*	*C. albicans*	*C. tropicalis*	*C. glabrata*
O. kaiheirei	3.00	2.76	3.00	3.78	3.13	3.87	-	-	-
A. sfikasiana	0.0029	0.0029	0.0050	0.0057	0.0047	0.0047	0.0060	0.0056	0.0057
O. graeca	0.0021	0.0020	0.0033	0.0037	0.0039	0.0040	0.0051	0.0048	0.0047
O. erecta	3.50	2.95	3.75	4.22	3.87	3.96	-	-	-
C. columnae	3.70	3.10	3.97	4.57	4.10	4.56	-	-	-
Deoxyalkannin (1)	0.0029	0.0027	0.0034	0.0035	0.0038	0.0040	0.0057	0.0055	0.0051
2''-(*S*)-α-Methylbutyrylalkannin (2)	0.0024	0.0022	0.0032	0.0034	0.0037	0.0039	0.0053	0.0051	0.0048
Isobutyrylalkannin (3)	0.0022	0.0020	0.0029	0.0032	0.0034	0.0036	0.0055	0.0051	0.0043
Propionylalkannin (4)	0.0022	0.0022	0.0028	0.0032	0.0035	0.0038	0.0057	0.0052	0.0050
Acetylalkannin (5)	0.0026	0.0024	0.0034	0.0035	0.0038	0.0039	0.0056	0.0055	0.0050
β-Hydroxyisovalerylalkannin (6)	0.0020	0.0019	0.0027	0.0032	0.0033	0.0036	0.0049	0.0048	0.0043
Mix of β,β-dimethylacrylalkannin (7) + (2) + (3)	0.0020	0.0019	0.0024	0.0030	0.0033	0.0035	0.0048	0.0048	0.0043
5-Fluocytocine	-	-	-	-	-	-	0.0001	0.001	0.01
Amphotericin	-	-	-	-	-	-	0.001	0.0005	0.0004
Amoxicillin	0.002	0.002	0.0024	0.0028	0.0022	0.002	-	-	-
Netilmicin	0.004	0.004	0.0088	0.008	0.008	0.01	-	-	-
Clavulanic acid	0.002	0.002	0.0024	0.0028	0.0022	0.002	-	-	-

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Effects of Gamma-Irradiation on the Antioxidant Potential of Traditional Bulgarian Teas

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Food irradiation technologies are used to reduce the risk of food borne diseases by eliminating pathogenic microorganisms, prolonging shelf life and reducing storage losses by delaying ripening, germination or sprouting. However, application of irradiation in food technology can negatively influence the biologically active compounds in foods. In this research, the effect of gamma-irradiation on the antioxidant activity of Bulgarian teas was investigated. The aim of the study was to evaluate the total phenolic and tannin content and antioxidant activity of ethanolic extracts of Bulgarian herbal teas before and after gamma-irradiation. Mursalski tea (*Sideritis scardica*), Mashterka tea (*Thymus serpyllum*), Good Night tea (tea mix), Staroplaninski tea (Balkan tea mix), Trakia tea (tea mix), and Mountain tea (Planinski tea mix) were selected for this study. Gamma-irradiation was applied at the absorbed dose of 5 kGy. Antioxidant activity of non-irradiated and irradiated teas was determined by measuring antiradical activity against $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ and the ability to reduce ferrous ions. The highest total phenolic content was found in Mursalski tea (268 mg/g), and the highest tannin content in Good Night tea (168 mg/g). FRAP, TEAC and DPPH assays revealed that the most active samples were Staroplaninski (2.78 mmol Fe (II)/g), Planinski (0.87 mmol Trolox/g) and Planinski (0.032 mg/mL), respectively. The radical scavenging activity of irradiated tea samples was maintained after gamma-irradiation. The most interesting extract from irradiated tea studied was Staroplaninski, which demonstrated a higher antioxidant potential in the irradiated sample compared with the non-irradiated sample.

Keywords: Gamma-irradiation, Teas, Ethanolic extracts, Antioxidant activity, Reduction power, Scavenging activity.

Herbal teas are traditional beverages consumed worldwide. They are a rich source of phenolic compounds associated with positive effects on human health, and exhibit antioxidant, anti-mutagenic, and immune response activities, as well as anti-allergic and anti-diabetic properties [1]. The antioxidant potential of herbals teas has been confirmed by several authors [2-5] using several chemical methods (ABTS, FRAP, DPPH assays).

Herbal teas can often be contaminated by pathogens such as aerobic spore-forming bacteria, non-fermenting bacteria (non-pathogenic) and *Aspergillus* species [1, 6]. One of the methods that can be utilized to protect herbal teas against pathogens is gamma-radiation. During this process, $OH^{\bullet}$ free radicals are created. These radicals interact with the DNA of microorganisms, leading to their death [7]. It is worth emphasizing that gamma-radiation can also inhibit mycotoxinogenic fungi and kill insects [8].

It is important to know what is the impact of gamma-irradiation on the antioxidant activity and total phenolic content of herbal teas, and this is the aim of the present study. Information about this influence is scarce in the literature.

Total phenolic content in the extracts and teas are presented in Table 1. Before irradiation, the extract of Mursalski tea had the highest total phenolic content (268 mg/g), and the extract of Mashterka tea the lowest (104 mg/g). Total phenolic content of traditional Bulgarian teas was several times lower than that reported for herbal teas in China [2]. Furthermore, similar values for total phenolic content were determined in plants used for herbal teas in Spain [3].

Irradiation had the most significant effect on the total phenolic content of Mashterka and Staroplaninski tea extracts, as well as of Mursalski, Staroplaninski and Trakia dry teas. These results agree with the findings of the majority of the literature. Gamma-radiation increased the total phenolic content in peanut skins [9], almond skins [10], rosemary [11], and seed coat colored soybean [12]. However, no significant effect has been observed so far on the total phenolic content of radiation-processed tea [13].

A high value for high-molecular tannin content was noted for Good Night tea (Table 1). Much lower values were determined for Planinski and Trakia teas. In other teas, tannins were not detected. The effect of gamma-radiation was not clear. Irradiation increased the tannin content in Planinski tea and decreased it in Good Night tea. De Camargo *et al*. [9] found that the tannin content in peanut skins after irradiation was higher than in the control samples. Also, in the study of Stajner *et al*. [14], gamma-irradiated soybeans exhibited a higher tannin content. The change in tannin content in irradiated plant material could be due to depolymerization [9]. According to some studies [9, 15], radiation can convert the B-type procyanidin dimer into the A-type.

The antioxidant activity of phenolic compounds present in the extracts was investigated using ABTS, FRAP, and DPPH assays. The results are depicted in Figures 1-3. The strongest antiradical activity against the ABTS radical cation and DPPH radical was

Table 1: Total phenolic and tannin content of extracts and teas.

Herbal tea	Total phenolic contents (mg/g extract)		Total phenolic contents (mg/g tea)		Tannin contents (mg/g extract)		Tannin contents (mg/g tea)	
	Control	Irradiated	Control	Irradiated	Control	Irradiated	Control	Irradiated
Mursalski	268±5	272±10	15.1±0.3	16.8±0.6*	nd	nd	nd	nd
Mashterka	104±7	121±5*	35.5±3	36.4±1.6	nd	nd	nd	nd
Good night	215±6	226±4	14.9±0.6	15.8±0.3	168±5	155±5*	11.6±0.4	10.8±0.2
Staroplaninski	209±6	241±5**	12.4±0.4	14.8±0.3***	nd	nd	nd	Nd
Trakia	176±3	174±4	15.3±0.2	17.0±0.4**	33.9±3.5	37.5±2.1	2.95±0.30	3.67±0.36*
Planinski	257±5	273±10	27.7±0.6	28.5±1.01	16.2±0.8	34.3±1.1***	1.75±0.08	3.59±0.12***

nd – non detected; values for control and irradiated samples differ significantly with $p<0.05$ (*), $p<0.01$(**) or $p<0.001$ (***).irradiated samples differ significantly with $p<0.05$ (*), $p<0.01$(**) or $p<0.001$ (***).

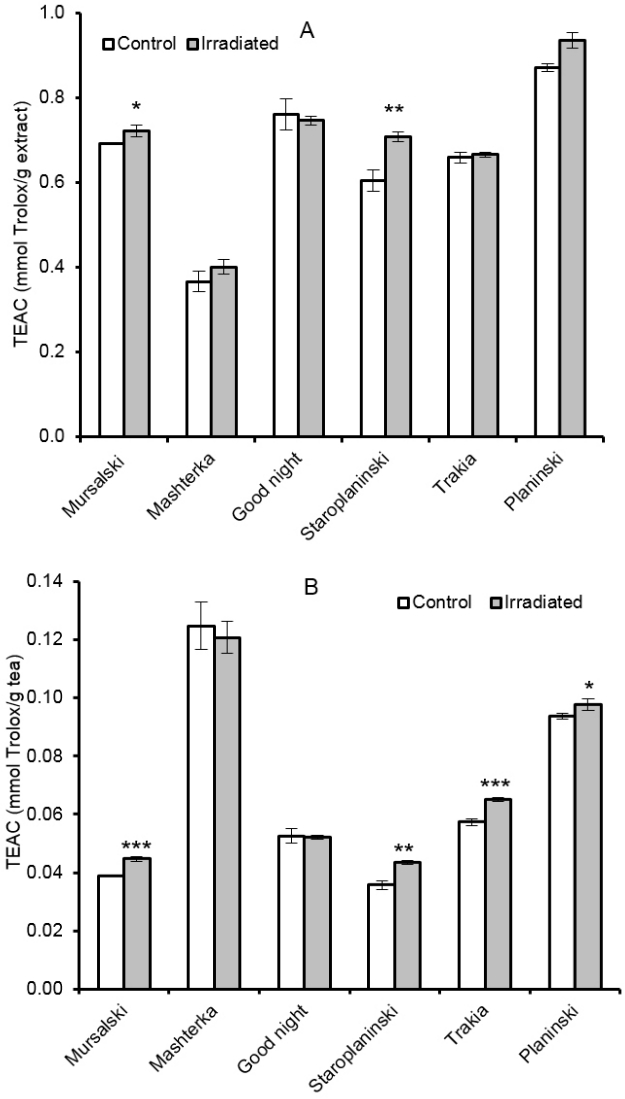

Figure 1: Antiradical activity against $ABTS^{\bullet+}$ in non-irradiated (control) and irradiated ethanolic extracts of tea extracts (A) and teas (B). Values for control and and irradiated sample differ significantly with $p<0.05$ (*), $p<0.01$(**) or $p<0.001$ (***).

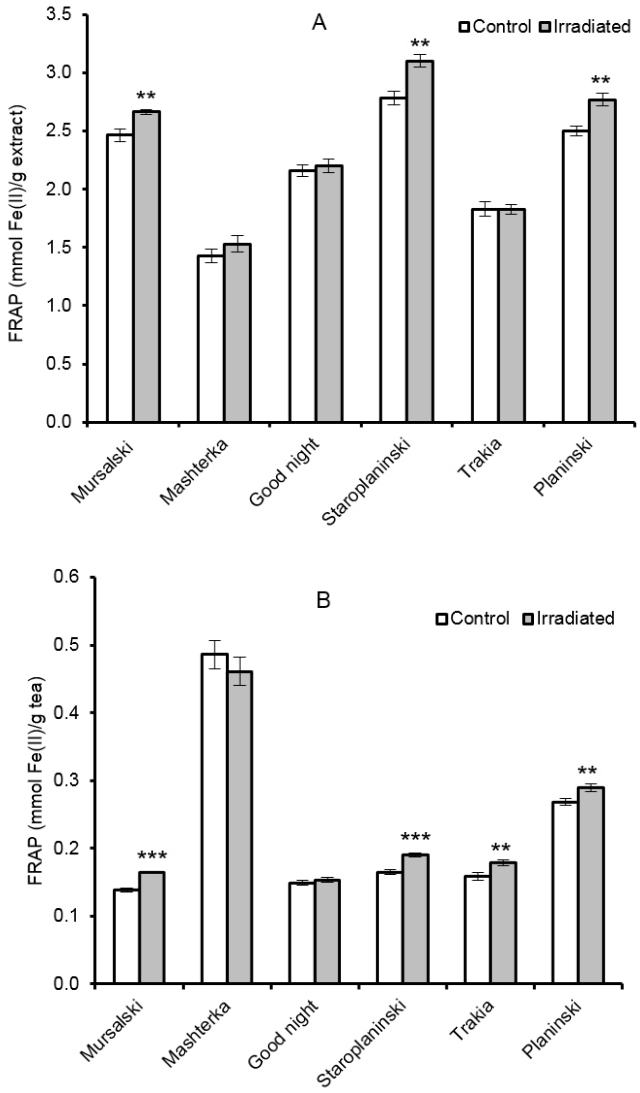

Figure 2: Ferric reducing antioxidant power (FRAP) non-irradiated (control) and irradiated ethanolic extracts of tea extracts (A) and teas (B). Values for control and irradiated sample differ significantly with $p<0.05$ (*), $p<0.01$(**) or $p<0.001$ (***).

observed in the extracts of Planinski tea. However, the highest values of TEAC were noted for Mashterka. The extract obtained from the Staroplaninski tea had the strongest reducing properties. The Mashterka tea had the highest FRAP value. Gamma-irradiation increased TEAC values of the extracts from Staroplaninski and Mursalski tea, and TEAC values increased in Mursalski, Staroplaninski, Trakia, and Planinski tea when compared with the other teas (Figure 1). Gamma-irradiation lead to increased FRAP values in extracts obtained from Mursalski, Staroplaninski and Planinski tea. The increase was observed for the above-mentioned teas and additionally for Trakia tea when the FRAP values were expressed in relation to the other teas (Figure 2). Gamma-irradiation increased antiradical activity against the DPPH radical in the case of the Mashterka and Staroplaninski teas (Figure 3). This positive effect of gamma-radiation on the antioxidant activity was previously reported in peanut skins [9], almond skins [10], rosemary [11], seed coat colored soybean [12], and peach [16].

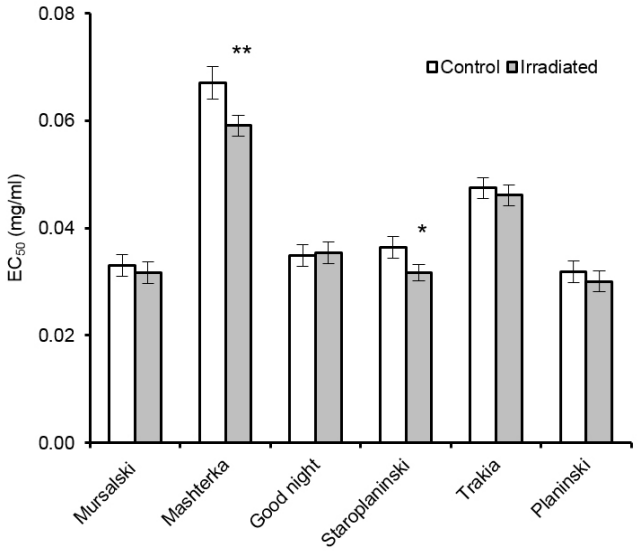

Figure 3: Antiradical activity against DPPH• in non-irradiated (control) and irradiated ethanolic extracts of teas. Values for control and irradiated samples differ significantly with $p<0.05$ (*), $p<0.01$(**).

Experiment

Reagents: Ethanol, methanol, disodium carbonate, potassium persulfate, hydrochloric acid, sodium acetate, 2,4,6-Tris(2-pyridyl)-S-triazine (TPTZ), and ferric (III) chloride hexahydrate were bought from POCH S.A., Poland, and [2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] diammonium salt (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), (+)-catechin, Folin-Ciocalteu reagent, 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox), and vanillin from Sigma-Aldrich, USA.

Samples: Herbal teas were purchased at local stores in Sofia, Bulgaria. Mursalski (*Sideritis scardica*; Mashterka *(Thymus serpyllum*); Good Night (mix: peppermint leaves, lemon balm leaves, hawthorn flowers and leaves, linden flowers and cone hops); Staroplaninski tea (Balkan mix: wild thyme, peppermint, marjoram, blackberry leaves and elderflowers); Trakia tea (mix: basil, blueberry, marjoram rosehips and coriander); and Planinski (Mountain mix: *Hypericum perforatum*, wild thyme, strawberry and blackberry leaves) teas were selected for the study.

Gamma-irradiation of the teas: Teas were irradiated with a 60-Co source with 8200 Ci activity. The gamma-ray facility has a mobile irradiation chamber with a 4.0 L volume and dimensions: 13.5 cm diameter and 22 cm height. The chamber rotates on its vertical axis during irradiation. For the study of absorbed dose distribution, Alanine dosimeters (Kodak BioMax) were used, measured by an ESR spectrometer E-scan Bruker and calibrated in units of absorbed dose in water. At each point, 3 dosimeters were placed. The maximum absorbed dose rate was 3.5 ± 0.02 kGy/h, the minimum average dose rate was 2.49 ± 0.02 kGy/h and the average dose rate was 2.98 kGy/h. All samples were irradiated in their commercial packaging with the minimum absorbed dose 5 ± 0.02 kGy and dose uniformity ratio $r = D_{max} / D_{min} = 1.25$. The absorbed dose of 5 kGy was chosen on the basis of preliminary microbiological tests made under the mentioned CRP, as it was found to be sufficient to reach the required level of "cleanness" in the teas.

Extraction: Non-irradiated and radiated teas were mixed with 95% ethanol in 1:20 solid: solvent ratio and left at room temperature for 24 h. The mixture was filtered, and the entire procedure was repeated for each sample. Extracts were combined, organic solvent was evaporated and the water residue was lyophilized.

Total phenolics content: The content of total phenolics in the examined teas was investigated using Folin and Ciocalteu's reagent [17] with (+)-catechin as a standard.

Tannin content: Tannin content was estimated using the modified vanillin assay [18]. The results were expressed as mg of (+)-catechin equivalents per 1 g of extract/tea.

TEAC: Antiradical activity against $ABTS^{\bullet+}$ was determined as Trolox equivalent antioxidant capacity and investigated according to Re *et al.* [19]. The results were expressed as mmol of Trolox equivalents per 1 g of extract/tea.

DPPH radical scavenging: Antioxidant capacity against DPPH radical was tested using the method described by Yen and Chen [20]. The results are expressed as EC_{50}, which was defined as the amount of antioxidant required to scavenge 50% of the radicals present in the reaction mixture.

FRAP: Ferric reducing antioxidant power for tea samples was conducted according to the method described by Benzie *et al*. [21]. The results were expressed as mmol of Fe (II) per g of extract/tea.

Statistical analysis: All analyses were triplicated. Mean values in the control and experimental groups were compared by Student's t-test using GraphPad Pris - Sowftware.

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Microelements and Heavy Metals Content in Frequently Utilized Medicinal Plants Collected from the Power Plant Area

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The effectiveness of medicinal plants is mainly associated with their active constituents, but one of the major quality problems frequently encountered is their high trace metals content that can be associated to extensive pollution of the environment where medicinal plants grow. Therefore the aim of this research was to evaluate the content of Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Zn and As in selected and frequently used medicinal plants, including chicory, broadleaf, common comfrey and dandelion. The plant material was collected from their wild habitats in the area of highly developed power plant activity during the summer of 2015. Plant analyses were done according to ICP methodology, using ICAP 6300 ICP optical emission spectrometer. The obtained results showed that the content of As, Cd, Co, Mn, Ni and Zn in the investigated medicinal plant species was below the maximum permissible concentration, while in all parts of all studied plants the concentration of Cr was toxic. The toxic concentrations of Cu were determined in root and aerial parts of chicory and common comfrey, and the toxic concentrations of Fe in root and aerial parts of dandelion and broadleaf plantain, and in aerial parts of common comfrey. However, high but not toxic content of Pb was found in aerial parts of chicory. It can be concluded that medicinal plants from the studied growing site are not appropriate for use in alternative medicine and that a determination of trace metals content in these plants must become a standard criterion for evaluation of their quality.

Keywords: Heavy metals, Chicory, Broadleaf, Common comfrey, Dandelion, Power plant.

For the majority of the world population medicinal plants represent the primary source of the health care. An effectiveness of medicinal plants is mainly associated with their constituents such as essential oils and secondary metabolites. As it was reported by the World Health Organization (WHO), about 80% of people in peripheral communities use only medicinal herbs for the treatment of many diseases [1]. When the herbs are used in the treatment of certain illnesses, it should be known that, besides the pharmacological effect they have, the medicinal plants could be toxic if the content of heavy metals in them is elevated. This can be associated to extensive pollution of the environment where medicinal plants grow since the plants can be easily contaminated by heavy metals in the course of cultivation or later during the processing stage [2]. Along with other pollutants, heavy metals can be added into the environment through industrial activities, municipal wastes, automobile exhaust, pesticides and fertilizers used in agriculture [3]. Several heavy metals such as Fe, Mn, Zn, Cu, Ni and Mo, in low concentrations, are considered to be essential micronutrients for plants. However, a high concentration of heavy metals causes several problems, including toxicity of plants, animals and humans [4]. As heavy metals pose a hazard to human and animal health, their content in plants used for consumption or medicinal purposes must be limited [5]. Therefore, controlling the heavy metal concentrations in both medicinal plants and their products should be made to ensure safety and effectiveness of herbal products.

Regarding the preceding comments, the main purpose of this research was to evaluate the content of cadmium (Cd), cobalt (Co), chromium (Cr), copper (Cu), iron (Fe), manganese (Mn), nickel (Ni), lead (Pb), zinc (Zn) and arsenic (As) in selected and frequently used medicinal plants, including chicory (*Cichorium intybus* L.), broadleaf (*Plantago major* L.), common comfrey (*Symphytum officinale* L.) and dandelion (*Taraxacum officinale* F. H. Wigg.).

The behavior of heavy metals and microelements in the soil is conditioned by many factors that may affect their mobility and accumulation by plants, and the most important are soil reaction, organic matter content and the percentage of colloidal clay [6].

Table 1 displays the data on total concentrations of the microelements and heavy metals in the study soil. The overall concentrations of Cd, Co, Cr, Cu, Fe, Mn, Pb, Zn and As in the soil samples correspond to the usual levels in agricultural soils and were within the permissible limits, except for the content of Ni which exceeds the maximum permissible concentrations (MPC) [7-10]. This may be the result of anthropogenic activity, where Ni enters the soil mostly due to atmospheric deposition by the coal, oil and diesel burning. In addition, the concentrations of Cr and Cu are high but do not exceed the MPC. Cr is mainly found in the soil in basic and ultrabasic rocks, which is the main source of geochemical origin of this metal in the soil. As for Ni, in industrial regions Cu mainly enters the soil by deposition from the atmosphere, so its high concentration in the soil is usually of anthropogenic origin [9]. Nevertheless, the impact of flood waters containing heavy metals may also be the reason for increased content of some trace metals in the soil from the study area which is characterized by highly developed power plant activities and affected by the great floods in May, 2014.

An analytical determination of heavy metals in medicinal plants is a significant part of quality control in order to establish the plants purity, safety and efficacy since human activities, such as industry and agriculture, promote trace metals release into the environment [11]. Figures 1-4 show the concentrations of microelements and heavy metals in the root and aerial parts of the studied medicinal plants compared to the the reference values for plants normal and toxic concentrations (Table 2).

Table 1: Total content of trace elements (mg kg^{-1}) in soil under studied plants.

Trace elements	Plant species				MPC (mg kg^{-1})
	C. intybus	*P. major*	*S. officinale*	*T. officinale*	
Cd	0.56±0.02*	0.57±0.03	0.55±0.02	0.48±0.02	3[a]
	0.54-0.58**	0.55-0.60	0.53-0.57	0.46-0.50	
Co	19.11±0.34	15.75±0.22	16.96±0.17	19.37±0.24	30[b]
	18.78-19.45	15.54-15.98	16.78-17.12	19.21-19.65	
Cr	78.71±0.23	82.11±0.34	84.61±0.42	81.21±0.66	100[a]
	78.56-78.97	81.78-82.45	84.15-84.98	80.56-81.87	
Cu	92.05±0.56	91.35±0.20	89.98±0.45	93.81±0.34	100[a]
	91.45-92.15	91.12-91.49	89.56-90.45	93.45-94.12	
Fe	29920±684	31345±579	30160±711	34491±612	50000[d]
	29150-30457	30978-32012	29457-30879	33897-35120	
Mn	724.67±5.03	428.67±9.29	481.00±7.55	749.33±16.92	1500-3000[c]
	720-730	421-439	474-489	735-768	
Ni	76.37±0.55	69.25±0.47	71.86±0.22	81.86±0.40	50[a]
	75.78-76.87	68.87-69.78	71.69-72.11	81.45-82.24	
Pb	21.81±0.42	20.68±0.32	19.93±0.29	14.16±0.35	100[a]
	21.41-22.24	20.34-20.98	19.65-20.23	13.78-14.48	
Zn	94.38±0.38	111.55±0.38	111.75±0.45	91.37±0.41	300[a]
	94.02-94.78	111.23-111.97	111.23-112.05	90.97-91.78	
As	8.60±0.37	3.46±0.37	3.80±0.21	9.86±0.38	25[a]
	8.23-8.97	3.04-3.94	3.59-4.01	9.48-10.24	

*means ± standard deviation; ** intervals; MPC - maximum permissible concentrations: [a][7], [b][8], [c][9], [d][10].

Table 2: Reference values for trace elements normal and toxic concentrations in plants.

Element	*Normal concentrations*	*Toxic concentrations*
	(mg kg^{-1})	
Cu	3-15[a]	20[b]
Ni	0.1-5[a]	30[b]
Pb	1-5[a]	20[b]
Cr	<0.1-1[a]	2[b]
Cd	<0.1-1[a]	10[b]
Mn	15-100[c]	400[b]
Zn	15-150[a]	200[b]
Co	0.05-0.5[c]	30-40[d]
Fe	50-250[f]	(>500)[f]
As	10-60[c*]	<2[c]

*μg kg^{-1}; reference values: [a][12], [b][13], [c][14], [d][9], [e][15], [f][16].

One of the most important role of Mn in plants is its involvement in the breakdown of water molecules with the releasing of oxygen, although, for its full metabolic activity, Mn is only required at low concentration [17].

Zn and Cu are considered to be an essential elements for humans, animals and plants growth and play a significant role in various metabolic processes [18]. The normal Zn concentration in plants ranges from 15 to 150 mg kg^{-1} [12], while the plant toxic concentrations of this element is 200 mg kg^{-1} [13]. The concentrations of Cu in many plant species varies between 20 and 30 mg kg^{-1} on dry weight basis. If its concentration in dry plant material is higher than 20-100 mg kg^{-1}, it becomes phytotoxic [19].

The appropriate concentration of Fe in all plant species is essential both for the health of plants and for the nutrient supply to humans and animals. The normal Fe concentration in plants used for animal nutrition ranges from 50 to about 250 mg kg^{-1}, while the nutritional requirements of grazing animals for this element are generally present at concentrations within the range of 50-100 mg kg^{-1} [9, 16]. Pb and Cd are trace metals which are not essential for either humans, animals or plants, and in humans could easily induce toxic effects at low concentrations. Pb as a pollutant can be detected in all environmental and biological systems. Sources of Pb contamination are the products of combustion in the metallurgical and chemical industry, transportation, industrial waste waters and landfills [20]. Similarly to Pb, Cd is a widespread metal in nature characterized as toxic industrial pollutant with expressed mobility [21]. The maximum permissible concentration of Cd in edible plants is as low as 1 μg kg^{-1} [22]. Hence, low levels of Cd in the studied plants is a highly desirable outcome.

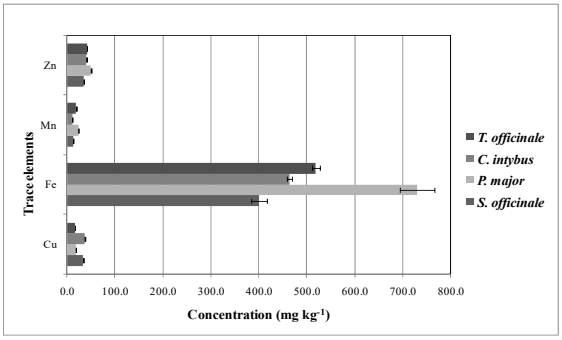

Figure 1: Concentrations of zinc (Zn), manganese (Mn), iron (Fe) and copper (Cu) in the plants root (mg kg^{-1}).

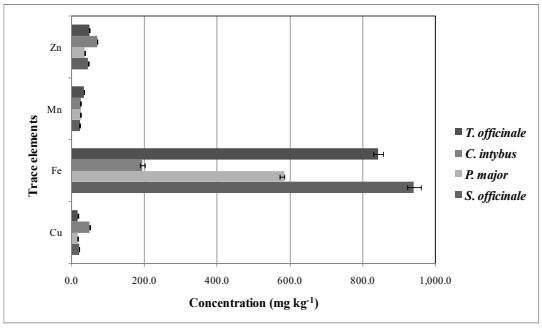

Figure 2: Concentrations of zinc (Zn), manganese (Mn), iron (Fe) and copper (Cu) in the plants aerial parts (mg kg^{-1}).

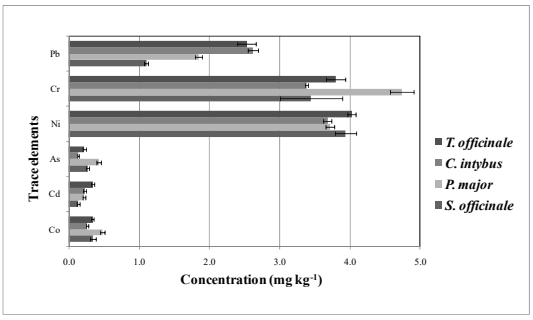

Figure 3: Concentrations of cobalt (Co), cadmium (Cd), arsenic (As), nickel (Ni), chromium (Cr) and lead (Pb) in the plants root (mg kg^{-1}).

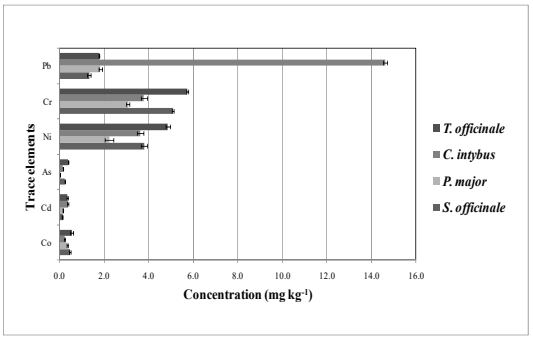

Figure 4: Concentrations of cobalt (Co), cadmium (Cd), arsenic (As), nickel (Ni), chromium (Cr) and lead (Pb) in the plants aerial parts (mg kg^{-1}).

The compounds of As are highly toxic and after Pb they represent the highest toxicological risk to humans and domestic animals [23]. As for most plant species, it is their common constituent and could be passively taken up by them with the water flow. Concentrations of As in edible plants vary highly, most commonly in the range from 10 to 60 μg kg^{-1}, while the tolerance for this element in plants is established as 2 mg kg^{-1} [14, 24]. As for Cd and Pb, the

undetectably low levels of As in tested medicinal plants in this study is highly acceptable because of its high toxicity to both humans and animals.

Ni is an essential element required for growth and absorption of Fe, but its presence in high concentrations can disturb the life processes causing chlorosis, intercostal necrosis and reduced root growth. The average Ni content in plants is 0.1-5.0 mg kg^{-1} of dry matter [13].

Cr is an essential element for humans as a nutritional enhancement to glucose metabolism, and for animals due to its essential role in normal metabolism of carbohydrates and lipids. As for the plant growth, Cr was never considered as an essential element, but some of its stimulative effects were reported. Toxic effects in plants is manifested in the form of chlorosis [23, 25]. The phytotoxic concentrations of Cr in tops of plants were as follows: 18 to 24 (mg kg^{-1}) in tobacco, 4 to 8 (mg kg^{-1}) in corn and 10 (mg kg^{-1}) in barley seedlings [9].

Cobalt is essential to humans and animals as the vital trace mineral and the main constituent of cobalamin, also known as vitamin B12, but required in small amounts for daily body growth and maintenance. For plants Co is not classified as an essential element, however, it is usually described as "beneficial". This trace element can be a contaminant in soils due to agricultural additives or metal refineries [26]. Regarding its toxicity in plants, commonly reported critical Co levels range from 30 to 40 mg kg^{-1} [9].

Results concerning the concentrations of Zn, Mn, Fe and Cu in the plants root and aerial parts (Figures 1 and 2) showed the following: Mn and Zn concentrations were below the maximum permissible [12-13, 15] in all the analyzed parts of the tested plant species; the toxic concentrations of Cu [13] were determined in root and aerial parts of chicory and common comfrey; the toxic concentrations of Fe [16] were registered in root and aerial parts of dandelion and broadleaf plantain, and in aerial parts of common comfrey.

Results on the content of Pb, Cr, Ni, Cd, Co and As in the plants root and aerial parts (Figures 3 and 4) showed the following: concentrations of As, Cd, Co and Ni were below the maximum permissible [9, 13-14] in all the analyzed parts of the tested plant species; the toxic concentrations of Cr [13] were determined in all parts of all studied plants; high but not toxic content of Pb [12-13] was found in aerial parts of chicory.

Accessibility, adoption and phytotoxicity of heavy metals are not only conditioned by their total content in the soil, but also by chemical form, affinity of plant, and individual or interactive effect of different soil properties. A dominant influence on increased and toxic content of trace metals determined in the studied plant species has a pH value of soil. Generally, in soils with low pH, the mobility of metal cations is increased, whereas in soils with high pH the mobility is decreased [9].

Floodwater could also be an important source of determined high and toxic levels of heavy metals in plants. The plants that were in or near flooded areas, should not be used in alternative medicine. Further control of trace metals content in the plants from the studied area is necessary in order to prevent their entry into the food chain and to ensure the healthy food production.

Experimental

Study area: The investigation was conducted at wild habitats in the city of Obrenovac, located about 30 km southwest of Belgrade in Serbia, during the summer of 2015. The study area is characterized by highly developed industrial activities since it is located in the middle of the power plants A and B (TENT A and TENT B), at the distance of about 5 km from each of them. Obrenovac is also known as a city mostly affected by the floods during May, 2014. The studied soil type, under all plant species sampled, was Calcic Gleysol [27]. This soil is a hydromorphic black soil, developed under the influence of groundwater and classified in A-G class. It is characterized with both humic and gleyic horizons which give this soil its name [28]. It is clayey soil with the following granulometric composition: the content of sand fractions (>0.02 mm) - 38.0%, the content of silt fractions (0.02-0.002 mm) - 25.4%, the content of clay fraction (<0.002 mm) - 36.6% [29]. According to the reference values [30], the soil analyzed in this study is characterized as slightly acid in reaction, having high levels of available potassium, low levels of available phosphorus, containing medium levels of SOM and medium to well provided with total nitrogen.

Sampling, preparation and analysis of the soil and plant material: Four soil samples, in triplicates, were taken from the rhizosphere of the tested species, from the depth of 0-30 cm. The soil samples were air-dried, crushed and passed through a sieve (≤ 2 mm). In order to provide the representative subsampling for analysis, soil fractions smaller than 2 mm were crushed into dust by hand. The total contents of Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Zn and As in soil samples were determined by inductively coupled plasma-atomic emission spectrometry - THERMO iCAP 6300 Duo (radial/axial view versions) ICP-OES, after the digestion of the samples with aqua regia [31-32]. For checking the accuracy of analytical results, the Certified European Reference Material ERM® - CC141 for loam soil was used. The following plant species, both aerial parts and root, were sampled: *C. intybus*, *P. major*, *S. officinale* and *T. officinale*. The sampled plant material were dried at 105°C for a period of 2 hours, using gravimetric method for determination of dry matter contents of plant tissues [33]. Plant material was then ground to 0.5 to 1.0 mm particle size to ensure homogeneity and to facilitate organic matter digestion. The contents of Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Zn and As in aerial parts and root of the selected medicinal plants were determined in triplicates with THERMO iCAP 6300 Duo (radial/axial view versions) ICP-OES after the digestion of the samples with concentrated HNO_3 and redox reaction with H_2O_2 for total forms extraction [34].

Calibration standards for both soil and plant material were in the range of 0-10 ppm, except for iron (0-25 ppm). The ICP detection limits (LOD) for soil and plant material are given in Table 3.

Table 3: The ICP detection limits for soil and plant material.

Trace elements	LOD for soil (mg kg^{-1})	LOD for plant material (mg kg^{-1})
Cd	0.0111	0.013
Co	0.0201	0.041
Cr	0.0931	0.053
Cu	0.1483	0.088
Fe	3.4387	0.430
Mn	0.1902	0.025
Ni	0.1493	0.029
Pb	0.0731	0.062
Zn	0.1987	0.060
As	0.0499	0.055

LOD - low limits of detection

Data analysis: The obtained data on microelements and heavy metals concentration in the soil studied represent the arithmetic means of three replicates of each sampling, their ranges and standard deviations values. The data on microelements and heavy metal concentrations in the studied plant species are presented by figures as the bar charts with standard deviation values.

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Molecular Characterization of *Verbascum anisophyllum* (Scrophulariaceae) Genetic Resources Through Inter-Simple Sequence Repeat (ISSR) Markers

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Verbascum (Mullein) flowers are highly valued as natural remedy for various respiratory diseases. *Verbascum anisophyllum* Murb. is a Balkan endemic, protected by law and included in the Bulgarian Red Data Book as "Critically Endangered". Thus, a strict conservation policy and a reliable evaluation of its genetic resources are required, considering its narrow distribution range and the increasing risk from destruction of its habitats. Here, we used Inter-simple sequence repeat (ISSR) markers to characterize the genetic diversity and to assess the genetic differentiation between the existing populations of *Verbascum anisophyllum* in Bulgaria. The level of genetic diversity found herein clearly indicates a long-term potential for adaptability of this endangered plant. Our findings provide important knowledge of population genetic structure of this species, thus representing a strategy for its efficient conservation and utilization.

Keywords: *Verbascum anisophyllum*, Genetic diversity, ISSR markers, Conservation.

The Earth is losing at least one undiscovered new drug every two years. Overharvesting and destruction of habitats have placed many medicinal and aromatic plants (MAPs) at risk of extinction. For this reason, their conservation has become increasingly urgent [1]. Understanding of the genetic variation within and among MAPs' populations is essential for establishment of conservation strategies and sustainable utilization of their available genetic resources. Therefore, the molecular and chemical authentication of endangered MAPs should go hand in hand, rather than in isolation in order to identify the 'elite' population for further bioprospecting.

The genus *Verbascum* L. (common name mullein) comprises about 360 species of flowering plants in the Scrophulariaceae family [2]. Mulleins have been used in traditional folk medicine for treatment of a wide range of human ailments. *Verbascum* leaves, flowers and whole aerial parts have been widely used for wound healing and treatment of respiratory and inflammatory disorders. So far, several groups of bioactive metabolites from *V. lychnitis*, *V. nigrum*, *V. phlomoides*, *V. thapsiforme*, *V. thapsus,* etc. have been reported to posses various biological activities. Other systematically examined species as *V. salviifolium*, *V. lasianthum*, *V. mucronatum* and *V. wiedemannianum* are rather well studied. On the other hand, the knowledge on some species as *V. songaricum*, *V. sublobatum* and *V. tzar-borisii* is relatively limited [3a,3b].

Around 770 species or 19% of all plant species in Bulgaria are of pharmaceutical value. A large number of rare species are included in the Red Data Book of the Republic of Bulgaria (http://susherbsbg.eu/en/medicinalplants/).*Verbascum anisophyllum* Murb. is one of the rarest plant species in the Bulgarian flora. Comprehensive chemical fingerprinting is needed in order to investigate its pharmaceutical potential. Along with the studies for chemical characterization of the species, a reliable evaluation of its genetic resources is required, considering its restricted distribution and the small size of its habitats.

DNA markers play an important role to portray the genetic diversity profile of rare MAPs, due to the number of advantages: they are not influenced by environmental factors; the tests can be performed during any stage of plant development; a small amount of plant sample is sufficient for analysis [4,5]. Inter-simple sequence repeats (ISSRs) require a comparatively low amount of DNA [6a], the utilization of long primers allows more stringent annealing temperatures and reveals more polymorphic fragments [6b,6c]. In addition, the development of ISSR markers does not need prior knowledge of the genome to be analyzed. The aim of the present study was to determine the level of ISSR variation in two existing populations of *V. anisophyllum* in Bulgaria. To the best of our knowledge there are no reports on genetic diversity of this endangered plant. Such data will provide important implications for the effectiveness of any programs devised to conserve and utilize the available genetic resources of this species.

It is generally assumed that plant species with small population sizes have lower genetic diversities than larger populations and vice versa [7a,7b]. Because of the small size and limited area of *V. anisophyllum* populations, we hypothesized that its level of genetic diversity will be low.

Among 35 ISSR primers used herein, 10 were highly polymorphic and amplified well-distributed fragments with good distinction. The polymorphic loci at the population level ranged from 56.9% to 60.2%, with an average of 58.5%. The Shannon's information index (SI) ranged from 0.211 to 0.354, with an average of 0.283 (Table 1).

Table 1: Genetic diversity indices based on the ISSR data for *V. anisophyllum*.

Population name	N	P (%)	SI	Private bands
Tsarvenyano village	20	60.21	0.354 (0.027)	12
Vukovo village	20	56.87	0.211 (0.029)	4
Mean (SE)		58.54	0.283 (0.026)	

*Standard error is shown in parentheses. N: number of individuals from each population, P: percentage of polymorphic loci, SI: Shannon's information index.

Based on the ISSR profiling, we estimated lower genetic diversity indices in the smaller population of the species in Vukovo village. Both populations of *V. anisophyllum* had a total of 16 private alleles, twelve in Tsarvenyano and four in population Vukovo. The high number of private alleles indicates restricted exchange of genes between populations. The genetic differentiation between the populations, F_{ST} is 0.585 ($P < 0.001$). Based on the estimated F_{ST} value, a low level of gene flow ($N_m = 0.250$; $N_m < 1$) was found. The AMOVA analysis points to higher levels of variation among populations (58%) than within populations (Table 2).

Table 2: Analysis of molecular variance based on ISSR markers for the two populations of *V. anisophyllum*.

Source of variation	df	Sum of squares	Mean sum of squares	Variance components	Variation (%))	F_{ST}	P
Among populations	1	45.071	45.071	3.064	58		<0.001
Within populations	26	56.571	2.176	2.176	42		<0.001
Total	27	101.643		5.240	100	0.585	<0.001

The STRUCTURE analysis revealed a strong separation between the individuals from population Vukovo (1–20) and individuals from population Tsarvenyano (21–40) (Figure 1).

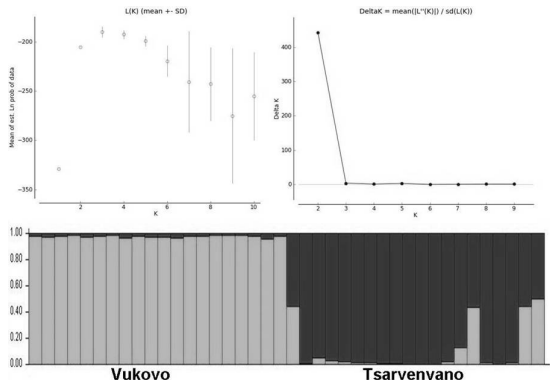

Figure 1: Results of the Bayesian model-based clustering STRUCTURE analysis of 40 individuals of *V. anisophyllum*.

In the two-dimensional PCoA, the individuals clustered strongly according to their population assignation. The first and second principal coordinates responded for 47.5% and 11.0% of total genetic variation, respectively (Figure 2).

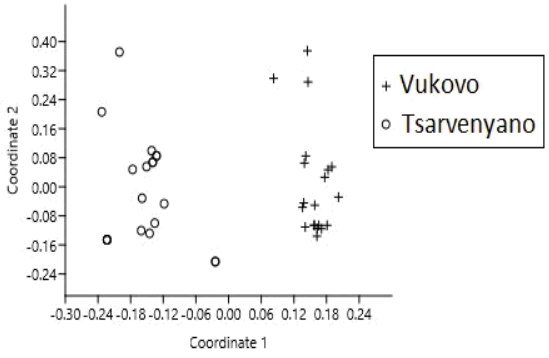

Figure 2: A two-dimensional plot of the PCoA of 40 *V. anisophyllum* individuals.

One possible explanation of our findings is that the outcrossing and long-lived seed plants maintain most of the genetic variations within populations, while predominantly selfing, short-lived species harbor comparatively higher variation among populations [8].

V. anisophyllum is an insect pollination outcrossing biennial plant. The age of its populations is unbalanced, since only a small number of plants reach maturity in the second year [9a-9d]. Hence, the observed differences in genetic variations could be contributed to the difference in the number of individuals in both populations, as well as to the low reproductive potential of the species, due to variation of seed production and flowering among the populations.

The ecogeographical differences of investigated populations should also be considered, since they can affect the levels of genetic diversity. Geographic isolation, e.g., by mountains and rivers, was noted among different populations of various plant species, and explained why the genetic diversity differed among the populations [10a-10d, etc.]. In this sense, Bulgarian populations of *V. anisophyllum* are not geographically distant from each other. Hence, the differentiation among them could be mainly due to isolation originated by its life-history and, most recently, by habitat fragmentation.

Conservation implications: Knowledge of genetic diversity pattern of *V. anisophyllum* is critical for the conservation and utilization of the available genetic resources of this endangered plant species. Based on the current molecular analysis of its natural populations, it can be assumed that the conservation strategies should be oriented towards *in situ* protection from overexploitation. It is well known that plant metabolites have appeared over the course of evolution as plants adapted to their environments. Therefore, high-throughput efforts are required for further phytochemical analyses with individuals belonging to the Tsarvenyano habitat. This higher-density population deserves special management, since it exhibited more genetic diversity and higher allelic richness, which increase its ability to adapt to changing environmental conditions.

Experimental

Study species: *Verbascum* is a genus of flowering plants belonging to the family Scrophulariaceae. The genus has palaeartic origin and consists of more than 360 species, native to Southeastern Europe and Southwestern Asia, with the highest species diversity in the Mediterranean [11].

Plant material: In Bulgaria, the species occurs in Mt. Konyavska and numbers some 300 individuals on an area of about 20 ha near Tsarvenyano village and about 100 individuals on an area of 5 ha near Vukovo village (Figure 3).

Figure 3: Geographic locations of the two Bulgarian *V. anisophyllum* populations sampled.

A small set of *V. anisophyllum* leaf samples were collected during the flowering stage (Table 3). The distance between the collected samples within the respective population was at least 5 m.

Table 3: Location and sampling information for two localities of *V. anisophyllum* in Bulgaria.

Population name	Geographical position	Number of samples collected
Tsarvenyano village	Latitude: 42.33966° Longitude: 22.80171°	20
Vukovo village	Latitude: 42.20414° Longitude: 22.97331°	20

DNA extraction: Genomic DNA was extracted from 50 mg of dried leaf tissue following a modified CTAB protocol [12] with no further purification.

***ISSR analysis*:** Ten ISSR primers (Microsynth, Switzerland) were selected after screening 35 primers on a small subset of samples (Table 4). The PCR and amplification product analysis followed [13].

Table 4: Primer sequences, total number and number of polymorphic bands, and annealing temperature (T_a) for ISSR primers used in this study.

Primer sequence (5'→ 3')	Total number of bands	Number of polymorphic bands	Polymorphism (%)	T_a (°C)
$(GA)_8T$	15	15	100	60
$(GA)_8A$	13	13	100	60
$(CA)_8G$	15	15	100	60
$(AC)_8T$	17	17	100	60
$(AC)_8C$	14	14	100	60
$(AC)_8G$	15	15	100	60
$(AG)_8YT$	17	17	100	55
$(AG)_8YC$	16	16	100	55
$(GA)_8YG$	15	15	100	55
$(AC)_8YT$	19	19	100	55
	156	156	100	

Software data analysis: The ISSR were treated as dominant markers and each locus was considered as a bi-allelic locus with one amplifiable and one null allele. The well resolved and consistently reproducible amplified DNA fragments as bands were scored with regards to their presence (1) or absence (0). The assignment of ISSR bands to genetic loci was performed semi-automatically using the GelAnalyzer 2010a image analysis software (http://www.gelanalyzer.com). The binary data were used for determining the genetic diversity among the populations. Genetic diversity was measured based on the percentage of polymorphic loci (P) and Shannon's information index (SI) using GenAlEx v.6.5 [14a,14b] and ARLEQUIN v.3.5.1 [14c]. GenAlEx was used for AMOVA [14d], to calculate the partitioning of genetic variation between and within the populations. Spatial genetic relationships among the samples were visualized by Principal Coordinate Analysis (PCoA) using Nei's genetic distance [14e]. To investigate for patterns among the populations, we used STRUCTURE v.2.2 [14f]. A total of 10 independent runs were performed for each set with K ranging from 1 to 10, a burn-in algorithm was applied (1×10^5 interactions and 1×10^5 subsequent Markov Chain Monte Carlo steps) [14g]. The mean likelihood was plotted for each cluster L(K) against the cluster number (K). To establish the optimal number of clusters, the relationship between K and ΔK, the second order rate of change of the likelihoods, was plotted [14h]. The best-fit number of groupings was evaluated using ΔK by STRUCTURE HARVESTER v.0.6.8 [14i].

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Effect of Drought and Salinity on Volatile Organic Compounds and Other Secondary Metabolites of *Citrus aurantium* Leaves

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Research was carried out in order to evaluate the effect of drought and salinity on *Citrus aurantium* L. plant physiological characteristics, total phenolic, flavonoid and ascorbic acid contents, and volatile organic compounds. *C. aurantium* plants were exposed to different levels of drought and salinity for an experimental period of 60 days. Moderate water deficit (MWD) and 100 mM NaCl increased significantly leaf total phenolic, flavonoid and ascorbic acid contents. Both drought and salinity promoted the accumulation of essential oil in leaves, while MWD and 100 mM NaCl resulted in the highest concentrations of essential oil. The main compounds of the essential oil were linalool, linalyl acetate, neryl acetate, geranyl acetate and α-terpineol. MWD and severe water deficit (SWD) reduced the concentration of hydrocarbon monoterpenes and promoted the accumulation of oxygenated compounds, while treatment with 50 and 100 Mm NaCl, promoted the accumulation of hydrocarbon monoterpenes and reduced oxygenated monoterpene concentrations in *C. aurantium*.

Keywords: *Citrus aurantium*, Drought, Essential oils, Salinity, Secondary metabolites.

Citrus aurantium L. (Rutaceae family), commonly known as sour or bitter orange, is often used as a rootstock for other *Citrus* species, due to its tolerance to cold, resistance to several viral diseases and the improvement of the fruit quality of the grafted plants. However, the fruits, leaves and flowers are also used by the food and cosmetic/pharmaceutical industries. Immature fruits are used for fruit jellies, spoon sweets production and as a condiment, while flowers and leaves are used as a source of fibers, substances for weight loss and flavored sweets [1]. Due to the anxiolytic and sedative effect and to the high price of the oils in the international market of aromatherapy, perfume and cosmetic industries, they are considered as one of the main by-products of sour orange [2].

Drought is one of the most significant limiting factors in many regions of the world, which seriously affects plant growth and development. Many physiological and biochemical processes in plants are affected, which often causes oxidative stress and increases the concentration of different secondary compounds [3]. In Greece, *Citrus* species are mostly grown or cultivated in coastal areas and in islands, where problems arise due to high concentrations of Na^+ and Cl^-. Saline soils are more pronounced in areas with semi-arid and arid climate, due to high levels of evapotranspiration and the low level of rainfall, which is insufficient for leaching salts from the soil. Salt stress often creates both ionic and osmotic stress in plants, resulting in either accumulation or decrease of specific secondary metabolites in plants [3]. Under stress conditions plants are forced to develop enzymatic and non-enzymatic mechanisms in order to resist the production of toxic free radicals and enhance their defense system [4]. To maintain a balance between ions in the vacuoles and cytoplasm, low molecular mass compounds referred to as osmolytes are produced in the cytoplasm [5].

Recently we reported linalool, α-terpineol and linalyl- and geranyl-acetates as the major compounds of the essential oil from *C. aurantium* leaves grown in Greece [6]. However, no data exist on the effect of drought and salinity on the content and composition. The aim of this study was to investigate the volatile organic compounds and other secondary metabolites of *C. aurantium* leaves under the influence of drought and salinity conditions.

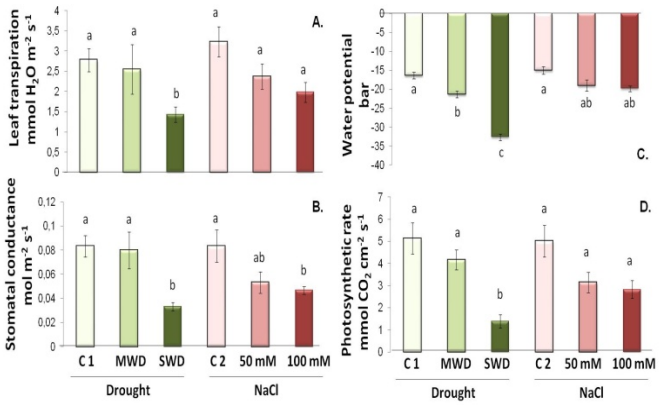

Figure 1: The effect of drought and salt stress on leaf transpiration (A), stomatal conductance (B), water potential (C) and photosynthetic rate (D) of *C. aurantium*. Data represent means ± SE of 5 replicates. Different letters indicate significant differences according to Duncan's multiple range tests ($P \leq 0.05$).

The photosynthetic parameters, leaf transpiration, stomatal conductance, water potential and photosynthetic rate, represent the physiological state of plants under the various levels of stress conditions; drought and salinity, applied in this experiment (Figure 1 A, B, C and D). Under the influence of both drought and salinity, these parameters were decreased compared with control plants (Figure 1A, B and D). In addition, there was a decrease in water potential under drought and salinity, while this decrease was 2 times higher (-32.67 bar) under the influence of SWD (Figure 1C). According to Lowlor and Cornic, the rate of photosynthesis in higher plants is decreased when relative humidity and leaf water potential are restricted [7]. Moreover, drought stress induces

stomatal closure, which in our study was manifested by reduced leaf transpiration and photosynthetic rate. Drought can generally suppress biochemical processes of assimilation and utilization of carbon through the activity of Rubisco [8].

The concentration of total carbohydrates (CHs) was limited under drought and salinity conditions, while significant differences were observed in the SWD and in 50 and 100 mM NaCl, compared with the control plants (Figure 2A). Proline almost doubled in SWD-treated plants and increased significantly in plants treated with 100 mM NaCl (Figure 2B). It is well documented that CHs are necessary for cell growth and they are produced mainly through the process of photosynthesis, while they have important functions, such as osmoregulators, carbon storage and deactivation of free radicals [9]. However, some studies have shown that osmotic stress increases the CH concentration [5] while others reported a reverse effect [10, 11]. On the other hand, one possible role of proline is that it may stabilize DNA, membranes and protein complexes and can act as an energy source providing carbon and nitrogen, in order to relieve the stress [12].

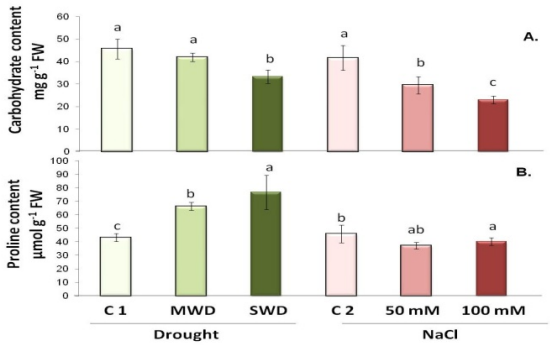

Figure 2: The effect of drought and salt stress on leaf CHs (A) and proline content (B) of *C. aurantium*. Data represent means ± SE of 5 replicates. Different letters indicate significant differences according to Duncan's multiple range tests ($P \leq 0.05$).

The two levels of drought and salinity stress increased the concentration of total phenols by 20 and 25% under MWD and 100 mM NaCl, respectively (Figure 3A). In addition, all the stressed plants accumulated higher amounts of flavonoids compared with the control (Figure 3B). The total ascorbic acid concentration was increased by 37 and 88%, under the influence of MWD and SWD, while 50 and 100 mM NaCl increased it by 22 and 36%, respectively (Figure 3C). These changes in phenol and total ascorbic acid were positively correlated with the level of drought (r=0.753 and 0.987, $P \leq 0.01$) and salinity (r=0.899 and 0.945, $P \leq 0.01$), respectively. It has been reported that various stress conditions enhanced phenylpropanoid metabolism, phenolic concentration and increased synthesis of flavonoids [13, 14]. In addition these changes could be due to an activation of enzymes like phenylalanine ammonia lyase, chalcone synthase and phosphoenolpyruvate-carboxylase, suggesting a shift from sucrose production to processes in support of defense and adaption [15]. Ascorbic acid acts as a co-factor for many enzymes such as ascorbate peroxidase (APX), which uses it as an electron donor [16]. A high level of endogenous APX is required, as it maintain effectively the antioxidant mechanism and protects plants from oxidative stress [17].

Under MWD conditions, *C. aurantium* yielded the highest essential oil in our experiments (Table 3). Similar positive effects have been reported in parsley [18], sage [19], mint and rosewood [20]. Moreover, treatment with 50 and 100 mM NaCl increased significantly the leaf essential oil content. Our results are also in agreement to those of Hendawy and Khalid (2005) [21], AbouEl-Fadl *et al*. (1990) [22] and Neffati et al. (2011) [23], who reported an increase of essential oil in salvia, mint and coriander, respectively, under the influence of salinity. According to Dow *et al*. [24], salinity causes a decrease of essential oils in plants of the family Lamiaceae, probably due to a limited absorption and transport of Ca from the roots to shoots and by changing the ratio of Ca/ABA in the leaves. Moreover, the salinity may affect the accumulation of essential oils indirectly, through the effects on assimilation of substances which are necessary for plant growth. While, salinity seems to inhibit the synthesis of essential oils in mint and chamomile [25, 26], in other plants the synthesis seems to be induced [27-39].

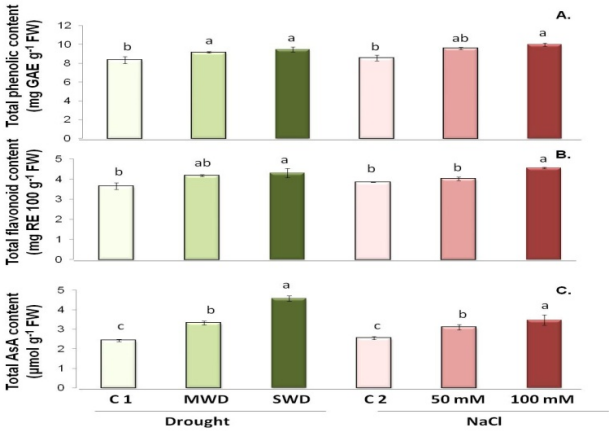

Figure 3: The effect of drought and salt stress on leaf total phenolic (A), total flavonoid (B) and total ascorbic acid (AsA) (C) of *C. aurantium*. Data represent means ± SE of 5 replicates. Different letters indicate significant differences according to Duncan's multiple range tests ($P \leq 0.05$).

Significant differences were observed in the composition of the examined oils, especially under SWD conditions. Fourteen compounds, accounting for 95.4-99.5% of the total essential oil, were detected in all the treatments and among them; the most abundant were linalool, α-terpineol, and linalyl-, geranyl- and neryl-acetates (Table 3). Generally, SWD reduced significantly the total amount of monoterpene hydrocarbons and increased the amount of oxygenated compounds in the essential oil of *C. aurantium* leaves. More specifically, SWD increased the concentration of linalyl acetate and β-caryophyllene, and limited the amounts of linalool, α-terpineol and all the hydrocarbon monoterpenes. Similar effects of salinity stress on the accumulation of sesquiterpenes have also been reported [23]. In contrast, 50 and 100 mM NaCl, promoted the accumulation of hydrocarbon monoterpenes and reduced the oxygenated monoterpenes concentration. In addition, both levels of NaCl provoked the accumulation of carene, limonene and *trans*-ß-ocimene. The accumulation of monoterpenes could have an ecological significance in the defense mechanism of plants. Monoterpenes are secondary metabolites formed in the chloroplasts so their concentration may depend on CO_2 levels and the metabolic intermediates formed during the process of photosynthesis [30]. Among other factors, the characteristics of photosynthesis and the productivity of plant tissues have a core role in carbon utilization for essential oil anabolism. Moreover, the recovery of glycerin aldehyde-3P and pyruvate on the plastidic pathway enhanced the hypothesis of close association of photosynthesis with the biosynthesis of essential oil [31]. However, in this study, the accumulation of essential oil increased, while the rate of photosynthesis was decreased, under the influence of oxidative stress. A possible explanation could be that the accumulation of monoterpenes could alleviate the damage in chloroplasts caused by ROS, as well as terpenes could limit plant respiration as a protection

Table 1: Composition of leaf essential oil (%) from *C. aurantium* under the effect of drought and salinity.

			Control 1	MWD	SWD	Control 2	50 mM NaCl	100 mM NaCl
Compounds[a]	RI[b]	RIL[c]	Concentration [d, e] %					
β-pinene	979	980	0.4 ± 0.0a	0.4 ± 0.0a	0.2 ± 0.0b	0.4 ± 0.0	0.4 ± 0.0	0.5 ± 0.01
β-myrcene	993	991	2.7 ± 0.0a	2.6 ± 0.0a	1.8 ± 0.1b	2.8 ± 0.0	2.8 ± 0.0	2.8 ± 0.01
3-δ-carene	1012	1011	0.8 ± 0.0a	0.8 ± 0.0a	0.4 ± 0.0b	0.7 ± 0.0c	0.8 ± 0.0b	1.0 ± 0.04a
limonene	1032	1031	1.5 ± 0.1a	1.5 ± 0.0a	1.0 ± 0.1b	1.5 ± 0.0c	1.7 ± 0.0b	1.9 ± 0.01a
***cis*-β-ocimene**	1041	1040	1.0 ± 0.0a	1.0 ± 0.0a	0.8 ± 0.0b	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.01
***trans*-β-ocimene**	1051	1050	2.5 ± 0.0a	2.5 ± 0.0a	2.1 ± 0.1b	2.5 ± 0.0b	2.6 ± 0.0a	2.7 ± 0.03a
terpinolene	1089	1088	0.7 ± 0.0a	0.7 ± 0.0a	0.5 ± 0.0b	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.0
linalool	1103	1104	46.5 ± 0.6a	45.7 ± 0.2a	44.0 ± 0.3b	46.1 ± 0.3	46.4 ± 1.0	42.1 ± 3.9
α-terpineol	1191	1189	11.0 ± 0.1a	11.0 ± 0.0a	10.5 ± 0.2b	10.9 ± 0.2	11.2 ± 0.1	11.1 ± 0.3
nerol	1231	1228	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.1 ± 0.1
linalyl acetate	1260	1257	20.1 ± 0.6b	20.8 ± 0.1b	25.4 ± 0.3a	20.7 ± 0.1	19.4 ± 0.6	19.3 ± 1.1
neryl acetate	1368	1365	3.3 ± 0.0	3.4 ± 0.1	3.8 ± 0.0	3.2 ± 0.0	3.3 ± 0.1	3.4 ± 0.0
geranyl acetate	1387	1383	6.3 ± 0.1	6.4 ± 0.1	6.5 ± 0.1	6.1 ± 0.1	6.3 ± 0.1	6.5 ± 0.2
β-caryophyllene	1419	1418	0.2 ± 0.0b	0.3 ± 0.0b	0.5 ± 0.0a	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
Hydrocarbons monoterpenes			9.6 ± 0.2a	9.5 ± 0.1a	6.8 ± 0.0b	9.6 ± 0.1b	10.0 ± 0.2a	10.6 ± 0.1a
Oxygenated monoterpenes			89.2 ± 1.4b	89.3 ± 0.6b	92.2 ± 1.0a	89.0 ± 0.8a	88.6 ± 1.9a	84.5 ±5.6b
Sesquiterpenes			0.2 ± 0.0	0.3 ± 0.0	0.5 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
Main constituents			99.0	99.1	99.5	98.9	98.9	95.4
Essential oil content (%)			0.7 **b**	0.9 **a**	0.9 **a**	0.7 **b**	0.8 **a**	0.8 **a**

Notes: [a] order of elution on HP-5 capillary column; [b] Calculated relative to C9-C22 *n*-alkanes, on HP-5 capillary column; [c] Literature Retention Indices on similar column. [d] Percentage of the total peak area. Components with percentage ≥ 0.2% are presented; e: ± SE

mechanism against stress factors as suggested [32], although this hypothesis needs further investigation. Generally, it has been reported that the production of secondary metabolites is stimulated by oxidative stress [15, 31]. However, there are only few experimental data to support this hypothesis as there are no studies on the effect of oxidative stress on citrus essential oil in the literature. The osmotic stress can indirectly affect the accumulation of essential oil through its effect on the assimilation of photosynthetic products. In the present study, the limited growth caused by low leaf water potential can potentially modify the supply of extra carbon skeletons required for the biosynthesis and accumulation of monoterpenes in leaves.

Experimental

Plant material and experimental design: water deficit and salinity treatments: The experiment was conducted in a greenhouse at the Experimental Farm of Aristotle University of Thessaloniki (40°34'35" N 22°57'19" E) in 2013, using 3 years old *C. aurantium* plants obtained by cutting propagation. The experimental layout was Completely Randomized Design, with 5 replications (pots) for each treatment, as follows: (a) control 1 (100% of FC), (b) 34% MWD of FC, and (c) 67% SWD of FC, (d) control 2 (0 mM NaCl), (e) moderate salinity (50 mM NaCl) and (f) severe salinity (100 mM NaCl). In all treatments the plants were irrigated with 50% Hoagland prepared with deionized water. MWD and SWD were determined in the pots by weight. For the salinity treatments, the appropriate quantity of NaCl was added each time in nutrient solution and the plants were irrigated with 250 mL of the solution. The experiment lasted 60 days, from April to June, and the leaves were collected, when leaf symptoms of toxicity appeared on the stressed plants. When the experiment was completed, the leaves were harvested and the following parameters were determined in 5 replicates.

Photosynthetic rate and water potential: The leaf photosynthetic rate was measured with an LC Pro+ (ADC BioScientific Ltd., UK). The measurements were performed 45 days from the beginning of the experiments, between 10 and 12 a.m. at steady light intensity (> 900 μmol m^{-2} s^{-1}), while leaf temperature varied between 28 and 32°C. Leaf water potential was measured 1 day prior to the termination of the experiment, between 6 and 7 a.m., on the first fully expanded leaf of each plant, with a Pressure Chamber type PMS-1000.

Determination of total phenols, flavonoids and ascorbic acid: For total phenols and flavonoids determination, 0.2 g of fresh leaves were extracted with 80% methanol in a cool mortar. The concentration of total phenols was determined as described by Scalbert *et al*. [33]. The values were expressed as mg gallic acid equivalents (GAE)/g fresh weight (FW).

For the determination of total flavonoid content the method described by Zhisen *et al*. [34] was used. The values were estimated using a standard curve of rutin and expressed as mg rutin equivalents (RE)/g FW. For the ascorbic acid determination 0.1 g fresh leaves were extracted with 5% meta-phosphoric acid. The values were estimated using a standard curve with a range of 0-50 μM and expressed as μmol/g FW [35].

Essential oil yield: Fresh leaves (50 g) were comminuted and subjected to hydrodistillation for 4 h with a distillation rate of 3 to 3.5 mL/min, in a Clevenger-type apparatus. The essential oil content was determined as mL/100 g FW. The obtained essential oil was dried over Na_2SO_4 and stored at 4-6 ºC for further analysis.

Gas chromatography: Gas chromatographic (GC) analysis of the essential oil was carried out on a CE Instruments TRACE™ Gas Chromatograph (ThermoQuest, Italy) equipped with a Flame Ionization Detector and HP-5ms capillary column (Bonded and cross-linked (5 %-Phenyl-methylpolysiloxane), non-polar, 30 m x 0.25 mm ID x 0.25 μm film), as previously described [36].

Gas chromatography/mass spectrometry: The essential oil was also analyzed by Gas Chromatography/Mass Spectrometry (GC/MS) on a fused silica DB-5 column, using a GC 17A Ver. 3 interfaced with a MS Shimadzu QP-5050A supported by Class 5000 software, as previously described [6]. The identification of the compounds was based on comparison of their Kovats Retention Indices, determined with reference to a homologous series of C_9-C_{22} *n*-alkanes, with corresponding literature data and by matching their mass spectral fragmentation patterns with those in MS libraries [37].

Statistical analysis: The data were analysed with Analysis of Variance (ANOVA), using the statistical package SPSS 11 17.0 (SPSS Inc., USA). Means were compared by the least significance test at the 0.05 level of confidence using Duncan's multiple range tests. For the correlations, the Pearson Product Moment was used. The data are presented as means ± SE.

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Insights into the Essential Oil Compositions of Brazilian Red and Taiwanese Green Propolis

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The objective of the present study was to characterize chemically the essential oils of two distinct propolis types: Brazilian red and Taiwanese green. Unlike the non-volatile chemical composition of these types of propolis, which has been extensively studied, the knowledge of the essential oils is scarce or even not investigated. The essential oils were obtained by hydrodistillation of raw propolis samples using a Likens-Nickerson type apparatus and then analyzed by GC/MS. The main volatile components of Brazilian red propolis were the phenylpropanoids: elemicin (26.1-27.5%), methyl eugenol (16.3-23.8%), *trans*-methyl isoeugenol (9.2-11.6%), isoelemicin (6.1-7.1%) and *trans*-anethole (4.4-7.1%), while the major constituents of Taiwanese green propolis essential oil were: β-eudesmol (13.9%), 6-methyl-3,5-heptadiene-2-one (12.2%), γ-eudesmol (4.4%), geranial (4.1%) and 6-methyl-5-heptene-2-one (3.7%).

Keywords: Essential oil, Brazilian red propolis, Taiwanese green propolis, Phenylpropanoids, β-Eudesmol.

Propolis (bee glue) is a bee product of plant origin, which is used in the hive as building material and a defensive substance. It is a complex mixture of resin, collected by bees from plant buds and exudates, and beeswax. Since ancient times, propolis has been widely used in folk medicine. Nowadays, its beneficial properties and biological activities have been extensively studied and it is proved that propolis exhibits antimicrobial, antioxidant, anti-inflammatory, immunostimulating, hepatoprotective as well as cytotoxic activity as it contains a large number of bioactive molecules [1, 2]. The chemical composition of bee glue is highly variable and depends mainly on the local flora, but despite the differences in origin and composition, propolis samples show very similar biological activity [3]. This leads to the continued increase of propolis use, for example in OTCs (over-the-counter preparations), "bio"-cosmetics and functional foods. Raw propolis is composed of about 50% resin and vegetable balsam (consisting mainly of phenolics and di- and triterpenoids), about 30% wax, 10% essential and aromatic oils, 5% pollen, and other substances [4]. In spite of their low concentrations volatile compounds of propolis are of importance because they are known to possess valuable biological activities, especially antibacterial, which contribute to the biological activity of propolis. This fact, along with their pleasant aroma, makes them of high relevance for cosmetics and especially for the food industry as preservatives [5, 6]. Therefore, the present work focuses on the determination of the essential oil chemical compositions of two distinct propolis types: Brazilian red and Taiwanese green. Unlike the non-volatile chemical composition of these types of propolis, which has been extensively studied, their essential oils are little or not investigated. There are only data on the chemical composition of volatiles obtained by a dynamic headspace technique from Brazilian red propolis [7]. Till now the chemical composition and biological activity of Taiwanese green propolis essential oil have never been investigated.

The essential oils of Brazilian red (three samples) and Taiwanese green (one sample) propolis were obtained by hydrodistillation of raw propolis samples in a Likens-Nickerson type apparatus and then analyzed by GC/MS. In Brazilian red propolis essential oil (samples R-1, R-2 and R-3), for which the main plant source is *Dalbergia ecastophyllum*, 69 volatiles were identified, which constitute 92.1%, 97.8% and 96.4% of the oil, respectively. The data obtained are summarized in Table 1. The quantities measured correspond to the percentage of total ion current and are not a true quantitation. However, they are completely reliable in the case of comparison between samples having similar qualitative composition [8, 9].

In all three studied samples the main volatile components, comprising significant parts of the total oil, were the phenylpropanoids: elemicin (26.1-27.5%), methyl eugenol (16.3-23.8%), *trans*-methyl isoeugenol (9.2-11.6%), isoelemicin (6.1-7.1%) and *trans*-anethole (4.4-7.1%), a mixture which we have already isolated from Brazilian red propolis [10]. Partially our results corroborate those of Nunes *et al*., who investigated the volatile fraction of Brazilian red propolis, obtained by a dynamic headspace technique, and also found phenylpropanoids as the major constituents [7]. However, as expected, the differences were both quantitative and qualitative, which once again proves that the chemical composition of an essential oil is dependent on the extraction method [11]. The presence of high amounts of phenyl propanoids in Brazilian red propolis essential oil seems to be unique comparing it with other types of propolis volatiles [12]. As regards the other components of the oil, in all three samples, as in poplar type propolis essential oil, sesquiterpenes predominate over monoterpenes. Monoterpene hydrocarbons are missing and oxygenated monoterpenes, except for linalool, are contained only in sample R-3. β-Caryophyllene and spathulenol (R-1), α-copaene (R-2 and R-3) and β-bisabolene (R-3) were also in appreciable percentages.

Taiwanese green propolis is a Pacific type and its plant source is *Macaranga tanarius*. Till now its essential oil composition is unreported. Hydrodistillation of raw propolis produced a colorless volatile oil, which was analyzed by GC/MS. The chemical composition of the essential oil is summarized in Table 2.

Among 91 identified constituents, representing approximately 77% of the total oil, β-eudesmol (13.9%), 6-methyl-3,5-heptadiene-2-one (12.2%) and γ-eudesmol (4.4%) were the most abundant.

Table 1: Chemical composition of Brazilian red propolis essential oil

No	Compounds	RI[a]	RI[b]	% of TIC[c]		
				R-1	R-2	R-3
	Oxygenated monoterpenes			**0.1**	**0.2**	**2.1**
1	Linalool	**1 101**	1 101	0.1	0.2	0.1
2	*p*-Mentha-1,5-dien-8-ol	**1 167**	1 166	-	-	0.2
3	Terpinen-4-ol	**1 186**	1 187	-	-	0.4
4	*p*-Cymen-8-ol	**1 191**	1 193	-	-	0.5
5	α-Terpineol	**1 199**	1 198	-	-	0.4
6	Eucarvone	**1 216**	1 223	-	-	0.3
7	Thymol	**1 296**	1 296	-	-	0.1
8	Carvacrol	**1 303**	1 304	-	-	0.1
	Sesquiterpene hydrocarbons			**9.0**	**6.7**	**12.7**
9	α-Cubebene	**1 352**	1 351	0.4	0.4	0.2
10	α-Copaene	**1 383**	1 382	1.5	2.5	3.5
11	β-Bourbonene	**1 391**	1 391	0.5	-	-
12	α- Gurjunene	**1 416**	1 412	-	-	0.1
13	α-*cis*-Bergamotene	**1 418**	1 420	0.3	0.7	1.3
14	β-Caryophyllene	**1 429**	1 428	2.6	0.5	0.9
15	α-*trans*-Bergamotene	**1 438**	1 438	0.3	0.2	0.2
16	α-Caryophyllene	**1 466**	1 465	0.6	0.1	0.2
17	*cis*-Muurola-4(14),5-diene	**1 472**	1 470	0.2	tr[d]	0.1
18	*trans*-Cadina-1(6),4-diene	**1 480**	1 477	tr	0.1	0.2
19	γ-Muurolene	**1 483**	1 485	0.3	0.1	0.2
20	α-Curcumene	**1 485**	1 486	tr	0.1	0.3
21	*trans*-Muurola-4(14),5-diene	**1 491**	1 491	0.3	-	-
22	β-Bisabolene	**1 513**	1 513	0.4	0.9	2.0
23	γ-Cadinene	**1 522**	1 514	0.2	tr	0.1
24	δ-Cadinene	**1 525**	1 525	0.9	0.7	1.5
25	*trans*-Calamenene	**1 530**	1 529	0.3	0.3	1.7
26	*trans*-Cadina-1(2),4-diene	**1 541**	1 537	0.2	0.1	0.2
27	β-Calacorene	**1 572**	1 573	tr	-	tr
	Oxygenated sesquiterpenes			**5.6**	**2.7**	**2.9**
28	E-Nerolidol	**1 564**	1 565	0.3	tr	0.1
29	Spathulenol	**1 589**	1 590	2.2	0.5	0.6
30	Caryophyllene oxide	**1 595**	1 593	1.3	0.3	0.2
31	Salvial-4(14)-en-1-one	**1 605**	1 603	0.1	-	-
32	*epi*-Cedrol	**1 626**	1 632	0.1	0.4	0.5
33	1-*epi*-Cubenol	**1 639**	1 629	0.4	0.3	0.7
34	*epi*-α-Cadinol	**1 653**	1 651	0.2	0.1	0.7
35	α-Cadinol	**1 666**	1 666	0.7	tr	0.1
36	α-Eudesmol	**1 669**	1 654	-	1.1	-
37	Eudesma-4(15),7-diene-1-β-ol	**1 703**	1 690	0.3	-	-
	Phenylpropanoids			**68.0**	**82.6**	**71.2**
38	Isoanethole	**1 202**	1 201	0.1	0.2	0.2
39	*trans*-Anethole	**1 291**	1 290	4.5	7.1	4.4
40	Eugenol	**1 358**	1 358	0.3	0.3	0.4
41	Methyl eugenol	**1 403**	1 402	16.3	23.8	20.4
42	*trans*-Isoeugenol	**1 454**	1 453	0.6	0.9	0.6
43	*trans*-Methyl isoeugenol	**1 499**	1 500	9.4	11.6	9.2
44	Elemicin	**1 550**	1 552	26.1	27.5	27.3
45	Methoxyeugenol	**1 600**	1 609	3.6	4.4	2.6
46	Isoelemicin	**1 649**	1 649	7.1	6.8	6.1
	Acids, esters, amides			**3.4**	**2.0**	**1.7**
47	Caproic acid	**988**	984	0.4	-	0.1
48	Octanoic acid	**1 175**	1 176	0.4	-	0.3
49	Nonanoic acid	**1 270**	1 271	0.2	-	-
50	*trans*-Cinnamyl acetate	**1 450**	1 452	0.2	0.2	0.3
51	Benzyl benzoate	**1 779**	1 774	0.6	-	-
52	*cis*-Benzyl cinnamate	**1 961**	1 959	0.6	-	-
53	*trans*-Benzyl cinnamate	**2 115**	2 102	0.3	-	-
54	Oleamide	**2 365**	2 362	0.7	1.8	1.0
	Alcohols, aldehydes, ketones			**1.3**	**0.4**	**1.7**
55	Benzyl alcohol	**1 040**	1 039	0.2	-	-
56	*n*-Decanal	**1 208**	1 207	tr	0.1	-
57	*m*-Guaiacol	**1 232**	1 235	0.5	0.1	0.5
58	*p*-Anisaldehyde	**1 263**	1 258	0.1	-	-
59	*trans*-Cinnamaldehyde	**1 282**	1 272	0.5	0.2	1.2
	Aliphatic hydrocarbons			**4.7**	**3.2**	**4.1**
60	*n*-Dodecane	**1 199**	1 200	0.3	0.4	0.6
61	*n*-Tridecane	**1 300**	1 300	0.1	0.3	0.3
62	*n*-Heptadecane	**1 700**	1 700	0.4	0.6	0.5
63	*n*-Octadecane	**1 800**	1 800	0.1	0.2	0.2
64	*n*-Nonadecane	**1 900**	1 900	0.1	0.2	0.2
65	*n*-Heneicosane	**2 102**	2 100	0.6	0.3	0.5
66	*n*-Tricosane	**2 301**	2 300	0.9	0.4	0.7
67	*n*-Pentacosane	**2 501**	2 500	1.0	0.4	0.6
68	*n*-Heptacosane	**2 701**	2 700	1.0	0.4	0.5
69	*n*-Nonacosane	**2 901**	2 900	0.2	-	-

[a] Retention indices on apolar column (HP-5MS); [b] Retention indices on apolar column of literature ([15] and NIST); [c] The total ion current (TIC) generated depends on characteristics of the compound concerned and is not a true quantification; [d] Traces of compound identified. In quantification of different classes of compounds the amounts of traces are not included.

Table 2: Chemical composition of Taiwanese green propolis essential oil

No	Compounds	RI[a]	RI[b]	% of TIC[c]
	Monoterpene hydrocarbons			**0.2**
1	Carvomenthene	**1 023**	1 025	0.2
2	*p*-Cymenene	**1 093**	1 093	tr[d]
	Oxygenated monoterpenes			**12.2**
3	Bergamal	**1 056**	1 057	0.4
4	*trans*-Dihydrorose oxide	**1 074**	1 073	1.7
5	*cis*-Vertocitral C	**1 080**	1 080	0.1
6	*cis*-Linalool oxide	**1 089**	1 087	tr
7	Linalool	**1 102**	1 101	0.5
8	*cis*-*p*-Mentha-2,8-dien-1-ol	**1 141**	1 138	0.1
9	Borneol	**1 178**	1 177	0.2
10	*p*-Cymen-8-ol	**1 191**	1 183	0.5
11	γ-Terpineol	**1 199**	1 199	0.6
12	*iso*-Dihydro carveol	**1 208**	1 215	0.1
13	Verbenone	**1 214**	1 205	0.4
14	Neral	**1 242**	1 238	1.6
15	Geraniol	**1 252**	1 253	0.3
16	Piperitone	**1 260**	1 253	0.1
17	Geranial	**1 271**	1 267	4.1
18	Thymol	**1 295**	1 290	0.2
19	Piperitenone	**1 347**	1 343	0.1
20	Ethyl-Z-geraniate	**1 351**	1 354	0.2
21	*trans*-Geranyl acetone	**1 450**	1 451	1.0
	Sesquiterpene hydrocarbons			**2.3**
22	α-Copaene	**1 383**	1 377	0.2
23	9-*epi*-*trans*-Caryophyllene	**1 470**	1 467	tr
24	β-Chamigrene	**1 481**	1 482	0.3
25	α-Curcumene	**1 486**	1 486	tr
26	Viridiflorene	**1 499**	1 497	tr
27	*trans*-γ-Cadinene	**1 523**	1 514	tr
28	δ-Cadinene	**1 526**	1 528	0.1
29	*trans*-Calamenene	**1 530**	1 529	0.2
30	α-Calacorene	**1 552**	1 550	0.1
31	β-Calacorene	**1 572**	1 574	tr
32	Cadalene	**1 684**	1 684	1.4
	Oxygenated sesquiterpenes			**20.9**
33	β-Agarofuran	**1 516**	1 516	0.3
34	Italicene ether	**1 535**	1 538	tr
35	Ledol	**1 584**	1 588	0.1
36	Caryolan-8-ol	**1 589**	1 573	0.3
37	Gleenol	**1 595**	1 595	tr
38	10-*epi*-γ-Eudesmol	**1 630**	1 624	0.2
39	Citronellyl pentanoate	**1 630**	1 625	0.2
40	1-*epi*-Cubenol	**1 639**	1 629	0.2
41	γ-Eudesmol	**1 643**	1 632	4.4
42	Hinesol	**1 652**	1 642	1.0
43	*epi*-α-Muurolol	**1 655**	1 654	0.1
44	δ-Cadinol	**1 657**	1 646	0.1
45	α-Cadinol	**1 667**	1 654	0.1
46	β-Eudesmol	**1 669**	1 651	13.9
	Phenylpropanoids			**0.5**
47	*E*-Anethole	**1 292**	1 285	tr
48	*trans*-Isoeugenol	**1 455**	1 453	0.1
49	*trans*-Methyl isoeugenol	**1 498**	1 499	tr
50	Elemicin	**1 549**	1 552	0.3
51	*cis*-Isoeugenol acetate	**1 567**	1 568	0.1
	Alcohols, aldehydes, ketones			**19.7**
52	2-Methylcyclopentanone	**845**	843	0.1
53	6-Methyl-5-hepten-2-one	**987**	986	3.7
54	6-Methyl-5-hepten-2-ol	**995**	995	0.1

No	Compounds	RI[a]	RI[b]	% of TIC[c]
55	Benzyl alcohol	**1 040**	1 039	0.2
56	o-Guaiacol	**1 091**	1 092	0.6
57	6-Methyl-3,5-heptadiene-2-one	**1 107**	1 107	12.2
58	Phenyl ethyl alcohol	**1 118**	1 117	0.1
59	*E*-Cinnamaldehyde	**1 282**	1 270	0.2
60	2-Heptadecanone	**1 902**	1 900	1.9
61	Octadecanol	**2 089**	2 078	0.1
62	2-Nonadecanone	**2 107**	2 101	0.4
63	2-Heneicosanone	**2 311**	2 309	0.1
	Acids, esters			**3.1**
64	4-Methylpent-2-enolide	**952**	952	0.1
65	Caproic acid	**991**	984	0.3
66	Heptanoic acid	**1 083**	1 080	0.1
67	Octanoic acid	**1 176**	1 176	0.2
68	Pelargonic acid	**1 274**	1 271	tr
69	*o*-Anisic acid methyl ester	**1 337**	1 336	0.9
70	*trans*-Methyl cinnamate	**1 393**	1 392	tr
71	Phenyl ethyl 2-methylbutanoate	**1 490**	1 487	tr
72	*n*-Decanoic acid	**1 367**	1 367	0.5
73	Benzyl benzoate	**1 779**	1 760	0.3
74	Benzyl salicylate	**1 883**	1 866	0.7
	Aliphatic hydrocarbons			**17.7**
75	Dodecane	**1 200**	1 200	0.6
76	Tridecane	**1 300**	1 300	0.7
77	Tetradecane	**1 400**	1 400	1.6
78	Pentadecane	**1 500**	1 500	1.6
79	Hexadecane	**1 600**	1 600	1.0
80	Heptadecane	**1 699**	1 700	1.5
81	Octadecane	**1 800**	1 800	0.6
82	Eicosane	**1 999**	2 000	0.1
83	Heneicosane	**2 100**	2 100	0.5
84	Docosane	**2 201**	2 200	0.1
85	Tricosane	**2 301**	2 300	3.5
86	Tetracosane	**2 401**	2 400	0.1
87	Pentacosane	**2 501**	2 500	2.4
88	Hexacosane	**2 601**	2 600	0.1
89	Heptacosane	**2 701**	2 700	1.2
90	Squalene	**2 816**	2 790	1.9
91	Nonacosane	**2 901**	2 900	0.2

[a] Retention indices on apolar column (HP-5MS); [b] Retention indices on apolar column of literature ([15] and NIST); [c] The total ion current (TIC) generated depends on characteristics of the compound concerned and is not a true quantification; [d] Traces of compound identified. In quantification of different classes of compounds the amounts of traces are not included.

Oxygenated mono- and sesquiterpenes predominate over terpene hydrocarbons and sesquiterpenes are more than monoterpenes. The oxygenated monoterpenes represent 12.2% of the oil, with geranial as the main part. Unlike Brazilian red propolis volatiles, the Taiwanese green propolis essential oil contains a significant amount of aliphatic alcohols, aldehydes and ketones (19.7%) and very low amounts of phenylpropanoids (0.5%). The amount of aliphatic hydrocarbons is significant (17.7% of the total oil), with tricosane the principal compound. The major compound, β-eudesmol, is very typical for poplar type propolis volatiles. The main components of popular Brazilian green propolis essential oil, β-caryophyllene and *trans*-nerolidol, are missing [13, 14]. An appreciable part of the compounds remained unidentified, so further research is needed for their identification.

The present work provides additional insights into Brazilian red and Taiwanese green propolis concerning the chemical composition of their volatiles. The phenylpropanoids, identified as main components of Brazilian red propolis essential oil, can be eventually used as its chemical markers. As far as it concerns Taiwanese green propolis this is the first report about the chemical composition of its essential oil. However, further research is needed to reveal the biological activity of the investigated volatiles, as well as their potential and contribution to the biological activity and medicinal application of propolis.

Experimental

Propolis samples: The investigated propolis samples are commercial. Their plant sources and propolis type are determined by TLC comparison with authentic samples.

Essential oil extraction: The raw propolis samples were grated after cooling and subjected to hydrodistillation in a Likens-Nickerson type apparatus for 4 h. The collected distillates were extracted with diethyl ether/*n*-pentane (1:1). The extracts obtained were dried over anhydrous Na_2SO_4, evaporated and stored in a freezer until GC/MS analysis. The essential oil content of the Brazilian red propolis samples was 0.12% (R-1) and 0.11% (R-2, R-3) and of the Taiwanese green- 0.05% based on the weight of raw propolis.

GC/MS analysis: Analysis of the oils was performed on a Hewlett-Packard gas chromatograph 5890 series II Plus linked to a Hewlett–Packard 5972 mass spectrometer system equipped with a HP5-MS capillary column (30 m x 0.25 mm and 0.25 μm film thickness). The ion source was set at 250°C and the ionisation voltage at 70 eV. The temperature was programmed from 40 – 280°C at a rate of 6°C/min, and helium was used as the carrier gas at a flow rate of 0.8 mL/min. The split ratio was 1:10, the injector temperature 280°C. Acquisition mass range m/z 35–750; scan time 49 min. The identification of the compounds was based on their retention indices (RI), obtained using *n*-alkanes (C8–C40), and by comparison of their EI-mass spectra with NIST library spectra and literature [15].

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Essential Oil Content, Composition and Bioactivity of Juniper Species in Wyoming, United States

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The objective of this study was to evaluate variations in leaf essential oil (EO) content and composition of *Juniperus* species in the Bighorn Mountains {*J. communis* L. (common juniper), *J. horizontalis* Moench. (creeping juniper), and *J. scopulorum* Sarg. (Rocky Mountain juniper)} in Wyoming, USA. The EO was extracted via steam distillation of fresh leaves (needles). The EO composition of the three Juniper species varied widely. Overall, the essential oil content of fresh leaves was 1.0% (0.4-1.8% range in different accessions) in *J. communis*, 1.3% (1.2 to 1.6% range) in *J. horizontalis*, and 1.1% (0.7-1.5% range) in *J. scopulorum*. The EO chemical profile of *J. communis* was very different from that of the other two species. The concentration of α-pinene in the oil was 67-80% in *J. communis*, 2.8-6% in *J. horizontalis*, and 2.3-13% in *J. scopulorum*. The concentration of sabinene was 57-61% of the oil of *J. horizontalis* and 13-59% in oil of *J. scopulorum*, whereas sabinene was either below 1% or not detected in *J. communis*. The oils of *J. scopulorum* and *J. horizontalis* had higher antioxidant capacity than that of *J. communis*. The oils of the three junipers did not show significant antimicrobial activity against 10 organisms. The diversity of the essential oil composition of these three junipers may encourage diverse industrial applications of *Juniperus* leaf essential oil.

Keywords: *Juniperus communis*, *Juniperus horizontalis*, *Juniperus scopulorum*, Essential oils.

Junipers are some of the most widespread species on earth, including North America [1-3]. According to Adams [1], the genus *Juniperus* includes around 68 species and 36 different varieties. These species are evergreen, mostly dioecious gymnosperms. Junipers are widely used as ornamentals, but also as medicinal plants, with a long history of medicinal uses by Native Americans [4, 5]. Although junipers are found throughout North America, most grow slowly and are not extensively used for lumber production. However, juniper wood is very durable and valued for its color, aroma, antimicrobial properties and used for interior paneling, furniture, various novelties, and fence posts. Junipers are mostly found in the arid and semi-arid regions of North America and are important for wildlife species such as deer, elk, small mammals, and birds, which use it for cover, and as a food source [2, 6]. Birds and mammals foster the juniper seed dispersal. In some states, Junipers are considered undesirable, and are subject to targeted removal from pastures and agricultural land.

Rocky Mountain juniper (*J. scopulorum* Sarg.) is found in the western US, and in Canada, and Mexico [1-3]. Creeping juniper (*J. horizontalis* Moench) is mostly found in the northcentral and northwestern US, and in all provinces of Canada [3]. Common juniper (*J. communis* L.) is found in most US states [3], and is one of the most widely distributed woody species in the Northern Hemisphere due to its great ecological adaptation. Juniper leaves, juniper wood, and juniper berries contain relatively high concentrations of essential oil with pleasant aroma that has applications as an aromatic agent in a number of consumer products and also in aromatherapy. Among the three species, common juniper, *J. communis* has been subject to a number of research projects and hence, there have been numerous reports on its essential oil profile and bioactivity [7-10]. Research reports on *J. scopulorum* essential oil are also available [11-15]. However, there are relatively fewer reports on the essential oil content and composition of *J. horizontalis* [16-18]. Also, there are no reports on the relative essential oil composition of the three species of junipers from the same ecological region. Therefore, the objective of this study was to evaluate variations in leaf essential oil (EO) content and composition of *Juniperus* species in the Bighorn Mountains in Wyoming, USA; *J. communis*, *J. horizontalis*, and *J. scopulorum*.

The three junipers differed in essential oil content and composition. The gas chromatography (GC) analysis revealed that the essential oil of the three juniper species had different chemical profiles. Seventy-six constituents were identified in the oil of *J. scopulorum*, 77 in the oil of *J. communis*, and 43 in the oil of *J. horizontalis*. The overall essential oil content and composition of the three junipers are presented in Table 1. The major essential oil constituent of *J. communis* was α-pinene, whereas that of *J. horizontalis* and *J. scopulorum* was sabinene (Table 1). The concentration of sabinene was higher in the oil of *J. horizontalis*, whereas limonene was found in higher concentration in the oil of *J. scopulorum* (Table 1). Myrcene, α-terpinene, and terpinolene were present in the oils of the three junipers, but *J. communis* oil had greater concentrations of myrcene and α-terpinene compared with the ones in the oil of the other two junipers. Conversely, the oils of *J. horizontalis* and *J. scopulorum* had greater concentration of terpinolene than the oil of *J. communis* (Table 1). Also, while the concentrations of 4-terpinenol, pregeijerene B, elemol, and 8-α-acetoxyelemol in the oils of *J. horizontalis* and *J. scopulorum* were well above 1%, these compounds were either under 1% or not detected in the oil of *J. communis* (Table 1). The concentrations of 4-terpinenole and 8-α-acetoxyelemol were greater in the oil of *J. horitontalis*, whereas the concentrations of pregeijerene B and elemol were higher in the oil of *J. scopulorum*. The results suggest that while the essential oil composition of *J. horizontalis* and *J. scopulorum* is similar, the profile of the *J. communis* oil is quite different from those of the oils of the other two junipers.

Table 1: Mean EO content and concentration (%) of main compounds from three species and their accessions showing significant differences. Accession nested within species effect (top 25 rows) and significant species effect (bottom 3 lines). *JC = J. communis, JH = J. horizontalis, JS = J. scopulorum*.

Species/ Accessions	EO content %	α-Thujene	α-Pinene	Sabinene	Myrcene	α-Terpinene	Limonene	Unknown	λ-Terpinene	Terpinolene	4-Terpinenol	Pregeijerene B	Elemol	8-α-Acetoxyelemol	β-Pinene
JC/101	0.4 i*	n.d.	66.6 f	n.d.	8.5 b	n.d.	n.d.	2.0 abc	n.d.	1.5 b	n.d.	n.d.	n.d.	n.d.	3.8 f
JC/102	1.0 e	n.d.	79.6 a	n.d.	3.3 fg	n.d.	n.d.	1.8 d	n.d.	0.8 k	n.d.	n.d.	n.d.	n.d.	4.1 bc
JC/103	0.4 i	n.d.	69.6 e	n.d.	11.9 a	n.d.	n.d.	2.2 a	n.d.	0.4 m	n.d.	n.d.	n.d.	n.d.	4.7 a
JC/106	0.6 h	n.d.	71.0 e	n.d.	3.4 f	n.d.	n.d.	2.1 ab	n.d.	1.4 de	n.d.	n.d.	n.d.	n.d.	3.9 e
JC/110	1.0 e	n.d.	74.6 bc	n.d.	3.3 f	n.d.	n.d.	1.9 cd	n.d.	0.9 ij	n.d.	n.d.	n.d.	n.d.	3.9 de
JC/111	0.9 ef	n.d.	75.2 b	n.d.	7.7 c	n.d.	n.d.	2.0 bc	n.d.	0.9 ij	n.d.	n.d.	n.d.	n.d.	3.3 g
JC/112	0.9 ef	n.d.	72.9 d	n.d.	2.9 hi	n.d.	n.d.	2.1 ab	n.d.	1.0 hi	n.d.	n.d.	n.d.	n.d.	3.3 g
JC/113	1.8 ab	n.d	72.9 d	n.d	3.0 gh	n.d	n.d	1.9 cd	n.d	0.9 ij	n.d	n.d	n.d	n.d	3.7 f
JC/114	1.6 c	n.d.	73.3 cd	n.d.	4.8 e	n.d.	n.d.	2.1 ab	n.d.	0.9 jk	n.d.	n.d.	n.d.	n.d.	4.0 cd
JC/115	0.6 h	n.d.	76.1 b	n.d.	5.3 d	n.d.	n.d.	2.1 ab	n.d.	0.7 l	n.d.	n.d.	n.d.	n.d.	4.1 b
JC/121	1.6 bc	n.d.	71.3 e	n.d.	3.4 f	9.4 a	n.d.	2.1 ab	n.d.	1.1 gh	n.d.	n.d.	n.d.	n.d.	4.1 b
JH/116	1.3 d	1.9 b	6.1 hi	57.6 a-d	2.0 l	2.1 fg	3.4 g	n.d.	3.5 de	1.4 cd	4.4 def	4.3 de	5.6 ab	1.9 cd	n.d.
JH/117	1.6 c	1.8 bc	2.4 k	61.0 a	2.0 l	1.7 hi	2.8 ij	n.d.	2.9 f	1.3 def	3.9 fg	4.4 cde	3.5 cd	2.0 cd	n.d.
JH/118	1.3 d	1.8 bc	6.6 h	56.6 b-e	2.6 ij	2.3 de	2.7 jk	n.d.	3.8 bc	1.4 cd	5.5 ab	2.3 g	1.7 f	1.2 d	n.d.
JH/119	1.2 d	1.9 b	3.6 jk	60.2 ab	2.9 hij	2.2 ef	2.9 hi	n.d.	3.7 cde	1.8 a	5.2 abc	4.4 cde	2.3 ef	1.6 d	n.d.
JS/104	0.8 efg	1.6 cd	2.8 k	55.4 cde	2.6 j	2.6 b	2.7 jk	n.d.	4.5 a	1.7 a	5.9 a	4.6 cd	5.1 ab	3.1 b	n.d.
JS/105	0.7 gh	1.3 e	2.3 k	43.3 hi	2.0 l	1.9 gh	7.2 e	n.d.	3.4 e	1.4 de	4.1 ef	4.8 c	6.0 a	3.3 b	n.d.
JS/107	0.8 efg	1.4 de	3.6 jk	51.2 f	1.9 lm	2.3 de	3.1 h	n.d.	3.9 bc	1.6 b	5.5 ab	8.5 a	5.6 ab	4.3 a	n.d.
JS/108	0.9 efg	1.7 cd	3.2 jk	53.3 ef	2.3 k	2.2 ef	5.0 f	n.d	3.8 bcd	1.4 de	4.7 c-e	4.0 ef	2.5 ef	2.7 bc	n.d
JS/109	0.9 ef	0.7 f	13.1 g	13.2 j	2.8 hij	1.1 j	10.8 d	n.d.	1.9 h	1.2 fg	4.2 ef	n.d.	n.d.	n.d.	n.d.
JS/125	1.5 c	1.9 b	2.9 k	58.8 abc	1.8 mn	1.8 hi	10.1 d	n.d.	2.9 f	1.2 f	3.9 fg	3.8 f	3.2 cde	1.5 d	n.d.
JS/126	1.8 a	1.5 de	5.4 hi	47.3 g	1.6 n	1.6 i	22.0 a	n.d.	2.6 g	1.3 ef	3.7 fg	4.1 ef	2.0 f	1.6 d	n.d.
JS/127	0.9 ef	1.8 bc	6.3 h	41.0 i	1.7 mn	2.6 bc	15.3 c	n.d.	4.4 a	1.6 b	5.1 bcd	2.7 g	3.2 de	2.0 cd	n.d.
JS/128	1.2 d	1.9 b	4.6 ij	46.4 gh	1.6 n	2.1 efg	17.2 b	n.d.	3.5 de	1.5 b	4.0 ef	4.7 cd	3.2 cde	1.7 d	n.d.
JS/129	0.8 fgh	2.3 a	5.3 hi	54.3 def	1.8 mn	2.4 cd	2.5 k	n.d	4.0 b	1.5 bc	3.3 g	6.5 b	4.3 bc	2.8 bc	n.d.
JC	1.0 c	n.d.	73.0 a	n.d.	4.8 a	n.d./9.4 a	n.d.	2.0	n.d.	1.0 b	n.d.	n.d.	n.d.	n.d.	3.9
JH	1.3 a	1.9 a	4.8 b	58.8 a	2.3 b	2.1 b	2.9 b	n.d.	3.5 a	1.5 a	4.8 a	3.9 b	3.1 b	1.7 b	n.d.
JS	1.1 b	1.6 b	4.9 b	44.6 b	2.0 c	2.1 b	7.6 a	n.d.	3.5 a	1.4 a	4.4 b	4.8 a	3.8 a	2.5 a	n.d.

* within each column and each effect (Accession nested within species, and species), means sharing the same letter are not significantly different at the 5% level using Tukey's method.

The oils of *J. scopulorum* and *J. horizontalis* had significantly higher antioxidant capacity (p<0.05, Tukey's method) than the oil of *J. communis* measured using $ORAC_{oil}$ method (58.7, 59.5, and 11.8 μM/g, respectively). Also, the essential oil of the three junipers did not exhibit significant antimicrobial activity against the 10 microorganisms *Candida albicans*, *C. glabrata*, *C. krusei*, *Aspergillus fumigatus*, *Cryptococcus neoformans*, *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Mycobacterium intracellulare* (data not shown).

J. communis. In this study, the major oil constituent of *J. communis* was α-pinene, whereas sabinene was the major oil constituent of *J. horizontalis* and *J. scopulorum* essential oils. The results of this study agree with those reported previously by Adams *et al.* [7] who conveyed that the oil of several varieties of *J. communis* from the US contained high concentration of α-pinene (up to 60%) and moderate concentrations of β-pinene. The concentration of β-pinene in our samples of *J. communis* was similar to that reported by Adams *et al*. [7]. Previous reports indicated α-pinene as the major constituent of *J. communis* leaf essential oil [19]. Interestingly, in a recent study of *J. communis* in Canada, Kilic *et al*. [20] did not find much α-pinene in the oil; the authors reported that the main compounds were limonene (26.1%), benzene (15.6%), β-myrcene (9.1%) and β-pinene (7.3%). Other authors also reported sabinene rather than α-pinene as the major oil constituent of *J. communis* leaf oil [21-23]. Stoyanova [24] reported β-pinene as the main constituent of *J. communis* leaf oil from Bulgaria. Indeed, Adams *et al*. [7] reported variations in α-pinene concentration of *J. communis* oil depending on the variety and the collections location. Filipowicz *et al.* [10] reiterated that the essential oil composition of *J. communis* and its major constituents may vary significantly depending on the environment (geography). In our study, the essential oil content (yield) of *J. communis* varied broadly, from 0.4 to 1.8% in fresh herbage. Butkiene *et al*. [25] reported that oil content of *J. communis* can reach up to 0.5%.

J. horizontalis. The results from a recent study from Canada [26] deviate substantially from other reports on *J. horizontalis* oil composition. According to the latter study [26] the main compounds found in the oil of leaves were linalool (33.8%), P-cymene (23.2%), and λ-terpinene (8.7%). Ehsani *et al*. [27] reported other main components in the essential oil as sabinene (30.2%-38%), followed by bornyl acetate (10.7%) and that the essential oil showed significant antimicrobial effect against 12 species from 13 tested bacteria species. Another study by Vinutha and von Rudloff [28] found that sabinene was the main constituent in the essential oil of *J. horizontalis* and that the constituents identified in the essential oils of *J. horizontalis* and *J. scopulorum* were similar, suggesting a close phylogenetic relationship.

Adams [17] reported a rather unusual EO profile of *J. horizontalis* from Saskatchewan River bank, Saskatoon, Saskatchewan, Canada, with the major oil constituents sabinene (37.2%) and limonene (3.5%), and with α-pinene only 1.7% [17]. In our study, the essential oil from *J. horizontalis* collected from the Bighorn Mountains in Wyoming had approximately 1.6 times higher concentration of sabinene (57-61%), higher α-pinene, and similar limonene concentrations compared with the results reported by Adams [17].

J. scopulorum. Adams and Hagerman [29] reported sabinene as the major oil constituent of greenhouse grown *J. scopulorum* ranging from 33 to 39% of the oil. Sabinene was the major constituent in the essential oil of *J. scopulorum* collected in the Bighorn Mountains in Wyoming. Adams [30] reported sabinene (46.3%) as the major oil constituent of *J. scopulorum* sampled from Durango, CO. Sabinene concentration in the essential oil obtained from most of the *J. scopulorum* accessions ranged from 41 to 55%, whereas in one of the accessions, sabinene concentration was only 13%. Adams and Powell [11] reported very small differences in leaf essential oil composition between male and female trees. Earlier studies also reported sabinene as the major constituent of the oil from leaves of *J. scopulorum* [31].

Studies emphasized the importance of the duration of the distillation time on *J. scopulorum* oil profile [14, 15, 32]. Cantrell *et al*. [32] reported that the major oil constituent, sabinene, varied from 30 to 47% of the oil, limonene from 29-31%, and α-pinene from 4.2 to 9.6%, among other constituents [32]. Zheljazkov *et al*. [14] reported most oil constituents changed in their concentration depending on the duration of distillation; e.g. sabinene varied from 51 to 77% of the oil of a single female tree [14], while sabinene concentration in male trees varied from 45 to 75% of the total oil [15]. *J. scopulorum* oil composition has also been shown to depend on sex [11, 33]. In the latter studies, the authors reported that the concentrations of α-pinene, α-terpinene, λ-terpinene, terpinolene, pregeijerene B, elemol, β-eudesmol/α-eudesmol, and 8-α-acetoxyelemol were greater in the oil of the female tree than in that of the male tree. Conversely, the concentrations of α-thujene, sabinene, myrcene, limonene, and δ-cadinene were greater in the oil of the male tree than in that of the female tree [33]. Samples of *J. scopulorum* accessions collected in the Bighorn Mountains in Wyoming in this study included female and male trees.

In conclusion, the leaf essential oil composition of *J. scopulorum* and *J. horizontalis* is similar, whereas that of *J. communis* is different. The oils of *J. scopulorum* and *J. horizontalis* had higher antioxidant capacity than the oil of *J. communis.* There is large variation in the chemical composition of the leaf oil within each of the three species, suggesting the occurrence of chemotypes within the area of Bighorn Mountains in Wyoming. The existence of various oil profiles within a species may be used for cultivar selection with desirable oil composition to meet industry needs utilizing juniper oil.

Experimental

Plant collection: The collection of *Juniperus* was conducted in August and September 2011 across the Bighorn Mountains in Wyoming at altitudes between 1,450 m and 2,884 m above sea level. We collected approximately 3.5 kg of fresh material (leaves and branches less than 5 mm thick), from the lower, middle and upper level of each individual, naturally occurring, visually healthy plants in sites away from roads. GPS coordinates (altitude, latitude, longitude) and a digital photo were taken and recorded for every single tree/site. In addition, we recorded data on tree height and sex. Representative subsamples from each sample were identified by Ms Bonnie Heidel, a botanist at the Wyoming Natural Diversity Database, University of Wyoming. Subsamples from each collection site were deposited in the University of Wyoming Rocky Mountain Herbarium. From each sample, we generated 3 sub-samples, for essential oil extraction.

Extraction of the essential oil: The steam distillations were conducted as described previously [14, 34]. Each distillation sample was 500 g of fresh biomass (collected at different heights and from different sides of the tree), chopped into approximately 2.5 cm pieces, and immediately distilled in 2 L steam distillation units (Hearthmagic, Rancho Santa Fe, CA). The duration of the steam distillation for each sample was 360 min; this time was established in previous research on *J. scopulorum* juniper leaves [14-15]. The beginning of the distillation process was recorded when the first drop of oil was deposited in the Florentina part of the distillation apparatus. At the end of each distillation, the power was switched off, the stillhead removed from the rest of the apparatus, the oil collected, measured on an analytical scale, and kept in a freezer at -5°C until the gas chromatography analyses could be performed. The essential oil content (also indicated as yield) was calculated by weight, as g of oil per 100 g of fresh herbage, and expressed as percentage of oil in the fresh juniper biomass.

Gas chromatography analysis of the juniper essential oil: The essential oil of all junipers were analyzed using a Hewlett Packard gas chromatograph (GC) model 6890, fitted with a GC column HP-INNOWAX (cross-linked PEG; 30 m × 0.32 mm × 0.5 μm) and an auto sampler. The FID detector temperature was 275 °C, and the carrier gas was helium {40 cm/sec, 11.7 psi (60°C), 2.5 mL/min constant flow rate; injection: split 60:1, 0.5 μL, inlet 220°C; oven temperature program: 60°C for 1 min, 10°C/min to 250°C). The identification of the compounds was made by GC-MS, and by comparison of mass spectra with the ones reported in the mass spectral database of the National Institute of Standards and Technology.

Antioxidant capacity of J. communis, J. horizontalis, and J. scopulorum oils: Antioxidant capacity of *J. communis* (accession #114)*, J. horizontalis* (accession #116), and *J. scopulorum* oils (accession #120) (in 3 replicates plus 3 internal replicates = 9 total replications per sample) were determined using the oxygen radical absorbance capacity (ORACoil) method [35-36]. Specific details regarding this method were previously described by Zheljazkov *et al.* [33]. The antioxidant capacity of the oil samples of the 3 junipers was measured using Trolox as a standard (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid); the results are expressed as μmole Trolox g^{-1}.

Antimicrobial activity testing: The antimicrobial testing of essential oils from the 3 juniper species was performed at the NCNPR, University, MS. The essential oils (all in 2 replicates) were tested for activity against *Candida albicans*, *C. glabrata*, *C. krusei*, *Aspergillus fumigatus*, *Cryptococcus neoformans* and for antibacterial potential against Gram +ve bacteria *Staphylococus aureus*, methicillin-resistant *S. aureus* and *Mycobacterium intracellulare* and Gram–ve bacteria *Escherichia coli* and *Pseudomonas aerogenosa* at a concentration of 50 μg/mL, and percent inhibition was calculated following the method published previously [37]. The antifungal activity was tested using amphotericin B, and the antibacterial drug control was ciprofloxacin.

Statistical analysis: The effect of species and accession on the essential oil content and the concentrations of α-thujene, α-pinene, sabinene, myrcene, α-terpinene, limonene, limonene/β-phellandrene (unknown), λ-terpinene, terpinolene, 4-terpinenol, pregeijerene B, elemol, 8-α-acetoxyelemol, and β-pinene was completed using a Nested design with species, and accession nested within species effects in the model. The analysis was completed using the Mixed Procedure of SAS [38], and the validity of model assumptions (normal distribution and constant variance of the error terms) was verified by examining the residuals as described in Montgomery [39]. Transformations were applied for some of the constituents to achieve normality; however, the means shown in Table 1 are back-transformed to the original scale. Since both species and accession effects were significant, multiple means comparisons of the species, and the accession nested in species (both shown in Table 1) were completed using Tukey's Multiple Means Comparison method at the 5% level of significance.

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Chemical Composition and Antibacterial Activity of *Angelica archangelica* Root Essential Oil

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Roots of wild growing *Angelica archangelica* L. from Mt. Ozren (Serbia) were subjected to hydrodistillation and GC-MS analysis. The roots contained 0.10% of essential oil with α-pinene (29.7%), δ-3-carene (14.2%), and a mixture of β-phellandrene and limonene (13.2%) as main compounds. The modified resazurin microtiter-plate assay was used to evaluate the antibacterial activity of the essential oil against *Staphylococcus aureus* and *Escherichia coli*. The minimum inhibitory concentration (MIC) values were 14.2 µL/mL for *S. aureus* and 28.4 µL/mL for *E. coli*, while the minimum bactericidal concentrations (MBC) were 56.8 µL/mL and 113.6 µL/mL, respectively. According to the obtained results, the angelica root essential oil can be applied as a natural preservative in food and as a natural antibiotic for the treatment of several infectious diseases caused by these two bacteria.

Keywords: Angelica, *Staphylococcus aureus*, *Escherichia coli*, MIC, MBC.

Angelica archangelica L. (syn. *A. officinalis* Hoffm.),Apiaceae, is distributed throughout Northern Europe and Eastern Siberia, and is cultivated in Europe. The wild grown type is rare in the Serbian flora and there are attempts at its cultivation in the mountainous regions of central Serbia [1, 2]. Angelica has been used in folk medicine and as a food ingredient. The rhizome with roots is used for treatment of gastrointestinal problems [3]. However, it is established that angelica also possesses anxiolytic, hepatoprotective, antimicrobial and antioxidant effects [4].

Essential oils possess different biological properties due to their chemical diversity. The aim of our investigation was to determine the chemical composition and antibacterial activity of root essential oil of a population of *A. archangelica* from Serbia. A total of 59 compounds were detected in the essential oil (AREO) (99.3% of the total oil), including 15 that were unidentified (3.6%). The main components were α-pinene (29.7%), δ-3-carene (14.2%), and a mixture of β-phellandrene and limonene (13.2%). Other important compounds were sabinene, α-phellandrene, myrcene, *p*-cymene and *trans*-β-ocimene. All other compounds constituted less than 2% (Table 1).

Essential oil composition varies depending on many factors, including origin and variety. The AREO from France contained α-pinene (32.2%) and δ-3-carene (16.2%) as the main compounds [5], whereas in Italy they wereα-pinene (21.3%) and δ-3-carene (16.5%), followed by limonene (16.4%) and α-phellandrene (8.7%) [6]. The dominant compounds in the AREO from Siberia were β-phellandrene (30.5%) and α-pinene (23.6%) [7]. According to [8], there are two chemotypes of AREO, differing mainly in either the absence or presence of β-phellandrene.

The antibacterial activity was assessed by the MIC using the resazurin assay [9]. *S. aureus* was more sensitive to AREO than *E. coli*. The oil at a concentration of 14.20 µL/mL inhibited growth of *S. aureus*, while the MIC for *E. coli* was higher, 28.4 µL/mL (Table 2).

Table 1: Angelica root essential oil composition.

No	Compound*	Rt	RI	%
1	α-Thujene	5.651	927	0.5
2	α -Pinene	5.844	934	29.7
3	Camphene	6.227	948	1.1
4	Thuja-2,4(10)-diene	6.373	954	0.1
5	Sabinene	6.898	973	6.1
6	β-Pinene	7.005	977	1.8
7	Myrcene	7.385	990	4.1
8	δ-2-Carene	7.723	1002	tr
9	α-Phellandrene	7.840	1005	5.7
10	δ-3-Carene	8.038	1011	14.2
11	α-Terpinene	8.248	1017	0.5
12	*p*-Cymene	8.502	1024	3.8
13,14	β-Phellandrene + Limonene	8.670	1028	13.2
15	*cis*-β-Ocimene	8.945	1036	1.4
16	*Trans*-β-Ocimene	9.318	1046	3.6
17	γ-Terpinene	9.729	1058	1.0
18	Terpinolene	10.858	1086	1.2
24	*p*-Mentha-1,5-dien-8-ol	14.081	1165	0.3
25	Terpinen-4-ol	14.518	1175	1.1
27	Cryptone	14.922	1184	0.3
30	Bornyl acetate	19.299	1283	0.4
32	Cyclosativene	22.935	1364	tr
33	Cubenene	23.118	1374	tr
34	α-Copaene	23.312	1378	0.7
35	β-Elemene	24.038	1391	tr
36	*trans*–Caryophyllene	25.198	1418	0.1
37	β-Copaene	25.631	1429	0.1
38	β-Barbatene	26.165	1441	0.1
39	α-Humulene	26.653	1453	0.6
40	*Trans*-Muurola-4(14),5-diene	27.845	1481	0.2
41	α-Muurolene	28.662	1501	0.3
42	Cuparene	28.868	1506	0.1
43	β-Bisabolene	29.033	1510	0.3
45	δ-Cadinene	29.625	1524	0.2
46	α-Copaene-11-ol	30.317	1540	1.3
48	β-Gemacrene	30.979	1558	0.1
49	β-Copaene-4-alpha-ol	31.198	1562	0.4
50	Spathulenol	31.817	1577	0.1
54	Humulene epoxide II	33.072	1608	0.3
55,56	Oxacyclotetradecane-2-one + NI	33.777	1626	0.5
57	β-Eudesmol	34.679	1650	0.1
58	Cyclopentadecanolide	41.271	1830	0.2
59	Osthole	51.751	2150	0.1

*Compounds listed in order of elution on a HP-5MS column (Rt - retention time, RI - retention index), tr- compound present less than 0.1%, NI – Unidentified compound.

Table 2: The minimum inhibitory concentration (MIC) of angelica root essential oil*.

Essential oil concentration (µL/mL)	454.40	227.25	113.62	56.81	28.40	14.20	7.10	3.55	1.77	0.88	0.44	0.22
Escherichia coli												
Staphylococcus aureus												

*Plates after 24 h in modified resazurin assay (grey color indicates growth and white means inhibition of growth)

According to published data, the principal constituents in our AREO, α-pinene, and limonene showed considerable activities against *E. coli* and *S. aureus* [10, 11] while δ-3-carene was inactive [11]. The AREO from central Italy, similar in composition to our sample, possesses activity against *Clostridium difficile*, *C. perfringens*, *Enterococcus faecalis*, *Eubacterium limosum*, *Peptostreptococcus anaerobius* and *Candida albicans*, with MIC values of 0.25, 0.25, 0.13, 0.25, 2.25, and 0.50 v/v, respectively [6]. The MBC values in our study were slightly higher than the MIC ones. The lowest concentration of AREO which reduced the viability of the initial bacterial inoculums for *S. aureus* was 56.8 µL/mL, while for *E. coli* the MBC was 113.6 µL/mL. As a positive control, gentamicin was used with MIC/MBC of 0.25/0.75 µg/mL for *S. aureus* and 0.50/1 µg/mL for *E coli*. Determination of MIC by gentamicin MIC Test Strip (Liofilchem®) also provided a MIC value of 0.25 µg/mL for *S. aureus* and 0.5 µg/mL for *E. coli.*

Experimental

Plant material: Roots of *A. archangelica* were collected from wild plants near Aleksinac at Mt. Ozren (Serbia). A voucher specimen (No 2-1575) were confirmed and deposited at the BUNS Herbarium, University of Novi Sad. Roots were dried and ground. The powdered material was subjected to distillation in a Clevenger apparatus; the yield of essential oil was 0.10%.

GC-FID and GC-MS analyses were carried out with an Agilent 7890A apparatus equipped with a 5975C mass-selective detector, a flame ionization detector, and a HP-5MS fused-silica capillary column (30 m x 0.25 mm i.d., 0.25µm film thickness). Temperature program: 60°C to 285°C at a rate of 4.3°C/min. Carrier gas He; inlet pressure 25 kPa; linear velocity 1 mL/min at 210°C. Injector temperature: 250°C; splitless. MS conditions: source temperature, 200°C; interface temperature, 250°C; energy, 70 eV; mass scan range, 40-350 amu. Compound identification was made based on retention index, retention times, and by comparison with reference spectra (Wiley and NIST databases). The percentage of each compound was calculated from peak area obtained by FID.

The antimicrobial activity was evaluated using control strains obtained from the American Type Culture Collection: *Escherichia coli* (ATCC 8739) and *Staphylococcus aureus* (ATCC 25923). The activity was tested by a modified broth microdilution method according to the National Committee for Clinical Laboratory Standards [12]. A serial doubling dilution of the tested essential oil was prepared in a 96/well microtiter plate over the range of 454.4-0.22 µL/mL in inoculated Mueller-Hinton broth (MHB, HiMedia). The mixture was discharged from the last well in row 100 µL. The test was performed in a total volume of 110 µL/mL with final microbial concentration 10^6 CFU/mL per well. The plate was incubated for 24 h at 37°C. The same tests were performed simultaneously for growth control (MHB + test organism), sterility control (MHB + test oil), and positive control (MHB + gentamicin + test organism). Gentamicin was prepared in sterile water and diluted in MHB to obtain concentrations in a range of 16 to 0.016 µg/mL. Additionally, susceptibility to gentamicin was confirmed using a quantitative assay for determining the MIC (gentamicin MIC Test Strip (Liofilchem®) according to the manufacturer's instructions. Microbial growth was determined by adding 10 µL of 0.01% resazurin (7-Hydroxy-3*H*-phenoxazin-3-one 10-oxide, HiMedia) aqueous solution. The MIC was defined as the lowest concentration of the samples inhibiting visible growth (blue colored pellet on the bottom of the wells after the addition of resazurin). To determine the MBC, the broth was taken from each well without visible growth and inoculated in Mueller-Hinton agar (MHA) for 24 h at 37°C. The MBC was defined as the lowest samples concentration killing 99.9% of bacterial cells.

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Gas Chromatography-Mass Spectrometry (GC-MS) Combined with Retention Index Prediction for the Rapid Identification of Halogenated monoterpenes from a Namibian *Plocamium* species

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Plocamium species collected from the Namibian coast display morphological features similar to those of both *P. rigidum* and *P. suhrii* which makes identification of these species a difficult task. It has been reported that the major secondary metabolites found in various *Plocamium* species are unique to each species [1]. In this study GC-MS combined with a retention index (RI) prediction strategy was used for the rapid identification of halogenated monoterpenes characteristic of a particular Namibian *Plocamium* species. The RIs of the metabolites were matched with the predicted RIs of halogenated monoterpenes for which similar MS data have been reported for the same species of *Plocamium*. Based on the identification of the major secondary metabolite, 1*E*,3*R*,4*S*,5*E*,7*Z*-1-bromo-3,4,8- trichloro-7-(dichloromethyl)-3-methylocta-1,5,7-triene [2], it was proposed that these Namibian samples are closely related to that of *P. suhrii*. From this, it was determined that the proposed *P. suhrii* specimens collected in Namibia contain four additional metabolites (with molecular formulae $C_{10}H_{16}Br_2Cl_2$, $C_{10}H_{11}BrCl_4$, $C_{10}H_9BrCl_6$ and an unknown compound) previously not reported in *P. suhrii* species. In addition, a compound previously identified in South African *P. suhrii* was not present in the Namibian *Plocamium* specimens.

Keywords: *Plocamium*, GC-MS, Retention index prediction, Chemotaxonomy, Halogenated monoterpenes.

A wide variety of polyhalogenated monoterpenes are found in red algae (Rhodophyta) of the genus *Plocamium*. These compounds display a range of biological activities including antimicrobial, antitubercular and anticancer activities [3-8]. Although the major secondary metabolites found in various *Plocamium* species are unique to each species, some degree of overlap occurs with regards to some of the minor secondary metabolites [1]. Certain seaweed species can also be similar morphologically, but genetically different, making taxonomic identification by visual inspection difficult. In contrast to this, some specimens may display varied morphologies, while in actual fact they are the same species [9]. Chemotaxonomy can therefore play an important role in distinguishing different species and perhaps also variants of the same species from one another. In a recent study, *Plocamium* specimens collected from the Namibian coast displayed morphological features similar to those of both *P. rigidum* and *P. suhrii*, but an unequivocal visual taxonomic identification was not possible. The major metabolite extracted from these specimens was identified as 1*E*,3*R*,4*S*,5*E*,7*Z*-1-bromo-3,4,8-trichloro-7-(dichloro-methyl)-3-methylocta-1,5,7-triene (compound **8** in Table 1) [2]. This is the major metabolite previously identified in *P. suhrii*, along with six other halogenated monoterpenes [8], suggesting that these Namibian specimens are chemically related to *P. suhrii*.

Similar types of halogenated monoterpenes have been isolated from other *Plocamium* species such as *Plocamium hamatum* [6], *Plocamium cartilagineum* [5] and *Plocamium costatum* [7]. However, none of these secondary metabolites have the exact structures of the compounds described and proposed in this paper. This illustrates the considerable diversity of this genus in terms of the chemical variation of halogenated monoterpenes. It also supports the notion that different species of *Plocamium* can be identified by chemotaxonomy because they produce unique major metabolites. For example, *Plocamium hamatum* [6], *Plocamium cartilagineum* [5] and *Plocamium costatum* [7] all had major metabolites which differed from one another and were unique to the species being studied (according to the amounts extracted in terms of mg). In addition, several minor metabolites characterised in these studies have been previously discovered and reported from other species. This supports the perception that there is some overlap between the species with regards to some of the minor secondary metabolites. Variations in the minor metabolites between variants of *P. suhrii* that differ morphologically have not yet been fully investigated. Although rapid identification of halogenated monoterpenes could for instance be facilitated with the use of an HPLC-PDA-MS-NMR approach [10], the equipment used to achieve this is much more expensive than that of a GC-MS instrument. In addition, there are many challenges associated with performing LC-NMR analysis and skilled operators are necessary to perform these experiments as well as interpret the data. Therefore, in this study GC-MS was used for the rapid identification of halogenated monoterpenes in proposed *P. suhrii* specimens collected on the Namibian coastline in order to investigate how their minor metabolites compare to those found in South African *P. suhrii* specimens (previously investigated by Antunes and co-workers [8]. Unfortunately, limited MS data is available for halogenated monoterpenes found in *Plocamium* species (e.g. the mass spectra of most of these compounds do not appear in the latest NIST mass spectra database) while no retention index (RI) data have been reported to date. In the absence of reference standards, confidence in the identities of the compounds can be gained from *predicted* (or estimated) retention indices (RIs) [11], published MS data and knowledge of the structural properties of compounds that have already been identified from known *Plocamium* species [1, 8, 12-14]. This approach has been used successfully in the past for a number of applications [15].

According to Stegenga *et al*. [16] *P. suhrii* plants are richly branched and complanate and can reach a height of up to 15 cm. It is commonly found in the lower intertidal pools and sublittoral where it can be recognized by its bright red colour, sometimes fading to pink especially under water. While it is not easily distinguished from other *Plocamium* species due to the overlap in characteristics, its axes never exceeds 2 mm in width. In addition, the groupings consist mainly of two laterals although apically the laterals can be up to three, although unusual. Based on our observations, *P. suhrii* grows along the central coast of Namibia (Swakopmund to Henties Bay), and according to Stegenga *et al*. [16] it should at least extend to the southern part of Namibia. However, a prior study conducted by Lluch [17] along the northern half of the Namibian coastline failed to observe any *P. suhrii*, attributing all the forms of *Plocamium* collected in the area to either *P. rigidum* or *P. glomeratum*. In actual fact most of our samples resemble a cross between *P. suhrii* and *P. rigidum* although the forms were generally inclined towards *P. suhrii* (Figure 1). As pointed out by Stegenga *et al*. [16], the taxonomy of *Plocamium* is in need of clarification and thus we cannot be certain that the specimens used in this study are indeed *P. suhrii* until DNA analysis has taken place.

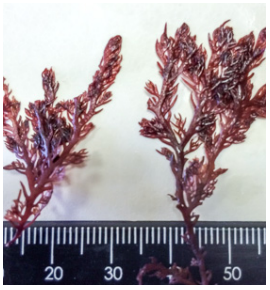


Figure 1 Photograph of a Namibian *Plocamium* sample

GC-MS analyses of the five Namibian *Plocamium* extracts revealed that they were qualitatively identical and quantitatively very similar. The total ion chromatogram (TIC) of one of the extracts is depicted in Figure 2. A GC equipped with a flame ionisation detector (FID) was used to determine the relative amounts of the one major and 11 prominent minor compounds (see Supporting Information). The results confirmed that all compounds were present in similar relative concentrations in all five samples (Table 1). The twelve compounds could be tentatively identified as halogenated monoterpenes (Table 1), by comparison of their electron ionisation mass spectra with MS data found in the literature [12-13]. Comparison of the mass spectra with those in one of the latest mass spectra databases (NIST 11 Mass Spectral Library) did not yield any suitable library matches. Furthermore, the molecular ions could not be observed in any of the mass spectra of the detected compounds. In addition, no RI values could be found for these compounds, either in the NIST RI database or any other published literature.

However, in a recent study the major metabolite (relative abundance >88%; GC-FID) was identified as 1*E*,3*R*,4*S*,5*E*,7*Z*-1-bromo-3,4,8-trichloro-7-(dichloro-methyl)-3-methylocta-1,5,7-triene using NMR [2]. This is the major metabolite previously identified in *P. suhrii* [8] and had also previously been isolated as a minor metabolite from *Plocamium cartilagineum* [12]. By comparison of the MS data and retention time of compound **8** with those of authentic reference material (from the previous study by Knott and co-workers [2]), compound **8** was identified as 1*E*,3*R*,4*S*,5*E*,7*Z*-1-bromo-3,4,8-trichloro-7-(dichloro-methyl)-3-methylocta-1,5,7-triene. With the

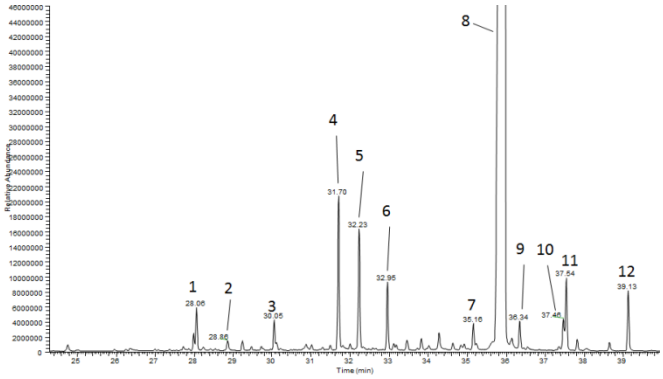
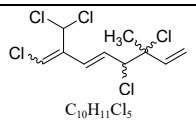
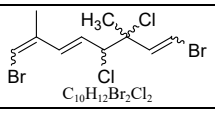
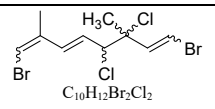
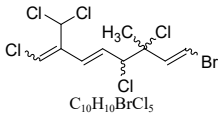
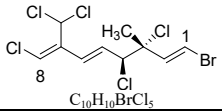
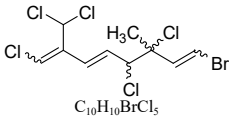
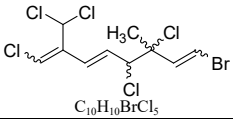
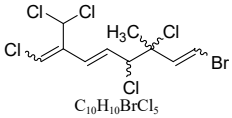


Figure 2 Total ion chromatogram of the methanolic extract of a Namibian *Plocamium* specimen.

structure of compound **8** known, it was possible to determine its RI value experimentally, compare it to the predicted value calculated by the group contributions approach (as described by Stein and co-workers [11]), and subsequently to predict the RIs of the remaining 11 halogenated monoterpenes in order to facilitate their rapid identification. According to Zenkevich and co-workers [18], predictions should be more accurate when adding or subtracting group contributions from the experimentally determined RI of a compound of which the structure is already known. The predicted RI value calculated for compound **8**, 2076 iu (h = 0), compared very well with the experimentally determined value, 2053 iu. The difference between these values was set as the correction factor, h = -23, and was used when calculating the predicted RI values for all the other compounds. Apart from possible errors inherent in the linear group increment approach (as described by Stein *et al*. [11]), this difference could also be observed due to the fact that the major limitation of the retention index prediction model is that it cannot distinguish between different isomers [11]. For instance, halogenated monoterpenes that are either diastereomers or *E/Z* isomers of compound **8** will be separable on a regular GC column, however, their predicted RIs calculated from group contributions will be identical. In this study, the structure of compound **8** was known, including which *E/Z*-isomer and which diastereomer it represents. The low resolution mass spectra of compounds **6**, **7**, **8**, **9**, **10**, **11** and **12** exhibited the same diagnostic ion at *m/z* 167, 169 (base peak), 171 (relative abundance: 3:4:1). This ion corresponds to a formula of $C_4H_5BrCl^+$ which is likely formed by the homolytic cleavage of the 3,4-bond of, for instance, compound **8** [12]. The mass spectra of compounds **7**, **9**, **10** and **11** are very similar to that of compound **8**. In light of the preceding arguments and the fact that their experimentally determined RI values only differ by -38, 24, 85 and 89 iu, respectively, from the predicted RI of compound **8** (after correction) suggested that these compounds are most probably isomers. Indeed, in addition to the major metabolite, two isomers of compound **8** have also been identified in *P. suhrii* [8]. One is the 3*R**,4*R** diastereomer of compound **8**, while the other is the 1*Z* isomer (but with 3*R**,4*S**). The remaining two isomers detected in the current study could possibly be diastereomers of the 7*E* isomer of compound **8**, previously identified in *P. rigidum* [14] and *P. cartilagineum* [12]. Compound **6** eluted significantly earlier and is therefore more likely a halogenated monoterpene with less halogen substituents. Subsequently, the predicted RI value of a compound with one less Cl than compound **8** was calculated. The resulting predicted RI value is only 42 iu less than the experimentally determined value, a difference which can again be ascribed to the fact that the prediction does not take isomers into account. The following molecular formula was proposed for compound **6**: $C_{10}H_{11}BrCl_4$. Although the mass spectrum of compound **12** also displayed some similarities with that of compound **8** (including the

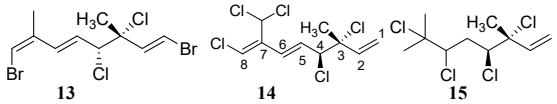
Table 1: Halogenated monoterpenes tentatively identified in the methanol extract of proposed *P. suhrii* found on the Namibian coastline

	Rt[a]	Predicted RI[b]	RI[c]	Diagnostic ions (*m/z*)	Proposed ion formula	Proposed molecular structure and/or molecular formula	Identification	Relative amounts[d] (*n* = 5)
1	28.06	1698	1674	133	$C_{10}H_{13}^+$	$C_{10}H_{16}Br_2Cl_2$	MS, RI	0.52 ± 0.40
2	28.86	-	1706	162, 164 (3:1)[e]	unknown	unknown	MS	0.40 ± 0.16
3	30.05	1743	1766	89, 91 (3:2)[e]	$C_4H_6Cl^+$	 $C_{10}H_{11}Cl_5$	MS, RI	0.64 ± 0.14
4	31.70	1788	1840	114, 116 (3:1)[e]	$C_6H_7Cl^+$	 $C_{10}H_{12}Br_2Cl_2$	MS, RI	2.34 ± 0.51
5	32.23	1788	1867	114, 116 (3:1)[e]	$C_6H_7Cl^+$	 $C_{10}H_{12}Br_2Cl_2$	MS, RI	1.74 ± 0.46
6	32.95	1860	1902	167, 169, 171 (3:4:1)	$C_4H_5BrCl^+$	$C_{10}H_{11}BrCl_4$	MS, RI	1.17 ± 0.23
7	35.16	2053	2015	167, 169, 171 (3:4:1)[e]	$C_4H_5BrCl^+$	 $C_{10}H_{10}BrCl_5$	MS, RI	0.58 ± 0.07
8	**35.98**	2053	2053	**167, 169, 171 (3:4:1)**[e]	$C_4H_5BrCl^+$	 $C_{10}H_{10}BrCl_5$	**MS, Rt comparison**	88.79 ± 1.61
9	36.34	2053	2077	167, 169, 171 (3:4:1)[e]	$C_4H_5BrCl^+$	 $C_{10}H_{10}BrCl_5$	MS, RI	0.54 ± 0.11
10	37.46	2053	2138	167, 169, 171 (3:4:1)[e]	$C_4H_5BrCl^+$	 $C_{10}H_{10}BrCl_5$	MS, RI	0.78 ± 0.30
11	37.54	2053	2142	167, 169, 171 (3:4:1)[e]	$C_4H_5BrCl^+$	 $C_{10}H_{10}BrCl_5$	MS, RI	1.28 ± 0.09
12	39.13	2207	2227	167, 169, 171 (3:4:1)[e]	$C_4H_5BrCl^+$	$C_{10}H_9BrCl_6$	MS, RI	1.23 ± 0.23

[a]Retention time of the compounds in the GC-MS total ion chromatogram; [b]Predicted retention indices, calculated according to the procedure described by Stein and co-workers [11], using the formula $RI = \sum_n f_n g_n + h$ with h = -23; [c]Kováts retention indices determined relative to the retention times of a series of alkanes analysed under the same conditions; [d]Relative amounts calculated as a percentage of the total compounds detected by GC-FID. The mean ± standard deviation is reported. [e]Relative intensities of isotope peaks.

base peak), additional ions are observed, for instance a very prominent ion at *m/z* 225 (isotope peaks at *m/z* 227 and 229; relative intensity 4:8:3; $C_6H_4BrCl_2^+$). In addition, compound **12** eluted significantly later and may therefore be an analogue which contains more halogen substituents. The following molecular formula was proposed for compound **12**: $C_{10}H_9BrCl_6$ (predicted and experimental RI values differ by only 20 iu). The base peak in the mass spectra of compounds **4** and **5** was observed at *m/z* 114 (together with its isotope peak at *m/z* 116; relative intensity ratio 3:1). Based on the isotope peak ratio, this peak was attributed to the presence of an ion with a formula $C_6H_7Cl^+$. The mass spectra of compounds **4** and **5** therefore correspond well to the MS data of compound **13** (Figure 3) and its diastereomer reported by Mynderse and Faulkner [12]. The predicted RI value for compound **13**, 1788 iu, corresponds well with the experimental values for compounds **4** and **5** (Table 1). Compound **13** and its corresponding diastereomer were also identified in *P. suhrii* [8] and therefore compounds **4** and **5** could possibly be these two diastereomers.

A prominent peak in the mass spectrum of the compound **3** was observed at *m/z* 89 (isotope peak at *m/z* 91; relative abundance 3:1). Mynderse and Faulkner [12] reported that the mass spectrum of compound **14** (Figure 3) exhibited the same peak (but as the base peak) which was attributed to the presence of an ion with a formula $C_4H_6Cl^+$ due to cleavage of the 3-4 bond. The fact that this compound has been identified in *P. suhrii* before [8] provided additional certainty that compound **3** is the same as (or an isomer of) compound **14**. Finally, the predicted RI of compound **14** compares very well to the experimentally determined RI of compound **3**, which provides additional corroborating evidence.



13 **14** **15**

Figure 3 Chemical structures of compounds **13 - 15**

The mass spectrum of compound **1** has a base peak at m/z 133 in addition to a series of other diagnostic ions at *m/z* 169 (isotope peak

m/z 171; intensity ratio 3:1), *m/z* 213 and 215 (1:1), *m/z* 249, 251 and 253 (3:4:1) and *m/z* 293, 295 and 297 (1:2:1). Based on the isotope peak ratios, these peaks represent ions with formulae $C_{10}H_{13}^+$, $C_{10}H_{14}Cl^+$, $C_{10}H_{14}Br^+$, $C_{10}H_{15}ClBr^+$ and $C_{10}H_{15}Br_2^+$. Hence the ion observed at m/z 213 and 215, $C_{10}H_{14}Br^+$, may be formed by the loss of HCl and HBr from $C_{10}H_{15}ClBr^+$ and $C_{10}H_{15}Br_2^+$, respectively. The ion observed at m/z 169 and 171, $C_{10}H_{14}Cl^+$, may therefore be formed by the loss of HBr from $C_{10}H_{15}ClBr^+$, while the ion observed at m/z 133, $C_{10}H_{13}^+$, may be formed by the loss of HCl and HBr from $C_{10}H_{14}Cl^+$ and $C_{10}H_{14}Br^+$, respectively. The formation of all these ions provides evidence that compound **1** is a mono-unsaturated compound that contains at least one chlorine and two bromine and atoms. Calculation of an RI based on such a monoterpenoid, but with an additional chlorine atom, yielded a predicted RI value, 1698 iu, close to the experimentally determined RI value of compound **1**, 1674 iu. The following molecular formula was therefore proposed for compound **1**: $C_{10}H_{16}Cl_2Br_2$. It is conceivable that a loss of a chlorine radical from this molecule, followed by the loss of HCl or HBr, will yield the $C_{10}H_{15}Br_2^+$ or $C_{10}H_{15}ClBr^+$ ions, respectively. A related compound, compound **15** (Figure 3), also with only one degree of unsaturation, was previously identified in *P suhrii* [8]. This compound was also previously isolated from *P. corallorhiza*, although the exact stereochemistry was not determined [13]. However, this compound was not detected in the samples investigated in our study.

The base peak in the mass spectrum of compound **2** is observed at *m/z* 162. The isotope peak of this ion is detected at *m/z* 164 with an intensity ratio of 3:1, indicating the presence of one chlorine atom in the ions. In addition, a series of peaks is observed at *m/z* 197, 199 and 201 (8:5:1), *m/z* 233, 235 and 237 (3:3:1) and *m/z* 268, 270 and 272 (3:4:2) and their isotope peak ratios indicate the presence of two, three and four chlorine atoms in each of the fragment ions respectively. Unfortunately it was not possible to rationalise the composition of these formulae, based on the assumption that the chlorine atoms are present on a monoterpene hydrocarbon skeleton. No match could be found for this mass spectrum either in the NIST database or the relevant literature on halogenated monoterpenes. In the absence of any MS data, it is therefore not possible to propose a possible formula or structure for compound **2**.

These findings indicate that in addition to the major metabolite, compound **8**, the Namibian *Plocamium* samples investigated in this study also contain all the compounds previously identified in *P. suhrii*, collected in South Africa, except for compound **15**. However, a possible analogue of compound **15**, not found in *P. suhrii*, has been detected (i.e. compound **1**). In addition, three more compounds, **2**, **6** and **12**, were present that were not previously detected in either *P. suhrii* or *P. rigidum*. Finally, these specimens also contained a total of four isomers related to compound **8**, while Antunes and co-workers only reported two [8]. Based on the isolated yields reported by Antunes and co-workers [8] compound **8** was present in 45% relative abundance, while the two isomers related to compound **8** were present in relative amounts of 7% each in the *P. suhrii* samples investigated in their study (the compounds were isolated using silica gel column chromatography followed by HPLC). Compound **3** comprised 6% of the total isolated mass, while compound **13** and its corresponding diastereomer were present in relative amounts of 12% and 13%, respectively. Compound **15** was present at a relative abundance of 9% [8]. Since the isolation of compounds using column chromatography is prone to losses of material, the isolated yields of these compounds does not give an accurate indication of their true relative abundancies in the extract. In our study, on the other hand, GC-FID was used, which facilitates the accurate determination of the relative amounts of volatile compounds that are present in the mixture. Using this approach it was determined that compound **8** is present at a relative abundance of almost 90% in the methanol extracts of the Namibian *Plocamium* specimens, while the minor metabolites are present in relative amounts lower than 3% (Table 1).

In conclusion, for the first time, the experimentally determined RI of compound **8**, the major metabolite of *P. suhrii*, is reported. This will enable fast confirmation of the presence of this compound in future investigations and will facilitate this species' rapid chemotaxonomic identification. Using GC-MS in combination with a RI prediction approach it was possible to confirm the presence of known minor metabolites, but also to tentatively identify four additional metabolites (compounds **1**, **2**, **6** and **12**) not reported before in either *P. suhrii* or *P. rigidum*. In addition, a compound with formula of $C_{10}H_{16}Cl_4$ previously identified in *P. suhrii* was not present in these samples. Without corroboration of the predicted RI values, it would not have been possible to confidently propose structures for these detected compounds. In the absence of any literature MS data for compound **2**, however, it was not possible to propose a formula for this compound. From this detailed chemical profile it could be confirmed that these proposed Namibian *P. suhrii* specimens contain compounds that have not previously been identified in South African samples of the same species of marine algae. Although this approach using GC-MS in combination with RI prediction proved to be useful, the identities of these compounds could not be confirmed, since no reference standards are available. It would therefore still be necessary to isolate the individual constituents for further characterisation by accurate mass MS and NMR in order to elucidate their chemical structures unequivocally.

Experimental

General experimental procedures: GC-MS analyses were performed as previously described [2]. Quantitative analysis was performed using a Perkin Elmer Clarus 580 GC-FID using TotalChrom software, version 6.3.2, for data acquisition. A SGE capillary GC column (30 m x 0.32 mm i.d.) coated with 100% dimethyl polysiloxane stationary phase (0.25 μm film thickness) was used with hydrogen as carrier gas at a flow rate of 1.4 mL/min (constant flow). The GC inlet and detector temperatures were maintained at 220 °C and 300 °C respectively. Samples were injected in the split mode using a split ratio of 1:10. The oven temperature was programmed at 5 °C/min from 40 °C to 280 °C. A volume of ~2 μL of each sample solution was analysed. The alkane standard mixture, used for the experimental RI determinations was analysed under the same conditions as the sample solutions. HPLC grade methanol and hexane 85% CP (Merck) were purchased from Biodynamics (Windhoek, Namibia) and the alkane standard mixture (C_{10} – C_{40} *n*-alkanes, all with an even number of carbons) was obtained from Sigma-Aldrich (Taufkirchen, Germany). The authentic reference material 1*E*,3*R*,4*S*,5*E*,7*Z*-1-bromo-3,4,8-trichloro-7-(dichloro-methyl)-3-methylocta-1,5,7-triene was available from a previous study in our laboratory [2]. Sample solutions for GC-MS analysis were prepared in dichloromethane (DCM) at *ca.* 7 mg/mL (~10 mg in 1.5 mL).

Collection, extraction and analysis: The Namibian *Plocamium* samples used in this study were collected in May and December 2014 from Swakopmund and Henties Bay, Namibia, at low tide. The collected material was transported to Windhoek on ice and then stored at -20 °C until processed for analysis. A voucher specimen (collection code: LK320) is housed in the herbarium of the Sam Nujoma Campus, University of Namibia, Namibia. Five different samples of wet Namibian *Plocamium* samples (of the same species) were steeped in 100 mL MeOH overnight. Concentrated methanolic

extracts were filtered and partitioned three times with hexane (3 x 30 mL) to yield ca. 10 mg of dried extract for each sample.

The Kováts retention indices (RIs) of compounds **1 - 12** were determined by analysing a series of n-alkanes under the same GC-MS conditions and then calculating their RIs from their retention times relative to those of the n-alkanes. The predicted RIs were calculated from

$$\mathrm{RI} = \sum_{n} f_n g_n + h$$

where f_n is the number of times group n appears in the molecule, while g_n is the RI increment value for group n. An adjustable parameter h is included in order to correct the predicted RI for any uniform prediction error [11]. The group contributions that were needed for this series of compounds were for the following functional groups (contribution values in brackets): -CH_3 (+112), >CH- (+22), =CH- (+102), =CH_2 (+98), =C< (+67), >CH_2 (+99), >C< (-14), -Cl (+189), 1-Cl (+236), 2-Cl (+217), 3-Cl (172), -Br (+306). Symbols > and < denote two single bonds each [11]. For chlorides attached to C8 of the monoterpene, the generic -Cl contribution (+189) was used.

Conflict of interest - The authors declare that they have no conflict of interest.

Authors' contributions - *Plocamium* samples used in this study were collected and identified in May and December 2014 (from Swakopmund and Henties Bay) by LK. MGK processed the algal material and prepared the samples. SL performed the GC-MS and GC-FID analysis as well as the RI predictions. SL and MGK processed and interpreted the experimental data and wrote most of the manuscript while LK wrote the section on the taxonomic description of the algal material.

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Supplementary data – Mass spectra and chromatograms.

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Rapid and Efficient Extraction and HPLC Analysis of Sesquiterpene Lactones from *Aucklandia lappa* Root

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The root of *Aucklandia lappa* Decne, family Asteraceae, is widely used in Asian traditional medicine due to its sesquiterpene lactones. The aim of this study was the development and optimization of the extraction and analysis of these sesquiterpene lactones. The current Chinese Pharmacopoeia reports a monograph for "Aucklandiae Radix", but the extraction method is very long and tedious including maceration overnight and ultrasonication. Different extraction protocols were evaluated with the aim of optimizing the maceration period, solvent, and shaking and sonication times. The optimized method consists of only one hour of shaking plus 30 minutes of sonication using 100% MeOH as solvent. ^{1}H NMR spectroscopy was used as a complementary analytical tool to monitor the residual presence of sesquitepene lactones in the herbal material. A suitable LC-DAD method was set up to quantify the sesquiterpene lactones. Recovery was ca. 97%, but a very high instability of constituents was found after powdering the herbal drug. A loss of about 20% of total sesquiterpenes was found after 15-20 days; as a consequence, it is strongly endorsed to use fresh powdered herbal material to avoid errors in the quantification.

Keywords: *Aucklandia lappa*, Sesquiterpene lactones, Recovery, HPLC-DAD, NMR, Quality control.

Aucklandia lappa Decne. (Family *Asteraceae*) is a perennial plant, native to East Asia, growing in the Himalayas, Burma, China and India. The root (木香, Mu Xiang, also called Costus Root) is widely used in Traditional Chinese Medicine and a monograph is reported in the current Chinese Pharmacopoeia [1]. It is mainly used for treatments of digestive ailments, including gastric and abdominal pain, loss of appetite, indigestion, diarrhoea, anorexia, nausea and vomiting [2]. The plant is also utilized to treat asthma and cough, coronary heart disease, acute pancreatitis, acute cholecystitis and hepatitis [3]. The sesquiterpenes costunolide and dehydrocostus lactone are considered the major active compounds [4, 5] of "Aucklandia root", and many pharmacological activities have been attributed to their presence, such as anti-ulcer [6], anti-cancer [7], hepatoprotective [8] and cytotoxic properties [5]. Furthermore, they have also been found to exhibit antiangiogenic [9], anti-inflammatory [10], antimicrobial, fungicidal [10, 11] and immunomodulatory activities [12].

At present, this plant is widely used in the European market, which justifies the need for a simple and rapid HPLC method for quality control of the herbal drug. The HPLC assay described in the Chinese Pharmacopoeia monograph reports the quantification of the two major sesquiterpene lactones, but the sample extraction method is very long and tedious including a maceration overnight and diverse steps of ultrasonication. The main purpose of our work was the optimization of the extraction method for the sesquiterpene lactones in the roots and the consequent quantitative analysis of commercial samples of "Aucklandia root".

Different extraction methods of the powdered herbal material were evaluated. They were all identified with a number (1, 2, 3, 4, 5) and a letter indicating the type of extraction, namely "S" for sonication bath and "U" for ultrasonication probe. In parentheses, the time of extraction expressed in minutes and/or hours is reported. In all the experiments 0.30 g of powdered roots was tested after addition of 50 mL of MeOH.

The following methods were investigated:
-Method 1S (30): 24 hours of maceration with shaking, plus 30 minutes in the sonication bath
-Method 1U (30): 24 hours of maceration with shaking, plus 30 minutes of ultrasonication with a probe
-Method 2S (30): 30 minutes in the sonication bath
-Method 2U (30): 30 minutes of ultrasonication with a probe
-Method 3S (15+15): 15 minutes of sonication plus 1 hour of maceration with shaking, plus 15 minutes of sonication
-Method 3S (30): 1 hour of maceration with shaking, plus 30 minutes of sonication
-Method 3U (15+15): 15 minutes of ultrasonication with a probe plus 1 hour of maceration with shaking, plus 15 minutes of ultrasonication with a probe
-Method 3U (30): 1 hour of maceration with shaking, plus 30 minutes of ultrasonication with a probe
-Method 4S (15+15): 15 minutes of sonication plus 16 hours of maceration with shaking, plus 15 minutes of sonication
-Method 4U (30): 16 hours of maceration with shaking, plus 30 minutes of ultrasonication with a probe
-Method 5E-S: 48 hours of maceration with shaking, plus 30 minutes in a sonication bath
-Method 5E-U: 48 hours of maceration with shaking, plus 30 minutes of ultrasonication with a probe

The first set of experiments was started with *Method 1U (30),* which is that described in the Chinese Pharmacopoeia monograph for "Aucklandiae Radix". This method was used as a reference for a preliminary analysis of the data. Recovery of costunolide and dehydrocostus lactone with this method was considered to be 100%. Five different methods of maceration were tested using increasing times (0, 1, 16, 24, 48 hours) of mechanical stirring and followed by 30 minutes of ultrasonication, as reported in the official monograph of the Chinese Pharmacopoeia. A considerable loss of MeOH due to evaporation was observed in all samples and, consequently, ultrasonication was replaced by sonication.

Preliminary experiments gave contradictory results, probably because the sesquiterpene lactones degraded very quickly after powdering the herbal material due to atmospheric oxidation. Consequently, HPLC analyses, in triplicate, of the same sample during different days after powdering were carried out. A gradual loss in the content of the main constituents was observed. After 12 days the residual percentage of active ingredients was ca. 90%, and after 18 days, ca. 80%. The herbal material, therefore, when powdered undergoes rapid degradation.

Accordingly, sample Auck1 was tested immediately after the pulverizing process, using: Method 1S (30), Method 1U (30), Method 2S (30), Method 2U (30), Method 3S (15 + 15), Method 3U (15 + 15), Method 4S (15 + 15), and Method 4U (15 +15). Table 1 reports the results of the quantitative analyses performed by HPLC-DAD expressed as percentages of the active constituents.

Table 1: Quantitative results obtained from sample Auck1 after extraction assays based on different times of maceration combined with centrifugation or ultracentrifugation.

Sample Auck1	% costunolide (g/100g)	% dehydrocostus lactone (g/100g)	% costunolide plus % dehydrocostus lactone (g/100g)
Method 1S (30)	1.00± 0.05	1.38± 0.09	2.37± 0.07
Method 2S (30)	0.98± 0.03	1.34± 0.05	2.32± 0.04
Method 2U (30)	0.85± 0.07	1.18± 0.08	2.02± 0.08
Method 3S (15+15)	0.96± 0.09	1.35± 0.07	2.31± 0.08
Method 3U	0.87± 0.02	1.25± 0.04	2.12± 0.03
Method 3S (30)	1.01± 0.03	1.44± 0.02	2.45± 0.02
Method 3U (30)	0.85± 0.08	1.22± 0.05	2.07± 0.06
Method 4S (15+15)	0.96± 0.05	1.38± 0.07	2.33± 0.06
Method 4U	0.85± 0.03	1.20± 0.03	2.05± 0.03
Method 4S (30)	0.86± 0.03	1.27± 0.09	2.13± 0.06
Method 4U (30)	0.96± 0.04	1.41± 0.06	2.37± 0.05

According to the above results:

1. The sonication bath seems to be able to extract both costunolide and dehydrocostus lactone to either the same extent or even better than the ultrasonication probe.
2. Sonication or ultrasonication gave the same quantitative results
3. The best extraction method was 3S (30), with only one hour of shaking, followed by 30 minutes in the sonication bath.

To assess further the influence of maceration time, the extraction methods 3S (30), 2 S (30), and 1S (30) were repeated with samples Auck2 and Auck3. Data are reported in Tables 2 and 3.

Table 2: Repeated assays with sample Auck2.

Sample Auck2	% costunolide (g/100g)	% dehydrocostus lactone (g/100g)	% costunolide plus % dehydrocostus lactone (g/100g)
Method 2S (30)	0.91± 0.08	1.36± 0.06	2.27± 0.07
Method 3S (30)	1.01± 0.06	1.47± 0.04	2.47± 0.05
Method 1S (30)	0.83± 0.02	1.18± 0.01	2.01± 0.01

Table 3: Repeated assays with sample Auck3.

Sample Auck3	% costunolide (g/100g)	% dehydrocostus lactone (g/100g)	% costunolide plus % dehydrocostus lactone (g/100g)
Method 2S (30)	1.85± 0.07	1.76± 0.08	3.61± 0.05
Method 3S (30)	1.78± 0.09	1.68± 0.07	3.46± 0.06
Method 1S (30)	2.17± 0.03	2.03± 0.02	4.21± 0.02

The best extraction method for Auck3 was 1S (30), corresponding to 24 hours of maceration with shaking, plus 30 minutes in the sonication bath. For Auck2 it seems that method 3S (30) was the best. Similar results were obtained with Auck4 (Table 4).

Table 4: Extraction assays with sample Auck4.

Sample Auck4	% costunolide (g/100g)	% dehydrocostus lactone (g/100g)	% costunolide plus % dehydrocostus lactone (g/100g)
Method 2S (30)	0.82± 0.09	1.31± 0.08	2.13± 0.07
Method 2U (30)	0.82± 0.03	1.28± 0.06	2.09± 0.04
Method 1S (30)	0.89± 0.05	1.37± 0.04	2.26± 0.05
Method 1U (30)	0.86± 0.08	1.32± 0.07	2.18± 0.04

^{1}H NMR experiments were performed directly on the pulverized herbal drug to confirm the exhaustive extraction of active constituents. The exhausted powdered Auck samples were treated with DMSO-d6. In the investigated samples no characteristic signals of costunolide or dehydrocostus lactone were found in the range between δ 8 and 5.5, after extraction of the sample using the method 3S (30).

In conclusion, sonication is less invasive then ultrasonication and the extraction method 3S (30) is the best one for the extraction of the main active constituents of Aucklandia root. The optimized method is able to extract more than 97% of the total of active principles.

A simple HPLC-DAD method was used for the evaluation of the sesquiterpene lactones. Both costunolide (Rt 7.55) and dehydrocostus lactone (Rt 8.83 minutes) were easily identified by comparison of their retention times with those of reference standards.

Six different commercial samples of *A. lappa* were evaluated. The Chinese monograph for "Aucklandiae Radix" reports a minimum content of 0.6% of costunolide and a minimum of 1.8% for the sum of costunolide and dehydrocostus lactone with respect to the dried herbal drug. Quantitative analyses were performed by HPLC–DAD and the optimized method 3S (30) was used for the extraction of the roots. All results are reported in Table 5.

Table 5: Extraction assays with further samples.

Sample	% costunolide (g/100g)	% dehydrocostus lactone (g/100g)	% costunolide plus % dehydrocostus lactone (g/100g)
Auck5	0.65± 0.02	0.95± 0.03	1.61± 0.02
Auck6	1.06± 0.05	1.30± 0.6	2.36± 0.05
Auck7	0.81± 0.03	0.68± 0.04	1.49± 0.02
Auck8	0.02± 0.04	2.15± 0.06	2.17± 0.05
Auck9	1.31± 0.06	1.35± 0.03	2.66± 0.05
Auck10	1.53± 0.08	1.46± 0.07	2.99± 0.04

Of the tested samples, only Auck5 and Auck7 contained less than 1.8% of costunolide plus dehydrocostus lactone.

HPLC-DAD and NMR spectroscopy were used as integrative analytical tools to develop the best extraction method of sesquiterpene lactones from *A. lappa* root. A very high instability of both constituents was found after powdering the herbal drug with 80% residual percentage of active constituents after 15-20 days. Accordingly, it is strongly recommended to use fresh powdered herbal drug material to avoid errors in the quantification of constituents. The optimised, rapid and efficient extraction method is 3S (30), namely the powdered material is macerated for 1 h with shaking, followed by 30 minutes of sonication (total time of extraction is 1 h and 30 min).

The developed extraction and HPLC analytical methods were adequate for the quality control of Aucklandia root in order to guarantee the integrity and stability of the products and assess efficacy and safety.

Experimental

Apparatus: Extractions were performed using an electronic Sonorex RH 100 SH ultrasonic bath (Bandelin, Berlin, Germany) and a Bandelin electronic Sonoplus, using the mechanical shaker HS 250 BASIC (Ika Labortechnik, Staufen, Germany). A mortar was used to powder the roots before extraction. An Agilent 1100 HPLC system coupled with DAD detector was used for chemical profile and quantitative analysis. NMR spectra were recorded using a Bruker DRX (Koln, Germany) spectrometer operating at 400.13 MHz and a Bruker Avance-600 spectrometer operating at 600.13 MHz (14.1T), both using a 5 mm inverse probe equipped with a z-shielded gradient. Data processing was achieved using TOPSPIN software package 1.3.

Chemical and reagents: Dimethylsulfoxide-d_6 (99.9% purity) and MeOH were HPLC grade from Sigma Aldrich (Seelze, Germany). Water was purified by a Milli-Qplus system from Millipore (Milford, MA, USA). The HPLC column used was a Zorbax® Eclipse XDB C18, 150 × 4.6 mm, 5 µm (Agilent, Palo Alto, CA, USA). The following standards were used: Costunolide, code Y0001307 and dehydrocostus lactone, code 38384 both CRS and given by EDQM (Strasbourg, France).

Herbal drug samples: Two samples (46393, named Auck1 and 31401, named Auck2) were commercial herbal drugs from Shenyang, China, and sent by EDQM. A commercial Chinese sample, Auck3, was donated by Phytax (Schlieren, Switzerland) while 5 samples from China (Auck5=410079, Auck6=750079, Auck7=110079, Auck8=671375, Auck9=130079), one from Austria (Auck10=112014) and one from India (Auck11= 21657100) were provided by Plantasia (Oberndorf, Austria).

Preparation of extracts: All the investigated samples were whole roots, which were firstly cut into transverse slices and subsequently powdered in a porcelain mortar. Extracts were obtained by macerating 0.3 g of herbal drug with 50 mL of MeOH, with mechanical shaking for 1 h. After maceration overnight, the samples were than sonicated for 30 min using an ultrasonic bath. The total weight of preparations was monitored at the beginning and end of the extraction process (after cooling of the sample) and MeOH was replenished in the case of loss of weight. The obtained liquid extracts were mixed well and filtered through a membrane filter (nominal pore size 0.45 µm) before analysis.

Preparation of samples for HPLC-DAD analysis: The extracts of *A. lappa* were sonicated for 10 min in an ultrasonic bath and then centrifuged for 4 min at 14,000 rpm, prior to injection. Subsequently, the standard solutions were sonicated for 2 min before injection. Reference solutions were prepared as follows: (a): 5.0 mg of costunolide CRS was dissolved in 5 mL of MeOH, shaken well, diluted to 50 mL with the same solvent and finally mixed well. b): 2.5 mg of dehydrocostus lactone CRS was dissolved in 5 mL of MeOH, shaken well and diluted to 25 mL with the same solvent and finally mixed well.

Preparation of samples for ^{1}H NMR analysis: The herbal material, both before and after extraction, was freeze-dried for 12 h in order to remove the residual water. Fifty mg of the dried powdered herbal material was put in glass tubes and treated with 0.6 mL of DMSO-d_6. Tubes were manually shaken and finally filtered before analysis.

Sonication and ultrasonication process: Sonication was performed using a bath and the temperature never exceeded 25°C. Ultrasonication was performed by immersion of the probe directly into the sample with a maximum amplitude of 50% and a frequency of 20 KHz, at room temperature. At the end of the process, the samples became very hot; as the extracts were in direct contact with the air there was a possible increase in the oxidative processes and evaporation of the solvent.

Qualitative and quantitative HPLC–DAD analysis: The analysis was performed using a Zorbax® Eclipse XDB C18, 4.6 x 150 mm, 5 µm column, at 24°C. The mobile phase was composed of methanol (solvent A) and water at pH 3.2 (solvent B), 13:7 v/v. The flow rate was 1.0 mL/min and the detection wavelength was set at 225 nm. The injection volume was 10 µL. Identification of the 2 main constituents was performed by comparison with the retention times and UV spectra of the reference standards and of the data reported in the literature. Quantitative analysis of the constituents was performed using external standards. Costunolide and dehydrocostus lactone were used to obtain the calibration curve in a range of 0.220-2.200 µg/mL and 0.407-4.070 µg/mL, respectively. The standards were weighed accurately and dissolved in MeOH to obtain stock solutions, which were then diluted. The linearity of the calibration curves were expressed by the values of R^2 (0.99992) for both standards.

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Antimalarial Activity of some Kaurenes

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The antimalarial activity of sixteen *ent*-kaurenes was assayed on male albino mice infected with *Plasmodium berghei*. *Ent*-kaur-16-en-19-oic acid (kaurenic acid), 15α-hydroxy- *ent*-kaur-16-en-19-oic acid, 15α-acetoxy- *ent*-kaur-16-en-19-oic acid, and *ent*-kaur-9(11)16-en-19-oic acid, natural kaurenes isolated from two species of Espelletiinae, were modified by semisynthesis to obtain methyl esters, glucopyranosyl esters, epoxides, 17-hydroxy, and isokaurenes (compounds with a 15,16-double bond). The kaurenes were first submitted to an *in vitro* test to measure their capacity to inhibit the formation of β-hematin. Compared with chloroquine (95.7%), the best effect was shown by 16,17-epoxy-*ent*-kauran-19-oic acid α-D- glucopyranosyl ester (**2a**), which produced 92.6% inhibition. Three other kaurenes showed good inhibition levels: *ent*-kaur-16-en-19-oic acid (**1a**, 73.5%), 17-hydroxy- *ent*-kaur-15-en-19-oic acid methyl ester (**3b**, 76.5%), and 15-oxo-16,17-epoxy-*ent*-kaur-16-en-19-oic acid α-D-glucopyranosyl ester (**4b**,76.1%). These four compounds were assayed in a four day suppressive test *in vivo* (Peters´ test) using chloroquine as a positive control. Two hours after infection the mice received the first treatment and then every 24 hours during four consecutive days. Blood smears from the tails were prepared on the fourth day and parasitemia was determined microscopically. Survivals were followed up to the 30th day post-infection, Once again compound **2a** performed best, showing 4.5% of parasitemia on the fourth day post-infection (chloroquine 0.2%) and a survival time of 25.5 days (chloroquine 29.5 days; **1a** 18.8 days, **4b** 12.7 days and **3b** 10.3 days). A comparative examination of the effect of all compounds on the *in vitro* test permitted the inference that the presence of a C-19 carboxylic moiety was a requirement for the antimalarial activity and that a 16,17 epoxy group enhanced such activity.

Keywords: Antimalarial activity, Kaurenes, *Plasmodium berghei*, β-Hematin, Peters´ test.

Malaria is an infectious disease caused by protozoan parasites of the genus *Plasmodium* [1a]. It has afflicted humans since ancient times affecting each year, in tropical areas of the world, over 300 million people and it is fatal to more than one million every year, mainly children under five years old. Four species of *Plasmodium* are infectious to humans and are transmitted through the bite of an infected female mosquito of the *Anopheles* genus. Nowadays, for either the prevention or treatment of malaria several synthetic compounds are used, like 4-aminoquinolines, 8-aminoquinolines, arylamino-alcohols, artemisinins, antifolates, antibiotics and inhibitors of the respiratory chain [1b]. The need for such a wide arsenal of drugs against malaria has arisen because the *Plasmodium* parasites have developed resistance, which consists of a mechanism to expel the drugs from their cytoplasm. Therefore, the search for new substances capable of controlling malaria has become a global need [1c].

Kaurenes are diterpenes with a rigid tetracyclic skeleton. *Ent*-kaurenic acid has been reported to have moderate anti-microbial activity and to be active against *Trypanosoma cruzi.* It was also shown to have molluscicidal properties, but one of the most interesting biological properties of kaurenes is their moderate *in vitro* activity against several cancer cell lines [1d]. To assay their possible antimalarial activity sixteen kaurenes were tested on male mice infected with *Plasmodium berghei*, a rodent parasite. The tested kaurenes (Figure 1) were: *ent*-kaur-16-en-19-oic acid (**1a**), *ent*-kaur-16-en-19-oic acid α-D-glucopyranosyl ester (**1b**), 15α-hydroxy-*ent*-kaur-16-en-19-oic acid (**1c**), 15α-hydroxy-*ent*-kaur-16-en-19-oic acid α-D-glucopyranosyl ester (**1d**), 15α-*O*-acetyl-*ent*-kaur-16-en-19-oic acid α-D-glucopyranosyl ester (**1e**), 16,17-epoxy-*ent*-kauran-19-oic acid α-D-glupyranosyl ester (**2a**), 15α-*O*- acetyl-16-17-epoxy-*ent*-kauran-19-oic acid α-D-glucopyranosyl ester (**2b**),

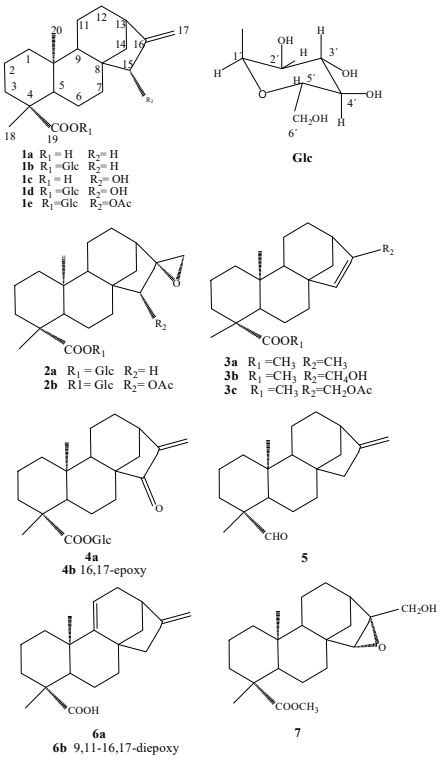

Figure 1: Structure of tested kaurenes.

ent-kaur-15-en-19-oic acid methyl ester (**3a**), 17-hydroxy-*ent*-kaur-15-en-19-oic acid methyl ester (**3b**), 17-*O*-acetyl-*ent*-kaur-15-en-19-oic acid methyl ester (**3c**), 15-oxo-*ent*-kaur-16-en-19-oic acid

α-D-glucopyranosyl ester (**4a**), 15-oxo-16,17-epoxy-*ent*-kauran-19-oic acid α-D-glucopyranosyl ester (**4b**), *ent*-kaur-16-en-19-al (**5**), *ent*-kaur-9(11)16-dien-19-oic acid (**6a**), 9,11-16,17-diepoxy-*ent*-kauran-19-oic acid (**6b**), and 15,16-epoxy-17-hydroxy-*ent*-kauran-19-oic acid-methyl ester (**7**).

Ent-kaur-16-en-19-oic acid (kaurenic acid, **1a**) and *ent*-kaur-16-en-19-al (**5**) were obtained from *Espeletia semiglobulata*, and 15α-hydroxy-*ent*-kaur-16-en-19-oic acid (**1c**), *ent*-kaur-(9,11)16-dien-19-oic acid (**6a**) and 15α-*O*-acetyl-*ent*-kaur-16-en-19-oic acid from *E. schultzii*. Both are Venezuelan species of the *Espelletiinae subtribe* (Compositae) [2a]. The other kaurenes were produced by semisynthesis from the above mentioned natural compounds. Isokaurenic acid (*ent*-kaur-15-en-19-oic acid) was obtained by isomerization of kaurenic acid [2b]; treatment with diazomethane yielded the methyl esters; and reaction with dimethyl oxirane yielded the epoxy derivatives. Oxidation of **1c** with Sarett's reagent rendered 15-oxo-*ent*-kaur-16-en-19-oic acid. The glucopyranosyl esters were obtained using the technique described by Visbal *et al* [2c].

To assay the possible antimalarial activity of the kaurenes, the inhibition of β-hematin synthesis *in vitro* was tested according to Baelman's procedure [2d], using chloroquine as a positive control. Results are presented in Table 1. In the first column the kaurenes are identified according to the numbering system used in Figure 1. In the second column, the inhibition of β-hematin formation (% IβHF) obtained by each kaurene is presented. Compared with chloroquine (95.7%) the best inhibitory effect was shown by 16,17-epoxy-*ent*-kauran-19-oic acid α-D-glucopyranosyl ester (**2a**), which produced 92.6% inhibition. Three other kaurenes attained more than 70% inhibition: *ent*-kaur-16-en-19-oic acid (**1a**, 73.5%), 17-hydroxy- *ent*-kaur-15-en-19-oic acid methyl ester (**3b**, 76.5%), and 15-oxo-16,17-epoxy-*ent*-kauran-19-oic acid α-D-glucopyranosyl ester (**4b**,76.1%). On the other hand, two compounds performed very poorly: 15α-hydroxy-*ent*-kaur-16-en-19-oic acid (**1c**, 8.1%), and *ent*-kaur-16-en-19-al (**5**, 6.4%). Some conclusions could be drawn from this experiment. The first would be that the presence of a carboxylic acid moiety at C-19 is a requirement for good antimalarial activity; if the carboxyl group is replaced by an aldehyde group the capacity to inhibit β-hematin synthesis disappears. On the other hand, the presence of a hydroxyl at C-15 seems to be negative, but at C-17 to be positive, even more so if there is an epoxy group at C16-C17.

Table 1: Percentage inhibition of β-hematin formation (IβHF) produced by the tested kaurenes using chloroquine as a positive control.

Compounds	% IβHF
Chloroquine	95.7+0.01
1a	73.5+0.04
1b	48.4+0.1
1c	8.1+0.07
1d	37.7+0.07
1e	57.6+0.03
2a	92.6+0.04
2b	53.3+0.07
3a	50.2+0.09
3b	76.5+0.03
3c	53.8+0.03
4a	51.4+0.02
4b	76.1+0.05
5	6.4+0.1
6a	63.5+0.01
6b	50.8+0.1
3c (10%) + **7**(90%)	57.8+0.07

The results are expressed as the mean ± SEM. n=4

Only those kaurenes that showed inhibition of β-hematin synthesis *in vitro* higher than 70% were submitted for *in vivo* evaluations. Therefore, compounds **2a**, **1a**, **3b**, and **4b** were assayed by the four-day suppressive Peters´ test [2e] using chloroquine as the positive control. Mice were infected intraperitoneally with parasitized red blood cells. Two hours after infection, animals received by ip the first treatment of 25 mg/kg of an individual kaurene, which was repeated every 24 hours for four consecutive days. Parasitemia and survivals were determined. Results are shown in Table 2.

Table 2: *In vivo* antimalarial activity of kaurenes **2a**, **1a**, **3b**, and **4b**.

Compound	% P	SDPI
Chloroquine	0.2±0.06	29.5±1.7
2a	4.5±0.02***	25.5±1.3***†
1a	8.5±0.05***	18.8±0.8**
3b	12.7±1.2***	12.7±1.7
4b	15.2±1.7**	10.3±1.3
Saline	27.6±2.4	9.0±1.9

The results are expressed as the mean ± SEM. %P: percentage of parasitemia at 4th day post-infection; SDPI: survival days post-infection. ***p<0.001; **p<0.01 and †p>0.05 compared with non-treated infected mice (saline solution). n=5.

Peters´ test indicated that 16,17-epoxy-*ent*-kauran-19-oic acid α-D-glucopyranosyl ester (**2a**) showed the best antimalarial properties among the group of tested kaurenes. Indeed, this compound was able to decrease the parasitemia levels and increased the survival time post-infection in the most significant manner, showing the same survival rates as the control, chloroquine. Thus, we suggest that this compound might be suitable for consideration for further studies in malaria research for inhibition of the β-hematin synthesis mechanism.

Experimental

General procedures: Melting points, Fisatom D 430 hot stage; IR, Perkin Elmer FT spectrometer; NMR, Bruker Advanced DRX 400 spectrometer; GC-MS, Hewlett-Packard MSD 5973 spectrometer; HRMS, Agilent 6210 LCTOF instrument; CC, silica gel Merck 60 (230-400 mesh) and TLC on silica gel Merck 60 F254 plates.

Isolation of* ent-*kaurenic acid (1a): This was obtained from the aerial parts of *Espeletia semiglobulata* collected at Páramo of Piedras Blancas, Mérida State, Venezuela and compared with an authentic sample (mmp, TLC, ^{1}H NMR).

Preparation of* ent-*kaur-16-en-19-oic acid α-D-glucopyranosyl ester (1b): This compound was obtained as described by Visbal *et al.* [2c]. Kaurenic acid silver salt was obtained by treating a solution of sodium kaurenate with an aqueous solution of $AgNO_3$. A solution of silver kaurenate (2 g, 4.88 mmol) in dry benzene was shaken during 12 h in the dark with 2.1 g (5.1 mmol) of 2,3,4,6-tetra-*O*-acetyl-D-glucopyranosyl bromide obtained by the method of Redemann and Niemann [3a]. The kaurenic acid ester of 2,3,4,6-tetra-*O*-acetyl-D-glucopyranose (1.92 g, MP 120-122°C), obtained by flash chromatography over silica gel eluting with *n*-hexane/acetone mixtures, was subjected to mild hydrolysis in a dry MeOH solution of anhydrous NH_3. Upon evaporation of the solvent, **1b**, MP 108-110°C, was obtained.

Isolation of 15α-hydroxy-*ent-*kaur-16-en-19-oic acid (1c): This compound (MP 224-228°C) was obtained from the aerial parts of *E. schultzii*, as described by Brieskorn and Poehlmann [3b], and compared with an authentic sample (mmp, TLC, ^{1}H NMR, MS).

Preparation of 15α-hydroxy-*ent-*kaur-16-en-19-oic acid α-D-gluco-pyranosyl ester (1d): One g (3.3 mmol) of 15α-hydroxy-*ent*-kaur-16-en-19-oic acid (**1c**), isolated from *Espeletia schulzii* [3b], was used as starting material. To obtain the glucopyranosyl ester, the procedure described by Villasmil *et al.* was used [3c]. **1d** crystallized from MeOH, MP 110°C. The compound was identical to that reported by Villasmil *et al.* (mmp, IR, ^{1}H and ^{13}C NMR).

Isolation of 15α-O-acetyl-*ent-*kaur-16-en-19-oic acid α-D-gluco-pyranosyl ester (1e): To obtain this compound, 15α-*O*-acetyl-*ent*-

Table 3: ^{13}C NMR and ^{1}H NMR spectroscopic data of **2a**, **2b**, and **4**[a].

	2a	**2a**	**2b**	**2b**	**4a**	**4b**
	δc	δ_H (*J*,Hz)	δc	δ_H (*J*,Hz)	δc	δ_H (*J*,Hz)
1	41.2	0.80, dt (4, 12) 1.81, m	38.5	0.78, dt (4, 12) 1.74, m	40.4	0.75, dt (3, 12) 1.71, m
2	19.9	1.40, m; 1.50, m	19.9	1.42, m; 1.55, m	19.5	1.43, m; 2.14, m
3	38.8	1.09, m; 2.42, s	36.7	1.08, m; 2.37, s	38.5	1.01, m; 2.35, m
4	44.5		44.4		44.4	
5	57.9	1.10, m	57.4	1.07, m	57.0	1.16, m
6	22.9	2.08, m; 2.45, m	23.5	1.90, m; 2.36, m	20.8	2.38, m; 2.08, m
7	37.9	1.40, m; 2.04, m	31.0	1.42, m; 1.69, m	34.7	2.05, m; 1.62, m
8	45.3		48.3		53.0	
9	56.6	1.04, m	53.6	1.13, m	52.2	1.28, m
10	40.4		35.4		40.9	
11	19.4	1.52, m; 1.68, m	19.8	1.50, m; 1.66, m	19.0	1.57, m; 2.14, m
12	29.0	1.52, m; 1.85, m	41.4	1.32, m; 1.90, m	33.6	1.50, m; 1.58, m
13	46.2	2.40, bs	40.6	3.19, bs	38.7	2.38, bs
14	43.2	1.48, m	39.4	1.79, m; 2.24, m	36.9	1.02, m; 2.46, m
15	54.1	1.74, m; 1.86, m	85.7	5.12, s	210.2	
16	82.0		67.0		147.0	
17	66.8	4.12, d (10.9) 4.15, d (10.9)	62.6	5.09, d (3) 5.19, d (3)	114.0	5.18s ,6.04, s
18	27.0	CH_3 1.27, s	27.5	CH_3 1.26, s	28.8	CH_3 1.26, s
19	177.3		177.2		177.1	
20	16.1	CH_3 1.21, s	16.5	CH_3 1.20, s	16.1	CH_3 1.26, s
CH_3COO			170.7			
CH_3COO			21.5	CH_3 2.10, s		
C-1'	96.1	6.28, d (8)	96.3	6.25, d (8)	96.2	6.25, d (8)
C-2'	74.4	4.22, t (5.7)	71.5	4.23, t (6)	72.4	4.20, dt (3, 8)
C-3'	79.5	4.26, t (4.7)	79.5	4.26, t (4.8)	79.5	4.25, t (8, 9)
C-4'	71.5	4.34, t (9.2)	74.5	4.36, t (8.9)	71.7	4.35, m
C-5'	79.9	4.02, m	79.8	4.06, m	79.7	4.03, bm
C-6'	62.5	4.42, dd (4, 26) 4.48	68.8	4.46, dd (4, 24) 4.89, m	62.5	4.29, m 4.48, m

kaur-16-en-19-oic acid was first obtained from *E. schultzii* resin [3b]. The pyranosyl ester **1e** was obtained from15α-*O*-acetyl-*ent*-kaur-16-en-19-oic acid as described by Villamil *et al.* [3c].

16,17-Epoxy-kauran-19-oic acid α-D-glucopyranosyl ester (2a): *Ent*-kaur-16-en-19-oic acid α-D-glucopyranosyl ester (**1b,** 100 mg, 0.215 mmol) was dissolved in acetone and treated overnight at room temperature with a dimethyloxirane solution in acetone, as described by Adams *et al.* [4a], using potasium peroxysulfate as oxidizing agent to afford **2a** as a white powder.
MP: 80-85°C.
IR (KBr): 3370 (OH), 2929, 1723 (C=O), 1448, 1071 (C-O-) cm^{-1}.
^{1}H and ^{13}C NMR: Table 3.
HRMS *m/z*: [M^+] 503.2615 ($C_{26}H_{40}O_8.Na^+$).

Preparation of 15α-O-acetyl-16,17-epoxi-ent-kauran-19-oic acid α-D-pyranosyl ester (2b): Compound **1c** was dissolved in acetone and treated overnight at room temperature with dimethyloxirane, as described above. The epoxide derivative crystallized from acetone.
MP: 155-159°C.
IR (KBr): 3392 (OH), 2936, 1713 (C=O), 1071 (C-O-) cm^{-1}.
^{1}H and ^{13}C NMR: Table 3.
HRMS *m/z*: [M^+] 561.6153 ($C_{28}H_{42}O_{10}.Na^+$).

Ent-kaur-15-en-19-oic acid methyl ester (3a): Kaurenic acid (**1a**) was methylated with diazomethane, dissolved in dry CH_2Cl_2 and treated with 10 drops of trifluoroacetic acid under reflux, as described by Rojas *et al.* [4b]. Under these conditions the methyl ester of **1a** isomerized to yield 66.6% of iso-kaurenic acid (**3a)**, which was separated from its isomer on a column of silica gel impregnated with 20% $AgNO_3$. Compound **3a** was identical to that reported by Rojas *et al.* [4b].

17-O-Acetyl-ent-kaur-15-en-19-oic acid methyl ester (3c): One g of kaurenic acid methyl ester (3.17 mmol) was dissolved in 5 mL dry benzene and refluxed with 80 mL of glacial acetic acid and 3.0 g (0.67 mmol) of lead tetra-acetate under an argon atmosphere. The course of the reaction was followed by TLC using *n*-hexane: EtOAc (3:1). After 30 min, H_2O was added and the mixture shaken with $CHCl_3$. The organic layer was dried over dry Na_2SO_4 and the $CHCl_3$ removed by distillation. The reaction product was submitted to a silica gel column (80 g) containing 20% $AgNO_3$. Fractions (50 mL each) were inspected by TLC and GC-MS. Fractions 1-30 eluted with *n*-hexane yielded 69 mg of kaurenic acid methyl ester. Elution was continued with *n*-hexane: 5% EtOAc. Fractions 31-46 yielded 457 mg of *ent*-kaur-16-en-15α-*O*-acetyl-19-oic acid methyl ester, identical to the methyl ester of the *O*-acetyl derivative of grandiflorolic acid obtained from *E. schultzii* resin. Fractions 47-60 yielded a mixture. Finally, fractions 61-81 yielded 472 mg of **3c**, identical to that obtained by Rojas *et al.* [4b].

Table 4: ^{13}C NMR and ^{1}H NMR spectroscopic data of **4b, 5 and 6b**.

	4b	**4b**	**5**	**5**	**6b**	**6b**
	δc	δ_H (*J*,Hz)	δc	δ_H (*J*,Hz)	δc	δ_H (*J*,Hz)
1	41.2	0.78, dt (4, 12) 1.84, m	39.9	0.79, dt (4, 15) 1.15, m	39.5	1.36, dt (4, 15) 1.71, m
2	20.1	1.40, m; 1.52, m	18.4	1.40, m; 1.67, m	19.8	1.43, m; 1.56, m
3	39.0	1.08, m 2.19, s	34.2	0.98, ddt (1.5, 3, 10) 2.37, td (11, 3)	37.9	1.01, td (18, 3) 2.35, td (11, 3)
4	44.4		48.4		44.2	
5	57.9	1.09, m	56.7	1.13, m	48.7	1.72, d (10)
6	22.5	1.85, m; 2.25, m	19.9	1.85, m; 1.67, m	19.0	2.85, m; 1.95, m
7	38.7	1.08, m; 2.37, m	31.0	1.50, m; 1.59, m	31.8	2.05, m; 1.62, m
8	45.9		44.0		43.4	
9	55.3	1.02, m	54.6	1.08, d (7.4)	69.8	
10	40.2		39.4		38.0	
11	19.9	1.64, m; 2.05, m	18.3	1.61, m; 1.54, m	53.7	1.57, m; 2.14, m
12	29.5	1.41, m; 1.75, m	41.4	1.44, m; 1.58, m	29.1	1.50, m; 1.58, m
13	43.3	2.35, bs	39.2	2.63, bs	38.9	1.54, bt
14	42.0	1.43, m 1.61, m	39.9	1.94, dd (7, 11, 4) 1.82, m	38.9	1.48, m 2.46, m
15	209.1		49.0	2.05, m; 2.06, m	45.6	2.48, d (20) 1.76, s
16	66.5		155.5		69.0	
17	50.3	2.78, m; 2.86, m	62.6	4.74, s; 4.79, s	50.6	2.79, d (7); 2.84, d (7)
18	28.9	CH_3 1.26, s	24.3	CH_3 0.98, s	28.6	CH_3 1.92, s
19	177.1		205.8	CHO 9.73	183.7	
20	16.4	CH_3 1.20, s	16.5	CH_3 0.87, s	15.0	CH_3 0.65, s
C-1'	96.1	6.25, d (8)				
C-2'	74.4	4.18, t (5.9)				
C-3'	79.4	4.23, t (5.2)				
C-4'	71.5	4.31, t (8.5)				
C-5'	79.8	4.02, bm				
C-6'	62.5	4.32, m; 4.44, m				

17-Hydroxy-ent-kaur-15-en-19-oic acid methyl ester (3b): Three hundred mg (0.8 mmol) of **3c** was dissolved in dry MeOH and treated overnight at room temp with anhydrous NH_3. The methanolic soln was then concentrated to yield 245 mg of **3b**, identical to the compound described by Rojas *et al.* [4b].

15-Oxo-ent-kaur-16-1n-19-oic acid α-D-glucopyranosyl ester (4a): One g (3.1 mmol) of 15α-hydroxy-*ent*-kaur-16-en-19-oic acid (**1c**) was dissolved in 20 mL of pyridine and treated overnight with 1.25 g (1.25 mmol) of CrO_3-pyridine complex (Sarett´s reagent). The following day, 100 mL of H_2O was added to the reaction mixture and the oxidation product was extracted with Et_2O. The Et_2O soln was concentrated to dryness and the residue submitted to flash chromatography over silica gel. Elution with *n*-hexane-diethyl ether yielded 0.83 g of 15-oxo-*ent*-kaur-16-en-19-oic acid, identical to the compound reported by Ruiz *et al.* [4c]. The glucopyranosyl ester (**4a)** was obtained as described previously for **1b**, **1d**, and **1e**; it was crystallized from MeOH. 15-Oxo-*ent*-kaur-16-en-19-oic acid (480 mg, 1.0 mmol) was neutralized to obtain the Na salt, which was treated with $AgNO_3$. The silver salt was treated with 2,3,4,6-tetra-*O*-acetyl-D-glucopyranosyl bromide in dry benzene solution to obtain the protected glucopyranosyl derivative, which was subjected to mild hydrolysis to obtain **4a**.
MP: 145-148°C.
IR (KBr): 3469 (OH), 2929 (CH), 1725 (CO), 1071 (C-O) cm^{-1}.
^{1}H and ^{13}C NMR: Table 3.
HRMS *m/z*: [M^+] 501.2457 ($C_{26}H_{38}O_8.Na^+$).

15-O-Acetyl-16,17-epoxy-ent-kauran-19-oic acid α-D-gluco-pyranosyl ester (4b): A solution (250 mg, 0.506 mmol) of **4a** in acetone was treated with a solution of dimethyloxirane. Crystal-ization from acetone yielded 252 mg of **4b**.

MP: 142-145ºC.
IR (KBr): 3369 (OH), 2930 (CH), 1733 (CO), 1070 (C-O) cm^{-1}.
^1H and ^{13}C NMR: Table 4.
HRMS *m/z*: [M^+] 517.2457 ($C_{26}H_{38}O_9.Na^+$).

Ent-*kaur-16-en-19-al (5):* This compound was also obtained from *E. semiglobulata* leaves. The *n*-hexane extract obtained from 5 Kg of dried leaves was concentrated to 2 L and shaken with a 0.5 M solution of NaOH. The remaining *n*-hexane solution was concentrated under vacuum to yield 54 g of solid, 10 g of which was submitted to flash chromatography over silica gel. Fractions eluted with *n*-hexane yielded 2.67 g of *ent*-kaur-16-en-al.
MP: 116ºC.
IR (KBr): 3060 (C=CH_2), 1710 (HCO), 1650 (C=C) cm^{-1}.
^1H and ^{13}C NMR: Table 4.
GCMS *m/z*: [M^+] 286 ($C_{20}H_{30}O$).

Ent-kaur-(9,11)16-dien-19-oic acid (6a): This was isolated from *E. schultzii* and shown to be identical to an authentic sample [3b].

9,11-16,17-Diepoxy*-ent-*kauran-19-oic-acid (6b): This compound was obtained by treatment of **6a** with dimethyloxirane, as described above. It was purified by flash chromatography yielding an oil that showed only one spot on TLC.
IR (KBr): 3450 (OH), 2928 (CH), 1695(CO), 1165,1150 (C-O) cm^{-1}.
^1H and ^{13}C NMR: Table 4.
GCMS *m/z*: [M^+] 332.3 ($C_{20}H_{28}O_4$).

15,16-Epoxy-17-hydroxy*-ent-*kauran-19-oic acid-methyl ester (7): This compound was obtained as described by Aparicio *et al.* [4d]. *Ent*-kaurenic acid methyl ester (100 mg, 0.32 mmol) was dissolved in 10 mL of dioxan and stirred with 46 mg of SeO_2 and 0.41 mL of 30% H_2O_2 during 4 h at room temperature. Diethyl ether extraction yielded a crude product, which was submitted to flash chromatography to yield 65 mg of **7**, which crystallized from MeOH. MP 114-116ºC; [M^+] at *m/z* 348 ($C_{21}H_{32}O_4$).

Inhibition of ß-hematin synthesis: The inhibition of ß-hematin synthesis assay was performed according to a previously described procedure [2d]. In short, a solution of hemin chloride in DMSO (50 µL, 5.2 mg/mL) was distributed into 96-well micro plates. Different concentrations of compounds dissolved in DMSO were added in triplicate to test wells (50 µL) at final concentrations between 100 and 5 µM. Controls contained either water (50 µL), DMSO (50 µL) or chloroquine (50 µL, 50-0.5 µM). β-Hematin synthesis was initiated by the addition of acetate buffer (100 µL, 0.2M, pH 4.4). Plates were incubated at 37ºC for 48 h to allow completion of the reaction and centrifuged (4000 RPM x 15 min). After discarding the supernatant, the pellet was washed 3 times with DMSO (200 µL), dissolved in NaOH (200 µL, 0.2N) and diluted 1:2 with NaOH (0.1N), recording the absorbances at 405 nm (Microplate Reader, BIORAD-550). The obtained results were expressed as percentage of inhibition of ß-hematin synthesis compared with control vehicle.

Experimental host and strain maintenance: Male albino mice (BALB/c, 18-22 g) were maintained on a commercial pellet diet and housed under conditions approved by the Ethics Committee, School of Pharmacy, Central University of Venezuela. *Plasmodium berghei* (ANKA strain), a rodent malarial parasite, was used for infection. Mice were infected intraperitoneally with 10^7 infected erythrocytes diluted in phosphate buffer saline solution (PBS 10 mM, pH 7.4, 0.1mL). Parasitemia was monitored by microscopic examination of Giemsa stained smears.

Four-day suppressive test (Peters´ test): Kaurene compounds were also evaluated in a malaria murine model by the four-day suppressive test [2e] using chloroquine as a positive control (20 mg/kg). In short, native BALB/c mice (18-22 g) were infected intraperitoneally (ip) with 10^7 parasitized RBCs on day 0. Kaurenes were freshly prepared, dissolved in water/DMSO (1:1) at a maximal concentration of 0.1 M and diluted with Saline-Tween 20 solution (2%). Two h after infection, mice received the first treatment (25 mg/kg, ip) and then every 24 h for 4 consecutive days. Blood smears from the tails were prepared on the fourth day and parasitemia was determined by microscopic examination of Giemsa-stained blood films. Survivals were followed until day 30 post-infection. The results were expressed as percent of parasitemia on the fourth day post-infection and as survival days. Data were presented as the average of 4 independent experiments (n=5) and tested for statistical significance using unpaired t-tests for specific group comparisons assuming 95% confidence limits using GraphPad Prism 4.02 software. The maintenance of the experimental animals complied with the guidelines of the human use of laboratory animals.

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Briarenol B, a New Polyoxygenated Briarane from the Octocoral *Briareum excavatum*

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A new polyoxygenated briarane diterpenoid, briarenol B (**1**), was isolated from the octocoral *Briareum excavatum* and its structure determined from spectroscopic data. In RAW264.7 cells, a macrophage-like murine cell line, briarane B (**1**) was found to enhance the protein expression of pro-inflammatory cyclooxygenase (COX-2) and inducible nitric oxide synthase (iNOS) in cells stimulated by lipopolysaccharide (LPS).

Keywords: *Briareum excavatum*, Octocoral, Briarane, Briarenol, COX-2, iNOS.

Over the past four decades, more than 600 diterpenoids possessing the briarane carbon skeleton, most of which contain a γ-lactone moiety in a bicyclo[8.4.0] system, have been isolated from marine coelenterates, mainly octocorals [1–5]. Increasing interest is being paid to these briaranes, not only due to their complex structures, but also owing to their anti-inflammatory activities [6–8]. In an ongoing survey of Taiwanese marine invertebrates possessing promising novel and bioactive briaranes, the octocoral *Briareum excavatum* (family Briareidae) is being systematically investigated. In this paper, we report the isolation, structure determination and bioactivity of a new polyoxygenated briarane, briarenol B (**1**) (Figure 1), following further study of *B. excavatum*.

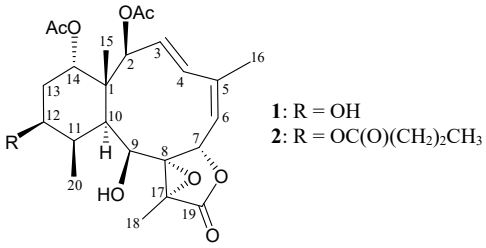

Figure 1: The structures of briarenol B (**1**) and briarenolide ZII (**2**).

Briarenol B (**1**) was obtained as a white amorphous powder. From the HRESIMS, the molecular formula of **1** was determined to be $C_{24}H_{32}O_9$ from the ion at *m/z* 487.19396 (calcd for $C_{24}H_{32}O_9$ + Na, 487.19441), indicating nine degrees of unsaturation. IR spectrum analysis showed that **1** had absorption peaks at 3479, 1766 and 1732 cm^{-1}, suggesting that the structure of **1** included hydroxy, γ-lactone and ester groups. In the ^{13}C and DEPT NMR spectra (Table 1), the signals of four carbons at δ_C 126.3 (CH-3), 138.6 (CH-4), 140.5 (C-5) and 118.4 (CH-6) indicated the presence of a disubstituted and a trisubstituted olefin in **1**; this result was further supported by the ^1H NMR spectrum of **1**, which showed three olefin proton signals at δ_H 5.80 (1H, dd, *J* = 16.0, 9.6 Hz, H-3), 6.76 (1H, d, *J* = 16.0 Hz, H-4) and 5.43 (1H, dq, *J* = 4.4, 1.2 Hz, H-6) (Table 1). In addition, three carbonyl resonance signals at δ_C 171.2 (C-19), 170.3 and 170.0 (2 × ester carbonyls) confirmed the presence of a γ-lactone and two esters; two acetyl methyls (δ_H 2.07, 1.98, each 3H × s) were also noted in **1**. Based on the aforementioned unsaturation data, the structure of **1** was determined to be that of a diterpenoid with four rings. Furthermore, the signals of two oxygenated quaternary carbons at δ_C 70.1 (C-8) and 63.4 (C-17) suggested the presence of a tetrasubstituted epoxide that contained a methyl substituent, and the proton signal of a methyl singlet at δ_H 1.49 (3H, s, H_3-18) further supported this observation.

The ^1H–^1H COSY spectrum of **1** revealed ^1H NMR coupling information showing the existence of H-2/H-3/H-4, H-6/H-7, H-9/H-10/H-11/H-12/H_2-13/H-14 and H-11/H_3-20 units (Table 1), which were established with the assistance of an HMBC experiment. Additionally, this experiment enabled clarification of the correlations between protons and quaternary carbons of **1**, such as H-2, H-9, H-10, H_3-15/C-1; H-3, H-4, H-7, H_3-16/C-5; H-6, H-9, H-10, H_3-18/C-8; H-9, H_3-18/C-17; and H_3-18/C-19, which provided confirmation of the carbon skeleton (Table 1). An allylic coupling between H-6/H_3-16 in the ^1H–^1H COSY spectrum and

correlations between H_3-16/C-4, -5, -6 and H-6/C-16 in the HMBC experiment demonstrated the presence of a methyl group at C-5. The 8,17-epoxide group was further confirmed by the HMBC correlations between H_3-18/C-8, -17 and H-9/C-8, -17. From the HMBC correlations between H-2/C-15; H-10/C-15; and H_3-15/C-1, -2, -10, -14, the C-15 methyl group was positioned at C-1. In addition, the carbon signals at δ_C 170.0 and 170.3 were correlated with the signals of the methyl protons at δ_H 1.98 and 2.07, respectively, in the HMBC spectrum and were consequently assigned as the carbon atoms of the acetate carbonyls. Additionally, the acetate positioned at C-2 was confirmed by the connectivity between H-2 (δ_H 5.37) and the carbonyl carbon (δ_C 170.0) of the acetate. Furthermore, the ^{1}H–^{1}H COSY spectrum demonstrated that a hydroxy proton signal at δ_H 2.35 (1H, d, J = 9.2 Hz) was associated with H-9 (δ_H 4.42, 1H, d, J = 9.2 Hz), which suggested that this hydroxy group could be positioned at C-9; this was further supported by HMBC correlations between OH-9/C-8, -9. Therefore, although no HMBC correlation was noted between H-14 and the acetate carbonyl, data from the analyses of ^{1}H–^{1}H COSY correlations and characteristic ^{1}H NMR signals for H-14 (δ_H 4.90) and H-12 (δ_H 3.99) indicated that the remaining acetoxy group and hydroxy group were positioned at C-14 and C-12, respectively.

Table 1: ^{1}H and ^{13}C NMR spectroscopic data, and ^{1}H–^{1}H COSY and HMBC correlations for **1**.

C/H	δ_H [a]	δ_C [b]	^{1}H–^{1}H COSY	HMBC (H→C)
1		45.4 (C) [d]		
2	5.37 d (9.6) [c]	75.9 (CH)	H-3	C-1, -3, -4, -14, -15, acetate carbonyl
3	5.80 dd (16.0, 9.6)	126.3 (CH)	H-2, H-4	C-5
4	6.76 d (16.0)	138.6 (CH)	H-3	C-2, -3, -5, -6
5		140.5 (C)		
6	5.43 dq (4.4, 1.2)	118.4 (CH)	H-7, H_3-16	C-8, -16
7	5.13 d (4.4)	77.1 (CH)	H-6	C-5, -6
8		70.1 (C)		
9	4.42 d (9.2)	74.6 (CH)	H-10, OH-9	C-1, -8, -10, -11, -17
10	1.99 m	38.8 (CH)	H-9, H-11	C-1, -2, -8, -11, -14, -15, -20
11	1.99 m	42.8 (CH)	H-10, H-12, H_3-20	C-10, -20
12	3.99 m	67.5 (CH)	H-11, H_2-13	n. o. [e]
13	1.82–1.94 m	29.6 (CH_2)	H-12, H-14	C-11, -12
14	4.90 dd (3.2, 2.8)	74.8 (CH)	H_2-13	C-10, -12
15	1.42 s	16.2 (CH_3)		C-1, -2, -10, -14
16	1.88 d (1.2)	23.5 (CH_3)	H-6	C-4, -5, -6
17		63.4 (C)		
18	1.49 s	10.2 (CH_3)		C-8, -17, -19
19		171.2 (C)		
20	1.20 d (6.8)	9.9 (CH_3)	H-11	C-10, -11, -12
OAc-2		170.0 (C)		
	1.98 s	21.3 (CH_3)		Acetate carbonyl
OAc-14		170.3 (C)		
	2.07 s	21.2 (CH_3)		Acetate carbonyl
OH-9	2.35 d (9.2)		H-9	C-8, -9

[a] Spectra measured at 400 MHz in $CDCl_3$ at 25°C. [b] Spectra measured at 100 MHz in $CDCl_3$ at 25°C. [c] J value (in Hz) in parentheses. [d] Multiplicity was deduced by ^{13}C, DEPT and HSQC experiments. [e] n. o. = not observed.

Based on previous studies of briarane derivatives [1–5], all naturally-occurring briarane-type natural products that have been identified include the C-15 methyl group *trans* to H-10, and these two groups are designated β- and α-oriented, respectively. NOESY (Figure 2) and vicinal proton coupling constant analysis revealed the configuration of **1**. From the NOESY experiment, the correlations of H-10 with H-2, H-9 and H-12, but not with H_3-15, showed that these protons (H-2, H-9, H-10 and H-12) were positioned on the same face of the molecule, and therefore they were assigned as α protons, as Me-15 was a β-substituent at C-1. H-14 was found to be correlated with H_3-15, but not with H-10, suggesting that this proton was of a β-orientation at C-14; H_3-20 was observed to be correlated with H_3-15, but not with H-10, demonstrating that the methyl group at C-11 was β-oriented; and H_3-18 was correlated with H-11 and H_3-20, indicating the β-orientation of the C-18 methyl in the γ-lactone ring. The configuration at C-9 is worthy of comment. H-9 was found to exhibit correlations with H-10, H-11, H_3-18 and H_3-20, as well as a lack of coupling between H-9 and H-10, indicating that the dihedral angle between H-9 and H-10 is approximately 90° and the 9-hydroxy group has a β-orientation. In addition, as the NOESY experiment showed a response between H-7 and the C-9 hydroxy proton, H-7 in **1** was determined to be β-oriented; a 16.0 Hz coupling constant between H-3 (δ_H 5.80) and H-4 (δ_H 6.76) indicated the *trans* geometry of the C-3/4 double bond; and a correlation between the C-6 olefin proton (δ_H 5.43) and the C-16 vinyl methyl (δ_H 1.88) suggested a *Z*-configuration of the C-5/6 double bond. Moreover, H-3 was found to be associated with H_3-15, but not with H-2 and H-4, and H-4 was associated with H-2, but not with H_3-16; these results demonstrated the 3*E*,5*Z*-configuration of $\Delta^{3,5}$. Therefore, based on the above findings, the *s-trans* diene moiety in **1** was elucidated. The NMR data of **1** were found to be similar to those of briarane derivatives with a 3*E*,5*Z*-diene system, such as briaexcavatin M [9], briaranolides I and J [10], excavatolide F [11] and briarenolide ZII (**2**) (Figure 1) [12]. Thus, briarane **1** was identified as the 12-*O*-debutyryl derivative of **2**.

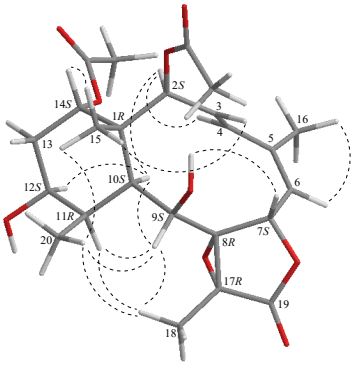

Figure 2: Selected protons with key NOESY correlations of **1**.

Since the first briarane-type diterpenoid, briarein A, was isolated from the Caribbean octocoral *Briareum asbestinum* in 1977 [13], it has become apparent that all naturally-derived briarane-based diterpenoids found in octocorals belonging to the genus *Briareum* possess a C-15 methyl group at C-1 *trans* to H-10, and these two groups have been proven to be β- and α-oriented, respectively, by chemical conversion [10,14,15] and X-ray analysis [10]. Therefore, based on biosynthetic derivation, the absolute configurations of the stereogenic centers of **1** were assigned as 1*R*, 2*S*, 7*S*, 8*R*, 9*S*, 10*S*, 11*R*, 12*S*, 14*S* and 17*R*.

Table 2: Effects of briaranes **1** and **2** on LPS-induced COX-2 and iNOS protein expression in macrophages.

Compounds	COX-2	iNOS
	Expression (% of LPS group)	Expression (% of LPS group)
Control	1.00 ± 0.02	0.79 ± 0.01
LPS	100.0 ± 18.4	100.0 ± 7.50
1	132.0 ± 33.5	158.1 ± 13.7
2 [a]	89.5 ± 4.00	47.2 ± 7.20

[a] These data were reported by Su et al., please see ref. [12].

In order to assess the anti-inflammatory activities of compound **1**, an *in vitro* assay using RAW264.7 cells (a macrophage-like murine cell line) stimulated with lipopolysaccharide (LPS) was performed to evaluate the effects of these compounds, and Western blotting was used to quantitate the changes in the protein expression levels of pro-inflammatory cyclooxygenase 2 (COX-2) and inducible nitric oxide synthase (iNOS) in the cells. The results showed that treatment of cells with 10 μM briarenol B (**1**), a 12-*O*-debutyryl

derivative of **2** (briarenolide ZII), was found to exhibit a much weaker anti-inflammatory activity than **2** [12] (Table 2), significantly enhancing the expressions of COX-2 and iNOS to 132.0 and 158.1%, respectively, at a concentration of 10 μM. These results suggested that a small structural variation could influence the bioactivity of compounds of this type, and further studies may be warranted in the future.

Experimental

General: Melting point was determined using a Fanchum melting point apparatus and was uncorrected. Optical rotation was measured using a Jasco P-1010 digital polarimeter. IR spectra were obtained with a Thermo FT-IR iS5 spectrophotometer; peaks are reported in cm^{-1}. NMR spectra were recorded on a Varian Mercury Plus 400 spectrometer using the residual $CHCl_3$ signal (δ_H 7.26 ppm) as the internal standard for ^1H NMR and $CDCl_3$ (δ_C 77.1 ppm) for ^{13}C NMR. Coupling constants (*J*) are given in Hz. ESIMS and HRESIMS data were recorded using a 7 Bruker Tesla SolariX FTMS. Column chromatography was performed on silica gel with a mesh size of 230–400. TLC experiments were performed on precoated Kieselgel 60 F_{254} (0.25 mm); the plates were sprayed with 10% H_2SO_4 solution followed by heating to visualize the compounds. Normal phase HPLC (NP-HPLC) was performed using a HPLC system equipped with a Rheodyne 7725 injection port and a Hitachi L-7110 pump. The column used for HPLC was a semi-preparative normal phase LiChrospher 250 mm × 10 mm column (Hibar, Si 60, 5 μm; Merck). Reverse phase HPLC (RP-HPLC) was performed using a system equipped with a Rheodyne 7725 injection port, a RP-18e column (5 μm, 250 × 21.2 mm; Luna), a Hitachi L-7100 pump and a Hitachi L-2455 photodiode array detector.

Animal material: Specimens of *B. excavatum* (Nutting 1911) were hand-picked by scuba divers in an area off the coast of southern Taiwan in July 2011. After harvest, the specimens were stored in a freezer immediately. A voucher specimen was deposited in the specimen bank of the NMMBA (Specimen number: NMMBA-TW-SC-2011-77) [16].

Extraction and isolation: *B. excavatum* (wet weight, 6.32 kg; dry weight, 2.78 kg) samples were sliced and then extracted with a mixture of methanol (MeOH)/dichloromethane (DCM) in a 1:1 ratio. The extract was partitioned between ethyl acetate (EtOAc) and H_2O. The EtOAc layer was first separated on silica gel, followed by elution and chromatography with a mixture of *n*-hexane/EtOAc (stepwise, 100:1 to pure EtOAc) to yield 26 sub-fractions, fractions A–Z. Fraction V was chromatographed on silica gel and eluted using a mixture of DCM/EtOAc (stepwise, 20:1 to pure EtOAc) into 14 sub-fractions, V1–V14. Fraction V9 was separated by NP-HPLC using DCM/EtOAc (1:1) to afford 25 sub-fractions, V9A–V9Y. Fraction V9G was further purified by RP-HPLC, using MeOH:H_2O (45:55, flow rate: 4.0 mL/min) as the mobile phase to afford **1** (3.0 mg, t_R = 87 min).

Briarenol B (1)
White amorphous powder.
MP: 185–186°C.
$[\alpha]_D^{24}$: −12 (*c* 0.2, $CHCl_3$).
IR (neat): ν_{max} 3479, 1766, 1732 cm^{-1}.
^1H NMR (400 MHz, $CDCl_3$): Table 1.
^{13}C NMR (100 MHz, $CDCl_3$): Table 1.
ESIMS: *m/z*: (%) = 487 $[M + Na]^+$.
HRESIMS: *m/z* $[M + Ma]^+$ calcd for $C_{24}H_{32}O_9$ + Na: 487.19441; found 487.19396.

In vitro anti-inflammatory assay: RAW264.7 (TIB-71) cells, a macrophage-like cell line derived from mice, was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The *in vitro* anti-inflammatory activity of compound **1** was measured by investigating its inhibition effects on pro-inflammatory iNOS and COX-2 protein expressions in LPS-stimulated RAW264.7 cells using Western blot analysis [17–19]. Briefly, an inflammation response in RAW264.7 cells was induced by incubating cells in medium containing only LPS (10 ng/mL) without test compounds for 16 h. For the anti-inflammatory activity assay, 10 μM of either compound **1** or dexamethasone (as a positive control) were added to the cells 10 min before LPS treatment. After incubation, the cells were lysed and the protein lysates were subjected to Western blotting analysis. The protein expression levels were determined based on the immunoreactivity of proteins to antibodies, and were calculated with respect to the average optical density of the signals on the film to that of the corresponding LPS-stimulated cells. Moreover, the cytotoxic effects of compound **1** in RAW264.7 cells were also evaluated by examining cell viability by the trypan blue exclusion test [18,19]. For statistical analysis, the data were analyzed by one-way analysis of variance (ANOVA), and then the Student–Newman–Keuls post hoc test was used for multiple comparisons. A significant difference was defined as a *p*-value of < 0.05.

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Triterpenes with Anti-invasive Activity from Sclerotia of *Inonotus obliquus*

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The methanolic extract [inhibition (%): 61.2±3.8 (p<0.01) at 100 μg/mL] and its EtOAc-soluble fraction [inhibition (%): 82.5±1.7 (p<0.01) at 100 μg/mL] from the sclerotia of *Inonotus obliquus* collected in Japan significantly inhibited invasion of human fibrosarcoma HT1080 cells through matrigel-coated filters. In addition, the methanolic extract significantly inhibited lung tumor formation fifteen days after injection of B16F10 melanoma cells in mice [inhibition (%) 66.1 ± 12.8 (p < 0.05) at 500 mg/kg/d, p.o.]. Lanostane-type triterpenes were isolated as the common principal constituents from Japanese and Russian *I. obliquus*. Furtheremore, we examine the inhibitory effects of the constituents on the invasion of HT 1080 cells. Interestingly, 3β-hydroxylanosta-8,24-dien-21-al [inhibition (%) 37.9 ± 3.0 (p < 0.05) at 30 μM] significantly inhibited the invasion, and no cytotoxic effect at 30 μM was observed.

Keywords: *Inonotus obliquus*, Lanostane-type triterpene, Anti-metastatic activity, Invasion inhibitor**,** Traditional medicine.

Inonotus obliquus (Hymenochaetaceae) has been distributed in north regions of Japan, Russia, and so on. The sclerotia of this fungus (Kabanoanatake in Japanese, Charga in Russian) have been used for treating cancer as a folk medicine. In the previous study, we reported the isolation and structure elucidation of several triterpenes from the sclerotia of *I. obliquus* collected in Japan [1]. Furthermore, several chemical and biological studies on *I. obliquus* have been reported [2–6]. For example, triterpenes were isolated as chemical constituents with cytotoxic effects from the sclerotia of *I. obliquus* [2–5]. Recently, polysaccharides fraction derived *I. obliquus* was reported to inhibit the invasion in B16F10 melanoma cells *in vitro* [6]. However, the biological study of the isolated compounds on metastatis of cancer was left uncharacterized. In addition, anti-metastatic experiment *in vivo* of the extract was also uncharacterized.

Metastatis of cancer, which is the major cause of death in cancer patients, occurs through a complex multistep process. On the metastasis, invasion into the circulation from the primary tumor through the extracellular matrix (ECM) and basement membrane (BM) is an essential step. Therefore, its blockade has been considered to enhance survival of cancer patients. In the previous study, we have reported the inhibitory effects of several rotenoids and phenylbutanoids on invasion of HT 1080 cells through matrigel-coated filters [7,8].

In the present study, to develop the invasion inhibitors and evaluate the traditional effects of *I. obliquus*, the inhibitory effects of the methanolic (MeOH) extract from the sclerotia of *I. obliquus* on the invasion of HT1080 cells through matrigel-coated filters as *in vitro* experiment were examined. In addition, the inhibitory effects of the MeOH extract on metastasis of B16F10 melanoma cells in mice as *in vivo* experiment were examined. Next, effects of triterpenes isolated from the sclerotia of *I. obliquus* collected in Japan and Russia on invasion of HT1080 cells and proliferation of HT1080 cells were examined.

To investigate the anti-metastatic activity of medicinal fungi, we examined the effects of the MeOH extracts from six medicinal fungi (the sclerotia of *Ganoderma lucidum*, *Polyporus umbellatus*, *Wolfiporia extensa*, *Lentinula edodes*, and *Agaricus subrufescens*) on the invasion of HT1080 cells through matrigel-coated filters. As a result, the MeOH extract from the sclerotia of *I. obliquus* collected in Japan showed the inhibitory effects [inhibition (%): 61.2±3.8 (p<0.01) at 100 μg/mL] (Table 1). The effect was strongest among six fungi.

Table 1: Effects of the MeOH extracts from the sclerotia of medicinal fungi on invasion of HT1080 cells *in vitro*[a].

Treatment	Inhibition (%)			
Conc. (μg/mL)	0	10	30	100
I. obliquus	0.0±7.6	−10.2±13.7	21.3±17.8	61.2±3.8**
G. lucidum	0.0±8.6	–17.0±13.7	17.0±12.6	16.7±9.0
P. umbellatus	0.0±2.7	–28.0±12.5	–12.9±13.6	43.5±11.4*
W. extensa	0.0±19.1	3.1±3.8	–12.5±13.8	18.0±2.8
L. edodes	0.0±11.0	4.6±13.5	–4.2±5.2	23.3±1.8
A. subrufescens	0.0±13.3	–15.2±8.9	0.4±6.5	42.5±3.1*

[a]Values represent the means±S.E.M. (n=4).
Significantly different from the control, *p<0.05, **p<0.01.

Table 2: Effects of the MeOH ext. from *I. obliquus* on metastasis of B16F10 melanoma cells in C57BL/6 mice[a].

	Dose (mg/kg/d, *p.o.*)	*n*	Numbers of Nodules	Inhibition (%)
Control	0	13	92.4±15.2	0.0±16.5
MeOH extract	250	12	66.3±18.0	28.2±19.5
	500	14	31.4±11.8	66.1±12.8*

[a]Values represent the means±S.E.M.
Significantly different from the control, *p<0.05.

Next, we examined the effects of the MeOH extract on metastasis of B16F10 melanoma cells in C57BL/6 mice. Fifteen days after injection of the B16F10 melanoma cells, 92 nodules were observed on the surfaces of both lungs in mice. On the other hand, after injection of the B16F10 melanoma cells, the MeOH extract was administered orally to mice for fourteen days once a day. The MeOH extract significantly suppressed the lung metastasis of the cells [inhibition (%) 66.1 ± 12.8 (p < 0.05) at 500 mg/kg/d, p.o.] (Table 2).

Therefore, the MeOH extract of the sclerotia of *I. obliquus* collected in Japan was partitioned into an EtOAc–H_2O mixture to furnish an EtOAc-soluble fraction and aqueous layer. The aqueous layer was further extracted with *n*-BuOH to give *n*-BuOH and H_2O-soluble fractions. The EtOAc-soluble fraction [inhibition (%): 82.5±1.7 (p<0.01) at 100 μg/mL] was found to show inhibitory effects on the invasion of HT 1080 cells through matrigel-coated filters (Table 3), whereas the *n*-BuOH- [inhibition (%): 18.8±18.0 at 100 μg/mL] and H_2O- [inhibition (%): 19.0±18.8 at 100 μg/mL] soluble fractions did not show significant inhibition.

Table 3: Effects of the fractions from the sclerotia of *I. obliquus* on invasion of HT1080 cells *in vitro*[a].

Treatment	Inhibition (%)		
Conc. (μg/mL)	0	30	100
EtOAc-souble fraction	0.0±4.0	10.1±9.6	82.5±1.7**
n-BuOH-souble fraction	0.0±11.1	–	18.8±18.0
H_2O-souble fraction	0.0±13.3	–	19.0±18.8

[a]Values represent the means±S.E.M. (n=4).
Significantly different from the control, **p<0.01

The EtOAc-soluble fraction, a predominant bioactive portion, was subjected to normal- and reversed-phase silica-gel column chromatography and repeated HPLC to give lanosterol (**1**) [1,9], 3®,25-dihydroxylanosta-8,23-diene (**2**) [1,10], inotodiol (**3**) [1,11], trametenolic acid (**4**) [1,11], 3β-hydroxylanosta-8,24-dien-21-al (**5**) [1,11], 3β,21-dihydroxylanosta-8,24-diene (**6**) [1,12], betulin (**7**) [13], ergosterol peroxide (**8**) [14], 3,4-dihydroxybenzaldehyde (**9**) together with lanosta-8,24-diene-3-one [12] (Figure 1). We have already reported the isolation of **1**–**6** [1]. In the present study, compounds **7**–**9** were isolated moreover. Next, for comparison of the constituents of *I. obliquus* collected in Japan with those of Russia, we examined the constituents from the sclerotia of *I. obliquus* collected in Russia. As a result, compounds **1**, **3**, **4**, **7**, and lanosta-8,24-diene-3-one, were isolated as well as those of Japan.

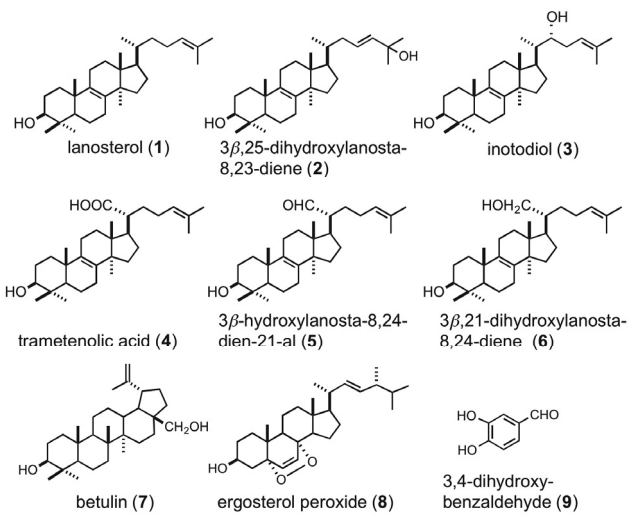

Figure 1: Structures of Constituents **1**–**9**.

The effects of the principal constituents from the sclerotia of *I. obliquus* on the invasion of HT1080 cells through matrigel-coated filters were examined (Table 4). Deguelin, which was known to display the inhibitory effects on the invasion and cytotoxicity on HT1080 cells was shown as positive control in Table 4 [7]. Among them, compounds **1**, **2**, **3**, **5**, and **6** showed the inhibitory effects on the invasion of HT1080 cells. Particularly, lanosterol (**1**), inotodiol (**3**), and 3β-hydroxylanosta-8,24-dien-21-al (**5**) significantly inhibited the invasion [inhibition (%) 52.7 ± 3.1 (p < 0.05) for **1**, 31.4 ± 10.3 (p < 0.05) for **3**, 37.9 ± 3.0 (p < 0.05) for **5**, at 30 μM].

Next, the cytotoxic effects of **1**, **3**, and **5** against HT-1080 cells by using WST-8 assay and calcein-AM assay were examined (Table 5). Interestingly, compound **5** did not show the cytotoxic effects at 30 μM against HT1080 cells after 24-72 h incubation [inhibition (%): less than 10% at 30 μM] although reference compound, deguelin, was known to exhibit the cytotoxic effects after 48-72 h at 10 μM.

Table 4: Effects of the constituents from the sclerotia of *I. obliquus* on invasion of HT1080 cells *in vitro*[a].

Treatment	Inhibition (%)			
Conc. (μM)	0	10	30	100
1	0.0±9.1	12.1±5.3	52.7±3.1*	79.6±9.0**
2	0.0±4.2	–12.2±7.0	–0.9±6.2	28.8±5.2**
3	0.0±7.6	2.5±10.4	31.4±10.3*	50.9±4.9**
4	0.0±9.9	0.8±22.9	–9.3±17.8	19.0±14.3
5	0.0±6.0	5.9±7.6	37.9±3.0*	62.3±1.3**
6	0.0±13.2	–22.2±6.1	17.4±10.2	44.4±9.6*
7	0.0±5.7	–2.2±6.4	13.1±10.8	26.4±3.6
8	0.0±5.0	–1.9±7.3	12.6±3.2	24.1±3.2
9	0.0±4.7	4.5±9.0	–6.3±12.9	15.5±6.7
Deguelin[b]	0.0±7.3	58.0±7.5**	57.9±10.5**	–

[a]Values represent the means±S.E.M. (n=4).
Significantly different from the control, *p<0.05, **p<0.01
[b]Reference compound. Data was cited from our report [7].

Table 5: Effects of the constituents **1**, **3**, and **5** on proliferation of HT1080 cells by WST-8 and calcein-AM assay[a].

Treatment	Inhibition (%)			
Conc. (μM)	0	10	30	100
WST-8 assay				
1				
24 h	0.0±1.0	10.3±0.9**	33.8±1.9**	98.6±0.3**
48 h	0.0±0.9	13.4±1.1**	33.1±1.7**	98.5±0.3**
72 h	0.0±1.5	11.8±1.6**	35.5±0.9**	97.0±1.5**
3				
24 h	0.0±0.6	3.5±1.3	9.2±1.9	87.5±1.5**
48 h	0.0±1.3	4.5±0.7	13.9±1.6*	90.5±1.3**
72 h	0.0±0.5	10.4±0.5**	16.6±1.4**	88.4±3.1**
5				
24 h	0.0±1.6	–0.7±0.2	–1.1±1.5	25.2±2.1**
48 h	0.0±0.9	0.3±1.7	–2.7±0.8	6.6±0.9**
72 h	0.0±0.6	–0.9±1.9	–2.0±0.6	24.5±4.8**
Clcein-AM Assay				
1				
24 h	0.0±5.6	20.5±1.7**	34.6±3.3**	84.7±2.9**
48 h	0.0±2.6	16.2±1.0**	40.4±0.7**	94.6±0.7**
72 h	0.0±1.1	14.8±1.8**	48.5±1.0**	90.9±2.5**
3				
24 h	0.0±6.1	6.1±3.7	–6.6±4.2	63.7±3.6**
48 h	0.0±2.4	12.2±1.1	17.0±1.1	66.9±12.6**
72 h	0.0±1.6	9.7±1.6	27.8±2.0**	85.4±7.7**
5				
24 h	0.0±5.4	–11.5±2.4	–4.2±4.1	22.4±11.0
48 h	0.0±3.0	0.2±3.0	2.4±1.8	47.6±4.5**
72 h	0.0±1.2	–2.7±0.8	2.6±2.3	42.2±6.8**
Deguelin[b]				
24 h	0.0±1.4	–1.8±5.1	17.0±1.4	–
48 h	0.0±1.6	51.0±2.1**	40.4±2.7**	–
72 h	0.0±0.6	98.5±0.1**	98.5±0.1**	–

[a]Values represent the mean±S.E.M. (n=4).
Significantly different from the control, *p<0.05, **p<0.01.
[b]Reference compound. Data was cited from our report [7].

In conclusion, the MeOH extract from the sclerotia of *I. obliquus* collected in Japan significantly inhibited invasion of HT1080 cells through matrigel-coated filters and lung tumor formation fifteen days after injection of B16F10 melanoma cells in mice. In additon, lanostane- and lupane-type triterpenes were isolated as the common constituents from the sclerotia of *I. obliquus* collected in Japan and Russia. Furthermore, 3β-hydroxylanosta-8,24-dien-21-al (**5**) significantly inhibited invasion of HT1080 cells at 30 μM, and no cytotoxic effect was observed at 30 μM. These results may be useful in the design of lead compounds for development of anti-metastatic drugs.

Experimental

General: The following instruments were used to obtain physical data: specific rotations, a Horiba SEPA-300 digital polarimeter (l = 5 cm); EIMS and HREIMS, JEOL JMS-GCMATE mass spectrometer; ^{1}H-NMR spectra, JEOL JNM-ECS400 (400 MHz), JEOL JNM-LA500 (500 MHz), and JEOL JNM-ECA600 (600 MHz) spectrometers; ^{13}C-NMR spectra, JEOL JNM-ECS400 (100 MHz), JEOL JNM-LA500 (125 MHz), and JEOL JNM-ECA600 (150 MHz) spectrometers with tetramethylsilane as an internal standard; HPLC, a Shimadzu RID-6A refractive index and SPD-10A*vp* UV-VIS detectors. COSMOSIL 5C18-MS-II (250 × 4.6 mm i.d. and 250 × 20 mm i.d.) columns were used for analytical and preparative purposes. The following materials were used for chromatography: normal-phase silica gel column chromatography, Silica gel BW-200 (Fuji Silysia Chemical, Ltd., 150–350 mesh); reversed-phase silica gel column chromatography, Chromatorex ODS DM1020T (Fuji Silysia Chemical, Ltd., 100–200 mesh); TLC, precoated TLC plates with Silica gel 60F_{254} (Merck, 0.25 mm) (ordinary phase) and Silica gel RP-18 F_{254S} (Merck, 0.25 mm) (reversed phase); reversed-phase HPTLC, precoated TLC plates with Silica gel RP-18 WF_{254S} (Merck, 0.25 mm). Detection was achieved by spraying with 1% $Ce(SO_4)_2$–10% aqueous H_2SO_4 followed by heating.

Animal: Female C57BL/6 mice aged 6 weeks were purchased from Kiwa Laboratory Animals Co., Ltd. (Wakayama, Japan) and maintained in an air-conditioned room at 23±2°C. Standard laboratory chow (MF, Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water were given freely.

Plant Material: The sclerotia of *Inonotus obliquus* in Japan were collected in 2007, which were identified by one of the authors (M. Y.). The sclerotia of *I. obliquus* in Russia were provided from KSA International Inc. (Kyoto, Japan) in 2016, which were identified by one of the authors (H. M.). The sclerotia of *G. lucidum*, *P. umbellatus*, and *W. extensa* were purchased from Aotsubu Co., Ltd. (Hyogo, Japan) in 2008, which were identified by one of the authors (H. M.). The sclerotia of *L. edodes* and *A. subrufescens* were purchased from Tochimoto Tenkaido Co. Ltd (Osaka, Japan) in 2008, which were identified by one of the authors (H. M.). A voucher of the material is on file in our laboratory [2007. Japan-IO-1, 2016. Russia-IO-1, 2008. A-GL-1, 2008. A-PU-1, 2008. A-WE-1, 2008. T-LE-1, and 2008. T-AS-1, respectively].

Reagents for Bioassay: Minimum essential medium Eagle's (MEM) and RPMI1640 were purchased from Sigma–Aldrich (MO, USA); fetal bovine serum (FBS) was from Roche Diagnostics (Basel, Switzerland); Matrigel (BD Matrigel^{TM}) was from BD Biosciences (NJ, USA); Cell Culture Insert^{TM} was from BD Falcon (NJ, USA); Cell Counting Kit-8^{TM} and Cell Counting Kit-F^{TM} were from Dojindo Lab. (Kumamoto, Japan); other reagents were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Twenty four-well multiplates were purchased from Sumitomo Bakelite Co., Ltd. (Tokyo, Japan); 96-well black microplates were from Nunc (Roskild, Denmark).

Extraction and isolation: The sclerotia of *I. obliquus* (1.4 kg) collected in Japan were extracted three times with MeOH under reflux for 3 h as reported previously [1]. Evaporation of the solvent under reduced pressure provided a MeOH extract (304 g, 21.7% from the sclerotia). The aliquot (267.5 g) from the extract was partitioned into an EtOAc–H_2O (1:1, v/v) mixture to furnish an EtOAc-soluble fraction (125.4 g, 10.2%) and an aqueous phase. The aqueous phase was further extracted with *n*-BuOH to give an *n*-BuOH-soluble fraction (54.0 g, 4.4%) and an H_2O-soluble fraction (88.1 g, 7.1%). The EtOAc fraction (125.4 g) was subjected to ordinary-phase silica gel column chromatography (CC) [3.0 kg, *n*-hexane-EtOAc (2:1 → 1:1, v/v) → EtOAc → $CHCl_3$–MeOH (50:1 → 20:1 → 10:1 → 5:1, v/v) → MeOH] to give 14 fractions {Fr. 1–Fr. 7, Fr. 8 (3.3 g), Fr. 9, Fr. 10, Fr. 11 (5.2 g), Fr. 12 (18.9 g), Fr. 13, and Fr. 14}. Fraction 8 (3.3 g) was subjected to reversed-phase silica gel CC [165 g, MeOH–H_2O (60:40 → 70:30 → 80:20 → 90:10, v/v) → MeOH] to afford seven fractions [Fr. 8-1–8-3, Fr. 8-4 (1.278 g), Fr. 8-5–8-7]. Fraction 8-4 (510 mg) was separated by HPLC [MeOH–H_2O (90:10, v/v)] to afford six fractions {Fr. 8-4-1–8-4-3, Fr. 8-4-4 (64 mg), Fr. 8-4-5, and Fr. 8-4-6}. Fraction 8-4-4 (64 mg) was purified by HPLC [MeOH–H_2O (85:15, v/v)] to furnish betulin (**7**, 14.0 mg, 0.0035%). Fraction 11 (5.2 g) was subjected to reversed-phase silica gel CC [260 g, MeOH–H_2O (10:90 → 20:80 → 40:60 → 60:40 → 80:20, v/v) → MeOH] to afford 19 fractions {Fr. 11-1–11-16, Fr. 11-17 (415 mg), Fr. 11-18, and Fr. 11-19}. Fraction 11-17 (275 mg) was purified by HPLC [MeOH–H_2O (90:10, v/v)] to furnish ergosterol peroxide (**8**, 30 mg, 0.0035%). Fraction 12 (18.9 g) was subjected to reversed-phase silica gel CC [950 g, MeOH–H_2O (20:80 → 40:60 → 60:40 → 80:20, v/v) → MeOH] to afford 16 fractions {Fr. 12-1, Fr. 12-2 (683 mg), Fr. 12-3–12-16}. Fraction 12-2 (150 mg) was purified by HPLC [MeOH–H_2O (20:80, v/v)] to furnish 3,4-dihydroxybenzaldehyde (**9**, 59 mg, 0.022%). The isolation method of compounds **1**–**6** was reported in the previously paper [1].

The sclerotia of *I. obliquus* (153.6 g) collected in Russia were extracted three times with MeOH under reflux for 3 h. Evaporation of the solvent under reduced pressure provided a MeOH extract (7.9 g, 5.1% from the sclerotia). An aliquot (7.6 g) from the extract was partitioned into an EtOAc–H_2O (1:1, v/v) mixture to furnish an EtOAc-soluble fraction (3.4 g, 2.3%) and an aqueous phase. The aqueous phase was further extracted with *n*-BuOH to give an *n*-BuOH-soluble fraction (1.7 g, 1.1%) and an H_2O-soluble fraction (2.5 g, 1.7%). The EtOAc fraction (3.4 g) was subjected to ordinary-phase silica gel CC [90 g, *n*-hexane → *n*-hexane-EtOAc (4:1 → 3:1 → 2:1 → 1:1 → 1:10,, v/v) → MeOH] to give 9 fractions {Fr. 1 (138 mg), Fr. 2 [= inotodiol (**3**, 134 mg, 0.09%)], Fr. 3 (55 mg), Fr. 4, Fr. 5 [= trametenolic acid (**4**, 31 mg, 0.021%), Fr. 6–Fr. 9]. Fraction 1 (138 mg) was also subjected to ordinary-phase silica gel CC [*n*-hexane-EtOAc (5:1, v/v) to give 3 fractions {Fr. 1-1 [= lanosta-8,24-diene-3-one (**10**, 6.7 mg, 0.0045%)], Fr. 1-2 (55 mg), Fr. 1-3 [= lanosterol (**1**, 13 mg, 0.0088%)]}. Fraction 1-2 (55 mg) was purified by ordinary-phase silica gel CC [*n*-hexane-EtOAc (10:1 → 2:1, v/v) to furnish **1** (15 mg, 0.010%). Fraction 3 (55 mg) was also purified by ordinary-phase silica gel CC [*n*-hexane-EtOAc (10:1 → 2:1, v/v) → EtOAc] and HPLC [MeOH–H_2O (85:15, v/v)] to give betulin (**7**, 0.3 mg, 0.00020%)]

Cell Culture: Human fibrosarcoma HT1080 cells (Cell No. JCRB9113) were obtained from Health Science Research Resources Bank (Osaka Japan). B16F10 melanoma cells were provided from Institute of Development, Aging and Cancer, Tohoku University. These cells were maintained in MEM and RPMI1640 supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin.

Invasion Assay: The invasion assay of HT1080 cells was performed using Cell Culture Insert^{TM} and 24-well multiplates as reported previously [7,8]. The upper side of each filter of Cell Culture Insert^{TM} was pre-coated with matrigel (25 μg/filter). Briefly, 100 μL of 0.25 mg/mL matrigel in PBS solution was added onto each filter (pore size 8 μm), incubated for 4 h at 37 °C, and dried at room temperature. Cell Culture Insert^{TM} with matrigel-coated filters

was inserted into the 24-well multiplates with 700 μL/well MEM supplemented with FBS [FBS (+)]. A mixture of HT1080 cells (1 x 10^6 cells/mL) suspended in 100 μL MEM without FBS [FBS (-)] and test compound solution in 100 μL MEM [FBS (-)] was then added onto the filters and incubated for 24 h. After incubation, the cells crossing the filters were collected after treatment of trypsin solution (0.25% trypsin and 0.02% EDTA in phosphate-buffered saline (PBS), and the invaded cells were resuspended in RPMI1640 [FBS (-), phenol red (-)] and seeded onto 96-well black microplates. After incubation for 4 h at 37 °C in 5% CO atmosphere, Cell Counting Kit-F^{TM} was used for counting of the invaded cells according to the manufacturer's instruction. The test compound was dissolved in dimethylsulfoxide (DMSO) and final concentration of DMSO in the medium was 0.1%.

Calcein-AM Assay: After 24, 48, or 72 h incubation of HT1080 cells (5 x 10^4 cells/100 μL/well) with test compounds in MEM [FBS (+)] in 96-well black microplates, the medium was exchanged for RPMI1640 [FBS (–), phenol red (–)], and then 10 μL of calcein-AM in PBS solution (Cell Counting Kit-F^{TM}) was added to each well. After a further 30 min in culture, the fluorescence intensity of each well was measured with a microplate reader (ex: 485 nm, em: 520 nm, FLUOstar OPTIMA, BMG Labtechnologies).

WST-8 Assay: After 20, 44, or 68 h incubation of HT1080 cells (5 x 10^4 cells/100 µL/well) with test compounds in RPMI1640 [FBS (+)] in 96-well microplates, 10 μL of WST-8 solution (Cell Counting Kit-8^{TM}) was added to each well. After a further 4 h in culture, the optical density of the water-soluble formazan produced by the cells was measured with a microplate reader (Model 550, Bio-Rad) at 450 nm (reference: 655 nm). Inhibition (%) was calculated by the following formula and IC_{50} value was determined graphically.

Inhibition (%) = (A – B)/A × 100

A and B indicate optical density or fluorescence intensity of vehicle and test compound-treated groups (n=4).

Assay for Experimental Lung Metastasis of the Melanoma Cells: A highly lung metastatic cell line desired from B16F10 melanoma cells was obtained by the *in vivo* selection method. Briefly, the melanoma cell suspension in PBS (2 x 10^5 cells/200 μL) was injected intravenously into tail of C57BL/6 mice. Fifteen days later, metastatic nodules in the mice were isolated. After treatment of trypsin solution (0.25% trypsin and 0.02% EDTA in PBS), and the melanoma cells were cultured in RPMI1640 supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. This same process was repeated, and the resultant to C57BL/6 mouse highly lung metastatic cells were obtained and used for the experiments.

B16F10 melanoma cells described above were resuspended in PBS. The melanoma cell suspension in PBS (2 x 10^5 cells/200 μL) was injected intravenously into tail of C57BL/6 mice. Fifteen days later, the mice were killed and the lungs were excised and tumor colonies were counted. Test samples were suspended in 5% acacia solution and given orally in a volume of 10 mL/kg once a day for fourteen days after the injection of melanoma cells.

Statistics: Values were expressed as means±S.E.M. One-way analysis of variance following Dunnett's test was used for statistical analysis. Probability (p) values less than 0.05 were considered significant.

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A New Cytotoxic Cyclolanostane Triterpenoid Xyloside from *Souliea vaginata*

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A new cyclolanostane triterpenoid xyloside, soulieoside P (**1**), and a known oleanane-type saponin, hederasaponin B (**2**), were isolated from the rhizomes of *Souliea vaginata*. Their structures were established by extensive spectroscopic and HRESIMS analysis, as well as chemical methods. Compound **1** showed significant inhibitory effects with IC_{50} values of 7.6−11.2 μM against three human cancer cell lines, while compound **2** exhibited no hepatoprotective effect on CCl_4-induced injury of human HepG2 cells, in the tested range of 0.1−100 μM.

Keywords: *Souliea vaginata*, Cyclolanostane triterpenoid glycoside, Soulieoside P, Hederasaponin B, Cytotoxicity, Hepatoprotective activity.

Souliea vaginata (Maxim.) Franch. (family Ranunculaceae), a perennial herbaceous plant mainly distributed in western China, is used in traditional medicine for the treatment of conjunctivitis, stomatitis, pharyngitis, and enteritis [1]. Chemical studies on the species showed the presence of cyclolanostane triterpenoid glycosides, alkaloids, and phenolic acids [2−4]. Among these constituents, cyclolanostane-type triterpenoid glycosides are characteristic of the genus *Souliea*, showing anti-complement, cytotoxic, and hepatoprotective activities [5−7]. Our previous study on the plant disclosed the presence of two new cyclolanostane triterpene glycosides [8]. Further investigation resulted in the isolation of another new cyclolanostane triterpenoid monoxyloside, soulieoside P (**1**) and a known oleanane-type saponin, hederasaponin B (**2**), from the ethanolic extract of the rhizomes (Figure 1). Compounds **1** and **2** were tested for their inhibitory activity against three human cancer cell lines and hepatoprotective effect on CCl_4-induced injury of human HepG2 cells, respectively.

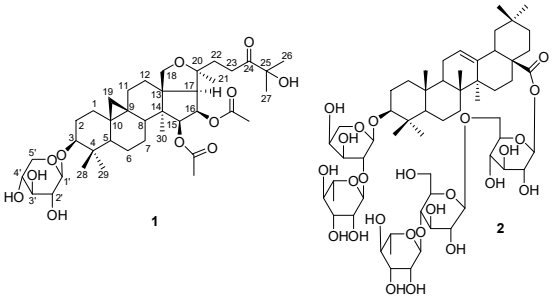

Figure 1: Structures of compounds **1** and **2** isolated from *Souliea vaginata.*

Soulieoside P (**1**), obtained as a colorless amorphous powder, exhibited a positive Liebermann–Burchard reaction. Its molecular formula was deduced as $C_{39}H_{60}O_{12}$ based on HRESIMS analysis (*m/z* 743.3948 $[M + Na]^+$, calcd 743.3982), consistent with ten degrees of unsaturation. The IR spectrum of **1** showed absorption bands at 3400 and 1720 cm^{-1} ascribable to hydroxyl and carboxyl groups. The ^1H NMR spectrum exhibited signals for two characteristic cyclopropane protons at δ_H 0.09 and 0.40 (1H each, d, J = 3.6 Hz), two acetyl methyl groups at δ_H 2.04 and 2.16, an oxygenated methylene at δ_H 3.97 and 4.04 (1H each, d, J = 9.0 Hz), six singlet methyl groups at δ_H 0.99, 1.12, 1.29, 1.50, 1.55, and 1.55, as well as ABX-type signals of three methine protons ascribable to H-15 (δ_H 5.89, d, J = 9.6 Hz), H-16 (δ_H 5.59, dd, J = 10.2, 9.6 Hz), and H-17 (δ_H 2.48, d, J = 10.2 Hz). In addition, the ^1H NMR spectrum also showed one anomeric doublet proton signal at δ_H 4.84 (J = 7.8 Hz) in the downfield region indicating the presence of a β-linked sugar and other signals for a pentose at δ_H 4.03 (t, J = 8.4 Hz, H-2'), 4.17 (t, J = 8.4 Hz, H-3'), 4.22 (m, H-4'), 3.73 (t, J = 10.8 Hz, H-5'), and 4.35 (dd, J = 10.8, 4.8 Hz, H-5'). The sugar was identified as xylose by acid hydrolysis followed by comparison with an authentic sample by TLC.

The ^{13}C APT NMR spectrum of **1** displayed 39 carbon resonances including a methylene carbon of a cyclopropane ring at δ_C 30.9 (C-19), one oxymethylene carbon at δ_C 74.3 (C-18), three oxygenated methine carbons at δ_C 88.7 (C-3), 80.1 (C-15), and 78.3 (C-16), two oxytertiary carbons at δ_C 86.6 (C-20) and 77.2 (C-25), one keto carbonyl group at δ_C 216.5 (C-24) and two ester carbonyls at δ_C 171.1 and 171.4. All carbon-bound protons were assigned based on HSQC and ^1H–^1H correlation spectra. The ^1H and ^{13}C NMR spectroscopic data (Table 1) of **1** confirmed that the compound was a cyclolanostane triterpene glycoside and was similar to those of

Table 1: ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR data of **1** in C_5D_5N (δ in ppm).

No.	δ_H (*J* in Hz)	δ_C	No.	δ_H (*J* in Hz)	δ_C
1	1.09 (m), 1.58 (m)	32.8	**21**	1.50 (s)	24.2
2	1.88 (m), 2.32 (m)	30.4	**22**	2.12, t (7.8)	39.0
3	3.48 (dd,11.4, 4.2)	88.7	**23**	3.19, t (7.8)	32.2
4		41.7	**24**		216.5
5	1.27 (m)	47.5	**25**		77.2
6	0.55 q (12.6), 1.35 (m)	21.1	**26**	1.55 (s)	27.7
7	1.07 (m), 1.60 (m)	26.5	**27**	1.55 (s)	27.7
8	1.52 (m)	49.0	**28**	1.29 (s)	26.1
9		20.3	**29**	0.99 (s)	15.7
10		26.9	**30**	1.12 (s)	15.2
11	1.24 (m), 0.92 (m)	26.3	**1′**	4.84 (d, 7.8)	108.0
12	1.64 (m), 2.10 (m)	29.7	**2′**	4.03 (t, 8.4)	75.9
13		59.1	**3′**	4.17 (t, 8.4)	79.0
14		42.4	**4′**	4.22 (m)	71.6
15	5.89 (d, 9.6)	80.1	**5′**	3.73 (t, 10.8), 4.35 (dd,10.8, 4.8)	67.5
16	5.59 (dd, 10.2, 9.6)	78.3	$CO\underline{CH_3}$-15	2.16	21.6
17	2.48 (d, 10.2)	52.7	$\underline{CO}CH_3$-15		171.1
18	3.97 (d, 9.0), 4.04 (d, 9.0)	74.3	$CO\underline{CH_3}$-16	2.04	21.4
19	0.09 (d, 3.6), 0.40 (d, 3.6)	30.9	$\underline{CO}CH_3$-16		171.4
20		86.6			

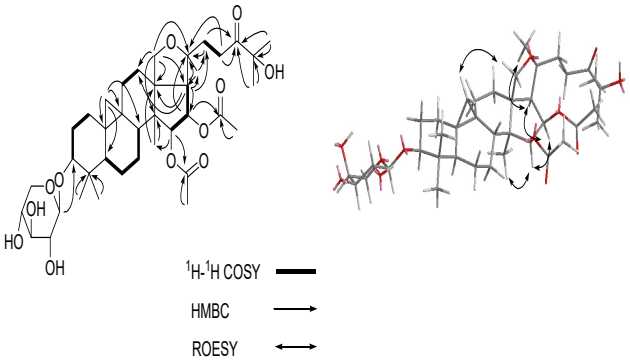

Figure 2: Key ^{1}H-^{1}H COSY, HMBC and ROESY correlations of **1**.

(20*S*)-15β,16β-diacetoxy-18,20-epoxy-3β,25-diol-9,19-cyclolanostan-24-one synthesized using beesioside I as substrate, except that a xylose group was attached at C-3 in **1** [9]. The HMBC experiment (Figure 2) was performed to establish the location of the functional groups and the full structure of **1**. The xylose residue was attached at C-3 due to the long-range correlation between the xylopyranosyl anomeric proton at 4.84 (1H, d, *J* = 7.8 Hz) and C-3 (δ_C 88.7). The HMBC spectrum of **1** showed long-range correlations between H-17 and C-16 (δ_C 78.3), C-20 (δ_C 86.6), H-16 and C-20 (δ_C 86.6), the carbonyl group (δ_C 171.1), and H-15 and C-30 (δ 15.2), the carbonyl group (δ_C 171.4), which confirmed the presence of the OAc-15 and OAc-16 groups. The HMBC experiment also showed the presence of key correlations between H-19 and C-9, C-10; between H-18 and C-12, C-13, C-14, C-17, C-20; between C-24 and H-22, H-23, H-26, H-27. The stereochemistry of **1** was resolved by analysis of the ROESY spectrum and ^{1}H–^{1}H coupling constant value (*J*). NOEs were detected between H_3-18/H_2-19; H-15/H-17, H_3-21; H-16/H_3-30, and H-17/H_3-21, H_3-30.

Thus, the configuration at C-20 must be *S*, and both H-15 and H-16 are α-oriented. The coupling constants between H-16 and H-17 (*J* = 10.2 Hz) suggested a *cis*-relationship of the acetoxyl group and the side chain [10]. Consequently, a 16β-acetoxyl configuration was indicated. The coupling constants ($J_{15,16}$ = 9.6 Hz) suggested the 15β-acetoxyl configuration. The D-configuration of the xylose unit was confirmed using GC-MS analysis after acidic hydrolysis of **1** following derivatization with L-cysteine methyl ester and silylation [11]. Therefore, the structure of compound **1** was established as (20*S*)-15β,16β-diacetoxy-18,20-epoxy-3β,25-diol-16-oxo-9,19-cyclolanostan-3-*O*-β-D-xylopyranoside and named soulieoside P, as illustrated in Figure 1.

The known triterpenoid derivative was identified as hederasaponin B (**2**) by analysis of its spectroscopic and MS data with those reported in the literature [12].

Compounds **1** and **2** were tested for their inhibitory activity against three human cancer cell lines and their hepatoprotective effect on CCl_4-induced injury of human HepG2 cells using MTT assay, respectively. Compound **1** showed significant inhibitory effects with IC_{50} values of 7.6−11.2 μM (Table 2), while compound **2** exhibited no hepatoprotective activity in the tested range of 0.1−100 μM.

Table 2: Cytotoxicity of compound **1** against three human cancer cell lines.

Compounds	IC_{50} (μM)		
	HT-29	A549	MDA-MB231
1	11.2 ± 2.9	9.7 ± 6.5	7.6 ± 3.6
5-FU[a]	57.6 ± 5.3	87.9 ± 7.7	40.5 ± 6.9

Values present mean ± SD of triplicate experiments.
[a] Positive control substance

Experimental

General: Optical rotations were obtained on a Perkin-Elmer 341 digital polarimeter (PerkinElmer, Norwalk, CT, USA), IR spectra on a FTIR-8400S spectrometer (Shimadzu, Kyoto, Japan), and NMR spectra, including APT (Attached Proton Test), with a Bruker AV III 600 NMR spectrometer (chemical shift values as δ values with TMS as the internal standard) (Bruker, German). HRESIMS spectra were performed on a LTQ-Obitrap XL spectrometer (Thermo Fisher Scientific, Boston, MA, USA). Silica gel (100~200, 200~300 and 400~500 mesh, Qingdao Marine Chemical plant, Qingdao, China) and C_{18} reversed-phase silica gel (40–60 μm, Merck, Darmstadt, Germany) were used for column chromatography. Precoated silica gel GF_{254} plates (Zhi Fu Huang Wu Pilot Plant of Silica Gel Development, Yantai, China) were used for TLC. All solvents used were of analytical grade (Beijing Chemical Works, Beijing, China).

Plant material: The rhizomes of *S. vaginata* were collected in August 2015 from Wen County, Gansu Province, China and identified by Prof. Junshan Yang, Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences and Peking Union Medical College. A voucher specimen (No. 150828) was deposited at the herbarium of the Institute of Medicinal Plant Development.

Extraction and isolation: The air-dried and powdered rhizomes of *S. vaginata* (1.2 kg) were extracted 3 times with 95% ethanol (3 × 3 L) at room temperature. Removal of the ethanol under reduced pressure yielded the extract (165 g), which was suspended in distilled water and the suspension partitioned with ethyl acetate and *n*-BuOH, successively. The EtOAc-soluble fraction (86 g) was subjected to silica gel (100 ~ 200 mesh) column chromatography using a light petroleum–EtOAc gradient (from 100:0 to 0:100) as eluent, to yield 5 fractions (Fr. A–E). Fr. D (28 g) was applied to a silica gel (200 ~ 300 mesh) column eluting with a dichloromethane–methanol gradient (from 50:1 to 0:1) gradient to afford 5 fractions (Fr. D1–D5). Fr. D3 (4.2 g) was isolated by repeated silica gel CC (400 ~ 500 mesh) eluting with a dichloromethane–methanol gradient (20:1-1:1), followed by preparative TLC eluting with CH_2Cl_2-MeOH-formic acid (15:1:0.1-10:1:0.1) to yield **1** (5.8 mg). Fr. D5 (3.8 g) was isolated by repeated silica gel CC (400 ~ 500 mesh) eluting with a dichloromethane–methanol gradient (10:1−1:1) to give **2** (11.6 mg).

Acid hydrolysis: Compound **1** (2 mg) was heated in 2 mL of 2M trifluoroacetic acid at 95°C for 2 h. The reaction mixture was extracted 3 times with 2 mL of $CHCl_3$. The remaining aqueous layer was concentrated to dryness with EtOH to give a residue, which was dissolved in anhydrous pyridine (2 mL). The sugar was derivatized with L-cysteine methyl ester hydrochloride (3 mg, 60°C, 1 h) and subsequently silylated with hexamethyldisilazane and chlorotrimethylsilane (Fluka) (2:1, 1.5 mL; 60°C, 30 min). Finally, the supernatant (0.5 mL) was analysed by GC-MS (Agilent 7890A/5975C, Agilent Technologies, Santa Clara, CA, USA) under the following conditions: capillary column HP-5 (30 m × 0.25 mm × 0.25 μm); temperature gradient: 150°C for 2 min, then 5°C/min to 210°C; carrier, helium gas (1.0 mL/min); and injection volume: 1.0 μL. The injection and detector temperature were set at 290°C, and the split ratio was 1/10. The presence of D-xylose in the acid hydrolysate of **1** was confirmed by comparison of its retention time with that of a standard sample. The retention times (t_R, min) of D-xylose were 8.17 and 9.11 min.

Soulieoside P

Colorless amorphous powder.

$[\alpha]_D^{25}$: +23 (*c* 0.10, MeOH).

IR (KBr) ν_{max}: 3400, 2920, 1720, 1650, 1238, 1065 cm^{-1}.

^1H (600 MHz, pyridine-d_5) and ^{13}C NMR (150 MHz, pyridine-d_5): Table 1.

HRESIMS *m/z*: $[M + Na]^+$ 743.3948 (calcd. for $C_{39}H_{60}O_{12}Na$ 743.3982).

Cytotoxic assays: The cytotoxicity of compound **1** was assessed against HT-29, A549, and MDA-MB231 human cancer cell lines by the MTT method. HT-29 cells were grown in RPMI 1640 medium, and MDA-MB231 and A549 cells in DMEM, supplemented with 10%, v/v, FBS and 1% penicillin–streptomycin solution, and were cultured at a density of 2×10^4 cells/mL per well in a 96-well microtiter plate (Corning, Suzhou, China) and incubated overnight at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Then, the cells were treated with test samples for 24 h. After removing the supernatant of each well, a total of 10 μL of MTT solution was added to each well at the time of incubation for 4 h. The formazan crystals in each well were dissolved in lysis buffer overnight at 37°C. The absorbance at 550 nm was measured by an Infinite M200 Pro spectrophotometer (Tecan, Switzerland). The data are expressed as the percentage of the control optical density (OD) values for each experiment.

Hepatoprotective activity assay: HepG2 cells were grown in DMEM (Dulbecco's modified Eagle medium) supplemented with 10% FBS (fetal bovine serum), 100 μg/mL penicillin, and 100 μg/mL streptomycin. Cells were incubated at 37°C in a moist atmosphere containing 5% CO_2. HepG2 cells (5×10^4/well) were plated in a 96-well plate for 16 h. Then, the medium was aspirated and replaced with serum-free medium of different concentrations of compound **2** for 6 h. After removing the supernatant of each well, 10 μL of MTT solution was added to each well at the time of incubation for 4 h. The formazan crystals were solubilized with 100 μL of MTT stop solution and measured using an Infinite M200 Pro spectrophotometer [13]. Cytoprotective activity of test samples on CCl_4-induced cell injury was investigated. In total, 5×10^4 cells were plated per well in 96-well plates with culture medium for 16 h and then exposed to different concentrations of compound **2** for 30 min before exposure to 0.4% CCl_4 for 6 h. Cell viability was determined as described above and the percentage cell viability was expressed as a percentage with the control cells treated with vehicle as 100%. Following treatment, the cells were harvested and re-suspended in 0.4% trypan blue solution. The number of blue-stained (dead) and unstained (viable) cells were counted using a hemocytometer.

Supplementary data: Spectral data relating to this article are available online.

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Polyhydroxy Sterols Isolated from the Red Sea Soft Coral *Lobophytum crassum* and their Cytotoxic Activity

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One new (**1**) together with four known sterols (**2 - 5**) and a sesquiterpene (**6**) were isolated from a polar extract of the Red Sea soft coral *Lobophytum crassum*. The compounds were identified as 24-methylenecholest-5-ene-1α,3β,11α-triol 1-acetate (**1**), 24-methylenecholest-5-ene-1α,3β,11α-triol (**2**), 24-methylenecholest-5-ene-3β-ol (**3**), 24-methylenecholestane-1α,3β,5α,6β,11α-pentol (**4**), 24-methylenecholestane-3β,5α,6β-triol (**5**) and alismoxide (**6**) based on extensive NMR analysis. The cytotoxicity of compounds **1** – **6** was evaluated *in vitro* using three human cancer cell lines *viz*., HepG2, Hep-2 and HCT-116. Compound **1** showed selective cytotoxic activity against HepG2, while **3** exhibited cytotoxicity against all tested cell lines.

Keywords: *Lobophytum crassum*, Red Sea, Polyhydroxy sterols, Cytotoxicity.

Alcyonaceans (soft corals, Phylum: Coelenterata) belonging to the genus *Lobophytum* form a rich source of cemberanoids having diversified macrocyclic skeletons [1]. *L. crassum* is distributed in the indo-Pacific and Red Sea regions. A few cemberanoids have been identified from the nonpolar extract of a sample collected previously from the Gulf of Suez in the Red Sea [2], while samples collected from the Indo-Pacific region contained cemberane diterpenes [3-5]. Other metabolites *viz*., glycolipids [6] and polyhydroxy sterols have also been reported [7a, b]. Different biological activities for these isolates e.g. HIV-inhibitory [8], cytotoxic [9-11], and anti-inflammatory [12, 13] have also been reported.

The Red Sea has a unique ecological nature containing diverse flora and fauna considered to be potentially one of the most important sources of bioactive compounds. Exploration of this untapped natural resource would certainly be regarded as a high priority venture due to the universally expected impending climatic and environmental changes which will no doubt also affect the ocean flora and fauna. The ongoing search by our group for bioactive metabolites from Red Sea marine organisms [14] directed our attention to the soft coral *L. crassum* collected near Hurghada, Red Sea. We now report on the isolation, purification, structural elucidation and biological evaluation of compounds found in a *L. crissum* extract.

The concentrated crude MeOH extract of *L. crassum* was partitioned between EtOAc and water. The water extract was evaporated and the residue re-extracted with methanol and added to the ethyl acetate fraction. The combined EtOAc fractions were chromatographed repeatedly to afford the pure metabolites **1-6** (see Experimental Section).

Compound **1**, obtained as an amorphous powder, had the molecular formula $C_{30}H_{48}O_4$ established by HRESIMS {*m/z* 473.6605 (M^++1),

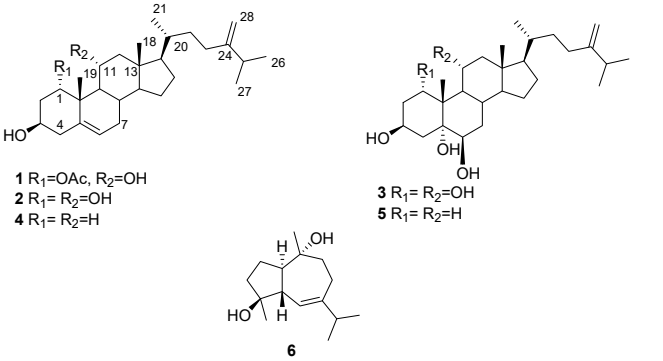

Figure **1**.Chemical structures of compounds **1-6**.

EIMS *m/z* 472 (M^+)}. Successive losses of 18 and 60 mass units {*m/z*, 454 (M^+– H_2O), 394 (M^+– OAc – H_2O)} suggested the presence of free hydroxyl group(s), while the fragment peak at *m/z* 412 (M^+– OAc – H^+) supported the presence of an acetyl group. The IR spectrum demonstrated the presence of hydroxyl (3500 cm^{-1}), carbonyl (1725 cm^{-1}) and a terminal methylene double bond (1646, 1250 and 880 cm^{-1}). The ^1H NMR spectrum ($CDCl_3$, 600 MHz) confirmed the presence of a terminal methylene group at δ 4.62 and 4.68 [each a singlet], an olefinic proton at δ 5.56 (d, *J* = 6.2), five methyl signals at δ 0.68 (s), 0.92 (d, *J* = 6.1), 1.00 (d, *J* = 6.8), 0.98 (d, *J* = 6.8), and 1.22 (s), and an acetoxy at δ 2.02 (s). Signals at δ 5.66 (1H, br s), 3.88 (1H, m), and 3.92 (1H, m) are assigned to protons attached to hydroxylated carbons based on HSQC spectra, the first one attached to an acetoxy group. ^{13}C and DEPT spectral analysis indicated the presence of 30 carbon atoms which are attributed to six methyls (including an acetoxy), nine methylenes (including an olefinic), ten methines (including an olefinic and three oxygenated), and five quaternary carbons (including two olefinic and a carbonyl groups). The above data suggested that this was a tri-hydroxylated 24-methylenecholest-5-ene derivative.

Furthermore, the NMR spectral data of **1** were remarkably similar to those for compound **2** [15] with the only difference being the presence of an additional acetoxy group in **1**. The large deshielding of H-1 at δ 5.66 vs that of δ 4.21 for compound **2** [15] suggested that the acetoxy group was situated at C-1, while the multiplet centered at δ 3.92 had a similar complexity normally seen for a 3 β-carbinol proton in a steroid skeleton [16]. The oxygenated methine signals at δ_C 78.3 (C-1) 66.7 (C-3) and 68.9 (C-11) are in similar positions as the 1α,3β,11α-triol system in the known compound **2**. Further confirmation for the position of the acetoxy group at C-1 was provided by HMBC correlations which showed cross-peaks (among others) between H-1/C-5, CO (carbonyl carbon); H-19/C-1, C-11, C-10; H-3/C-1, C-5. The relative configuration of the trihydroxylated position (C-1, C-3, and C-11) was established using NOESY, which showed correlations (among others) between Me-19/H-1, H-11; Me-18/H-11, acetyl group/H-3. The above data confirmed the chemical structure of **1** as 24-methylenecholest-5-ene-1α,3β,11α-triol 1-acetate.

The methanol extract also afforded five known compounds *viz*., **2**-**6**. Compound **2** (24-methylenecholest-5-ene-1α,3β,11α-triol) was previously isolated from both *Sinularia dissecta* [17] and *Palythoa tuberculosa* [15], while 24-methylenecholestane-1α,3β,5α,6β,11α-pentol (**4**) was isolated from both the Formosan soft coral *Sinularia gibberosa* [18] and the South China Sea soft coral *L. crassum* [19]. Compound **3** was only recently isolated from *Sinularia polydactyla* collected from the Red Sea [20], while 24-methylenecholestane-3β,5α,6β-triol (**5**) was reported from *Sinularia* sp. [21, 22]. Alismoxide (**6**) is a rare metabolite, identified previously from *Alisma rhizomes* [23], and was recently isolated from *Lithophyton arborium* [24].

The cytotoxic activity investigations against the growth of the human cancer cell lines HepG2, Hep2 and HCT-116 (Table 1) illustrated that compound **3** exhibited strong cytotoxicity toward the growth of HepG2, Hep-2 and HCT-116, with IC_{50} values of 1.90, 5.82 and 6.46 μM, respectively. HepG2 cell lines on the other hand showed a greater sensitivity towards compounds **1**, **5** and **6** than other cell lines (IC_{50} 1.90, 3.00 and 3.77 μM respectively).

Table 1: Cytotoxicity of compounds **1-6**.

Compound/extract	cancer cell line (IC_{50}, μM)		
	HepG2	Hep-2	HCT-116
EtOAc extract*	2.1	2.0	0.9
1	1.90	10.15	10.15
2	19.52	19.05	21.39
3	1.94	5.82	6.46
4	6.74	11.99	15.24
5	3.00	8.08	11.31
6	3.77	11.33	17.20
Doxorubicin	2.21		
Vinblastine		3.20	5.67

*μg/mL

The Red Sea is well known for its high salinity and low nutrient content. Such conditions generate different environments which are able to affect the secondary metabolites profile of the growing species in the same area. Earlier studies on the non-polar fraction of the same species by Kashman [2] demonstrated the presence of a cembrane diterpene. The difference between Kashman's work and this study could most likely be due to the extraction methods employed as well as the collection site. The chemical contents of *Lobophytum* species vary considerably depending on the geographical location and season of collection [11, 12, 25-26]. Generally, sterol patterns in marine invertebrates have a more complex profile than that of terrestrial organisms. The symbiotic relationships between organisms also complicates the sterol composition [27]. Fleshy soft corals, particularly the Alcyonarian corals of the genera *Lobophytum, Sinularia*, and *Sarcophyton* produce 3β,5α,6β−trihydroxy sterols. The isolation of Δ^5 steroid **4** together with 5,6-diol derivative **5** supports the assumption that the unoxidized Δ^5 sterol is the starting precursor [28].

Experimental

General experimental procedures: IR spectra were recorded on a JASCO FT/IR-8400S infrared spectrophotometer, UV spectra on a Shimadzu-265 spectrophotometer, and NMR spectra on a Jeol spectrometer at 600 MHz for ^{1}H and 150 MHz for ^{13}C using $CDCl_3$ with TMS as internal standard. Chemical shifts are given in δ (ppm) and coupling constants in Hertz (Hz). EIMS were recorded on a Shimadzu Qp-2010 (Tokyo, Japan) and Triple Quadropole TQD mass spectrometer (Waters, Milford, MA, USA) for ESI-MS. Perkin-Elmer model 343 plus polarimeter using a Na lamp at 25ºC (Shelton, CT, USA) used for optical rotation. Si gel 60 (Merck, 230-400 mesh) was used for column chromatography, and precoated Si gel plates (Merck, Kieselgel 60 F_{254}, 0.25 mm) for TLC analyses.

Animal materials: The soft coral *L. crassum* Marenzeller, 1886, was collected using scuba technology at Hurghada (Red Sea, Egypt) during May 2013 at a depth of 2-3 m. Upon collection the material was kept in MeOH. The organism was identified by Dr Abdel-Hamid Abdel-Rahman Mohamed Ali, co-author of this paper. A voucher sample (AB8-2013) is kept at the National Institute of Oceanography and Fisheries, Suez Branch, Egypt.

Extraction and isolation: Sliced bodies of *L. crassum* (2 kg fresh material) were exhaustively extracted with MeOH (4 L X 3). The organic layer was filtered and concentrated under vacuum and then partitioned between EtOAc and H_2O. The water layer was dried and washed with methanol (X3) and combined with the EtOAc fraction. The residue thus obtained (32.5 g) was subjected to CC on Si gel and eluted with a gradient of EtOAc in *n*-hexane in order of increasing polarity (0-100%) to yield 16 fractions. Fraction 9, eluted with *n*-hexane-EtOAc (8:2) yielded **1** (10 mg) and **6** (15 mg). Fraction 13, eluted with *n*-hexane-EtOAc (1:1), yielded 5 sub-fractions. The fourth and fifth sub-fractions were combined and subjected to preparative TLC using DCM-MeOH (9:1) to yield **2** (5 mg) and **5** (10 mg). Fraction 5 eluted with *n*-hexane-EtOAc (98:2) yielded **4** (23 mg), while fraction 15 eluted with DCM- MeOH (1 to 10%), gave 8 sub-fractions, of which 6 and 7 were combined and subjected to prep.-TLC using DCM-MeOH (9:1) to afford **3** (10 mg)

24-Methylenecholest-5-ene-1α,3β,11α-triol 1-acetate (1)
Amorphous white powder.
$[\alpha]_D^{25}$: 38.3 (*c* 0.28, MeOH).
IR (KBr) cm^{-1}: 3500, 1725, 1646, 1600, 1250, 880.
^{1}H NMR ($CDCl_3$ 600 MHz) δ_H: 5.66, (br s H-1); 1.75, 2.08 (m, each, H-2); 3.96 (m, H-3); 2.22, 2.37 (m each, H-4); 5.56, (d, *J* = 6.2 Hz, H-6); 1.99, 1.65 (m, each, H-7); 1.53 (m, H-8); 1.63 (m, H-9); 3.88 (m, H-11); 1.24, 2.31 (m, each, H-12); 1.13 (m, H-14); 1.24, 1.63 (m, each, H-15); 1.24, 2.00 (m, each, H-16); 1.14 (m, H-17); 0.68 (s, H-18); 1.22 (s, H-19); 1.76 (m, H-20); 0.92 (d, *J* = 6.1, H-21); 1.41, 1.05 (m, each, H-22); 1.24, 1.39 (m, each, H-23); 1.94 (m, H-25); 1.00 (d, *J* = 6.8, H-26); 0.98 (d, *J* = 6.8, H-27); 4.62, 4.68 (s, each, H-28); 2.02 (s, OAc).
^{13}C NMR (150 MHz): δ_C: 78.3, (CH, C-1); 35.6 (CH_2, C-2); 66.7 (CH, C-3); 41.8 (CH_2, C-4); 137.0 (C, C-5); 124.5 (CH, C-6); 31.3 (CH_2, C-7); 31.5 (CH, C-8); 48.9 (CH, C-9); 41.6 (C, C-10); 68.9 (CH, C-11); 51.1 (CH_2, C-12); 43.1 (C, C-13); 56.3 (CH, C-14); 24.0 (CH_2, C-15); 28.3 (CH_2, C-16); 55.7 (CH, C-17); 13.1 (CH_3, C-

18); 18.6 (CH_3, C-19); 35.6 (CH, C-20); 18.7 (CH_3, C-21); 34.5 (CH_2, C-22); 30.8 (CH_2, C-23); 156.8 C, C-24); 33.8 (CH, C-25); 21.9 (CH_3, C-26); 22.0 (CH_3, C-27); 106.0 (CH_2, C-28); 21.6,171.9 (CH_3, C respectively, OAc).
EIMS *m/z*: 472 $[M]^+$, 454, 394, 412; HRESIMS *m/z*: 473.6605 $[M^+ + 1]$.

Cytotoxicity assay: Human liver tumor cell lines (HepG-2), human colon tumor cells (HCT-116) and human epidermoid larynx carcinoma (Hep2) were obtained from the American Type Culture Collection and maintained in RPMI supplemented with 10% fetal bovine serum (FBS), 100 mg/L streptomycin and 100 IU/mL penicillin at 37°C in a humidified atmosphere of 5% CO_2. Metabolites **1**-**3** were dissolved in DMSO at a concentration of 1mg/mL, which were then diluted to appropriate concentrations with culture medium when used. Tumor cells ($5x10^4$-10^5 cells/ well) were incubated with serial dilutions of metabolites **1**-**6** in 96-well culture plates for 48 h, and their cytotoxicity was measured spectrophotometrically at 564 nm with an ELISA microplate reader (Meter tech. Σ 960, USA). All assays were performed in triplicate. The results were expressed as percentages, and the effective dose required to inhibit cell growth by 50% (IC_{50}) was determined. This work was carried at the Regional Center for Mycology and Biotechnology, Al-Azhar University, Cairo, Egypt.

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Raphanus sativus Sprout Causes Selective Cytotoxic Effect on *p53*-Deficient Human Lung Cancer Cells *in vitro*

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Raphanus sativus L. (Brassicaceae) is widely consumed in many different forms worldwide. Its sprouts, in particular, are commonly consumed as a health food. *R. sativus* sprout has recently been shown to have anti-tumor activity on human colon cancer cells, suggesting that it may have potential use in cancer prevention and treatment. The extent of this anti-tumor activity and its underlying mechanisms, however, remain to be investigated in other types of cancer cells. In this study, we showed that the MeOH extract from *R. sativus* sprout exhibits significant but variable cytotoxic effects on human lung adenocarcinoma cells depending on their *p53* status. The MeOH extract decreased the viability of *p53*-deleted human lung cancer cells (H1299 and Calu-6) by inducing apoptosis; this effect, however, did not occur for wild-type *p53* cancer cells (A549), for cells expressing a *p53* mutant lacking the C terminus (H1264), or for non-tumor fibroblast cells (NIH3T3). Phytochemical analyses of the MeOH extract allowed us to identify and isolate β-sitosterol as a major component of the MeOH extract. Direct treatment with β-sitosterol significantly reduced the viability of Calu-6 cells, suggesting that it may, in part, contribute to *R. sativus* sprout's anti-tumor activity. This work provides experimental evidence for a novel biological application of *R. sativus* sprout in treating human lung cancer, and it identifies the main component involved in this effect, further supporting its potential use as a functional food for cancer management.

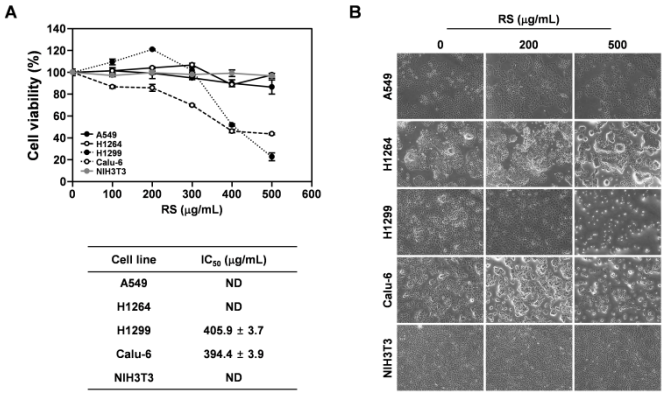
Keywords: *Raphanus sativus*, Brassicaceae, Sprouts, Lung adenocarcinoma, Cytotoxicity, Apoptosis.

Cancer is a lethal disease typically caused by mutations that result in uncontrolled cell growth that subsequently invades surrounding tissues; such systemic metastasis is now the second leading cause of death worldwide [1,2]. Natural products from many species (including microorganisms, marine organisms, and other plants) have proved valuable resources for securing molecular treatments for cancer [3-12]. Indeed, many anti-cancer drugs derived from natural products (such as paclitaxel, doxorubicin, and rapamycin) are currently either in clinical use or preclinical evaluation to treat cancer patients [4,5,13]. Therefore, screening natural product extracts for anti-cancer activity and then identifying useful compounds is an important avenue of research for cancer prevention and treatment.

Raphanus sativus L. (Brassicaceae), commonly known as radish, is widely consumed around the world [14]. It is one of the Brassica vegetables that contains many compounds that have positive effects in maintaining human health such as vitamins C and E, fiber, carotenoids, polyphenols, and glucosinolates [15,16]. The first Chinese pharmacopoeia, '*Tang Materia Medica,*' reported that *R. sativus* was used as a traditional herbal medicine for more than 1400 years [17]. All parts of the radish, including leaves, seeds, and roots, have been used for medicinal purposes, such as carminative, diuretic, expectorant, laxative, and digestive treatments [18]. Building upon our group's prior work investigating the biological properties and bioactive constituents of Korean medicinal resources, we examined the seeds of *R. sativus*, also known as Raphani Semen [17-19]. In our previous works, we demonstrated that the MeOH extract of Raphani Semen induces anti-tumor and anti-inflammatory effects, and we isolated and identified 4-methylthio-butanyl derivatives, phenylpropanoid sucrosides, and phenolic compounds that are the likely causative agents [17-19]. Here, we extend this work by investigating the effect of the MeOH extract of *R. sativus* sprouts on human lung adenocarcinoma cells.

R. sativus sprouts have long been considered a health food, as they contain high levels of protein, minerals, and vitamins [20], as well as antioxidant glucosinolates, including glucoraphasatin and glucoraphenin [21]. In Korea, in particular, they are consumed in many ways, such as in salads and "Bibimbap," a signature Korean dish. Previous studies have shown that *R. sativus* sprouts contain glucosinolates [21], sinapinic acid esters [22], flavonoids [22], and anthocyanins [23]. Sinapinic acid esters and several kinds of flavonoids in the sprouts have been shown to possess strong antioxidant activity [22]. Of particular note is a recent study in which 4-methylthio-3-butenyl isothiocyanate was isolated in the extract of *R. sativus* sprout and shown to inhibit cell proliferation in three human colon carcinoma cell lines (LoVo, HCT-116, and HT-29). Furthermore, this extract was shown to induce apoptotic cell death in those cancer cells *in vitro*, suggesting that it has excellent potential use in cancer management [15]. The anti-cancer activity of *R. sativus* sprout, however, must be further validated in other types of cancer in order to assess thoroughly its potential utility for cancer prevention and treatment.

In this study, we measured the effect of the MeOH extract of *R. sativus* sprout on human lung adenocarcinoma cells and found that it dramatically reduced cell viability by inducing apoptosis depending on *p53* status. Additionally, we phytochemically investigated the MeOH extract, which led us to isolate a major component, β-sitosterol. To the best of our knowledge, *R. sativus* sprout has not previously been shown to have anti-tumor effects against lung cancer. With this work, we characterized the anti-tumor effects of *R. sativus* sprout on human lung cancer cells and isolated the main component that is the likely causative agent.



Cell line	IC_{50} (μg/mL)
A549	ND
H1264	ND
H1299	405.9 ± 3.7
Calu-6	394.4 ± 3.9
NIH3T3	ND

Figure 1: MeOH extract of *R. sativus* sprouts selectively decreases human lung adenocarcinoma cell viability in the absence of *p53* expression. (A) The viability of human lung adenocarcinoma cells, A549, H1264, H1299 and Calu-6, and non-tumor cells, NIH3T3, was assessed by WST-1 assay after treatment with the MeOH extract (RS) of *R. sativus* sprouts at the indicated concentrations (*upper*). The IC_{50} value of the extract for each cell line is indicated (*lower*). (B) Representative bright-field images of cells taken at 100×magnification after MeOH extract treatment for 48 h at the indicated concentrations. Data are shown as mean ± SEM based on three independent replicate experiments. ND: not determined.

Lung cancer has been the leading cause of cancer death in both men and women worldwide for several decades [2,24]. As such, we evaluated the cytotoxic effect of the MeOH extract of *R. sativus* sprouts on human lung adenocarcinoma cells (Figure 1). Because chemoresistance in cancer patients has previously been shown to be correlated with *p53* status [25,26], we also characterized the effect of the MeOH extract on four human non-small cell lung cancer (NSCLC) cell lines with different *p53* statuses [27]. Furthermore, we characterized its cytotoxicity against a non-tumor cell line, NIH3T3, in order to determine whether this effect specifically targets cancer cells.

Treatment with the MeOH extract of *R. sativus* sprouts dramatically reduced cell viability in human lung cancer cells null for *p53* expression (Calu-6 and H1299) in a dose-dependent manner (IC_{50} values ranged from 394.4 ± 3.9 to 405.9 ± 3.7 μg/mL - Figure 1A). Interestingly, however, it failed to induce cytotoxicity in A549 and NIH3T3 cells (which harbor wild-type *p53*). Further, it failed to exhibit a cytotoxic effect on H1264 cells, which express a *p53* mutant that lacks both the nuclear localization signal (NLS) and the oligomerization domain (Figure 1). These data suggest that the MeOH extract of *R. sativus* sprouts induces selective cytotoxicity against human lung cancer cells depending on *p53* status. Because *p53* plays several important regulatory roles in the cytoplasm and in the nucleus [28], this suggests that the cytoplasmic function of *p53* confers resistance in H1264 cells against the MeOH extract of *R. sativus* sprouts. Further studies will be necessary to determine whether other *p53* mutations can similarly reverse the anti-tumor activity of the MeOH extract of *R. sativus* sprouts on human lung cancer cells.

Upon treatment with the MeOH extract of *R. sativus* sprouts, Calu-6 and H1299 cells exhibited significant morphological changes, including cell shrinkage and rounding up, membrane blebbing, and detachment from the substratum; these changes are known to be typical features of apoptotic cell death (Figure 1B) [29]. To verify further that the MeOH extract induced apoptosis in human lung cancer cells depending on their *p53* status, we assessed cell populations undergoing apoptosis in those cells using TUNEL staining after treating them with the extract at IC_{50} doses for 48 hours (Figure 2). As expected, apoptotic cells were significantly increased in Calu-6 and H1299 cells, but not in A549, H1264, and NIH3T3 cells, implying that the MeOH extract of *R. sativus* sprouts

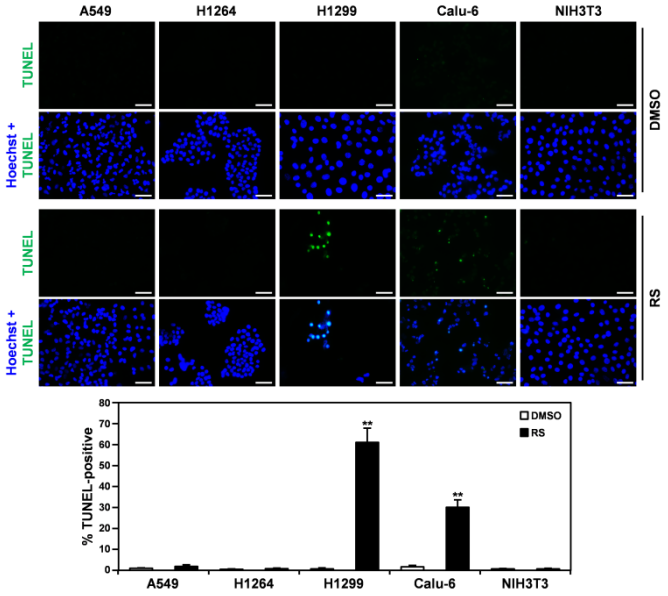


Figure 2: The MeOH extract of *R. sativus* sprouts induces apoptotic cell death in human lung adenocarcinoma cells deficient for *p53*. Representative images of TUNEL staining (*upper*) and quantitation of cells positive for TUNEL staining (*lower*) in human lung cancer cells and NIH3T3 cells treated either with *R. sativus* sprout MeOH extract (250 μg/mL) or with DMSO as vehicle control, for 48 hours. Data are shown as mean ± SEM based on three independent replicate experiments. Scale bar: 50 μM. ** $p < 0.01$.

exhibits anti-tumor activity by inducing apoptosis in the absence of *p53* expression. Additionally, it appears that only cytoplasmic *p53* is required to reverse the MeOH extract-induced apoptotic activity; further studies are needed to characterize fully the underlying molecular mechanism of this effect.

The MeOH extract of *R. sativus* sprouts was fractionated, yielding CH_2Cl_2 soluble and EtOAc fractions. Phytochemical investigation of the consolidated CH_2Cl_2 and EtOAc soluble fractions with column chromatography revealed the presence of β-sitosterol as a main component; this compound was identified by comparing spectroscopic data, including ^{1}H and ^{13}C NMR, with previously reported values and LC/MS analysis.

β-Sitosterol is a common steroid found in a wide range of plant species and in foods like vegetables, nuts, and salads and it has been shown to have various pharmacological activities (such as anti-herpes, anti-inflammatory, cytotoxic, and immunomodulation effects) [30,31]. β-Sitosterol has also been reported to inhibit the growth of human colon cancer cells and to induce apoptosis in human prostate cancer cells [32,33]. After identifying it at a high level in the MeOH extract, we next examined its direct impact on the viability and proliferation of human lung cancer cells (Figure 3). After treating Calu-6 cells with β-sitosterol, we found a significant and dose-dependent decrease in cell viability. Additionally, the cells exhibited the typical morphology of apoptotic cells (Figure 3). Although the proliferation of H1299 cells also decreased slightly, but statistically significantly, upon β-sitosterol treatment, this effect was not dose-dependent. Cell proliferation in A549 and H1264, where the MeOH extract of *R. sativus* sprouts did not exhibit any cytotoxicity, were also reduced at a similar level to that of H1299 cells after β-sitosterol treatment. Together with the measured IC_{50} values of the MeOH extract in H1299 and Calu-6 cells, these observations suggest that the distinct effect of β-sitosterol on H1299 versus Calu-6 cells is likely due to different cellular and genetic contexts. This work thus suggests that the anti-tumor activity of the MeOH extract of *R. sativus* sprouts is due, at least in part, to β-sitosterol, although other compounds also are likely to contribute to its cytotoxic activity on human lung cancer cells.

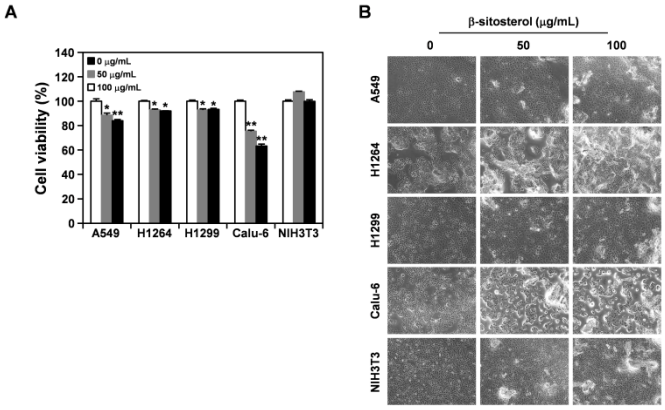


Figure 3: β-Sitosterol significantly reduces cell viability in Calu-6 cells, but not in other human lung cancer cells. (A and B) The viability of human lung cancer cells and NIH3T3 cells assessed by WST-1 assay (A); representative bright-field images taken at 100× magnification (B) after treatment with β-sitosterol for 48 hours at the indicated concentrations. Data are shown as mean ± SEM based on three independent replicate experiments. * $p < 0.05$, ** $p < 0.01$.

Together with recent prior work showing anti-tumor activity of *R. sativus* sprouts in human colon cancer cells [8], this study demonstrates that *R. sativus* sprouts hold strong and broad potential use as a functional food for the prevention and treatment of cancer.

Experimental

General experimental procedures: Column chromatography was performed using silica gel 60 (Merck, Darmstadt, Germany; 70-230 mesh and 230-400 mesh). Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AVANCE III 700 NMR spectrometer operating at 700 MHz (^{1}H) and 175 MHz (^{13}C), with chemical shifts measured in ppm (δ) (Bruker). Merck pre-coated silica gel F254 plates and reversed-phase (RP)-18 F254s plates (Merck) were used for thin-layer chromatography (TLC). TLC spots were detected under UV light or by heating after spraying with anisaldehyde-sulfuric acid. Semi-preparative high-performance liquid chromatography (HPLC) was performed using a Shimadzu Prominence HPLC System with SPD-20A/20AV Series Prominence HPLC UV-Vis Detectors.

Sample collection and extraction: *R. sativus* sprouts, purchased at E-mart, were cultivated and harvested in Garak-dong, Sonpa-gu, Seoul, in October 2015. A voucher specimen (SKK-MOO-2015) was identified by one of the authors (K. H. Kim) and deposited in the herbarium at the School of Pharmacy, Sungkyunkwan University, Suwon, Korea. The air-dried sprouts of *R. sativus* (155 g) were extracted 3 times with 100% MeOH at room temperature. To obtain a crude MeOH extract, the filtered extract was evaporated using a rotavapor. The MeOH extract was then dissolved in 50% dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO, USA), diluted in RPMI-1640 media (WelGENE, Seoul, Korea) and stored at -80°C until used for treating cells.

Cell lines and culture: Human lung adenocarcinoma cell lines A549, H1264, H1299, and Calu-6, and immortalized mouse embryonic fibroblasts, NIH3T3, were maintained in RPMI-1640 and DMEM media (WelGENE), respectively, both supplanted with 10% FBS (Gemini Bio-Products, West Sacramento, CA, USA), 2 mM L-glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin (WelGENE) at 37°C in a humidified atmosphere of 5% CO_2.

Cell viability analysis: For analysis, 5×10^3 cells were seeded in triplicate in 96-well tissue culture plates (Thermo Scientific, Waltham, MA, USA) and treated with either MeOH extract of *R. sativus* sprouts or β-sitosterol. The cells were also treated with equivalent amounts of either DMSO or EtOH (Merck) as vehicle control. After 48 h of treatment, cells were subsequently incubated with water-soluble tetrazolium salt-1 (WST-1) reagent at 37°C for 2 h, according to the manufacturer's instructions (Daeil Lab Service, Seoul, Korea). Absorbance was measured at 450 nm with a scanning multi-well spectrophotometer (Molecular Devices, Sunnyvale, CA, USA), and cell viability was determined as a percentage of the control cells. The 50% inhibitory concentration (IC_{50}) values were calculated from the dose-response curves of 3 independent replicate experiments.

TUNEL assay: For this analysis, 7.5×10^3 cells were plated in triplicate on coverslips and treated with either MeOH extract of *R. sativus* sprouts or DMSO (as a negative control) for 48 h. Cells undergoing apoptosis were assessed by terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining using the Dead-End labeling kit (Promega, Madison, WI, USA), according to the manufacturer's instructions. Briefly, cells were fixed with 4% formaldehyde (Sigma, St. Louis, MO, USA) in PBS at 4°C for 25 min, permeabilized in 0.2 % Triton X-100 (Sigma) at room temperature for 5 min, and then incubated in equilibration buffer at room temperature for 10 min. The cells were subsequently incubated with both fluoresceinated rdNTPs and terminal deoxynucleotidyl transferase at 37°C for 60 min in a humidified chamber; the reaction was then terminated by incubation in 2×SSC buffer at room temperature for 15 min. After counterstaining with 1 μg/mL Hoechst dye (Sigma) in order to visualize cell nuclei, the coverslips were mounted on slides with 90% glycerol (Sigma) in PBS and examined under a fluorescence microscope (Carl Zeiss, Jena, Germany). The percentage of apoptotic cells was determined as the number of cells positive for TUNEL staining divided by the total number of cells counted in 6 randomly selected high-power fields (400×) on each slide. More than 200 cells were counted per slide.

Statistical analysis: Statistical differences were assessed between cells treated with either MeOH extract of *R. sativus* sprout or β-sitosterol and their respective vehicle controls using unpaired Student's two-tailed *t* test. Data are presented as mean ± SEM, and *p* values less than 0.05 were considered statistically significant.

Isolation of main constituents: The crude extract (2 g) was fractionated by sequential liquid-liquid partitioning of H_2O (200 mL) with dichloromethane (CH_2Cl_2, 200 mL) and EtOAc (200 mL). Each layer was then consolidated to yield fraction A (332 mg) by TLC analysis. Fraction A was subjected to open silica gel column chromatography using a gradient solvent system of CH_2Cl_2-MeOH (200:1, 100:1, 30:1, 10:1, 5:1 and 2:1) to yield 6 fractions (A1-A6) by TLC analysis. Among these sub-fractions, A3 (48.3 mg) was purified by semi-preparative reversed-phase HPLC (87% MeOH) using a Phenomenex Luna HPLC phenyl-hexyl column (250 × 10 mm; flow rate: 2 mL/min) to yield compound **1** (5 mg, t_R = 30 min). Compound **1**, β-sitosterol, was then dissolved in EtOH and stored at -80°C until used for treating cells.

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Preparative and Rapid Purification of Saponins from *Asparagus racemosus* Root by High Performance Centrifugal Partition Chromatography

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High performance centrifugal partition chromatography (HPCPC) was applied to the rapid isolation and purification of saponin glycosides in *Asparagus racemosus* Willd. root. A two-phase solvent system composed of $CHCl_3$-MeOH-water (4:4:2, v/v) in descending mode was used for the separation, yielding shatavarin IX (**1**) and asparacoside (**2**) in one step. Asparanin A (**3**) and shatavarin V (**4**) were separated by repeated HPCPC fractionation using CH_2Cl_2-MeOH-water (4:4:2, v/v) as the solvent system, followed by either gel-filtration or TLC. Their structures were identified by NMR spectroscopy and ESI/MS. The *A. racemosus* extracts and **1**, **2**, **3** and **4** were cytotoxic towards human hepato- and prostate-carcinoma cell lines (IC_{50} 14-37 µM), while primary human fibroblasts were less vulnerable (IC_{50} 22-66 µM), i.e., every saponin glycoside showed selectivity towards carcinoma cells compared with normal fibroblasts. HPCPC has proven rapidity to separate complex mixtures of phytochemicals yielding quantities suited to biological studies.

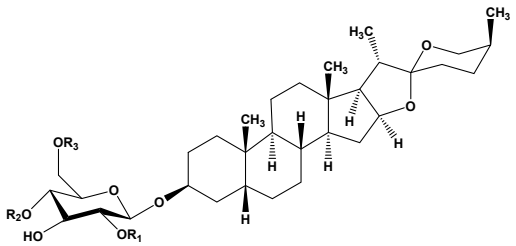
Keywords: *Asparagus racemosus*, Cytotoxicity, High performance centrifugal partition chromatography, Saponins, Carcinoma cells.

Asparagus racemosus Willd. or Shatavari (Asparagaceae family) is an important medicinal plant in Ayurvedic medicine. Shatavari roots are known as a tonic for woman [1]. *A. racemosus* has been reported to have phytoestrogenic [2], galactogogue [3], anti-fungal [4], anti-oxidant [5a,5b] and anti-cancer activities [6]. Major active constituents of *A. racemosus* roots are saponin glycosides such as shatavarin I and IV [7]. Several papers have reported the separation of the saponin glycosides from *A. racemosus* roots by various chromatographic techniques with a solid sorbent [8a-8c]. However, these methods are time-consuming, labour-intensive and have low recovery. High performance centrifugal partition chromatography (HPCPC) was developed as a solid support-free liquid chromatographic system [9]. HPCPC increases sample recovery and eliminates peak tailing on solid supports [9]. Based on these two advantages, HPCPC has been successfully used for separating some bioactive saponins [10a-10c].

Here, we aimed to isolate saponin glycosides from *A. racemosus* roots extract by HPCPC. Their cytotoxicities were tested for possible selective anti-cancer actions. For the first time, we showed that HPCPC efficiently isolated active components from *A. racemosus* and two compounds selectively killed hepatocarcinoma cells.

A two-phase solvent system for CPC separation was chosen based on partition coefficients (K values) of the target compounds. The appropriate range of K values is between 0.5-2 [11]. In addition, the ratio of two-phase solvent system should be "stable" during the run, which can be suggested from a settling time experiment. A settling time <20 s is preferred [11]. For the separation of saponin glycosides from *A. racemosus* roots extract, we found that $CHCl_3$-MeOH-water (4:4:2, v/v) gave K values around 1 and settling times <20 s. Therefore, we used this system for the first separation step.

By using HPCPC, the separation of shatavarin IX and asparacoside from a saponin-enriched *A. racemosus* root extract was successfully and rapidly achieved in only one step. Subsequently, asparanin A and shatavarin V were obtained with HPCPC followed by other chromatographic techniques. The four isolated saponin glycosides shared the same aglycone (sarsasapogenin), but differed in their sugar moieties (Figure 1). Asparacoside has previously been reported in *A. cochinchinensis* [12], but here, was isolated from *A. racemosus* for the first time. This study showed that HPCPC can isolate a series of closely related saponins whose physical properties differ only slightly by the sugar moiety. Most previous studies reported the preparative isolation of saponin glycosides from *A. racemosus* using preparative RP-HPLC [7, 13], HPTLC [8a] or silica column chromatography [8b]. The limitations of these techniques are the low sample loading and adsorption of glycosides on the solid sorbents. This is the first time that asparacoside separation could be achieved in one step. Our studies demonstrated that HPCPC is useful as a rapid preparative method for separating saponin glycosides from *A. racemosus*.



	Compound	R_1	R_2	R_3	[M-H]$^-$
1	Shatavarin IX	β-D-glucose	β-D-glucose	H	901.47
2	Asparacoside	β-D-glucose	α-L-arabinose	α-L-arabinose	1003.51
3	Asparanin A	β-D-glucose	H	H	739.42
4	Shatavarin V	α-L-rhamnose	β-D-glucose	H	885.47
-	Shatavarin IV	β-D-glucose	α-L-rhamnose	H	885.47

Figure 1: Structures of shatavarin IX, asparacoside, asparanin A, shatavarin V and shatavarin IV, and glycoside substituents.

Table 1: Cytotoxicity of *A. racemosus* extracts and some saponin glycosides.

Samples	HepG2		LNCaP		Fibroblasts
	IC_{50}	SI	IC_{50}	SI	IC_{50}
Defatted EtOH extract (μg.mL^{-1})	798 ± 54.6^a	1.44 ± 0.22^a	469 ± 7.50^a	2.43 ± 0.21^a	1140 ± 110^a
Saponin enriched extract (μg.mL^{-1})	207 ± 15.4^b	1.47 ± 0.15^a	87.3 ± 1.58^b	3.47 ± 0.10^b	303 ± 8.69^b
Shatavarin IX (μM)	13.7 ± 1.62^c	1.58 ± 0.19^a	16.3 ± 2.43^c	1.33 ± 0.22^c	21.4 ± 1.27^c
Asparacoside (μM)	13.6 ± 2.45^c	5.01 ± 1.12^b	29.3 ± 0.57^d	2.27 ± 0.11^{a,d}	66.5 ± 3.14^d
Asparanin A (μM)	14.1 ± 0.96^c	3.61 ± 0.29^b	36.6 ± 1.77^e	1.39 ± 0.13^c	50.9 ± 1.18^e
Shatavarin V (μM)	24.1 ± 2.50^d	1.77 ± 0.23^a	16.5 ± 0.33^c	2.57 ± 0.21^a	42.4 ± 3.25^e
Shatavarin IV (μM)	25.7 ± 4.08^d	1.24 ± 0.37^a	24.4 ± 0.93^d	1.26 ± 0.12^c	30.7 ± 2.52^c
Paclitexal (μM)	1.15 ± 0.11^e	1.62 ± 0.17^a	1.05 ± 0.21^f	1.79 ± 0.01^d	1.88 ± 0.38^f

Data are expressed as mean IC_{50} ± SD from 3 independent experiments, triplicate for each. Paclitaxel was used as positive control. Each letter in same column (a-f) represent a significant difference at $p < 0.01$ to other members of the column. Values having the same letter are not different (One-way ANOVA followed by Tukey's test). The selectivity index (SI) is the IC_{50s} ratios for fibroblasts: carcinoma cells.

A. racemosus root extracts were toxic towards HepG2 and LNCaP cells, and normal fibroblasts (Table 1). A defatted ethanolic extract (7.4 ± 0.5% of saponins, by competitive enzyme-linked immunosorbent assay (competitive ELISA)), showed weak cytotoxicity towards all three cell types, while a saponin enriched extract (46 ± 2% of saponins) was more toxic, suggesting that saponin glycosides contribute substantially to the cytotoxicity.

We sought to discover compounds which were more toxic to carcinoma cells and least damaging to normal cells (fibroblasts). While all the purified saponin glycosides were toxic (IC_{50s}, 21.4 to 66.5 μM), the HepG2 and LNCaP cell lines were consistently more sensitive (IC_{50s}, 13.6 and 36.6 μM) than the fibroblasts, i.e., the selectivity indices were consistently >1. Asparacoside and asparanin A were the most potent and showed high selectivity against HepG2 cells, whereas only shatavarin V was more potent against LNCap cells compared with fibroblasts, i.e., it showed potency and selectivity.

The standard, shatavarin IV, was also tested even though it was not detected in extracts, but is a major compound of *A. racemosus* collected from India. It was also cytotoxic, as noted previously [7, 13], but had the lowest selectivity of all the saponin glycosides. Asparanin A, in another study [14], was slightly more potent and induced apoptosis through cell cycle arrest.

This study demonstrates how HPCPC can expand the scope of phytochemistry particularly for complex mixtures of difficult compounds such as saponin glycosides isolated from *A. racemosus* roots in rapid and high yielding steps. Its application to phytomedicine was demonstrated by the selective cytotoxicity of five saponin glycosides towards carcinoma cell lines.

Experimental

General experimental procedures: A SIC CPC240 HPCPC (System Instruments Co., Ltd., Japan) was used for isolations. The effluent was collected by a Foxy R1 fraction collector (Teledyne Isco, Inc., USA). All organic solvents used were either analytical or HPLC grade. Standard grade shatavarin IV was purchased from Natural Remedies Pvt. Ltd. (India). Saponin contents in *A. racemosus* extracts expressed as saponin equivalent to shatavarin IV were evaluated by competitive ELISA using monoclonal antibody (MAb) against shatavarin IV [15]. Saponins in extracts and fractions were detected by TLC (TLC silica gel 60 F254 plates, Merck, Germany) developed using ethyl acetate:MeOH:water (7.5:1.5:1 v/v) as the mobile phase. The saponin glycosides were visualized by dipping the plate in vanillin-sulfuric acid reagent and heated until a yellow colour appeared. Identification of isolated compounds was carried out by ESI-MS, ^{1}H NMR, ^{13}C NMR, and 2D-NMR. ESI-MS experiments were performed in a negative-ion mode using an ESI-QTOF LC/MS (Agilent Technologies) system. NMR spectra were recorded on a Bruker Avance 400 MHz spectrometer at 25 ºC, using pyridine-d_5.

Preparation of the A. racemosus extract: *A. racemosus* roots were collected from Rayong Province, Thailand. A plant voucher specimen (Collection no. RKT 0005) is stored at the PBM herbarium, Faculty of Pharmacy, University of Mahidol, Bangkok, Thailand. Two different extracts were prepared as follows.

Defatted ethanolic extract: Dried powdered roots of *A. racemosus* (100 g) were defatted by maceration with *n*-hexane for 3 days, then filtered. The residue was then macerated with 95% ethanol for 3 days, twice. The filtrates were pooled and concentrated under vacuum to give the defatted ethanolic extract (10.94 g).

Saponin enriched extract: Powdered dry *A. racemosus* roots (100 g.) were extracted at room temperature (90% acetonitrile/water, 2000 mL), assisted by sonication (45 min), and then stirred overnight. The mixture was then filtered and the solvent removed under vacuum to give the saponin enriched extract (4.3 g.).

Measurement of partition coefficients and settling time: The K values of the two-phase solvent systems were estimated by comparing the target compounds of the upper phase with that of the lower phase from each pair of corresponding spots in the TLC.

The settling time of the two-phase solvent system was expressed as the time required to form clear layers between the two phases when the phases were mixed in the ratio 1:1, v/v. The settling time is correlated with the retention of the stationary phase.

Preparative separation procedure by HPCPC: The two-phase solvent system composed of $CHCl_3$-MeOH-water (4:4:2, v/v) and the second system composed of CH_2Cl_2-MeOH-water (4:4:2, v/v) was used for HPCPC separation. *A. racemosus* saponin enriched extract (2 g) was dissolved in 2 mL of each upper and lower phase prior to injection into the HPCPC.

The HPCPC was first entirely filled with the upper phase as the stationary phase (rotation speed at 300 rpm, flow rate at 20.0 $mL.min^{-1}$) and then the lower phase was pumped into the column using descending mode at a flow-rate of 1.5 $mL.min^{-1}$, and rotation speed of 800 rpm. After equilibration, the sample solution was injected and the fractions collected every 10 min. The HPCPC separation ran for 300 min. At the end of the separation, all solution was eluted. Fractions were pooled according to the TLC results and the solvent was evaporated at 45ºC under vacuum.

The HPCPC separation of *A. racemosus* saponin enriched extract (2.0 g) using $CHCl_3$-MeOH-water (4:4:2 v/v) system yielded **1** (29.2 mg) in fractions 13-15 and **2** (6.7 mg) in fractions 17-18 after recrystallization from MeOH. Fractions 7-12 (75.0 mg) were successively fractionated again by HPCPC using CH_2Cl_2-MeOH-water (4:4:2 v/v) as the solvent system. After TLC determination, fractions 16-20 and 21-25 were pooled separately, dried, and named as F1 and F2. F1 (24.0 mg) was dissolved in 0.5 mL MeOH and loaded onto a Sephadex LH-20 (GE Healthcare Bio-Sciences AB, Sweden) column using MeOH as the mobile phase. The fractions were collected every 10 mL and combined according to TLC. **3** (4.6 mg) was obtained from fractions 25-26. F2 (32.5 mg) was further purified by TLC. The sample was dissolved in MeOH and applied to the TLC plate and developed with the described mobile phase upward to 8 cm. **4** showed a band at R_f 0.51. The TLC band was cut-out and extracted using CH_2Cl_2-MeOH (1:1 v/v) producing **4** (3.2 mg). **1**-**4** were isolated as amorphous white powders and were identified as shatavarin IX, asparacoside, asparanin A and shatavarin V by comparing their NMR spectroscopic and ESI/MS data with those previous reported [12-13,16a,16b].

Human cell lines and cell cultures: The human hepatocarcinoma cell line HepG2 (ATCC® HB8065™), prostatecarcinoma cell line LNCaP clone FGC (ATCC®CRL1740™), and normal human fibroblast cells (see below) were used for cytotoxicity testing. The HepG2 cell line was obtained from Asst. Prof. Dr Sakonwun Prapertbut, Faculty of Pharmaceutical Science, Naresuan University. The LNCaP cell line was purchased from American Type Culture Collection (ATCC), USA. Normal human fibroblast cells were isolated from foreskin. Human foreskins were obtained from 3 anonymous donors (ages 1-2 years) provided by Buddhachinaraj Hospital (Phitsanulok, Thailand). The protocol was approved by Naresuan University Institutional Review Board (Ethical approval No. HE-55-Ex-0134). All cells were cultured followed the ATCC protocols and maintained at 37°C in a 5% CO_2 atmosphere with 95% humidity.

Cytotoxic assay: The MTT colorimetric assay [17] was conducted using Paclitaxel as the positive control. The cells were treated with tested compounds at various concentrations, for 24 h following by incubation with MTT solution (20 µL of 6 $mg.mL^{-1}$) at 37°C for 4 h. Then, the treated cells were lysed with 200 $\mu L.well^{-1}$ of 1:1 DMSO:MeOH. Absorbance was measured at 570 nm using a microplate reader. The IC_{50} values were calculated using GraphPad Prism 5 software. The selectivity index (SI) was calculated from the IC_{50} ratio of normal fibroblasts divided by that of cancerous cells (LNCaP, HepG2).

Statistics: Results were expressed as mean ± S.D (Statistical significance was determined by using one-way ANOVA followed by Tukey's test at $P<0.01$ (Statistical Package of Social Sciences (SPSS) 16, SPSS Inc, Chicago, IL).

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AKR1C3 Inhibitory Potency of Naturally-occurring Amaryllidaceae Alkaloids of Different Structural Types

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Aldo-keto reductase 1C3 (AKR1C3) is an important human enzyme that participates in the reduction of steroids and prostaglandins, which leads to proliferative signaling. AKR1C3 is frequently upregulated in various cancers, and this enzyme has been suggested as a therapeutic target for the treatment of these pathological conditions. The fact that the isoquinoline alkaloid stylopine has been identified as a potent AKR1C3 inhibitor has prompted us to screen a library of diverse types of Amaryllidaceae alkaloids, which biogenetically are isoquinoline alkaloids, on a recombinant form of AKR1C3. From the tested compounds, only tazettine showed moderate AKR1C3 inhibitory potency with an IC_{50} value of 15.8 ± 1.2 μM. Tazettine is a common Amaryllidaceae alkaloid, which could be used as a model substance for the further development of either analogues or related compounds with better inhibition potency.

Keywords: Amaryllidaceae alkaloids, Aldo-keto reductase 1C3, Tazettine.

Plants of the Amaryllidaceae family are known for producing an exclusive group of alkaloids, named Amaryllidaceae alkaloids, which are of great interest due to their wide range of biological activities, including antiviral, antimalarial, anticancer and anticholinesteratic [1,2]. The medical properties of these plants were already known in the fourth century B.C., when Hippocrates of Cos used oil from the daffodil, *Narcissus poeticus* L., for the treatment of uterine tumors. Some species of this family contain galanthamine, a long-acting, selective, reversible and competitive acetylcholinesterase inhibitor, which has been approved by the Food and Drug Administration for the treatment of mild to moderate Alzheimer's disease under the commercial name Reminyl© (galanthamine hydrobromide). Many Amaryllidaceae alkaloids have been reported to exhibit promising antitumor properties [3a,b]. Those that are cytotoxic at micromolar concentrations include lycorine [3c], narciclasine [3d], pancratistatin [4a] and haemanthamine [2a,4b].

Human enzyme AKR1C3, also known as 17β-hydroxysteroid dehydrogenase type 5, is a member of the aldo-keto reductase protein superfamily. It catalyzes the reduction of carbonyl groups of several steroids and prostaglandins, which leads to pre-receptor regulation of their action and proliferative signaling. AKR1C3 is involved in the development of several types of hormone-dependent {e.g. breast and castration resistant prostate cancer (CRCP)} and hormone-independent cancers [4c,d]. AKR1C3 is overexpressed in prostate tumors from CRCP patients [5,6]. Reduction of AKR1C3 activity significantly decreases the level of testosterone, 5α-dihydroxytestosterone, and androgen dependent gene expression e.g. prostate specific antigen. *In vivo* inhibition of AKR1C3 leads to reduction in growth of xenograft models of CRCP [6,7]. These research findings have made AKR1C3 a promising target for prostate cancers.

Twenty-eight Amaryllidaceae alkaloids, belonging to seven structural types, have been screened for their potency in inhibiting a recombinant form of AKR1C3. The tested alkaloids belong to belladine- (belladine); haemanthamine- (haemanthamine, haemanthidine, hamayne, epimaritidine); crinine- (ambelline, 11-*O*-acetylambelline, crinine, undulatine, buphanamine, buphanidrine, buphanisine, 1-*O*-acetylbulbisine), galanthamine- (galanthamine, chlidanthine), lycorine- (caranine, acetylcaranine, lycorine, 1-*O*-acetyllycorine, galanthine, 9-*O*-demethylgalanthine), homolycorine- (homolycorine, masonine, tetrahydromasonine, lycorenine, oduline, hippeastrine) and tazettine-structural types (tazettine) (Figure 1, Supplementary Material). Of the tested alkaloids, only tazettine showed moderate inhibitory potency (IC_{50} = 15.8 ± 1.2 μM) compared with the known AKR1C3 inhibitor, indomethacin (IC_{50} = 3.7 μM) [8]; eleven alkaloids showed weak inhibitory potency and the others were considered as inactive (Figure 1).

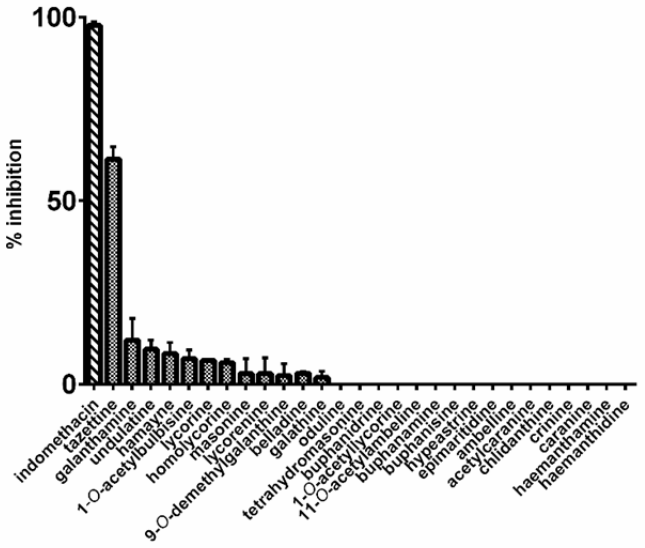

Figure 1: AKR1C3-inhibitory potency of various Amaryllidaceae alkaloids

Although tazettine has a lower inhibitory potency than those of known inhibitors (e.g. indomethacin and its analogues, and baccharin), next to another alkaloid stylopine, tazettine is the second alkaloid of interest since it can be used as a lead structure in the development of more potent AKR1C3 inhibitors. The advantage of tazettine is its good availability from natural sources, as it is one of the most abundant alkaloids within the family Amaryllidaceae.

Experimental

Amaryllidaceae alkaloids: All tested alkaloids have been previously isolated in the laboratory of the Department of Pharmaceutical Botany from *Zephyranthes robusta* [9a,b], *Chlidanthus fragrans* [2a, 9c], *Nerine bowdenii* [10], and *Narcissus poeticus* cv. Brackenhurst [11a]. The purity (≥ 95 %) of each isolated compound was confirmed by NMR spectroscopy. A stock solution (10 mM) of each alkaloid was prepared in DMSO.

Preparation of recombinant form of AKR1C3: A recombinant form of human AKR1C3 was prepared in an *Escherichia coli* expression system and purified to homogeneity, as described previously [11b].

AKR1C3-inhibitory potency assay of Amaryllidaceae alkaloids: Incubation mixtures, which contained 1.5 μg of pure recombinant AKR1C3, an NADPH-generation system (13 mM NADP+, 96 mM glucose-6-phosphate, 3.5 U glucose-6-phosphate dehydrogenase, 50 mM $MgCl_2$), either 20 μM (for screening purposes) or 4–100 μM (for IC_{50} determination) of test alkaloid, and 0.1 M Na-phosphate buffer, pH 7.4, were incubated for 10 min on ice and subsequently pre-incubated for 5 min at 37°C. The enzymatic reaction was initiated by the addition of Adion in a final concentration of 12 μM. The reaction mixture (100 μL) was incubated at 37°C for 30 min and then stopped by the addition of 40 μL of 25% NH_4OH and by cooling to 0°C. After 10 min on ice, testosterone was extracted with 1 mL of ethyl acetate by shaking for 15 min. Each sample was then centrifuged for 2 min at 13,000 rpm. The organic phases were transferred to new Eppendorf microtubes and evaporated to dryness under vacuum at 30°C. Control samples of identical composition containing only DMSO without test alkaloids were prepared in an identical manner. A similar procedure to that used for the tested substances was employed for the model inhibitor of AKR1C3, indomethacin. Sample residues were dissolved in 300 μL of mobile phase and subjected to HPLC analysis. The formed metabolite, testosterone, was determined using an HPLC Agilent 1100 Series system (Santa Clara, CA, USA), which was equipped with a BDS Hypersil C18 chromatography column (250 × 4.6 mm, 5 μm) and a BDS 10 × 4 mm i.d. 5 μm guard column (Thermo Electron Corporation, UK). A mobile phase consisting of methanol:water 70:30, v/v, at a flow rate of 0.6 mL/min was used; detection was performed using a diode array detector at 240 nm.

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Product Selectivity of Esterification of L-Aspartic Acid and L-Glutamic Acid Using Chlorotrimethylsilane

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TMSCl works as an acid catalyst precursor for selective esterification of L-aspartic and L-glutamic acids in the presence of primary, secondary and tertiary alcohols. Although excess TMSCl was required for the completion of esterification, the resulting alkyl TMS ether could be azeotropically removed by simple evaporation with alcohol.

Keywords: Esterification, TMSCl, Aspartic acid, Acid catalyst, Protective group.

For the chemical synthesis of peptides or glycopeptides, many useful and convenient methodologies such as solid-phase synthesis [1] or microwave assisted synthesis have been developed [2]; however, there are still many challenges relating to the selective protection and deprotection of several functional groups [3]. Regarding acidic amino acids (aspartic acid and glutamic acid), selective protection of the carboxy groups is necessary for most organic reactions. Belshaw *et al*. previously reported that chlorotrimethylsilane (TMSCl) mediated formation of omega-allyl esters of aspartic and glutamic acids in the presence of allyl alcohol as a solvent [4a], and Brook *et al*. reported the preparation of a variety of esters [4b]. We have also reported quantitative TMSCl mediated esterification of *N*-fluorenylmethyloxycarbonyl (Fmoc) amino acids in CH_2Cl_2 [4c]. Recently, Li *et al*. reported the esterification of amino acids with methanol using TMSCl [4d]. It is desirable, however, to obtain desired omega-monoesters selectively using various alcohols without heating at high temperature or purification by silica-gel column chromatography. In this study, we systematically explored 19 reaction conditions for the selective synthesis and purification of monoester and diester derivatives of L-aspartic and L-glutamic acids in the presence of TMSCl as an acid catalyst precursor.

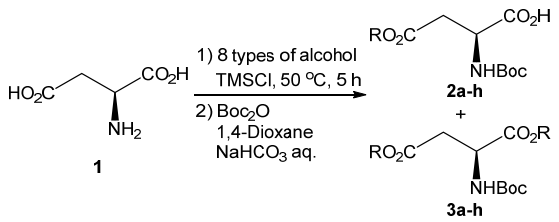


Figure 1: Selective esterification using TMSCl as an acid catalyst precursor.

Esterification proceeded at room temperature using TMSCl and alcohol as solvent, however, reactions proceeded more rapidly at 50°C. To a suspension of L-aspartic acid in various alcohols (methanol, ethanol, propanol etc.) was added TMSCl, followed by stirring at 50°C. After 5 h, aspartic acid was consumed as indicated by TLC (thin-layer chromatography). In the early stage of the reaction, only the β-monoester was observed. Furthermore, more than 10 equiv. of TMSCl was required for sufficient esterification of both aspartic acid carboxy groups. After completion, the mixture was concentrated under reduced pressure. In order to simplify the purification of esters, the amino group was protected using *tert*-butoxycarbonyl (Boc). Di-*tert*-butyl dicarbonate was added to a mixture of the residue in 1,4-dioxane-saturated aqueous $NaHCO_3$ solution (v/v 2:1) at 0 °C. The reaction mixture was stirred for 1 h and then at room temperature for 12 h. Monoesters and diesters were obtained with high selectivity after being converted to *N*-Boc-derivatives with subsequent extraction by $CHCl_3$ and aqueous HCl (Table 1) [5].

Table 1: Various conditions for selective esterification using TMSCl.

Entry	Solvent (5 mL)	TMSCl (eq.)	Yield (%)[a] Monoester (**2**)		Diester (**3**)	
1	MeOH	1		71		8
2	MeOH	5	**2a**	24	**3a**	75
3	MeOH	10		n.d.[c]		99
4	EtOH	1	**2b**	66	**3b**	18
5	EtOH	5		8		91
6	*n*-BuOH	1	**2c**	68	**3c**	7
7	*n*-BuOH	5		7		92
8	BnOH	1	**2d**	40[b]	**3d**	1[b]
9	BnOH	5		38[b]		61[b]
10	AllylOH	1	**2e**	59	**3e**	10
11	AllylOH	5		5		91
12	PropargylOH	1	**2f**	24	**3f**	17
13	PropargylOH	5		3		88
14	*i*-PrOH	1		30		23
15	*i*-PrOH	5	**2g**	46	**3g**	46
16	*i*-PrOH	10		46		54
17	*t*-BuOH	1		n.d.[c]		n.d.[c]
18	*t*-BuOH	5	**2h**	n.d.[c]	**3h**	n.d.[c]
19	*t*-BuOH	10		n.d.[c]		n.d.[c]

[a]The product did not need purification by silica-gel column chromatography, because no by-product was detected by ^{1}H-NMR and TLC for each reaction. [b]The product was purified by silica-gel column chromatography to remove BnOH. [c]Not detected on TLC.

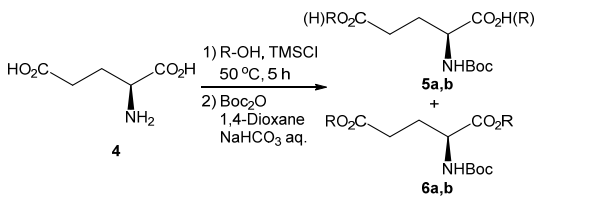
The mono and diesters could be easily separated by pH-controlled extraction. Fortunately, pure products were obtained as indicated by ^{1}H-NMR and TLC, without the need for purification by silica-gel column chromatography. Both the monoesters and diesters were obtained in high yields when benzyl alcohol was used as a solvent (Table 1, entry 8, 9). When isopropyl alcohol was used as solvent, esterification proceeded when using less than 3 equiv. of TMSCl; however, selectivity for either the monoester or diester was low (Table 1, 14-16). In the case of *tert*-butyl alcohol as solvent, the

addition of more TMSCl as acid and/or solvent did not seem to improve esterification yields. We suspect that *tert*-butyl ester was unstable under our conditions, and the tertiary carbocation, which was generated from *t*-BuOH by TMSCl, possibly reacted with *t*-BuOH to give di-*tert*-butyl ether via S_N1 reaction (Table 1, entry 17-19).

Our method was then applied to various alcohols in order to prepare building blocks for peptide synthesis. This included successful application of allyl esters as carboxy protecting groups (Table 1, entry 10, 11). The propargyloxycarbonyl group is stable when exposed to neat TFA (trifluoroacetic acid) but is readily cleaved by treatment with $Co_2(CO)_8$ and TFA in CH_2Cl_2 via formation of an alkyne-Cobalt complex [6a]. The propargyl ester similarly serves as a good protecting group for carboxy functions [4c, 6]. By using of propargyl alcohol as solvent, propargyl ester was also obtained in high yield (Table 1, entry 12, 13).

Glutamate esters were also obtained with high yield after being converted to *N*-Boc derivatives, however, unlike esterification of aspartic acid, both the gamma-monoesters and alpha-monoesters were formed. Therefore it was necessary to separate the glutamate esters using silica-gel column chromatography (Table 2). Alpha-monoesters were probably formed instead of gamma-monoesters due to the higher reactivity of the alpha-carbonyl group after formation of a six-membered cyclic anhydride intermediate under heat and acidic conditions.

Table 2: Various useful conditions for the esterification of L -glutamic acid using TMSCl.



Entry	Solvent (5 mL)	TMSCl (eq.)	Yield (%)[a] Monoester (**5**)		Diester (**6**)
1	MeOH	1	71 (only γ)[b]		8
2	MeOH	3	41 (α:γ = 3:11)[b]		59
3	MeOH	5	**5a** 24 (α:γ = 5:14)[b]	**6a**	75
4	MeOH	10	1 (α:γ = 5:3)[b]		92
5	EtOH	1	84 (only γ)[b]		10
6	EtOH	5	**5b** 18 (only γ)[b]	**6b**	69

[a]The product did not need purification by silica-gel column chromatography, because no by-product was detected by ^{1}H-NMR and TLC for each reaction. [b]Estimated by ^{1}H-NMR.

TMSCl works as an acid catalyst precursor for selective esterification of acidic amino acids with primary alcohols. Although excess TMSCl was required for the completion of esterification, by-product alkyl TMS ether could be removed by simple evaporation with alcohol. Our selective method can be applied to the esterification of various amino acids with primary, secondary and tertiary alcohols.

Experimental

General procedure for selective esterification: the case of methyl *N*-Boc-L-aspartate (**2a**) and dimethyl *N*-Boc-L-aspartate (**3a**); To a mixture of L-aspartic acid (0.13 g, 1.0 mmol) in CH_3OH (5 mL, 123 mmol) was added TMSCl (0.63 mL, 5.0 mmol). The resulting mixture was heated and stirred at 50 °C for 5 h, and then concentrated under reduced pressure. To a mixture of the residue in 1,4-dioxane (10 mL) and saturated aqueous $NaHCO_3$ solution (5 mL) was added di-*tert*-butyl dicarbonate (0.26 g, 1.2 mmol) at 0°C. The reaction mixture was stirred at 0 °C for 1 h and then stirred at room temperature for 12 h. The 1,4-dioxane was removed on a rotary evaporator and the residue was poured onto ice-water (50 mL) and the cold solution was washed with $CHCl_3$ (50 mL x 3) to separate nonacidic compounds. The combined $CHCl_3$ extracts, were washed with water and dried, and the solvent was removed on a rotary evaporator to afford the *N*-Boc-dimethyl ester as a colorless liquid (**3a**, 0.20 g, 0.75 mmol, 75%). The aqueous solution was acidified (pH 2.5) with a solution of 1 M HCl aq., and extracted with $CHCl_3$ (50 mL x 3). The combined $CHCl_3$ extracts were washed with water and dried. The solvent was removed on a rotary evaporator to give the *N*-Boc-monomethyl ester as a colorless liquid (**2a**, 0.06 g, 0.24 mmol, 24%).

Methyl *N*-Boc-L-aspartate (**2a**)
^{1}H NMR (400 MHz, $CDCl_3$) δ 1.45 (s, 9 H, *t*-Bu), 2.81 (dd, 1 H, *J* = 17.2, 4.8 Hz, Hβ-a), 3.01 (dd, 1 H, *J* = 17.2, 4.8 Hz, Hβ-b), 3.64 (s, 3 H, CO_2CH_3), 4.20 (t, 1 H, *J* = 5.5 Hz, Hα).

Dimethyl *N*-Boc-L-aspartate (**3a**)
^{1}H NMR (400 MHz, $CDCl_3$) δ 1.45 (s, 9 H, *t*-Bu), 2.81 (dd, 1 H, *J* = 17.2, 4.8 Hz, Hβ-a), 3.01 (dd, 1 H, *J* = 17.2, 4.8 Hz, Hβ-b), 3.64 (s, 3 H, CO_2CH_3), 3.77 (s, 3 H, CO_2CH_3), 4.20 (t, 1 H, *J* = 5.5 Hz, Hα).

The case of methyl *N*-Boc-L-glutamate (**5a**) and dimethyl *N*-Boc-L-glutamate (**6a**) using 3 equiv. of TMSCl (3 mmol).

Methyl *N*-Boc-L-glutamate (**5a**, 0.11 g, 0.41 mmol, 41%)
^{1}H NMR (400 MHz, $CDCl_3$) δ 1.45 (s, 9 H, *t*-Bu), 1.95-2.19 (m, 2H, Hβ), 2.33-2.47 (m, 2H, Hγ), 3.68 (s, 2.36 H, γ-CO_2CH_3), 3.77 (s, 0.64 H, α-CO_2CH_3), 4.20 (t, 1 H, *J* = 5.5 Hz, Hα).

Dimethyl *N*-Boc-L-glutamate (**6a**, 0.16 g, 0.59 mmol, 59%).
^{1}H NMR (400 MHz, $CDCl_3$) δ 1.45 (s, 9 H, *t*-Bu), 1.95-2.19 (m, 2H, Hβ), 2.33-2.47 (m, 2H, Hγ), 3.68 (s, 3 H, γ-CO_2CH_3), 3.77 (s, 3 H, α-CO_2CH_3), 4.20 (t, 1 H, *J* = 5.5 Hz, Hα).

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New Cyclopentyl Fatty Acid and Cyanohydrin Glycosides from Fruits of *Hydnocarpus hainanensis*

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Three new compounds, hydnohainanic acid (**1**), and hydnohainanin A (**2**) and B (**3**) were isolated from the fruits of *Hydnocarpus hainanensis* (Achariaceae). Their structures were determined by spectroscopic analysis, including 2D NMR, and MS. Compounds **1**-**3** were evaluated for their cytotoxic activity against the KB cell line. Compound **1** had a moderate cytotoxicity with an IC_{50} value of 32.5 μg/mL, while the two remaining compounds did not exhibit inhibition, even at a concentration of 128 μg/mL.

Keywords: *Hydnocarpus hainanensis*, Flacourtiaceae, Achariaceae, Hydnohainanic acid, Hydnohainanin A, B.

The genus *Hydnocarpus* has previously been described in the family Flacourtiaceae. However, it has now been transferred to the family Achariaceae in the molecular phylogeny-based classification, known as the APG III system [1]. The *Hydnocarpus* genus consists of about 40 species. Many *Hydnocarpus* species have been used in folk medicine [2]. A literature overview showed that the bioactive compounds of *Hydnocarpus* were assigned as flavonolignan [3a-e] and cyclopentyl fatty acids [4], which exhibited various pharmacological activities. Cyclopentenyl fatty acids were found to be the major constituents of the oil of several *Hydnocarpus* species and to have antileprotic activity [2]. Also, hydnocarpin, a flavonolignan isolated from *Hydnocarpus*, is reported to exhibit antimicrobial and anticancer activity [2,5a-b]. Biological screening of other species of *Hydnocarpus* revealed that plants of this genus could be a rich source of bioactive molecules [6a-e].

In continuation of our search for natural bioactive compounds, we examined the fruits of *H. hainanensis* (Achariaceae), collected in Quang-Tri, Vietnam, as the extract of the fruits exhibited a cytotoxic activity against KB cells. In this communication, we report the isolation and structural elucidation of three new compounds (**1**-**3**) (Figure 1) from the fruits of *H. hainanensis*.

Compound **1** was isolated as colorless oil. In the HR-ESI mass spectrum of **1**, the proton adduct molecular ion $[M+H]^+$ at *m/z* 267.1960 was observed. The ^1H NMR spectrum of **1** showed a singlet olefinic proton at δ_H 5.95 (H-2), aliphatic protons at δ_H 2.57 (2H, m, CH_2-4), 2.41 (2H, m, CH_2-5), 2.39 (2H, t, J = 7.5 Hz, CH_2-6), 2.34 (2H, t, J = 7.5 Hz, CH_2-15), 1.63 (2H, quint, J = 7.5 Hz, CH_2-14), and 1.57 (2H, quint, CH_2-7), and overlapped protons at δ_H 1.27-1.28. Analysis of the ^{13}C NMR spectrum, with the aid of the HSQC spectrum, revealed the presence of a ketone at δ_C 210.6 (C-3), three sp^2 carbons at δ_C 183.7 (C-1), 179.3 (C-16) and 129.4 (C- 2), and twelve methylenes. The COSY spectrum of **1** indicated

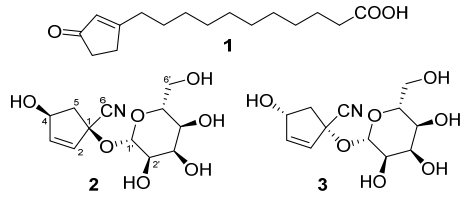

Figure 1: Structures of compounds **1** – **3**.

correlations between protons of CH_2-4 and CH_2-5, and those of the side chain starting from CH_2-6 to CH_2-15. Analysis of the HMBC spectrum revealed the presence of a cyclopentyl ring by cross-peaks of ketone carbon C-3 with H-2, CH_2-4 and CH_2-5, and those of protons of CH_2-5 with C-1 and C-2. In addition, the HMBC correlations of C-1 with the protons of CH_2-6 and CH_2-7 assigned the linkage of C-1 with C-6. Finally, the terminal carboxylic acid group was confirmed by HMBC cross-peaks of C-16 with the protons of CH_2-15 and CH_2-14 (Figure 2). Compound **1** was thus identified as 1-cyclopentene-3-one-1-undecanoic acid. This new cyclopentyl fatty acid was named hydnohainanic acid.

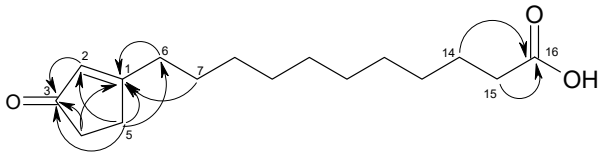

Figure 2: Selected HMBC correlations of **1**.

Compound **2**, obtained as an amorphous solid, was optically active {$[\alpha]_D$ -68 (*c* 0.16, MeOH)}. Its HRESI mass spectrum showed the proton adduct molecular ion $[M+H]^+$ at *m/z* 288.1091, consistent with the molecular formula $C_{12}H_{17}NO_7$.

Table 1: NMR data of compound **1** ($CDCl_3$).

position	δ_C	δ_H mult. (*J* in Hz)	position	δ_C	δ_H mult. (*J* in Hz)
1	183.7		9	29.3	1.28 m
2	129.4	5.95 s	10	29.3	1.28 m
3	210.6		11	29.3	1.28 m
4	31.6	2.57 m	12	29.3	1.28 m
5	35.3	2.41 m	13	29.0	1.32 m
6	33.5	2.39 t (7.5)	14	24.7	1.63 quint (7.5)
7	27.1	1.57 quint (7.5)	15	34.0	2.34 t (7.5)
8	29.5	1.27 m	16	179.3	

Table 2: NMR data of compounds **2** and **3** (CD_3OD).

	2		**3**	
position	δ_C	δ_H mult. (*J* in Hz)	δ_C	δ_H mult. (*J* in Hz)
1	82.3		81.9	
2	132.9	6.16 d (5.5)	131.8	6.16 dd (1.0, 5.5)
3	142.1	6.24 d (2.0, 5.5)	142.8	6.29 dd (2.0, 5.5)
4	74.1	4.85 m	74.6	4.83 m
5	47.9	2.23 dd (5.0, 14.5)	48.5	2.27 dd (5.0, 14.5)
		3.06 dd (7.0, 14.5)		3.04 dd (7.0, 14.5)
6	120.2		120.3	
1'	99.2	5.06 d (8.0)	98.7	5.01 d (8.0)
2'	71.9	3.38 dd (2.7, 8.0)	71.9	3.37 dd (3.0, 8.0)
3'	72.9	4.10 t (2.7)	72.9	4.09 t (3.0)
4'	68.5	3.53 dd (2.7, 9.5)	68.6	3.54 dd (3.0, 10.0)
5'	75.6	3.77 m	75.6	3.74 m
6'	62.8	3.68 dd (5.5, 12.0)	62.9	3.69 dd (5.5, 11.5)
		3.87 dd (2.0, 12.0)		3.86 dd (2.0, 11.5)

The presence was observed in the ^{1}H NMR spectrum of **2** of two olefinic protons at δ_H 6.16 (H-2) and 6.24 (H-3), an anomeric proton at δ_H 5.06, and seven protons in the carbohydrate region. The ^{13}C NMR and DEPT spectra of **2** presented two olefinic carbons at δ_C 132.9 (C-2) and 142.1 (C-3), one nitrile group at δ_C 120.2 (C-6), one oxygenated quaternary carbon at δ_C 82.3 (C-1), one oxygenated methine at δ_C 74.1 (C-4), and signals characteristic of a sugar unit [δ_C 99.2 (C-1'), 71.9 (C-2'), 72.9 (C-3'), 68.5 (C-4'), 75.6 (C-5') and 62.8 (C-6')]. Analysis of the COSY spectrum indicated two spin-spin coupling systems as follows: A) H-2 (δ_H 6.16)/H-3 (δ_H 6.24)/H-4 (δ_H 4.85)/CH_2-5 (δ_H 2.23 and 3.06), and B) correlations of a sugar moiety starting from H-1' (δ_H 5.06) to CH_2-6' (δ_H 3.68 and 3.87) via H-2' (δ_H 3.38), H-3' (δ_H 4.10), H-4' (δ_H 3.53) and H-5' (δ_H 3.77). The spectral features of the coupling systems A, B, the oxygenated quaternary carbon and the nitrile group deduced from HMBC data indicated structural similarity of **2** to cyclopentenoid cyanohydrin glucosides [7a-f]. However, significant differences were noted for the sugar moiety. Coupling constant analysis revealed that H-1' had an *anti*-coupling constant (J = 8.0 Hz), indicating a 1,2-diaxial relationship between H-1'/H-2'. Additionally, H-2' presented *anti* (J = 8.0 Hz) and *gauche* (J = 2.7 Hz) coupling constants. H-3' was thus determined to have an equatorial orientation. Similarly, H-4' also had *anti* (J = 9.5 Hz) and *gauche* (J = 2.7 Hz) coupling constants. This suggested an axial disposition for both H-4' and H-5'. The sugar moiety was thus identified as *β*-allopyranose for **2**. The same planar structure was determined for **3** from analyses of its spectral data. Furthermore, coupling constant analysis (Table 2) revealed also the presence of β-allopyranose in the structure of **3**. The sugar moiety from acidic hydrolysis of **2** and **3** gave positive optical rotation, assigning a β-D-configuration for the allopyranose moiety of both **2** and **3**. The difference between **2** and **3** was hence related to the aglycones. Previously, four isomers of 1,4-dihydroxy-2-cyclopeten-1-carbonitrile, tetraphylin B, volkenin, taraktophylin and epivolkenin (Figure 3) were reported [7a,c,e]. It was found that the ^{13}C NMR data are almost similar for these four isomers. However, the proton chemical shifts and proton coupling constants of CH_2-5 were significantly different between the 1,4-*trans* isomers (tetraphylin B and volkenin) and the 1,4-*cis* isomers (taraktophylin and epivolkenin) [7e]. Proton coupling constants of CH_2-5 of 1,4-*trans* isomers were reported to be around 15.0 Hz (J_{AB}), and 6.5 and 3.5 Hz for J_{AX} and J_{BX}, whereas, for the 1,4-*cis* isomers, these values were around 14.5 Hz (J_{AB}), and 4.8 and 7.2 Hz for J_{AX} and J_{BX}.

Compounds **2** and **3** had chemical shifts and proton coupling constants (Table 2) close to those of the 1,4-*cis* isomers (taraktophylin and epivolkenin). This observation suggested that the hydroxy at C-4 and the allosidic oxygen at C-1 had a *cis* relationship for the two compounds **2** and **3**. This is in accord with a previous report that Flacourtiaceae species predominantly produce 1,4-*cis*-dihydroxy-2-cyclopeten-1-carbonitrile [8]. As *β*-D-allopyranose was present in the structures of both **2** and **3**, their aglycones were thus enantiomers.

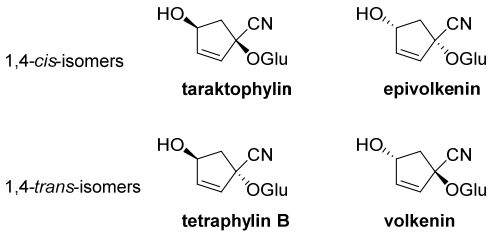

Figure 3: Isomers of 1,4-dihydroxycyclopenten-1-carbonitrile.

Since, the aglycones of **2** and **3** could not be obtained properly due to decomposition during acidic hydrolysis, the configurations of these aglycones were not directly determined. However, the absolute configuration of these aglycones could be suggested from application of the Brewster rules to these systems [9a-c]. Thus, the rotatory contribution of the allylic hydroxy group (OH at C-4), largely determined by its interaction with the double bond, should be negative for **2** (4*S*) and positive for **3** (4*R*) [7e,9c]. Additionally, the molecular rotation of **2** {$[M]_D$ -195} is more levorotatory in comparison with **3** ({$[M]_D$ +63}). The observed rotations of **2** (negative) and **3** (positive) could hence confirm the 4*S* and 4*R* configuration for **2** and **3**, respectively. As **2** had a negative rotation (same effect with allylic hydroxy for 4*S*) and **3** a positive one (same effect with allylic hydroxyl for 4*R*), the rotatory contribution of the substituents at C-1 did not cancel the rotatory contribution of the allylic hydroxy (whether the substituents at C-1 make either a negative or positive contribution to overall rotation). Probably, the observed rotations of **2** and **3** should be dominated by the contribution from the free allylic hydroxyl group, as noted for taraktophylin and epivolkenin [7e]. The absolute configurations of **2** and **3** are thus suggested as shown (1*R*, 4*S* for **2** and 1*S*, 4*R* for **3**). These two compounds are reported here for the first time and named hydnohainanin A and B, respectively.

The known compounds, hydnocarpic acid [10], taraktophylin [7e] and litchiol B [11] were also isolated from this plant. Their structures were determined by spectral data and comparison with those reported in literature data.

Compounds **1**-**3** were evaluated for their cytotoxic activity against the KB cell line. Compound **1** had a moderate cytotoxicity with an IC_{50} value of 32.5 μg/mL. Compounds **2** and **3** did not exhibit inhibition activity, even at the concentration of 128 μg/mL. Ellipticine was used as a positive reference compound.

Experimental

General: Optical rotations were measured on a Jasco P-2000 polarimeter, and HR-ESIMS on a FT-ICR 910-MS TQFTMS-7 T mass spectrometer. NMR spectra were recorded on a Bruker 500.13 MHz spectrometer operating at 125.76 MHz for ^{13}C NMR, and at 500.13 MHz for ^{1}H NMR. ^{1}H chemical shifts were referenced to $CDCl_3$ and CD_3OD at δ 7.27 and 3.31 ppm, respectively, while the ^{13}C chemical shifts were referenced to the central peak at δ 77.0 ($CDCl_3$), and 49.0 (CD_3OD). For HMBC experiments the delay

(1/2*J*) was 70 ms, and for the NOESY experiments the mixing time was 150 ms.

Plant material: *H. hainanensis* (Merr.) Sleum was collected in 2006 at Quang Tri, Vietnam, and identified by Dr Nguyen Quoc Binh. A specimen (VN 1761) was deposited at the Institute of Ecology and Natural Resources, Vietnam Academy of Science and Technology.

Extraction and isolation: Dried and ground fruit of *H. hainanensis* (650 g) was extracted with ethanol at room temperature (5 x 1.0 L). The extracts were combined and concentrated under diminished pressure. The residue (101 g) was suspended in water (250 mL) and extracted successively with *n*-hexane and EtOAc. The *n*-hexane and EtOAc solutions were concentrated under reduced pressure to afford 43.3 g and 20.3 g, respectively. The water solution was concentrated under vacuum to give 30.3 g of dry extract. The *n*-hexane extract (43.3 g) was subjected to silica gel column chromatography (CC) eluted with a solvent gradient of *n*-hexane/EtOAc to yield 8 fractions. Fractions 6 and 7 were combined (3.1 g) and subjected to CC on silica gel (gradient of *n*-hexane/EtOAc), affording compound **1** (32 mg). The water extract (30.1 g) was separated by CC on silica gel (0% to 100% of MeOH in CH_2Cl_2) to furnish 9 fractions. Fraction 4 (2.2 g) was subjected to CC on Sephadex LH-20 (MeOH/CH_2Cl_2: 9/1), yielding 2 sub-fractions. Sub-fraction 1 (1.1 g) was purified by preparative TLC (CH_2Cl_2/1,4-dioxane: 9/1) to obtain compounds **2** (7 mg) and **3** (9 mg).

Hydnohainanic acid (**1**)
Colorless oil.
Rf: 0.6 (*n*-hexane-EtOAc, 9:1).
^{1}H and ^{13}C NMR: Table 1.
HRMS-ESI: *m/z* [M + H^+] calcd for $C_{16}H_{27}O_3$: 267.1960; found: 267.1960.

Hydnohainanin A (**2**)
White amorphous solid.
$[\alpha]_D$: -68.0 (*c* 0.16, MeOH).
Rf: 0.5 (CH_2Cl_2 – 1,4-dioxane, 9:1).
^{1}H and ^{13}C NMR: Table 2.
HRMS-ESI: *m/z* [M + H^+] calcd for $C_{12}H_{18}NO_7$: 288.1083; found: 288.1075.

Hydnohainanin B (**3**)
White amorphous solid.
$[\alpha]_D$: +22.0 (*c* 0.16, MeOH).
Rf: 0.45 (CH_2Cl_2 -1,4-dioxane, 9:1).
^{1}H and ^{13}C NMR: Table 2.
HRMS-ESI: *m/z* [M + H^+] calcd for $C_{12}H_{18}NO_7$: 288.1083; found: 288.1086.

Acid hydrolysis of 2 and 3: A solution of either **2** or **3** (each 4 mg) was heated at 70°C for 3 h in 1N HCl (dioxane-H_2O, 1:1, 1.5 mL). The filtrates from the hydrolysate were neutralized with DOWEX HCR-S ion-exchange resin and filtered. The filtrate was concentrated under reduced pressure and purified by preparative TLC. The sugar had an identical Rf with that of the standard allose and had a positive optical rotation value {$[\alpha]_D$: +13.2 (*c* 0.12, H_2O).

Cytotoxic activity assay: The cytotoxicity assays were carried out in triplicate in 96-well microtiter plates against KB (mouth epidermal carcinoma) cells. Cells were maintained in Dulbecco's D-MEM medium, supplemented with 10% fetal calf serum, L-glutamine (2 mM), penicillin G (100 UI/mL), streptomycin (100 µg/mL) and gentamicin (10 µg/mL). Stock solutions of compounds were prepared in DMSO/H_2O (1/9), and the cytotoxicity assays were carried out in 96-well microtiter plates against either cancer or normal cells (3 x 10^3 cells/mL) using a modification of the published method [12]. After 72 h incubation at 37°C in air/CO_2 (95:5) with or without test compounds, cell growth was estimated by colorimetric measurement of stained living cells by neutral red. Optical density was determined at 540 nm with a Titertek Multiscan photometer. The IC_{50} value was defined as the concentration of sample necessary to inhibit the cell growth to 50% of the control. Ellipticine was used as a reference compound.

Supplementary data: 1D and 2D NMR spectra for compounds **1** - **3** are available.

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Chemical Constituents of the Roots and Rhizomes of *Saposhnikovia divaricata* and their Cytotoxic Activity

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Phytochemical investigation of the MeOH extract of the roots and rhizomes of *Saposhnikovia divaricata* (Umbelliferae) resulted in the isolation of six chromons (**1**-**6**) and five polyacetylene derivatives (**7**-**11**). Compounds **9** and **11** were isolated from *S. divaricate* for the first time. The chromon derivatives (**1**-**6**) were evaluated for their cytotoxic activity against HL-60 human promyelocytic leukemia cells. Compound **1** (3'-*O*-angeloylhamaudol) showed the most potent cytotoxic activity with an IC_{50} value of 4.41 μM and was found to induce apoptotic cell death in HL-60 cells. The loss of mitochondrial membrane potential, release of cytochrome *c* into the cytoplasm, and activation of caspase-9 in the **1**-treated HL-60 cells suggests that **1** induces apoptosis through the mitochondrial-dependent apoptotic pathway.

Keywords: *Saposhnikovia divaricata*, Umbelliferae, 3'-*O*-Angeloylhamaudol, HL-60 cell, Cytotoxicty, Apoptosis.

Saposhnikovia divaricata (Turcz.) Schischk. (Umbelliferae) is distributed throughout the northeastern region of China, and its roots and rhizomes have been used for the treatment of headache, migraine, and the common cold in Japan and China [1]. This crude drug is also used in Kampo prescriptions for its antipyretic effects. Coumarin, chromon, and polyacetylene derivatives have been isolated from *S. divaricata* as characteristic constituents [2, 3]. In the continuous search for natural products with anti-tumor activity, the MeOH-eluted fraction of *S. divaricata*, obtained by passing the MeOH extract of its roots and rhizomes through a column loaded with porous-polymer polystyrene resin, was found to show cytotoxic activity against HL-60 human promyelocytic leukemia cells. In this study, phytochemical examination of the MeOH-eluted fraction was performed, which resulted in the isolation of six chromons (**1**-**6**) and five polyacetylene derivatives (**7**-**11**). The cytotoxicity and apoptosis-induction activity of the chromon derivatives (**1**-**6**) against HL-60 cells were also examined.

The roots and rhizomes of *S. divaricata* were subjected to extraction with MeOH. The concentrated MeOH extract was passed through a porous-polymer polystyrene resin (Diaion HP-20™), and eluted with 30% MeOH, 50% MeOH, MeOH, EtOH, and EtOAc. The MeOH eluted fraction showed cytotoxic activity against HL-60 cells with an IC_{50} value of 3.85 μg/mL, and was thus repeatedly subjected to column chromatography (CC) on silica gel and octadecylsilanized (ODS) silica gel, yielding compounds **1**-**11**. Compounds **1**-**11** were identified as 3'-*O*-angeloylhamaudol (**1**) [4], ledebouriellol (**2**) [3, 4], divaricatol (**3**) [4], *sec*-*O*-glucosylhamaudol (**4**) [3-6], cimifugin (**5**) [3, 4, 6], 5-*O*-methylvisammioside (**6**) [4, 6], (9*Z*)-heptadeca-1,9-diene-4,6-diyn-3-ol (**7**) [7-10], (9*Z*)-heptadeca-1,9-diene-4,6-diyne-3,8-diol (**8**) [8-11], (9*Z*)-1-methoxy-9-heptadecene-4,6-diyn-3-ol (**9**) [12], (8*E*)-heptadeca-1,8-diene-4,6-diyne-3,10-diol (**10**) [13, 14], and (8*E*)-10-hydroperoxy-1,8-heptadecadiene-4,6-diyn-3-ol (**11**) [13, 14], on the basis of their physical and spectroscopic data (Figure 1). Compounds **9** and **11** were isolated for the first time from the roots and rhizomes of *S. divaricata.*

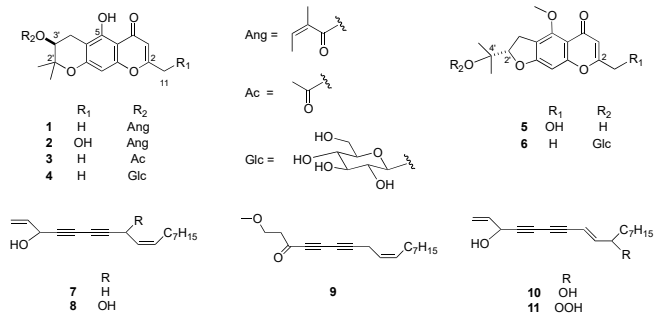

Figure 1: Structures of **1**–**10** isolated from the underground parts of *S. divaricata*.

The chromon derivatives (**1**-**6**) were evaluated for their cytotoxic activity against HL-60 cells by using a modified MTT assay method [15] (Table 1). Compounds **1** and **2** showed cytotoxic activity with IC_{50} values of 4.41 and 5.21 μM, respectively. Etoposide and cisplatin were used as the positive controls, and had IC_{50} values of 0.41 μM and 1.61 μM, respectively.

Table 1: Cytotoxic activity of **1**–**6** against HL-60 cells.

Compound	IC_{50} (μM)	Compound	IC_{50} (μM)
1	4.41±0.31	**5**	>100
2	5.21±0.62	**6**	>100
3	17.4±1.77	etoposide	0.41±0.15
4	>100	cisplatin	1.61±0.22

*Data are presented as the mean value ±S.E.M. of three experiments preformed in triplicate.

Compound **1** and the 11-hydroxy derivative (**2**) of **1** are chromon derivatives whose side-chain moiety forms a six-membered ring bearing an angeloyl group at the C-3' hydroxy group; they showed potent cytotoxic activity against HL-60 cells. Displacing the angeloyl group with an acetyl or glucosyl group diminished cytotoxic potency, suggesting that the presence of the angeloyl moiety enhances cytotoxic activity. The furanochromon derivatives (**5** and **6**) showed no cytotoxicity against HL-60 cells (> 100 μM).

Next, the apoptotic induction activity of **1** in HL-60 cells was evaluated. HL-60 cells were treated with 50 μM of **1** for 16 h and observed using fluorescence microscopy after staining with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI). The cells exposed to **1** and 15 μM of etoposide displayed apoptotic chromatin condensation and nuclear disassembly (Figure 2). In agarose gel electrophoresis of the DNA fraction of the **1**-treated HL-60 cells, an apoptotic DNA ladder pattern was observed (Figure 3). These results suggest that HL-60 cell death was mediated by **1** *via* the induction of apoptosis.

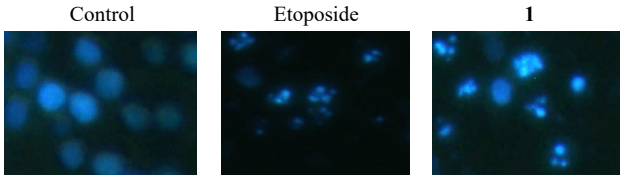

Figure 2: Morphological observations by fluorescence microscopy after DAPI staining. HL-60 cells were stained with DAPI after treatment with either 50 μM of **1** or 15 μM of etoposide for 16 h to evaluate fragmented and condensed nuclear chromatins.

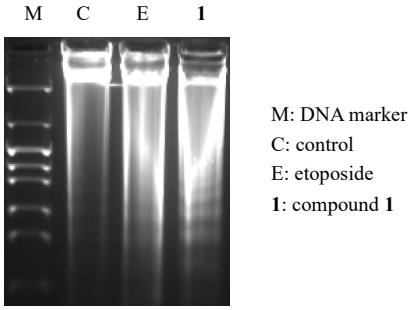

Figure 3: Induction of DNA fragmentation by either **1** or etoposide in HL-60 cells. HL-60 cells were incubated with either 50 μM of **1** or 15 μM of etoposide for 21 h. DNA was then extracted and applied to agarose gel electrophoresis.

Recently, mitochondrial dysfunction was found to play a key role during the early stage of apoptotic cell death [16]. Disruption of the mitochondrial membrane potential ($\Delta\Psi m$) is one of the intracellular events that occur during the induction of apoptosis. The $\Delta\Psi m$ was detected using the MitoCapture™ Apoptosis Detection Kit (BioVision, CA, USA). MitoCapture™ is a cationic dye that fluoresces differently in healthy and apoptotic cells. The apoptotic cells display diffused green fluorescence, whereas the normal control cell displays punctate red fluorescence. When HL-60 cells were treated with 50 μM of **1** for 6 h, a green fluorescence was observed (Figure 4).

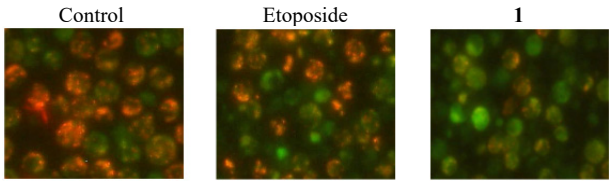

Figure 4: Morphological observations by fluorescence microscopy after MitoCapture™ reagent staining.
HL-60 cells were stained with MitoCapture™ reagent after treatment with either 50 μM of **1** or 15 μM of etoposide for 6 h.

Cytochrome *c* is located in the space between the inner and outer mitochondrial membranes. Apoptosis triggers the release of cytochrome *c* from the mitochondria into the cytosol [17]. In order to analyze the release of cytochrome *c* in the apoptotic HL-60 cells treated with **1**, the cytosolic and mitochondrial fractions were extracted, and cytochrome *c* was detected using Western blotting. As a result, cytochrome *c* was shown to be present in the cytosolic fraction of **1**-treated HL-60 cells (Figure 5).

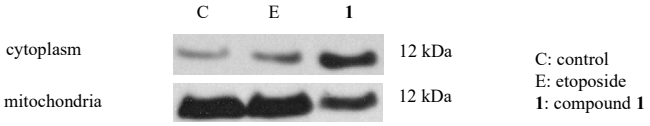

Figure 5: Release of cytochrome *c* from mitochondria in HL-60 cells treated with 50 μM of **1** or 15 μM of etoposide for 6 h by Western blot analysis.

Cytochrome *c* released into the cytosol interacts with Apaf-1, and the cytochrome c/Apaf-1 complex activates caspase-9, which then activates downstream caspase-3, a key protein in the execution of apoptosis [18]. When HL-60 cells were treated with 50 μM of **1** for 6 h, caspase-9 and caspase-3 were induced, as shown by the intermediate cleavage products (35 and 37 kDa) of caspase-9 (Figure 6) and that of caspase-3 (17 kDa) (Figures 7 and 8).

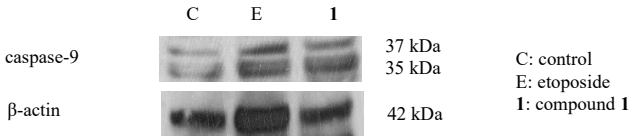

Figure 6: Effect on caspase-9 activation after treatment with 50 μM of **1** or 15 μM of etoposide for 6 h by Western blot analysis.

Figure 7: Effect on caspase-3 after treatment with 50 μM of **1** or 15 μM of etoposide for 17 h by Western blot analysis.

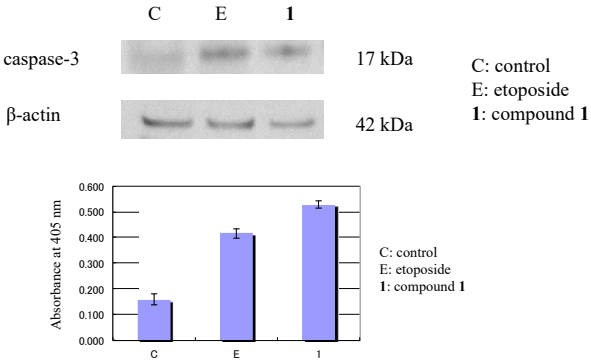

Figure 8: Caspase-3 activity in the lysates of cells treated with either **1** or etoposide. HL-60 cells were incubated with either 50 μM of **1** or 15 μM of etoposide for 17 h. Data represent the mean ± S.E.M. of three experiments.

The above-mentioned results implied that firstly **1** induced the release of cytochrome *c* into cytosol by loss of mitochondrial membrane potential, and then activated caspase-9 and caspase-3 (Figure 9). This is the first report on the cytotoxic and apoptotic induction activity of 3'-*O*-angeloylhamaudol (**1**).

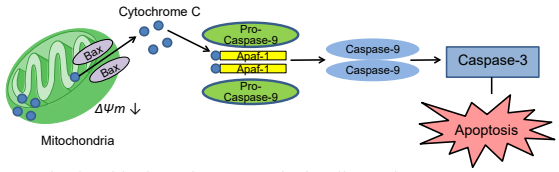

Figure 9: Mitochondria-dependent apoptosis signaling pathway.

Experimental

General experimental procedures: Optical rotations were measured using a JASCO P-1030 (Tokyo, Japan) automatic digital polarimeter. NMR spectra were recorded on a Bruker DRX-500 (500 MHz for ^{1}H NMR, Karlsruhe, Germany) spectrometer by

using standard Bruker pulse programs. MS-ESITOF data were recorded on a Waters-Micromass LCT mass spectrometer (Manchester, U.K.). Diaion HP-20 (Mitsubishi Chemical, Tokyo, Japan), silica gel (Fuji Silysia Chemical, Aichi, Japan), and ODS silica gel (Nacalai Tesque, Kyoto, Japan) were used for CC. TLC was carried out on silica gel 60 F254 (thickness: 0.25 mm; Merck, Darmstadt, Germany) and RP18 F254S plates (thickness: 0.25 mm; Merck), and compounds were visualized by spraying the plates with a 10% H_2SO_4 aqueous solution, followed by heating. HL-60 cells were obtained from the Human Science Research Resources Bank (JCRB 0085, Osaka, Japan). The following materials and reagents were used for cell culture assay: 96-well flat-bottom plate (Iwaki Glass, Chiba, Japan); RPMI 1640 medium, etoposide, DAPI, and MTT (Sigma-Aldrich, St. Louis, MO, U.S.A.); fetal bovine serum (Nichirei Biosciences, Tokyo, Japan); and penicillin G sodium salt and streptomycin sulfate (Gibco, Grand Island, NY, U.S.A.). All other chemicals used were of biochemical reagent grade.

Plant material: The roots and rhizomes of *Saposhnikovia divaricata* (Turcz.) Schischk. were purchased from UCHIDA WAKANYAKU Ltd. (Tokyo, Japan) in December 2011. A voucher specimen has been deposited in our laboratory (voucher no. KS-2011-001, Department of Medicinal Pharmacognosy).

Extraction and isolation: The roots and rhizomes of *Saposhnikovia divaricata* (dry weight: 5.0 kg) were extracted with MeOH (18 L). The MeOH extract was concentrated under reduced pressure, and the viscous concentrate (595 g) was passed through a Diaion HP-20 column and successively eluted with 30% MeOH, 50% MeOH, MeOH, EtOH, and EtOAc. The MeOH fraction exhibited cytotoxic activity against HL-60 cells (IC_{50} 3.85 μg/mL), while the 30% MeOH, 50% MeOH, EtOH, and EtOAc fractions did not show apparent cytotoxic activity (IC_{50} > 20 μg/mL). CC of the MeOH fraction (50 g) on silica gel and elution with a stepwise gradient mixture of $CHCl_3$/MeOH (19:1; 9:1; 4:1; 2:1), and finally with MeOH alone, gave 11 fractions (A-K). Fraction A was separated by silica gel CC eluted with *n*-hexane/acetone (9:1, 4:1, 2:1) and *n*-hexane-EtOAc (6:1, 3:1, 1:1), and by ODS silica gel CC eluted with MeOH-H_2O (7:3, 8:2) to give **1** (96.8 mg), **2** (2.8 mg), **3** (12.9 mg), **7** (6.2 mg), **8** (10.8 mg), **9** (12.2 mg), **10** (2.7 mg), and **11** (3.1 mg). Fraction B was separated using silica gel CC eluted with EtOAc/MeOH/H_2O (190:10:1, 40:10:1) and using ODS silica gel CC eluted with MeCN/H_2O (1:3) to give **4** (145 mg) and **5** (50.0 mg). Fraction E was separated using ODS silica gel CC eluted with MeCN/H_2O (1:3, 1:2) and using silica gel CC eluted with EtOAc/MeOH/H_2O (40:10:1, 20:10:1) to give **6** (52.0 mg).

Cell culture and assay for cytotoxic activity against HL-60 cells: HL-60 cells were maintained in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum and antibiotics (100 units/mL penicillin G sodium salt and 100 μg/mL streptomycin sulfate) in a 5% CO_2 humidified incubator at 37°C. The cells were washed and suspended in the medium (4×10^4 cells/mL), and 196 μL of this cell suspension was divided into 96-well flat-bottom plates. The cells were incubated in 5% CO_2/air for 24 h at 37°C. After incubation, 4 μL of EtOH/H_2O (1:1) solution containing the sample was added to give the final concentrations of 0.1-100 μM, and 4 μL of EtOH/H_2O (1:1) was added into control wells. The cells were further incubated for 72 h in the presence of each agent, and then cell growth was evaluated using a modified MTT reduction assay [17]. At the end of the incubation period, 10 μL of 5 mg/mL MTT in phosphate-buffered saline (PBS) was added to each well and the plate was further incubated in 5% CO_2/air for 4 h at 37°C. The plate was then centrifuged at 1,500 *g* for 5 min to precipitate the MTT formazan. An aliquot of supernatant (150 μL) was removed from each well, and 175 μL of DMSO was added to each aliquot to dissolve the MTT formazan crystals. The plate was mixed on a microplate mixer for 10 min, and then read on a microplate reader (Sunrise Rainbow RC-R, Tecan, Salzburg, Austria) at 550 nm. Each assay was carried out in triplicate and cytotoxicity was expressed as IC_{50}, which is the concentration that reduces the viable cell number by 50%.

DAPI staining: The cells (5×10^5 cells/well) were plated on coverslips in 96-well plates. After 24 h, HL-60 cells were treated with either 50 μM of **1** or 15 μM of etoposide for 16 h. The cells were fixed with 1% glutaraldehyde for 30 min at room temperature before staining with DAPI (0.5 μg/mL in PBS) at room temperature. They were then observed immediately through a CKX41 fluorescence microscope (Olympus, Tokyo, Japan).

Assay for DNA fragmentation: The cells were incubated with either 50 μM of **1** or 15 μM of etoposide for 21 h at 37°C. DNA was extracted with a commercially available kit (Wizard Genomic DNA Purification Kit, Promega, WI, U.S.A.). In brief, cells (2×10^6) were centrifuged for 5 min at 10,000 *g*. The cell pellet was suspended in 600 μL of nuclei lysis solution. Then, 3 μL of RNaseA solution was added to the cell lysate, and the solution was incubated at 37°C for 15 min. Protein precipitation solution (200 μL) was added to the RNaseA-treated cell lysate, and the mixture was incubated for 5 min on ice and centrifuged at 10,000 *g* for 5 min. The supernatant was transferred to a clean 1.5 mL microcentrifuge tube containing 600 μL of *iso*-PrOH and was mixed by inversion. After centrifugation at 10,000 *g* for 5 min, DNA was visible as a small white pellet, and was washed with EtOH/H_2O (7:3) solution. Finally, the pellet was suspended in 25 μL of DNA rehydration solution, incubated at 65°C for 1 h, and was stored at -20°C until use. The sample (15 μL) was used for 2% agarose gel electrophoresis in 40 mM Tris-acetate-EDTA buffer (pH 7.4) at 50 V for 1 h. A DNA molecular weight marker (pHY marker, Takara, Shiga, Japan) and DNA from apoptotic HL-60 cells induced by 15 μM of etoposide were used for calibration. The DNA fragmentation pattern was examined using the photographs taken under UV illumination.

Assay for mitochondrial membrane potential (ΔΨm): The mitochondrial membrane potential (*ΔΨm*) was investigated using the MitoCapture™ Apoptosis Detection Kit (BioVision, CA, U.S.A.), according to the manufacturer's procedure. HL-60 cells (5×10^5) were treated with either 50 μM of **1** or 15 μM of etoposide for 6 h, and the cells were centrifuged and collected. Cells were centrifuged at 500 *g* for 5 min. Cell pellets were resuspended in 250 μL of MitoCapture™ solution, incubated for 15 min at 37°C, and then centrifuged again for 5 min. Pellets were resuspended in 200 μL of incubation buffer and observed by fluorescence microscopy.

Release of cytochrome c to the cytosol: The release of cytochrome *c* into the cytosol was examined using the Cytochrome c Apoptosis Detection Kit (PromoKine, Heidelberg, Germany), according to the manufacturer's procedure. HL-60 cells (2.7×10^7) were treated with either 50 μM of **1** or 15 μM of etoposide for 6 h, and the cells were centrifuged and collected. Cells were homogenized and isolated as cytosolic and mitochondrial extractions by employing the appropriate reagents. The cytosolic and mitochondrial fractions (10 μg) were then loaded onto sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). A standard Western blot procedure was performed and probed with monoclonal mouse anti-cytochrome *c* antibody.

Detection of caspase-3 and caspase-9: HL-60 cells (2 x 10^6) were treated with either 50 μM of **1** or 15 μM of etoposide for either 6 h or 17 h, and the cells were centrifuged and collected. Cells were washed with PBS and lysed with RIPA buffer (0.05 M Tris-HCl at pH 8.0, 0.15 M NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS). Cell lysates were centrifuged at 20,000 *g* for 10 min at 4°C. The equalized amounts of proteins from each sample were mixed with NuPAGE™ LDS sample buffer (Thermo Fisher Scientific, MA, USA) and NuPAGE™ sample reducing agent (Thermo Fisher Scientific), boiled at 70°C for 10 min, and subjected to SDS-PAGE. The proteins were transferred from the gel to polyvinylidene difluoride (PVDF) membranes using an electroblotting apparatus (Power Station 1000XP and HorizeBLOT 2M, ATTO, Tokyo, Japan). Membranes were incubated in TBS with 0.1% Tween-20 (TBST) containing 5% skim milk for 30 min to inhibit nonspecific binding. The membranes were then incubated with the following primary antibodies: anti-β-actin, anti-caspase-3, and anti-caspase-9 (MBL, Aichi, Japan). After washing in TBST for 30 min, membranes were incubated for another 60 min with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (1:1,000). The membranes were then washed and visualized with ECL Western blotting detection reagents (GE Healthcare Biosciences, Buckinghamshire, U.K.). β-Actin expression was used as the internal control.

Assay for caspase-3 activation: The activity of caspase-3 was measured using the Appocyto Caspase-3 Colorimetric Assay Kit (MBL). HL-60 cells (2 x 10^6) were treated with either 50 μM of **1** or 15 μM of etoposide for 17 h, and the cells were centrifuged and collected. Cell pellets were suspended in 60 μL of ice-cold cell lysis buffer, and incubated on ice for 10 min. This cell pellet suspension was centrifuged at 10,000 *g* for 5 min and the supernatant was collected. The cell lysate (50 μL, equivalent to 200 μg protein) was mixed with reaction buffer (2 x 50 μL) containing the substrates for caspase-3 [DEVD-*p*NA (*p*-nitroanilide)]. After incubation for 2 h at 37°C, the absorbance of the liberated chromophore *p*NA was measured using a microplate reader at 405 nm. The activity of caspase-3 was evaluated in triplicate.

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Cytotoxic Activity of Compounds from *Styrax obassia*

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Cancer is a major public health burden in both developed and developing countries. Plant-derived compounds have played an important role in the development of useful anti-cancer agents. The current study was designed to evaluate the cytotoxic activity of chemical compounds from the stem bark of *Styrax obassia*. Seven known compounds (**1**−**7**) were isolated and identified. Compound **2** exhibited cytotoxic activity against the breast cancer cell line MCF-7 with an IC_{50} of 27.9 μM, followed by the human cervical cancer cell line Hela with an IC_{50} of 23.3 μM, and the human promyelocytic leukemia cell line HL-60 with an IC_{50} of 47.8 μM. Compound **7** exhibited cytotoxicity against Hela cells with an IC_{50} of 16.8 μM, followed by MCF-7 cells with an IC_{50} of 53.5 μM. This is the first study to investigate the significant anti-tumor properties of isolated compounds from the stem bark of *S. obassia*.

Keywords: *Styrax obassia*, Styracaceae, Benzofuran glucoside, Anti-cancer, Hela, MCF-7, HL-60.

Styrax is by far the largest genus in Styracaceae, which is a family of small trees and shrubs indigenous to tropical and subtropical regions. Chemical investigations into several *Styrax* species have revealed them to be rich sources of egonol, benzofurans, benzofuran glycosides, saponins, and triterpenoids [1a-1e]. *Styrax* species have been shown to have a variety of biological activities including insecticidal, fungicidal, antimicrobial, antidiabetic, antiproliferative, cytotoxic, and antioxidant [1f,1g]. Phytochemical studies have reported the presence of egonol derivatives in *S. obassia* extracts [1a, 1h, 1i]. Furthermore, *S. obassia* isolates have been found to exhibit inhibitory effects on nitric oxide (NO) production [1j].

Chemical investigation of the bark of *S. obassia* using efficient separation techniques led to the isolation of seven compounds (**1**−**7**), which were identified as 1"-hydroxylegonol gentiobioside (**1**), egonol glucoside (**2**), syringin (**3**), (+)-1-hydroxylpinoresinol-4'-β-D-glucoside (**4**), pinoresinol-4'-*O*-β-D-glucoside (**5**), (−)-lariciresinol 4'-(6"-*O*-feruloyl-β-D-glucopyranoside) (**6**), and castanoside B (**7**) by comparing their physicochemical and spectroscopic data with those reported in the literature [2a-2g]. Compound **7** was isolated from a *Styrax* species for the first time (Figure 1).

The seven isolated compounds (**1**−**7**) were tested for cytotoxic activity against MCF-7, Hela, and HL-60 cell lines. In the cytotoxic assay using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to Mosmann [3a] with adriamycin as the positive control, compound **2** revealed cytotoxic activities against MCF-7, Hela, and HL-60 cell lines with IC_{50} values of 27.9 ± 1.6, 23.3 ± 2.8, and 47.8 ± 2.5 μM, respectively. Compound **7** was inactive against HL-60, but displayed weak cytotoxic activity against MCF-7 and Hela cell lines with IC_{50} values of 53.5 ± 2.6 and 16.8 ± 1.0 μM, respectively, while the other compounds exhibited no cytotoxic activity. In comparison, adriamycin gave IC_{50} values of 7.7 ± 1.9, 0.61 ± 0.01 and 0.10 ± 0.01 μM, respectively. From the data, it seems that the benzofuran lignan and the flavonoid skeletons are essential for cytotoxic activity [3b,3c]. In addition, compound **1** is a benzofuran derivative, but showed no cytotoxic activity against MCF-7, Hela, and HL-60 cells (IC_{50} > 100 μM). The structure of compound **1** differs from that of **2** by more than one glycosyl group. The results suggest that more than one glycosyl group caused a decrease in cytotoxic activity against all cancer cell lines. Compound **7** has a free hydroxyl group at position 3 and a *para*-hydroxyl group in ring B, which probably increases the activity of this compound [3d]. Thus, this compound could be a useful anticancer agent. Unfortunately, the cytotoxic activity of all isolated compounds was less than that of the positive control.

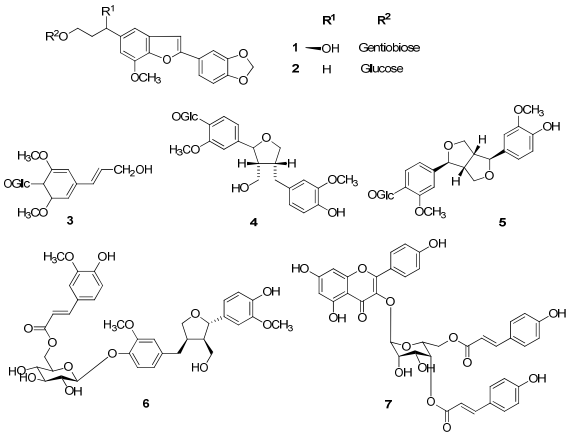

Figure 1: Chemical structures of isolated compounds (**1**−**7**) from *S. obassia*.

Experimental

Plant material: The aerial parts of *Styrax obassia* Sieb. *et* Zucc. were collected at Yosung, Daejeon, Korea, in May 2012, and identified by Prof. Ki Hwan Bae, Chungnam National University, Daejeon, Korea. A voucher specimen (CUD-2451-1) was deposited at the College of Pharmacy, Catholic University of Daegu, Keongbuk, Korea.

Extraction and isolation: The aerial parts (2.5 kg) were extracted with MeOH by reflux (3 times). After evaporation of the solvent

under reduced pressure, the crude MeOH extract (130 g) was obtained and suspended in hot water and partitioned with *n*-hexane, $CHCl_3$, EtOAc, and H_2O, successively, to afford *n*-hexane (53 g), $CHCl_3$ (35 g), EtOAc (6 g), and H_2O (35 g) soluble fractions, respectively. The EtOAc-soluble fraction (6.0 g) was subjected to a silica gel column with $CHCl_3$-MeOH (30:1→8:2) as eluents to acquire 12 fractions (E1 ~ E12). Fraction E7 (270.3 mg) was applied to a RP-C_{18} silica gel column and eluted with MeOH-H_2O (1:2) to obtain compound **2** (15.6 mg). Fraction E10 (521.7 mg) was applied to an RP-C_{18} silica gel column and eluted with MeOH-H_2O (1:3 → 2:5) to afford 19 sub-fractions (E10.1 ~ E10.19). Compound **5** (80.0 mg) was obtained from sub-fraction E10.2 (100.0 mg) by RP-C_{18} silica gel CC with MeOH-H_2O (1:3). Sub-fraction E10.9 (100.0 mg) was purified by high-performance liquid chromatography (HPLC) [eluted with a gradient solvent system of MeOH-H_2O (50:50 → 65:35) over 60 min; flow rate: 3 mL/min; UV detection at 210 nm; an YMC-Pack ODS-A, 250 × 20 mm column] to obtain compound **6** (4.8 mg, t_R = 36 min). Compound **7** (13.5 mg, t_R = 30 min) was obtained by HPLC [eluted with MeOH-H_2O (55:45 → 65:35) over 60 min; flow rate: 3 mL/min; UV detection at 210 nm] from sub-fraction E10.18 (97.3 mg). Fraction E12 (500.0 mg) was applied to an RP-C_{18} silica gel column and eluted with MeOH-H_2O (1:3 → 1:1) to afford compound **1** (200.0 mg) and 8 sub-fractions (E12.1 ~ E12.8). Sub-fraction E12.1 (50.0 mg) was chromatographed over a silica gel column and eluted with $CHCl_3$-MeOH (30:1) to obtain compound **3** (40.0 mg). Sub-fraction E12.3 (25.0 mg) was chromatographed over a silica gel column and eluted with $CHCl_3$ 100% to obtain compound **4** (6.2 mg).

Cytotoxic activity: The cytotoxic activity assay was carried out using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to Mosmann. MCF-7, Hela, and HL-60 cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F-12 with 15 mM HEPES buffer, L-glutamine, and pyridoxine hydrochloride supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin in a 96-well plate at a density of 6×10^4 cells/mL. After reaching confluence (2×10^5 cells/mL), the cells were treated with the compounds. The compounds were dissolved in dimethylsulfoxide (DMSO) and the final concentration of DMSO was 0.1%, v/v. Different concentrations of the compounds were prepared with serial dilutions. DMSO (0.1%) was used a control. The experiment was allowed to proceed for 48 h at 37°C in a humidified 5% CO_2 atmosphere. At the end of this period, supernatants were discarded. To minimize the interference of supernatant residue, the adherent cells were washed twice with Dulbecco's phosphate buffered saline (DPBS), and then 20 µL of MTT stock solution (5 mg/mL) was added to each well and the plates were further incubated for 3 h at 37°C. DMSO (100 µL) was added to each well to solubilize the water-insoluble purple formazan crystals. After 1 h, the absorbance was measured at 570 nm with a microplate reader. Adriamycin was used as a commercial standard anticancer agent. The 50% reduction in cell number relative to the control or IC_{50} was estimated visually. Values are expressed as mean ± SD.

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Isolation and Characterization of Antiangiogenesis Compounds from the Fungus *Aspergillus terreus* Associated with *Apostichopus japonicus* Using Zebrafish Assay

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Three compouds, (+)-butyrolactone IV (**1**), butyrolactone I (**2**) and terrelactone A (**3**) were isolated from the fungus *Aspergillus terreus* associated with *Apostichopus japonicus* from the Yellow Sea in China; their structures were elucidated by spectral methods. Compounds **1** and **2** were shown to have moderate antiangiogenesis activity when tested using the zebrafish assay. This is the first report of butyrolactones with antiangiogenesis activity.

Keywords: *Apostichopus japonicus*, *Aspergillus terreus*, Secondary metabolites, Antiangiogenesis activity.

As a rich source of novel and bioactive compounds, marine fungi continue to be of major research interest [1]. Sea cucumbers host a rich diversity and abundance of fungi [2a], which have produced many novel and bioactive secondary metabolites, such as diterpene glycosides from *Acremonium* sp. [2b], a benzofuran from *Alternaria* sp., an isopimarane diterpene from *Epicoccum* sp. [3], an acid anhydride from *Fusarium* sp., and polyhydroxy cyclohexanols and 12-membered macrolides from *Dendrodochium* sp. [4, 5]. During a study of the bioactive metabolites from fungi associated with sea cucumbers collected around Zhifu Island, Yantai City, China, the ethyl acetate extract of *Aspergillus terreus* (HS-Y-1) showed antiangiogenesis activity, producing 40% inhibition at 100 μg/mL. From this fungus, three compounds (Figure 1), (+)-butyrolactone IV (**1**), butyrolactone I (**2**) and terrelactone A (**3**) were obtained; both **1** and **2** showed strong antiangiogenesis activity.

Compound **1** was isolated as pale yellow glue producing an $[M+H]^+$ ion in its HRTOFMS at *m/z* 441.4290. Its UV spectrum showed absorption at 223 and 310 nm. Its NMR spectral data were consistent with (+)-butyrolactone IV [6]. Compound **2**, a pale yellow solid, displayed an $[M+H]^+$ ion at *m/z* 425.1570 in its HRTOFMS, consistent with a molecular formula of $C_{24}H_{24}O_7$. Its UV spectrum showed absorption at 223 and 309 nm. These, along with ^1H and ^{13}C NMR spectroscopic data, identified compound **2** as butyrolactone I [7]. The HRTOFMS of compound **3**, obtained as pale oil, exhibited an $[M+Na]^+$ ion at 465.1519, which was consistent with $C_{24}H_{26}O_8$. Its UV spectrum showed characteristic absorption of butyrolactones at 230 and 310 nm. Combined with ^1H and ^{13}C NMR spectral data, compound **3** was identified as terrelactone A [8a].

The positive optical rotations of **1** ($[\alpha]^{25}_D$ + 60, *c* 0.10, MeOH), **2** ($[\alpha]^{25}_D$ + 100, *c* 0.10, MeOH) and **3** ($[\alpha]^{25}_D$ + 60, *c* 0.10, MeOH) were consistent with the literature data, which confirmed the 4*R*-configuration [6,7,8a].

The antiangiogenic activities of compounds **1** and **2** were evaluated using zebrafish assay, in terms of the inhibition on the growth of intersegmental vessels, with PTK787 as positive control (IC_{50} 0.15 μg/mL). The results showed that both **1** and **2** could significantly inhibit the growth of intersegmental vessels of embryos compared to the control (0.1% DMSO in sterile salt water). The inhibition ratio of compound **1** was 43.4% at a concentration of 100 μg/mL and **2** was 28.7% at a concentration of 10 μg/mL (Table 1). Compound **3** has not been tested its antiangiogenesis activity for little amount.

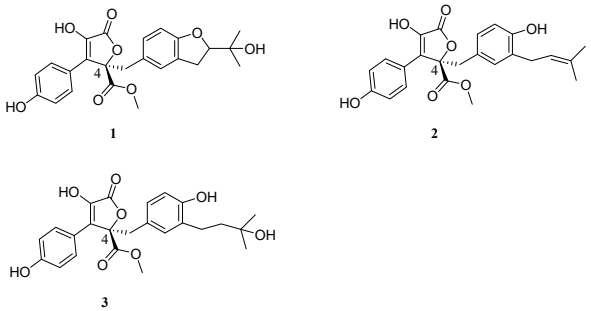

Figure 1: Structures of compounds **1, 2** and **3**.

Table 1: Antiangiogenesis activity of compounds **1** and **2** using zebra fish assay.

Compounds	Conc (μg/mL)	Number of vessels in zebrafish ($\bar{x}$ ± SD)	*P*
Control		25.8 ± 1.3	-
PTK 787	0.5	0.20 ± 0.45**	1.24508E-10
1	100	14.6 ± 3.7**	0.002
	10	19.2 ± 2.6**	0.001
	1	22.8 ± 1.8*	0.02
2	100	-	-
	10	18.4 ± 2.5**	0.001
	1	21.6 ± 3.4*	0.03

** $P \leq 0.01$ significantly different from the control. * $P \leq 0.05$ significantly different from the control. ¯, all of the zebra fish died, and the vessels were not calculated.

Compounds **1** and **2** exhibited strong antibacterial effects on *Staphylococcus aureus*, and moderate activities against *Enterobacter aerogenes* and *Bacillus subtilis* [8b]. Compound **2**

also showed mixed-type inhibitory activity against yeast α-glucosidase, antioxidant activity [8c], anti-H1N1 activity [8a], as well as inhibitory activities against eukaryotic cyclin-dependent kinase (CDK), preventing apoptosis [6]. There is no previous report available on the antiangiogenesis activity of butyrolactones. Angiogenesis inhibitors have been successfully used for cancer therapy in the clinic, and increasing attention has been paid to the development of marine-derived angiogenesis inhibitors [9].

Experimental

General: Optical rotations, Anton Paar MCP 300 (Anton Paar) polarimeter; NMR, Varian INOVA-600 MHz spectrometer; HRESIMS, Agilent QTOF-6530; CC, silica gel (200-300 mesh, Qingdao Haiyang Chemicals), octadecylsilyl silica gel (Unicorn, 45-60 um) and Sephadex LH-20 (Amersham Biosciences). Precoated silica gel plates (Yan Tai Zi Fu Chemical Group Co.; G60, F-254) were used for TLC. HPLC, Waters 2695 and DAD. The vessels of zebra fish were captured using an Olympus DP72.

Fungal material: A strain of *Aspergillus terreus* (HS-Y-1) was isolated from the surface muscle of *Apostichopus japonicus* from Zhifu Island, Yantai City, Yellow Sea, China. Voucher specimens are stored in the Biology Institute, Shandong Academy of Sciences, Jinan, PR China, and in China General Microbiological Culture Collection Center (CGMCC) with No. 11545.

Identification of the endophytic isolate: The fungus was grown on PDA for 5 days at 28°C. Genomic DNA was extracted and purified using the Fungal DNA Kit 50 (OMEGA, USA), according to the manufacturer's instructions, suitably modified. For identification and differentiation, the Internal Transcript Spacer regions (ITS4 and ITS5) and the intervening 5.8S rRNA region were amplified and sequenced. The ITS regions of the fungus were amplified by PCR with the universal ITS primers, ITS5 (5'- GGA AGT AAA AGT CGT AAC AAG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3'). The PCR products were then purified and desalted using the EZ Spin column PCR product purification kit (BBI) and sequenced. The sequencing results were aligned with the nucleotide-nucleotide database (BLASTn) of the U.S. National Center for Biotechnology Information (NCBI) for final identification of fungi.

Fermentation, extraction, and isolation: The fungal strain was cultivated in solid medium at 28°C for 28 days in 40 x 500 mL Erlenmeyer flasks, each containing 80 g rice in 120 mL of seawater. The combined cultures were extracted 3 times by shaking with an equal volume of ethyl acetate; the combined extracts were then concentrated below 50 °C to obtain the crude dry extract. This was subjected to silica gel CC, eluting with a gradient of light petroleum to ethyl acetate. Fractions (Fr. 5–8) of 30 mL were collected and combined by TLC examination. Fractions containing the desired compounds were further purified by Sephadex LH-20 chromatography, eluting with mixtures of light petroleum-$CHCl_3$-MeOH (2:1:1), which yielded **3** (1.0 mg). SFr. (5-8)-4 was further subjected to semi-preparative reverse-phase HPLC (octadecylsilane (ODS) (YMC, 250 × 10 mm) (30% MeOH-H_2O, v/v, 5 min; 30%–100% MeOH-H_2O, v/v, 45 min; 100% MeOH, 55 min) to collect compounds **1** (4.0 mg, t_R = 21.27 min) and **2** (10.0 mg, t_R = 21.82 min).

Antiangiogenesis assay: Fertilized eggs were treated with pronase to remove chorions, and raised in embryo medium (60 mmol/L NaCl, 2.4 mmol/L sodium bicarbonate, 0.8 mmol/L $CaCl_2$, 0.67 mmol/L KCl, and 10 mmol/L HEPES). Embryos were arrayed into 24-well plates (8 embryos per well) in 2 mL of embryo medium for 1 day postfertilization. Stock solutions (10 mg/mL) of all samples were prepared by dissolving the test compounds in 100% DMSO. These stock solutions were diluted in embryo medium to obtain working solutions with the test compounds dissolved in 0.1% DMSO. These working solutions were aliquoted into 24-well plates. After 24 h of treatment, the intersegmental vessels (ISV) of embryos were visualized by green fluorescent protein labeling under a fluorescent microscope. A fluorescent image of each embryo was captured using the DP72 imaging system. The length of the ISV on the captured image was measured by Image Pro Plus software. The antiangiogenesis activities of compounds were calculated from the inhibition ratio of angiogenesis. The positive control for this assay was 0.2 μg/mL PTK787, a VEGFR antagonist, and the negative control was 0.1% DMSO.

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Adsorptive Property of Food Materials and Chemicals to Cesium and Strontium

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In March, 2011, large amounts of radioactive materials were released from the Fukushima Daiichi nuclear power plant after the nuclear accident. Especially, for humans, internal exposure to ^{137}Cs and ^{90}Sr radionuclides presents very high risks because of their very long physical half-lives (^{137}Cs: 30.2 years, ^{90}Sr: 28.9 years). Therefore, it is important to inhibit the absorption of radioactive materials and to promote the excretion of them from the body through feces. The aim of this study was to explore foods, their components and various chemicals showing adsorption properties to Cs and Sr. Sodium alginate (ALA-Na) strongly adsorbed Cs and Sr compared with other samples. Chondroitin sulfate, carboxymethyl cellulose sodium (CMC-Na), methyl cellulose (MC) and apple polyphenols (AP; high molecule weight) also showed adsorption potency to Cs in that order. For Sr adsorption, kelp, CMC-Na, MC, AP (high molecule weight), laminaran and Jew's mallow exhibited adsorbing effects in that order. These samples might be useful and safe tools to protect from the adverse effects induced by internal exposure to these radioactive materials.

Keywords: Cesium, Strontium, Adsorption, Fiber.

Enormous amounts of radioactive materials (mainly ^{137}Cs, ^{129}I, ^{90}Sr) were released from the Fukushima Daiichi nuclear power plant after the Great East Japan Earthquake and tsunami on March 11th, 2011. Various foods and drink, such as vegetables, milk, meat, eggs, fish and drinking water, were contaminated by a large amount of radionuclides released from the power plant [1]. If these contaminated foods were to be consumed, internal radioactive contamination may be induced by radionuclides. Especially, ^{137}Cs and ^{90}Sr present high risks because of their long physical half-lives (^{137}Cs: 30.2 years, ^{90}Sr: 28.9 years) [2a,b]. At present, ferric ferrocyanide ($Fe_4[Fe(CN)_6]_3$), which is generally called Prussian blue, is recommended for protecting against internal exposure to radionuclides. Prussian blue binds Cs in the gut lumen by an ionic exchange reaction between iron and Cs [3]. Therefore, treatment with Prussian blue increases Cs excretion in the feces. However, Prussian blue tends to cause constipation as a side effect [4], so the intestinal tract might be locally exposed to radioactive materials, which increase the health risk. It has been reported that alginate (ALA) adsorbs Cs and Sr, inhibits absorption, and enhances excretion of them [5]. Therefore, the International Atomic Energy Agency (IAEA) has recommended ALA (dietary intake as sodium alginate (ALA-Na): 4 g/body/day) for treatment of patients exposed to radio strontium. However, when living environments and foods are suddenly polluted with radioactive materials, it is unknown whether it is possible to have ALA. Therefore, it is important to explore if materials other than ALA have the ability to capture radionuclide materials. In this study, we examined various food materials and chemicals showing adsorbing effects with Cs and Sr.

The adsorptive potency of samples to Cs and Sr were examined using *in vitro* systems. Figures 1 and 2 show the Freundlich adsorption isotherm of each sample for adsorption of Cs and Sr at 20°C. Tables 1 and 2 show some parameters (K, n and correlation coefficient value) from the Freundlich adsorption isotherm of each sample for absorption of Cs and Sr. From these results, micin, freeze-dried nameko, freeze-dried shiitake, chitosan, and chitin AP (low MW) SUN FIBER could not absorb Cs and Sr. However,

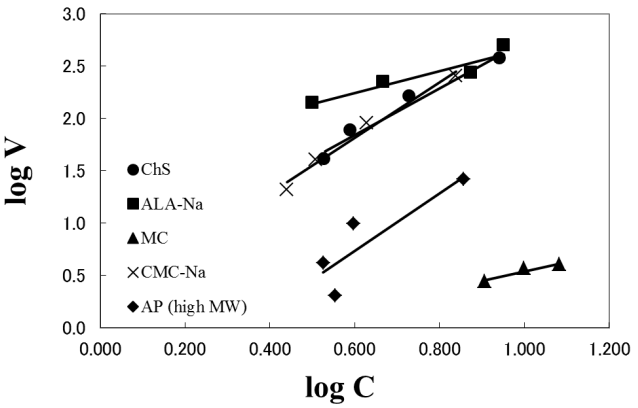

Figure 1: Freundlich adsorption isotherms. The X axis (log C) represents the logarithm of the residual amount of Cs in solution (mmol/L). The Y axis (log V) represents the logarithm of the amount of adsorbed Cs (μmol/g).

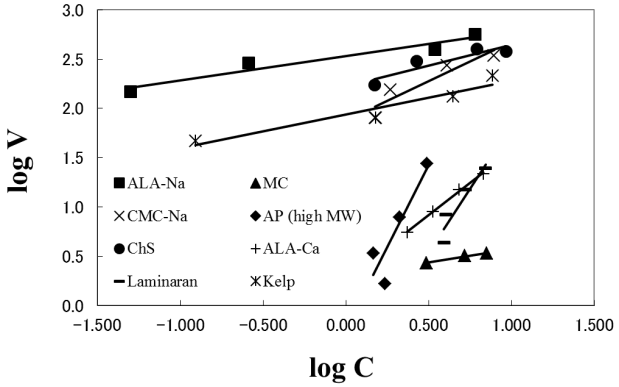

Figure 2: Freundlich adsorption isotherms. The X axis (log C) represents the logarithm of the residual amount of Sr in solution (mmol/L). The Y axis (log V) represents the logarithm of the amount of adsorbed Sr (μmol/g).

Table 1: Freundlich parameters of samples with Cs.

Sample	k	n	Correlation coefficient (R^2)
ALA-Na	41.6	0.96	0.87
ChS	3.20	0.45	0.97
CMC-Na	1.68	0.38	0.98
MC	4.47×10^{-1}	1.12	0.95
AP (high MW)	1.20×10^{-1}	0.36	0.76

Table 2: Freundlich parameters of samples with Sr.

Sample	k	n	Correlation coefficient (R^2)
ALA-Na	339	4.07	0.94
ChS	168	2.37	0.84
Kelp	86.6	2.92	0.91
CMC-Na	75.6	1.26	0.87
MC	1.99	3.61	0.98
ALA-Ca	1.81	0.76	0.99
AP (high MW)	0.56	0.30	0.81
Laminaran	1.72×10^{-1}	0.38	0.88

ALA-Na, chondroitin sulfate (ChS), carboxymethyl cellulose sodium (CMC-Na), methyl cellulose (MC) and apple polyphenols (AP: high MW) showed adsorption effects with Cs. Especially, ALA-Na exhibited a stronger effect to Cs than the other samples, and the k value of ALA-Na was the highest (41.6). The k values of each sample from the Freundlich adsorption isotherm for Cs were 3.2 (Chs), 1.68 (CMC-Na), 4.47×10^{-1} (MC) and 1.20×10^{-1} (AP) (Table 1). In Figure 2, ALA-Na, ChS, kelp, CMC-Na, MC, calcium alginate (ALA-Ca), AP (high MW) and laminaran showed adsorbing effects with Sr. The adsorptive activity of ALA-Na to Sr was the strongest of all the samples, as with Cs. The k values of each sample from the Freundlich adsorption isotherm for Sr were 339 (ALA-Na), 168 (ChsCMC-Na), 4.47×10^{-1} (MC) and 1.20×10^{-1} (AP) (Table 2).

Kelp showed a strong adsorbing effect for Sr, because kelp has ALA-Na in high concentration. Kelp is a common food in Japan, China and Korea, and is usually consumed as a flavored broth or a savory garnish for meals. Additionally, kelp is rich in iodine [6]. Iodine tablets are used to protect accumulation of radioactive iodine-131 in the thyroid gland and should be stocked by local governments in a 30 km zone around the nuclear power plants in Japan. Laminaran, which inhibits absorption of Sr, is also contained in kelp. It has been reported that beta-glucan including laminaran has immunostimulation activity and protects the body from radiation [7]. Therefore, kelp is a very useful food for preventing internal exposure because of the adsorbing effect on radioactive materials and because it contains iodine in high concentration. MC showed adsorptive properties for both Cs and Sr, and strongly adsorbed Cs in particular. MC has gel formation ability, induced by hydrophobic interaction, and can adsorb alkali and alkaline earth metals [8]. It is thought that MC holds a gel formation with Cs stronger than with K because the hydrated ionic radius of Cs is smaller than that of K [9]. Therefore, it may be said that MC has the potential ability to adsorb selectively to Cs. CMC-Na adsorbed Cs and Sr stronger than MC. CMC-Na has the same basic cellulose skeleton as MC, and also has carboxyl groups that are not present in the structure of MC. Because the carboxyl group has the ability to ion exchange, Cs and Sr were adsorbed to CMC-Na more strongly than to MC. ChS also adsorbed strongly to Cs and Sr. It is considered that the sulfate groups of ChS interacted with Cs and Sr. The adsorption k value (Cs) of ChS was approximately the same as that of ALA-Na, so it would be expected that ChS inhibits adsorption of Cs and Sr to the equivalent of ALA-Na. It has been reported that apple pectin reduced the ^{137}Cs whole body count in children [10]. Therefore, we also examined the adsorption effects of high and low molecular AP to Cs and Sr. Only high molecular AP showed the ability to adsorb Cs and Sr because of ionic exchange of the carboxyl group. In this experiment, the adsorbing potency was estimated by the detection of Cs and Sr concentrations in the filtrate after passage through a membrane filter (3000 NMWL). Because low molecular AP passed through the filter, we could not evaluate its adsorption activity. Therefore, it is necessary to consider a novel method for estimation of the potency of low molecular AP.

From these results, we found eight food samples and five compounds that adsorbed Sr and Cs. Carboxyl and sulfate groups in the structure were important for adsorption of Cs and Sr. These samples can be useful tools to defend against internal exposure by inhibiting absorption of Cs and Sr. In the future, it is necessary to estimate whether these samples might show the absorbing effects to Sr and Cs *in vivo,* using experimental animals.

Experimental

Adsorption experiments: Sodium alginate (ALA-Na) and calcium alginate (ALA-Ca) were bought from KIMICA Co. (Tokyo, Japan); chondroitin sulfate (ChS), apple-pectin (high molecular weight; MW 30000-100000) (AP high MW) and sodium carboxyl methyl cellulose (CMC-Na) from Sigma-Aldrich Japan Co. (Tokyo, Japan); mucin, methyl cellulose (MC), chitosan and chitin from Wako Pure Chemical Industries, Ltd. (Osaka, Japan); laminaran from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan); apple pectin (low molecular weight; MW 30000 less) (AP low MW) from apple pectin laboratory (Aomori, Japan); and SUN FIBER (guar gum degradation product) from Taiyo kagaku Co. Ltd. (Mie, Japan).

Pholiota microspora (nameko) and *Lentinula edodes* (Shiitake), that are Japan's most popular cultivated mushrooms, and kelp were bought from a supermarket in Shizuoka city. Nameko, shiitake and kelp were freeze-dried with FDU-2100 (Tokyo Rikakikai Co., Ltd., Tokyo, Japan) and used as samples. Each sample was prepared at the following concentration (mg/mL) using distilled water: ALA-Na 0.0025, 0.01, 0.025, 0.05 mg/mL; ALA-Ca 0.05, 0.2, 0.5, 1.0; ChS 0.0025, 0.01, 0.025, 0.05; freeze-dried kelp 0.0105, 0.042, 0.105, 0.21; mucin 0.05, 0.2, 0.5, 1.0; laminaran 0.05, 0.2, 0.5, 1.0; MC 0.1, 0.4, 1.0, 2.0; freeze-dried nameko 0.25, 1.0, 2.5, 5.0; freeze-dried shiitake 0.25, 1.0, 2.5, 5.0; chitosan 0.25, 1.0, 2.5, 5.0; chitin 0.25, 1.0, 2.5, 5.0; AP high MW 0.25, 1.0, 2.5, 5.0; AP low MW 0.25, 1.0, 2.5, 5.0; CMC-Na 0.005, 0.02, 0.05, 0.1; SUN FIBER 0.25, 1.0, 2.5, 5.0. Cesium chloride (CsCl; Wako Pure Chemical Industries, Ltd. (Osaka, Japan)) or strontium chloride ($SrCl_2$; Wako Pure Chemical Industries, Ltd. (Osaka, Japan)) was added to a final concentration of 0.02 mM [6]. These solutions were filtered by centrifugation (15,000 × *g*, 20 min, 37°C) through a membrane filter (A miconultra 3K device, 3000 NMWL, Merck Millipore Co., Tokyo, Japan). The amounts of Cs and Sr in filtrates were measured by ICP-MS (Varian 810/820-MS, Varian Medical Systems, Inc., Tokyo, Japan). Yttrium was used as an internal standard. Limits of quantification (LOQ) for measured metals were as follows: Cs 0.05 ppb, Sr0.05 ppb, All samples were analyzed in triplicate.

Adsorption isotherm: Adsorption ability of samples to Cs was evaluated by the Freundlich adsorption isotherm, expressed by the equation:

$$\log V = (1/n) * \log C + \log K \quad (1)$$

V = amount of adsorbed Cs or Sr (μmol/g); C = residual amount of Cs or Sr in solution (mmol/L), and K = adsorption constant.

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Germination and Clonal Propagation of the Endemic Shrub *Corema album*, a Vulnerable Species with Conservation Needs and Commercial Interest

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In this study, we aimed to explore regeneration possibilities of *Corema album* (L.) D. Don by determining germination mechanisms and testing vegetative propagation methods. We analyzed seed viability under natural conditions, carried out germination treatments and a greenhouse experiment to study clonal propagation. We confirmed that *C. album* seeds present physiological dormancy, broken by ingestion by natural dispersers (rabbits and foxes), and that seed viability under natural conditions is lost after one year. *In vitro* germination was better achieved with a 200 ppm gibberellic acid treatment. Clonal propagation proved to be a successful technique for the production of *C. album*. Treating cuttings with IBA 0.2, w/v, at 20% resulted in the highest rooting percentage, while planting rooted cuttings in a substrate of perlite with vermiculite 1:1 was essential for plant survival. Our results show that both germination pretreatments and cutting propagation are powerful tools for the production of this valuable species. Both methods could be incorporated for population regeneration in natural habitats, and for the potential establishment of the species as a new crop for consumption and pharmacological purposes.

Keywords: *Corema album*, Clonal propagation, Endangered species, *Ex vitro* rooting, Germination treatment, Gibberellic acid, Seed dormancy, Seed viability.

Corema album (L.) D. Don (*Ericaceae* subfam. *Ericoideae* tribu *Empetrae*) is a dioecious woody shrub endemic to the Atlantic coast of the Iberian Peninsula, commonly known as Camarina or Camarinha. *C. album* is an important species in sand dune ecosystems, being the dominant species in the areas where it grows. *C. album*'s branches and fruits have traditionally played a useful ecological role not only in landscape conservation but also economically for local communities both in Spain and Portugal, due to its use for fuel or for commercialization of its edible fruits [1]. *C. album* berries were traditionally used in popular medicine as an antipyretic [1, 2] and recent studies have discovered important pharmacological properties of its fruits and leaves [3-5]. According to León-González *et al.* [3], extracts from *C. album* berries and leaves are rich in hydroxycinnamic acids and contain different amounts of flavonoids and stilbenes; they also found that human colon cells pre-treated with *C. album* fruit and leaf extracts showed an outstanding protection against challenge-induced damage. These findings support the traditional use of *C. album* as a medicinal plant. Recently, the species has been proposed as a suitable crop for berry production for the food industry [6].

Several factors make the natural regeneration of *C. album* difficult. Seeds show low germination under natural conditions and are endozoocorous, presenting physiological dormancy, which is broken after consumption by vertebrates like seagulls [7, 8], rabbits, and foxes [7, 9-11]. *C. album* seedling mortality rate in natural conditions is high, reaching 99% during the summer season [10]. Moreover, the habitat of *C. album* is dwindling with its populations being fragmented [9, 10]. Sand dune ecosystems along the distribution area of the species are largely affected by the expansion of tourist resorts and other anthropogenic-origin disturbance, such as large-scale plantations of pine trees and *Retama monosperma* [12], and the invasive shrub *Acacia longiforme* [13]. *C. album* has consequently been classified as a vulnerable species because of habitat loss and is included in the regional Red List of threatened vascular plants in Andalusia, Spain [14].

Facing the ongoing habitat loss and disturbance in *C. album* communities, regeneration under natural conditions is really low, both in the northern [7, 10] and southern limits of its biogeographical distribution area [9, 10, 15]. Under this scenario it is necessary to gain knowledge on the mechanisms underlying *C. album* propagation, not only for maintaining and regenerating degraded *C. album* populations, but also for future feasible agricultural and pharmacological use [3-6]. So far there is very limited information on the germination responses of *C. album* [16] and, to our knowledge, no clonal propagation techniques have been described for this endemic and vulnerable species.

Clonal propagation by hardening off cuttings has been described for species related to *C. album* such as the American endemism *Ceratiola ericoides* [17], but studies involving *Ericaceae* propagation in the Iberian Peninsula or Mediterranean species are scarce [18]. In cuttings propagation, several phytohormones and growth factors such as indolebutyric acid (IBA) are described as effective rooting triggers [19]. The success of clonal propagation also depends on appropriate temperature, humidity, and substrate selection, which can be specific for each species, cutting type, season, and propagation system [20]. Various methods can be used for cuttings propagation of shrub species, but the one needed for *C. album* has not yet been described.

In this study we aimed to assess germination and clonal propagation requirements for *C. album* and to determine which regeneration constraints underlie its poor recruitment under natural conditions. We analyzed dormancy breaking and seed germination by means of different *in vitro* treatments (physical and physiological). We further evaluated the effectiveness of clonal propagation by cuttings hardening procedures in *C. album*, testing different over the counter growth regulators treatments and substrates. Our specific objectives were to: 1) establish a method to promote germination; 2) determine seed viability under natural conditions; and 3) evaluate the effectiveness of auxin treatments and different substrates on *C. album* clonal propagation under greenhouse conditions.

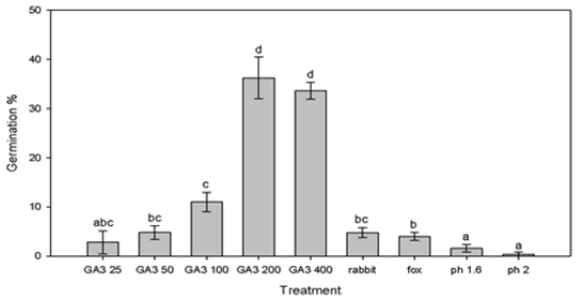

Figure 1: Total germination rates (%) for the different treatments. Treatments not included in the figure did not show any germinated seed (control, badger excrement, temperature shock, and scarification). Lower case indicates significant post-hoc Tukey test results.

We hypothesized that although this species can produce more than 40,000 seeds per plant every year [1,9,10], seed viability in the field is low and that clonal propagation is a feasible alternative procedure to produce *C. album* plants. Results can be relevant to establish a management protocol to foster the regeneration of *C. album* sand dune communities, as well as for its eventual commercial production for pharmaceutical and agronomic purposes.

Seed germination experiments: Gibberellic acid (GA_3) pretreatments broke dormancy successfully, reaching values near 40% of germinated seeds pretreated with 200 ppm and 400 ppm (Fig.1). Germination also occurred in seeds dispersed by rabbits (4.7%) and to a lower extent by foxes (4%), but not in seeds from badger excrements (ANOVA, F = 93.69; $P < 0.001$, Figure 1). pH treatments resulted in germination percentages below 2%, and the rest of the treatments (control, scarification, smoke, and temperature stratification) showed no germination (Figure 1).

Germination dynamics were similar for seeds from rabbit pellets and for all GA_3 treatments (29-30 days), with the exception of GA_3 25 ppm, which showed a longer time for t_0 (45 days). The time of first germination (t_0) was longer for seeds from fox excrements and for seeds under the pH treatments (63 and 129 days, respectively, Table 1).

Table 1: Germination parameters for the different treatments: t_0, t_{50}, and percentage of total germination. Treatments that are not shown in the table did not present any germination (control, pH 1.6, pH 2, smoke, scarification, and badger excrements).

Treatment	t_0	t_{50}	t_{total}	t_{mean}
GA_3 25 ppm	45	57	67	50.5
GA_3 50 ppm	29	67	69	59.5
GA_3 100 ppm	29	54	71	54.6
GA_3 200 ppm	29	48	71	51.8
GA_3 400 ppm	29	48	71	49.4
Rabbit	30	35	59	47.9
Fox	63	67	71	66.5
pH 1.6	128	131	143	137.6
pH 2	131	131	144	136.3

Viability test and seed bank experiment: The tetrazolium viability test showed that seeds extracted from mature fruits presented an initial viability close to 52% (Fig. 2). Seeds from rabbit pellets reached 46.5% of viability. Seed viability decreased significantly along the study period, with seeds from bags unburied after 8 months presenting 26% of viability, thus a 50% viability loss during that period (Chi-square=10, df=1, $P<0.01$); after 16 months, almost all seeds from buried bags were non-viable (96%), and presented signs of desiccation (Chi-square=40.3, df=1, $P< 0.001$, Figure 2).

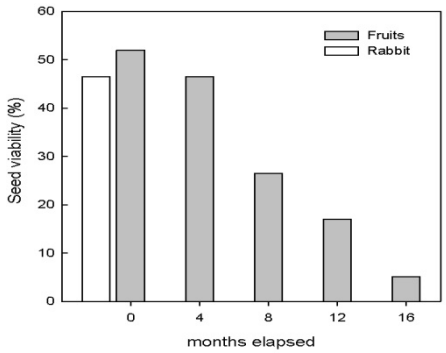

Figure 2: Seed viability from TZ test (%) for seeds extracted from fruits (initial), seeds from rabbit excrements (white bar), and seeds extracted from buried bags along the study period.

Clonal propagation experiment: Both the treatment and the substrate used for clonal propagation had a significant effect on the success of root production (Figure 3). The type of substrate strongly determined rooting success; PV substrate showed a higher percentage of rooted cuttings in relation to SP (Chi-square = 138.059, df = 1, $P < 0.001$). Hormonal treatments had a significantly different effect on root production (Chi-square = 15.847, df = 4, $P < 0.01$); IBA both at 0.2 and 0.4 %, w/v, was the treatment inducing higher root production, followed by Inabarplant, and control cuttings (Figure 4).

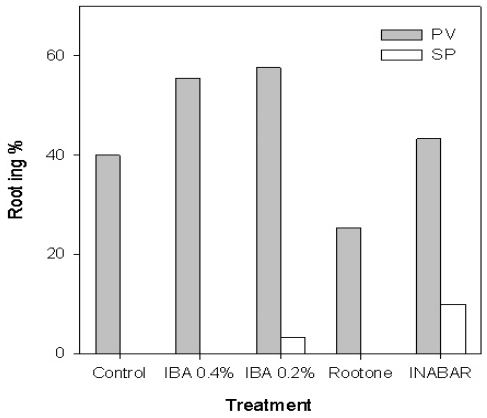

Figure 3: Total percentage of rooted cuttings for each treatment and substrate (PV perlite:vermiculite; SP sand:peat).

There were no significant differences in root production between cuttings from male and female plants (Chi-square = 0.218, P = 0.691). The most successful treatment was the combination of PV substrate with IBA treatment 0.2 and IBA 0.4%, w/v, with percentages of cuttings survival rates close to 60% (Chi-square = 17.161, df = 4, $P < 0.01$, Figure 3).

Within the surviving rooted cuttings, root number and length also differed between substrates, with cuttings planted in PV showing significantly higher number of roots than in the SP substrate (Mann Whitney U, Z = 11.78, df = 1, $P < 0.001$, Figure 4). Root number also varied among treatments (Chi-square = 15.16, df = 4, $P <$ 0.0044), with both IBA treatments showing the highest number of roots. Treatment also affected the total root length produced per cutting (ANOVA, F = 3.7, P < 0.01); roots were longer in control cuttings and in cuttings treated with Rootone (Figure 4).

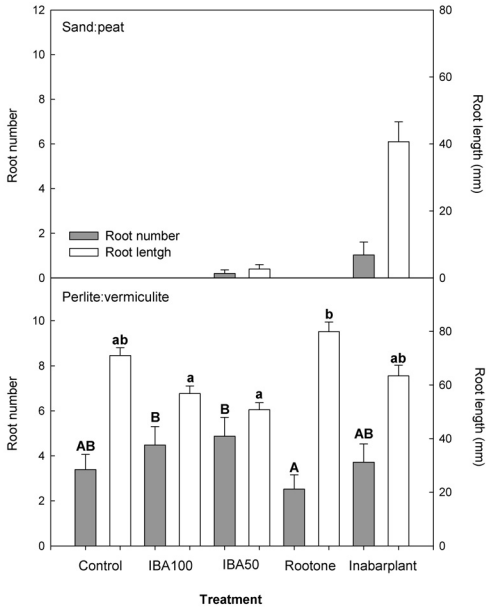

Figure 4: Number of roots per cutting and root length (mean ± standard error) per substrate (Perlite:vermiculite and sand:peat) and treatment. Gray bars represent root number and white bars root length. Significant post-hoc Tukey test results are shown for root length (lower case) and root number (upper case).

Plant survival three months after the transplant reached the highest value with the IBA treatment, with 52% survival, followed by control cuttings with 41%; while the treatment with Rootone had the lowest values (26%) (Chi-square = 16.55, $P < 0.01$). After three months, vegetative growth was similar for all treatments, reaching an average length of 23.5 ± 1.8 mm of new shoots produced per plant (Chi-square = 6.785, $P = 0.148$, Figure 5).

Germination constraints under natural conditions: We confirmed that *C. album* seeds present physiological dormancy [13, 16], which under natural conditions is broken by ingestion by natural dispersers such as rabbits and foxes (Figure 1). Fedriani and Delibes [13] described foxes and rabbits to be the most common dispersers of *C. album* seeds in the southern area of *C. album* distribution, the same as our study site, while Calviño-Cancela [10] described both rabbits and seagulls to be important but unspecialized dispersers. Our study corroborate these results, showing that rabbits and foxes are also much more efficient in breaking *C. album* seeds dormancy in relation to badgers, which did not increase germination percentages. We observed that the pattern of fruit digestion of the three dispersers differed significantly; seeds recovered from badger's droppings were surrounded by fruit pulp after digestion, which may reduce the potential digestion effects on seed dormancy release.

Viability tests in the field demonstrated that after 16 months *C. album* does not present a viable seed bank in the study site. These results would partly explain the lower population density in the southern area of the *C. album* distribution range, which has been attributed to lower precipitation and more severe drought during summers [15]. We found a strong decrease in seed viability within a year, with values under 5% of viable seeds after 16 months, which also presented clear signs of desiccation (Figure 2).

Our results reinforce the notion that *C. album* is an obliged endozoocorous species and unspecialized vertebrate dispersers such as rabbits and foxes play a key role in *C. album* regeneration in its

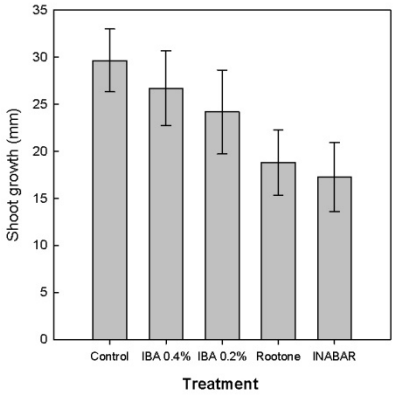

Figure 5: Shoot growth (mm) for the surviving plants from each treatment in the Perlite:Sand substrate (3 months after cutting planting).

southernmost distribution area, increasing germination by over 5%. This is in accordance with the study of Fedriani and Delibes [13], where rabbits were found to produce 6.7% of emerged seedlings in relation to no germination in control seeds.

Gibberellic acid pretreatment was the most successful method to germinate seeds of *C. album.* We found that GA_3 both at 200 and 400 ppm significantly increased germination in *C. album* seeds, reaching 45% of the total in relation to the other experimental treatments, which presented very low germination results (lower than 5% for pH treatments), and to control seeds, with no germination (Figure 1). Taking into account that under natural conditions the viability of seeds from fresh fruits is close to 50% (Figure 2), we can conclude that GA_3 treatment had an effect of almost 100% germination success. This treatment allowed us to produce seedlings in a relatively short period (2 months), and these we transplanted and grew in the greenhouse, proving that it is a powerful method that could be used for production of *C. album* plants for conservation and commercialization purposes. This method would be particularly useful when a genetically varied pool of individuals is needed, for example for transplants in a regeneration program or conservation of natural areas where *C. album* is the dominant shrub species.

GA_3 has been previously used for conservation purposes in endangered species [19, 21], and has been proposed as a successful method for *in vitro* production. In our study, the combined use of growth regulators in agar proved to be a key factor in the germination success. The same method was proposed by Rossini *et al.* [22] for the endemic shrub *Erica andevalensis* (Ericaceae). Our results also corroborate those of Santos *et al.* [16], who recently described hormonal methods as a means of germinating seeds from *C. album* from populations across the coast of Portugal. Nevertheless, our results differ from those of Santos *et al*. [16], who treated seeds with a combination of GA_3 at 1000 ppm and low pH, obtaining lower germination rates (30.3%) in a much longer period (175 days). Thus, we can conclude that treating seeds with 200 ppm GA_3 in agar medium is a successful method to promote germination of *C. album*.

Clonal propagation by cuttings hardening was a successful method to produce *C. album* plants. Both the substrate and the growth regulator treatment used in the cuttings hardening process had a significant effect on the rooting success and later cuttings survival.

The substrate of combined perlite and vermiculite (PV), that we choose for having been successful in the related American species

Ceratiola ericoides, was essential in the success of the experiment. Cuttings in PV showed an overall survival of 44% in comparison with cuttings grown in sand and peat (SP), with only 2% of surviving cuttings at the end of the experiment.

We also found that the treatment with IBA growth regulator was essential for the success in *C. album* plant production. Cuttings treated with IBA increased survival up to 60% in relation to controls, which resulted in only 40% of surviving plants (Figure 3). The surviving cuttings showed different degrees of root length and number; control and IBA- produced cuttings were the ones showing higher root number in relation to Rootone treated cuttings (Figure 4). Thus, we can conclude that the best treatment to produce long-living plants of *C. album* is to plant cuttings without the bottom leaves, treated with IBA 0.2, w/v, at 20% in a PV substrate.

In spite of the interest of producing *C. album* as a cultivar and the species vulnerable status due to habitat fragmentation, there have been very few attempts to develop a protocol of *ex situ* regeneration. We provide results for successful methods for the production of *C. album,* including both germination and clonal reproduction, which we believe could be useful tools in the conservation of this endemic species. Our results also showed that *C. album* seed germination under natural conditions is limited by both the seeds physiological dormancy and the lack of a viable seed bank. These results corroborate that *C. album* natural regeneration strongly depends on natural dispersers.

Together with habitat loss due to human activities, the ongoing global change projections of higher drought intensity and frequency in *C. album* distribution area threaten to constrain the populations of this vulnerable species even further [15]. The results in this study could constitute the methodological basis for protocols for the production of this valuable species. The production of *C. album* could be essential both for the species regeneration in natural habitats and for the species great potential as a new crop, due to the interest in its fruits for consumption and pharmacological purposes.

Experimental

Seed germination experiment: A population of *C. album* located in Doñana Natural Park (SW Spain) was chosen to collect ripe fruits and vertebrate excrements containing predated fruits in August. *C. album* is the dominant species in the study sand dune shrub community, and the only one that produces large amounts of fleshy fruits during the summer.

Germination treatments were carried out under laboratory conditions to determine factors breaking seed dormancy. *C. album* seeds were extracted from the fruits and excrements and washed carefully from any pulp debris. A disinfection treatment was applied to avoid fungal proliferation in the seeds, consisting of an immersion in NaOCl 1% for 1 min followed by ethanol 70% for 30 sec. After disinfection, seeds were washed thoroughly with distilled water and planted in an 8% agar medium [22] in Petri dishes, applying the following treatments:

1-5) Gibberellic acid treatments. Agar medium with dissolved gibberellic acid (GA_3) to concentrations of 25, 50, 100, 200, and 400 ppm before solidification. GA_3 regulates germination in numerous ways and its effect on germination has been observed in many species [22, 23].
6-8) Natural dispersers. Seeds were extracted from recent excrements from rabbits (*Oryctolagus cuniculus*), foxes (*Vulpes vulpes*), and badger (*Meles meles*), collected from the study site at the same time as fresh fruits. Seeds that presented predation signs were discarded.
9-10) pH treatments. We treated seeds with different concentrations of hydrochloric acid at pH 1.6 (similar to fox stomach pH), and pH 2 (similar to rabbit gut pH, [25]) during 10 min. Once treated, seeds were planted in the agar medium.
11) Smoke treatments: seeds were stored in a closed plastic container (10 x 10 cm) attached to a smoker device (used for apiculture) containing leaves and wood from shrubs from the *C. album* plant community (*Halimium halimifolium, H. commutatum, Corema album, Cistus salvifolium*, and *Stauracanthus genistoides*). We kept seeds in the smoke for 1 h before planting.
12) Cold stratification: seeds were kept at 4ºC for one month prior to planting (following [26]).
13) Scarification with sandpaper. Seeds were carefully sandpapered until the seed's woody endocarp was perforated.
14) Control. Seeds were extracted from ripe fruits and washed with distilled water before planting in the agar medium.

We prepared 5 Petri dishes per treatment, each with 50 seeds (5 replicates per treatment, 50 seeds, 14 treatments). Seeds were placed in a germination chamber with temperature-controlled conditions with a cycle of 12 h of light at 24ºC and 12 h in dark at 15ºC. We checked seeds periodically for germination during 4 months, daily during the first month, and weekly during the rest of the experiment. We considered seeds with an emerging radicle as germinated and removed them from the Petri dishes.

Germination values for each individual Petri dish (N = 5) were calculated. We then calculated the time of initial germination (t_0), time when 50% germination was reached (t_{50}), and final percentage of germination for each treatment. The parameter t_0 is useful to study germination dynamics when the germination rate is low [22].

Seed viability analysis: We tested seed viability by means of the tetrazolium test (TZ). The test was carried out in control seeds from the same collected fruits used for germination experiments and in the seeds extracted from rabbit pellets. In addition, to assess the existence of an active seed bank of *C. album* in natural conditions, seeds were placed inside mesh bags in the field (100 seeds per bag). We prepared 12 plastic mesh bags where the seeds were placed, and we buried them at a 15 cm depth in the study site. Three bags were collected every 4 months (month 4, 8, 12, 16) and seeds tested for viability. Seeds were cut longitudinally with a scalpel, and treated with a 1% solution of 2, 3, 5 tripiphenyl tetrazolium chloride (TZ) for 24 h at room temperature [26]. We visually evaluated the embryos under a dissecting microscope; dried or un-colored embryos were considered as non-viable and red-colored embryos as viable.

Clonal propagation experiment: In order to assess the possibility of producing this long-lived species for regeneration of natural populations, forest restoration and commercial purposes, we carried out a clonal propagation experiment under greenhouse conditions. In *C. album* both sexes present significant reproductive allocation differences that may alter vegetative growth [24]; thus, we collected shoots cuttings in the field site from 10 male and 10 female individuals (approx. 40 shoots per plant). Cuttings were obtained from mature plants in January, when the reproductive season had not yet started. We carefully selected yearly shoots after the previous node in the stem to ensure shoot age homogeneity; this is possible as in *C. album* each year's flowering leaves a node in the stem. Cuttings were kept watered in plastic containers until arrival at the laboratory. We then recut cuttings to a length of 9 cm, washed them with distilled water, and removed the leaves from the bottom 4

cm of each cutting. One set of cuttings was used as controls and the other set was treated with 5 different growth regulator treatments from commercially distributed agricultural components:

1) and 2) Liquid solution of indolebutyric acid (IBA) at 0.2 and 0.4%, w/v (Exuberone ®, Bayer).
3) Rooting powder growth regulator mix of 0.056%, w/w, IBA, 0.032%, naphthaleneacetic acid (NAA), and 0.078%, w/w, alpha naphthaleneacetamide (Rootone, Compo ®).
4) Rooting dust growth regulator mix of IBA 0.1% and NAA 0.1, w/w (Inabarplant, INABAR®).
5) Control shoots without any treatment but with leaf removal.
For the liquid treatment IBA 0.2 and IBA 0.4%, the base of each cutting was submerged in the solution for 10 s, and then left to dry before planting. For the powder treatments (Rootone and Inabarplant) the cuttings were previously soaked in distilled water and then covered with the powder.

We planted all shoots into 2 substrates, perlite with vermiculite 1:1 (hereafter PV) and sand with peat 1:1 (hereafter SP); these substrates were chosen for being the most successful in related *Ericaceae* species such as *Ceratiola ericoides* [17]. We prepared 3 cuttings per individual, treatment, and substrate (N = 600, 3 shoots x 10 individual x 2 sexes x 5 treatments x 2 substrates).

Cuttings were planted in 5 cm wide and 12 cm high pots (3 cuttings per pot). Trays were kept in the shade in an air-conditioned controlled-temperature greenhouse (22^{o}C) with high RH (minimum 60%), and were watered daily. Additionally, pots were watered every 15 days with the systemic fungicide polioxine-B 2%, w/v, to avoid proliferation of pathogenic fungi in the roots. Pots were relocated every week to avoid site-specific effects. After 3 months we checked for signs of rooting in each cutting and counted and measured all main roots when present.

Cutting survival was calculated as the total cuttings that rooted and survived until the end of the experiment per treatment. Dead or non-rooted cuttings were discarded.

To assess the success of each treatment, we planted the rooted cuttings in 10 cm wide pots containing a mix of sand from the natural distribution area with commercial peat in a 1:1 proportion, following Thetford *et al*. [17]. We placed the hardened cuttings in a greenhouse under natural environmental conditions; pots were relocated every week. Three months after planting, we calculated plant survival and final vegetative growth as the accumulated length of vegetative shoots produced after the last node in the stem [24].

Statistical analyses: We analyzed germination treatment effects by an ANOVA for the effect of treatment on the final germination percentage; we applied a Tukey post-hoc test to analyze significant differences between the different treatments.

We tested the effect of treatment on cutting propagation success with a Chi-square to analyze the percentage of rooted cuttings per treatment and substrate; we also tested for sex effects to discard any differences in rooting and growth between cuttings from male and female plants; g-tests were used to assess differences among treatments when necessary. Root number per cutting was analyzed by a Kruskal-Wallis non-parametric test for independent groups. Total root length produced per cutting was analyzed by a nested ANOVA with treatment and substrate as fixed factors and individual plant origin of the cuttings as within subject factor; we used a post-hoc Tukey test to compare pairwise differences. Final growth of plants obtained from the hardened cuttings was analyzed by a nested ANOVA with treatment as fixed factor and plant origin of cuttings as within subjects factor. We log-transformed the variables root length and vegetative growth to fit normality assumptions. We calculated the proportion of surviving plants from hardened cuttings as the plants that survived the transplant after 3 months and analyzed survival of plants with a Chi-square test.

All statistical analyses were performed with SPPS 17 software package (Chicago, IL, USA) and statistica 6 StatSoft (Tulsa, OK, USA).

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Essential Oil Profile, Phenolic Content and Antioxidant Activity of *Geranium kikianum*

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This work presents the first phytochemical investigation of *Geranium kikianum* Kit Tan & G. Vold (Geraniaceae), a species endemic to the southern Peloponnese, Greece. The essential oil from aerial parts of the plant was isolated by hydrodistillation and its chemical composition characterized by GC-MS. A total of 26 compounds were identified, constituting 98.3% of the oil composition. Oxygenated sesquiterpenes were the main group of constituents (75.4%). The major component of the oil was the sesquiterpene ketone germacrone (45.6%). Eleven alcohols represented the most diverse chemical class in the volatile oil (36.3%), and phytol was the most abundant acyclic diterpene alcohol (11.4%). The volatile compounds and residual water remaining after hydrodistillation were screened for their radical-scavenging activity by the DPPH method. IC_{50} values ranged from 0.2±0.03 mg/mL for the undiluted decoction to 69.7±0.5 mg/mL for the essential oil. The significant antioxidant activity of the residual water, comparable with that of the widespread synthetic antioxidant BHT (0.2±0.01 mg/mL), and almost tenfold higher than thymol (1.90 ± 0.04 mg/mL), correlates with a high content of total phenolic compounds (100.2±1.7 mg GAE/g), and flavonoids (11.9±1.2 mg GAE/g).

Keywords: *Geranium kikianum*, Essential oil, GC-MS, Antioxidant activity, DPPH, Total phenolics.

The genus *Geranium* L. (Geraniaceae) is represented by over 430 distinct species of annual, biennial, and perennial herbs, occasionally subshrubs, distributed mainly in mild-temperate regions of the northern hemisphere [1]. This genus is the largest one of the family Geraniaceae. Many taxa of the genus are well known ornamental plants due to their attractive flowers/leaves. A number of species from this genus are cultivated as medicinal plants, and extracts from the roots of some *Geranium* spp. are used in traditional medicine, tanning and dyeing. Recently, many articles about the composition, biological effects, and use in medicine, food-flavoring, perfumery, and cosmetics of extracts of *Geranium* taxa have been published [2a-e]. In general, the essential oil constituents of *Geranium* taxa have been poorly investigated, except for *G. macrorrhizum*, an ethnopharmacologically renowned species widely distributed in southern and central Europe, ranging from the southern Alps to southern Greece in the Balkan Peninsula [3a,b]. *G. macrorrhizum* is reputed for its wide range of medicinal properties. Some Balkan countries make use of both leaves and rhizomes of *G. macrorrhizum* mainly for their hypotensive, sedative, astringent, cardiotonic, and antiatheromatous effects [4]. This biological activity is due to its essential oil that makes this plant the most economically important species of *Geranium*.

G. kikianum, a closely related species to *G. macrorrhizum*, was described as a new species endemic to Greece [5]. However, no published reports of its chemical constituents can be found in the literature.

This work represents the first analysis of the volatile constituents of this endemic species. In addition, the aim of this study was to determine the antioxidant activity of the isolated essential oil, and the residual water retained after hydrodistillation; the latter has been examined for its content of total phenolics, and flavonoids.

The essential oil isolated by hydrodistillation from the aerial parts of *G. kikianum* was a light yellow color, easily solidifying, with a mild and pleasant scent. The percentage content of the essential oil, based on the dry weight of the plant material, was 0.71%. This yield was significantly higher than those for the oils from other *Geranium* species, such as *G. macrorrhizum*. The content of oil obtained from the herb of *G. macrorrhizum* varies from 0.02 to 0.13% depending on the plant's origin, and vegetative stage [6]

Hydrodistilled oil from *G. kikianum* was subjected to detailed GC-MS analysis to determine its chemical composition. A total of twenty-six compounds were identified, constituting 98.3% of the oil composition. The list of the identified volatile constituents with their relative percentages, and retention indices, as well as their attribution to different compound classes is given in Table 1.

The qualitative and quantitative composition of the *G. kikianum* essential oil revealed that it is typically sesquiterpenic (80.7%), while no monoterpene compounds was detected in the oil, not even in trace amounts. The dominant volatile class was oxygenated sesquiterpenes (75.4%). GC-MS analysis showed that the major constituent was a cyclic ketone germacrone (45.6%), following by other oxygenated sesquiterpenes. Elemane-type sesquiterpenoids amounted to 13.7% of the oil, elemol being the most abundant constituent of this type (8.6%), followed by *trans*-β-elemenone (4.2%), and γ-elemene (0.9%). One should be careful when considering the relative amount of compounds having an elemane skeleton since it is generally accepted that these are formed during a

Table 1: Chemical constituents of the essential oil of *Geranium kikianum*.

Compound	Class[a])	*RI*[b])	*RA*[c])	MOI[d])
β-Elemene	SH	1366	t	A[e]) MF[f]), MS[g])
γ-Elemene	SH	1408	0.9	A, MF, MS
γ-Muurolene	SH	1457	t	A, MF, MS
ar-Curcumene	SH	1461	t	A, MF, MS
α-Zingiberene	SH	1474	t	A
(*Z*)-α–Bisabolene	SH	1488	t	A, MF
cis-Sesquisabinene hydrate	SO	1515	t	A
Elemol	SO	1527	8.6	A, MF, MS
Germacrene B	SH	1531	4.4	A, MF, MS
trans-Sesquisabinene hydrate	SO	1545	t	A
Caryophyllene oxide	SO	1559	0.7	A, MF, MS
cis-β-Elemenone	SO	1580	t	A
trans-β-Elemenone	SO	1584	4.2	A
2-(7*Z*)-Bisaboladien-4-ol	SO	1597	1.3	A
γ-Eudesmol	SO	1610	3.1	A, MF, MS
β-Eudesmol	SO	1628	3.4	A, MF, MS
α-Eudesmol	SO	1631	3.0	A, MF, MS
Bulnesol	SO	1646	1.9	A, MF
Germacrone	SO	1675	45.6	A, MF, MS
β-Costol	SO	1741	3.6	A, MF
Cryptomeridiol	SO	1796	t	A
Phytol	DO	2113	11.4	A, MF, MS
Tricosane	A	2300	1.7	A, MF, MS
Tetracosane	A	2400	1.3	A, MF, MS
Pentacosane	A	2500	2.4	A, MF, MS
Hexacosane	A	2600	0.8	A, MF, MS
Sesquiterpene hydrocarbons			5.3	
Oxygenated sesquiterpenes			75.4	
Oxygenated diterpene (DO)			11.4	
Alkanes (A)			6.2	
Oil yield (%)			0.7	
Total identified (%)			**98.3**	

[a]) The abbreviations of the compound classes are given at the end of the table;
[b]) *RI*: Linear retention indices determined experimentally on the HP-5MS column relative to a series of *n*-alkanes (C_8–C_{26})
[c]) *RA:* relative area; t, traces (<0.1%)
[d]) MOI:Mode of identification
[e]) A: Reference [28].
[f]) MF: Mass Finder database.
[g]) MS: Mass spectra from our own laboratory database.

GC run as a consequence of thermal rearrangements of heat-sensitive germacrone-type precursors with the conversion taking place in the injector port, as well as in the column [7]. It might be possible that the compounds in question (e.g. *trans*-β-elemenone and γ-elemene) are not exclusively native metabolites of germacrone-containing plants [8].

The quantity of sesquiterpene alcohols, which represent the most diverse chemical class in *G. kikianum* oil, reached 24.9%, but three of the ten identified were present in trace amount. The most prominent alcohol and the third most abundant constituent was the tertiary alcohol elemol (8.6%). Phytol formed 11.3% of the total oil; this acyclic diterpene alcohol has not frequently been reported as an essential oil component, and was possibly formed by chlorophyll degradation during hydrodistillation and/or senescing of the plant material.

An extremely high content of terpenoids (92.1%) and the absence of fatty acid-derived compounds were found as the important features of *G. kikianum* essential oil. These results strongly support the hypothesis that essential oil rich taxa (oil yields much higher than 0.1%) are generally characterized by the specific production of mono- and sesquiterpenoids, and/or phenylpropanoids, while the main volatiles of essential oil poor species (yields less than 0.1%) are fatty acid- and carotenoid derived compounds [2d]. In addition, it is noticeable that the essential oil from *G. kikianum* almost entirely consists of oxygenated compounds (86.8%), namely oxygenated sesquiterpenes (75.4%), and an oxygenated diterpene (11.4%).

Based on the data published so far, our results appeared to be somewhat different from those previously reported for other *Geranium* essential oils. It is interesting to note that *G. kikianum* oil lacked even a trace of monoterpenoids, which were present in an extremely high percentage (82.2%) in *G. dalmaticum* [2b], or in a relatively significant amount (8.7%) in *G. phaeum* [2c], and (8.1%) *G. lucidum* [2d]. It is worth noting that in contrast to the similarity in the exceptionally high germacrone content of *G. macrorrhizum* (49.7%) [3a], and *G. kikianum* (45.6%), their essential oils significantly differ in the amount of monoterpenoids. Unlike *G. kikianum* essential oil, which is characterized by a complete absence of monoterpenoids, *G. macrorrhizum* oil contains a relatively notable amount of this chemical class (9.9%) [3a]. Similarly, according to Stoeva [9], germacrone comprised 50-55%, as a marker for quality control of the so called Bulgarian "zdravets oil", the essential oil of the cranesbill, *G. macrorrhizum,* and mono-terpenic compounds amounted to 7–10%, consisting of γ-terpinene, terpinolene, *p*-cymene, α-pinene, δ-3-carene, α-phellandrene, limonene and borneol. Moreover, some taxa of *Geranium* contain monoterpenoids as dominant constituents, e.g. piperitone (12.3%) in *G. phaeum* [3b], and linalool (22.9%), γ-terpinene (13.9%), limonene (5.3%), geraniol (4.4%), and α–terpineol (3.8%) in the essential oil from *G. robertianum* [10]. The recognized diversity of essential oil chemotypes with unique chemical characters within this genus continues to expand. The chemical polymorphism of *Geranium* species is not only limited to the main components, determining the specific chemotype, but also oil components can vary, dependent on several factors, accounting for quality variation. This first phytochemical investigation of *G. kikianum* has thus demonstrated considerable variability concerning the chemical composition of *Geranium* essential oils and confirms this plant as a unique species that requests further chemotaxonomic investigation.

The content of total phenolic and flavonoid compounds in the residual water remaining after hydrodistillation of *G. kikianum* essential oil, measured using the Folin-Ciocalteu method [11] is shown in Table 2. The content of total phenolic compounds in the residual water (100.2±1.7 mg GAE/g of dry extract) makes this plant a very rich resource of natural phenolics responsible for the total antioxidant capacity.

Table 2: Total phenolic and flavonoid content of residual water after hydrodistillation of *Geranium kikianum* essential oil.

	GAE[a] [mg/g] extract
Total phenolics	100.2±1.7[b]
Non-flavonoids	88.2±2.4
Flavonoids	11.9±1.2

[a]Gallic acid equivalents; [b]Each value is expressed as mean ± SD (n=3).

Since formaldehyde reacts with flavonoids and condenses them, these condensed molecules can be removed by filtration, and the residual non-flavonoid phenolics can be analyzed by the F-C method. The amount of flavonoids is calculated as the difference between total phenolics and non-flavonoids [12]. The content of flavonoid compounds in this sample was 11.9±1.2 mg GAE/g of dry extract and it was almost eightfold lower in comparison with the total content of non-flavonoid compounds (88.2±2.4 mg GAE/g of dry extract).

The antioxidant activity of *G. kikianum* essential oil and residual water remaining after hydrodistillation was evaluated by the DPPH radical-scavenging test. Concentrations of essential oil components that cause 50% scavenging (IC_{50}) were calculated. IC_{50} values ranged from 69.7±0.5 mg/mL for the essential oil and 0.20±0.03 mg/mL for residual water (butylated hydroxytoluene 0.21±0.01; thymol 1.9±0.04).

The essential oil obtained from aerial parts of *G. kikianum* showed a relatively weak reactivity in scavenging the DPPH radical. This is reasonable since the essential oil did not show a high concentration of a potent antioxidant. Still, the effectiveness in reduction of the stable DPPH radical by *G. kikianum* essential oil is probably due to the cyclic ketone germacrone and the diterpene allylic alcohol phytol, present as the major oil constituents, and previously suggested as possible antioxidants [13]. In addition, antioxidant activity of the oil may be attributed to a mixture of sesquiterpene alcohols present in significant content (24.9%), and also identified as potential antioxidants [14]. According to the aforesaid authors, a certain degree of activity may be due to allylic alcohols and so the presence of β-costol (3.6%) and 2-(7*Z*)-bisaboladien-4-ol (1.3%), alcohols having a hydroxyl group bonded to a carbon atom adjacent to a C=C double bond, may contributed to the observed antioxidant ability. The presence of available hydrogen atoms from allylic groups, similar to those of phenols, represents a good barrier against the oxidative process. Therefore, regarding the chemical complexity of *G. kikianum* essential oil, one can speculate that the antioxidant activity is mainly attributed to the high percentage of the main constituents, but also to the presence of other minor constituents or a synergy among different oil components.

The assessment of the DPPH radical scavenging capacity of these two samples demonstrated that most of the active compounds that showed significant antioxidant activity (IC_{50}=0.20±0.03 mg/mL) occurred in the residual water after hydrodistillation of the stems and leaves of *G. kikianum*. This value is comparable with that obtained under the same conditions for BHT, renowned as one of the most widespread synthetic antioxidants (IC_{50}=0.21±0.01 mg/mL), and even almost tenfold higher than that of thymol (IC_{50}=1.90±0.04 mg/mL), a natural phenolic compound which possesses good antioxidant properties [15]. The prominent antioxidant activity of the residual water could be explained by a high content of phenolic compounds in this sample (100.2±1.7 mg GAE/g of dry extract). Flavonoids, which comprise 11.9±1.2 mg GAE/g of dry extract of the total content of soluble phenolics could especially be taken into account for the activity observed since these polyphenolic compounds were confirmed as powerful antioxidants in many studies *in vitro* [16]. Total phenolic and flavonoid content often correlates well with antioxidant capacity. It has been reported that phenolic compounds have an important antioxidant role as a good barrier against oxidative processes, and a positive correlation was observed between antioxidant potential and total phenolic and flavonoid levels of the extract [17]. The presented results on antioxidant activity of the residual water remaining after hydrodistillation of *G. kikianum* essential oil are in agreement with this fact, as well as with previously published results on antioxidant activity of various plant extracts isolated from species of *Geranium* [4, 18, 19a-c]. However, the compounds responsible for the bioactive properties of *G. kikianum* remain unclear and so a detailed study of the phenolic composition in plant extracts is necessary.

Experimental

Plant material and chemicals: The plant material was taken from plants cultivated in the Copenhagen Botanic Garden under accession number *Kit Tan & G. Vold* 30690. Once harvested, the plant material was dried at ambient temperature, in a shaded, well-ventilated place. The plants had been collected from the foothills of Mt Taigetos in the southern Peloponnese, Greece, at moderate altitudes of 1400–1450 m. All reagents used were of the highest purity available and purchased from Sigma-Aldrich Chemical Company (Germany).

Sample preparation: Air-dried *G. kikianum* leaves and stems (10 g) were ground into small pieces and subjected to hydrodistillation for 2 h. The essential oil was extracted with dichloromethane, dried over anhydrous sodium sulfate, and stored at 4°C in the dark until analysis. For the GC/MS analysis a sample of essential oil was dissolved in dichloromethane, and for antioxidant assay in dimethyl sulfoxide (DMSO) in a concentration of 10.0 mg/mL. A sample of residual water obtained after hydrodistillation was prepared in a concentration of 1.0 mg/mL in distilled water. Thymol and butylated hydroxytoluene, BHT, were used as positive samples for the antioxidant assay, and prepared in the same way as the tested samples.

Gas chromatography-mass spectrometric analysis of essential oil: Volatile compounds from the aerial parts of the plant were analyzed by GC-MS using a Hewlett-Packard 6890 Series II gas chromatograph fitted with a fused silica HP-5 (5% phenyl methyl siloxane) capillary column (30 m × 0.25 mm, 0.25 μm film thickness), coupled to a HP 6890 Series II mass selective detector (MSD). The column temperature was programmed from 60°C to 240°C at a heating rate of 3°C/min, and helium was used as carrier gas (1.1 mL/min). Other operating conditions were as follows: inlet pressure 9.43 psi, injector temperature 250°C, detector temperature 280°C, split ratio 1:25, injection volume 1 μL. A mass selective detector was operated in the electron impact mode, EI, at the ionization energy of 70 eV, with scan range 20-555 amu, and scan time 1.60 s.

The linear retention indices, *RI*, for all compounds were determined by the Kovats method by injection of the sample with a *n*-hexane solution containing a homologous series of C_8-C_{26} *n*-alkanes as standards [20]. The identification of the essential oil constituents was accomplished by the visual interpretation, comparing their retention indices and mass spectra with literature data [21], by computer library search (HP Chemstation computer library NBS75K.L, NIST/EPA/NIH Mass Spectral Library 2.0 and Mass Finder 4 Computer Software - Essential Oils 4a), and with our laboratory own database. Compound concentrations (as % content) were calculated by integrating their corresponding chromatographic peak areas (TIC mode).

Total phenolic and flavonoid content: The total content of phenolic compounds was determined by a colorimetric assay that utilizes Folin–Ciocalteu (F-C) reagent. Sample solution (1 mL) was diluted with 60 mL of distilled water, and 5 mL of F-C reagent, previously diluted 2 times, and mixed. After 30 s, and before 8 min, 15 mL of 20% sodium carbonate solution was added, and the obtained solution was diluted to 100 mL. Prepared samples were incubated for 2 h at room temperature, and the absorbance was measured at 765 nm. The data were calculated according to a standard curve of gallic acid (0.01-0.20 mg/mL), and expressed as gallic acid equivalents (GAE) per g of dry extract. All measurements were performed in triplicate.

The total content of flavonoid compounds was calculated as the difference between total phenolic and non-flavonoid contents according to Kramling and Singleton [12]. A 1.0 mL sample solution was mixed with 0.5 mL of diluted HCl (1:3) and 5 mL of an 8 mg/mL formaldehyde solution to obtain the flavonoid fraction. Flavonoids were separated by centrifugation (3000 rmp, 10 min), and the supernatant was collected, containing all phenolic compounds except flavonoids (non-flavonoid phenolics). The non-flavonoid phenolics content was determined in the filtrate using F-C reagent. The flavonoids content represents the difference between total phenolics and non-flavonoids content.

1,1-Diphenyl-2-picrylhydrazyl radical-scavenging assay: A slightly modified method of [22] was used. A portion of sample solution (100 μL) was mixed with 1 mL of 5.25 x 10^{-5} M DPPH• in methanol. The decrease in absorbance of the test mixtures was monitored every 1 min for 30 min at 516 nm using a Perkin–Elmer Lambda 25 UV/Vis spectrophotometer. Methanol was used to zero the spectrophotometer; DPPH• solution was used as the blank sample, and thymol and BHT as positive probes. The DPPH• solution was freshly prepared daily, stored in a flask covered with aluminum foil, and kept in the dark at 4°C between measurements.

The radical-scavenging activity of the tested samples was determined by measuring the degree of absorbance quenching for varying sample concentration. Activity expressed as percentage inhibition of DPPH was calculated according to the formula:

$$IC(\%) = [(A_0 - A_t)/A_0] \times 100,$$

where A_0 and A_t are the absorbance values of the blank sample, and the test sample, at particular times, respectively. Percent inhibition after 30 min was plotted against concentration, and the equation for the line was used to obtain the IC_{50} value, a concentration required to quench 50% of DPPH radical. Experiments were carried out in triplicate.

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Integrated Analysis of the Bark Oil from *Cinnamosma madagascariensis* by GC(RI), GC-MS and NMR. ^{13}C NMR data of Cyclocopacamphene and Cyclosativene

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The composition of leaf and bark oils of *Cinnamosma madagascariensis* has been investigated by a combination of GC (RI), GC-MS and ^{13}C NMR. The leaf oil contained mainly monoterpenes: myrcene (17.9%), limonene (17.8%), β-phellandrene (15.3%) and linalool (12.2%). The bark oil, investigated for the first time, contained β-pinene (49.9%) and α-pinene (19.5%) as major components. Special attention was paid to the identification of cyclocopacamphene, an epimer of cyclosativene. ^{13}C NMR data of both compounds have been provided.

Keywords: *Cinnamosma madagascariensis*, Cyclocopacamphene, Cyclosativene, Leaf oil, Bark oil, ^{13}C NMR, 2D NMR.

The genus *Cinnamosma* (Canellaceae), endemic to Madagascar Island, contains three species, *C. fragrans* Baillon, *C. macrocarpa* H. Perrier and *C. madagascariensis* Danguy [1]. The three species, locally known as "mandravasarotra", are traditionally used in the treatment of malaria, fatigue and muscular aches [2].

C. fragrans leaf oil from Mariarano contained 1,8-cineole as its major component (45-55%) [2-4], whereas oil from Tsaramandroso contained mainly linalool (72.5%) [5]. Leaf oil samples from Katsepy and Tsaramandroso belonged to four chemotypes characterized by (i) linalool (ii) 1,8-cineole iii) geranial/neral and (iv) geranic acid (48.8%) [6]. An EtOAc extract of *C. macrocarpa* led to the identification of ten components, including cinnamacrins A-D, cinnamodial, cinnamolide, bemadienolide, and isopolygodial [1]. Concerning *C. madagascariensis*, thirteen components have been found in the EtOAc bark extract, including cinnamadin, cinnamodial, and cinnamonsmolide [7]. Twenty oil samples isolated from leaves harvested in Tampolo and Ambohitantely belonged to two chemotypes, the first containing caryophyllene oxide (26.6%) and the second 1,8-cineole (30.9%) as major components [6].

The aim of this work was to determine the chemical composition of essential oils (EOs) of *C. madagascariensis* isolated from leaves and bark harvested in northwestern Madagascar and separately subjected to hydro-distillation. Leaf and bark oils were obtained with yields of 0.45% and 0.19%, respectively. Special attention was paid to the identification of cyclocopacamphene, which is only rarely found in EOs.

1. Leaf essential oil: The composition of the leaf oil has been investigated by a combination of GC (Retention indices, RI), GC-MS and ^{13}C NMR (Table 1). Forty-two compounds were identified, accounting for 96.9% of the whole composition. The major components were myrcene (17.9%), limonene (17.8%), β-phellandrene (15.3%) and linalool (12.2%). α-Copaene (5.4%), (*E*)-β-caryophyllene (4.0%), δ-cadinene (3.3%) β-elemol (2.7%), caryophyllene oxide (2.1%), β-eudesmol (1.5%) and α-eudesmol (1.2%) were the main sesquiterpenenes. The chemical composition of our oil sample differed drastically from those reported in the literature, dominated by either caryophyllene oxide or 1,8-cineole. Our results confirmed the chemical variability previously observed for this plant [6].

2. Bark essential oil: The composition of the bark oil has been investigated as above (Table 1). Forty-two identified compounds accounted for 94.3% of the whole EO. The major compounds were β-pinene (49.9%) and α-pinene (19.5%). However, a component (2.0% of the whole composition) was not identified and its identification is detailed bellow.

2.1. Identification of cyclocopacamphene: Retention indices of the unidentified component ($RI_{a/p}$ = 1365/1472) suggested a sesquiterpene hydrocarbon. Computer matching against commercial and laboratory-made MS libraries proposed cyclosativene and effectively the mass spectrum fitted perfectly with that of the compound. RIs on apolar and polar columns were close to those of cyclosativene ($RI_{a/p}$ = 1368/1472). In contrast, none of the signals of cyclosativene were observed in the ^{13}C NMR spectrum of the EO. In order to elucidate its structure, we choose to concentrate this compound. The bark oil displayed a high content of monoterpene hydrocarbons (β-pinene, α-pinene and minor components, in total more than 76%). Monoterpene hydrocarbons were partially evaporated at reduced pressure and low temperature. Then, the remaining extract was subjected to column chromatography (CC) on silica gel (see experimental) and hydrocarbons were separated from oxygenated compounds. Thereby, the percentage of the unknown component was increased until it formed 33.7% in a CC fraction.

Table 1: Chemical composition of leaf and bark essential oils from *C. madagascariensis*.

No	Components[a]	RI lit[b]	RI_a[c]	RI_p[c]	RF[d]	Leaf oil[e]	Bark oil[e]	Identification
1	α-Thujene	932	923	1023	1.03	0.1	-	RI, MS
2	α-Pinene	936	933	1023	1.03	2.4	19.5	RI, MS, ^{13}C NMR
3	Camphene	950	946	1074	1.03	0.1	0.5	RI, MS, ^{13}C NMR
4	Sabinene	973	968	1129	1.03	0.9	0.5	RI, MS, ^{13}C NMR
5	β-Pinene	978	974	1120	1.03	0.6	49.9	RI, MS, ^{13}C NMR
6	Myrcene	987	982	1167	1.03	17.9	0.2	RI, MS, ^{13}C NMR
7	Mentha-1(7),8-diene	997[#]	997	1170	1.03	2.0	-	RI, MS, ^{13}C NMR
8	*p*-Cymene	1015	1014	1279	0.99	0.6	1.3	RI, MS, ^{13}C NMR
9	Limonene*	1025	1023	1208	1.03	17.8	2.1	RI, MS, ^{13}C NMR
10	β-Phellandrene*	1023	1023	1217	1.03	15.3	2.1	RI, MS, ^{13}C NMR
11	(*E*)-β-Ocimene	1041	1037	1249	1.03	0.2	-	RI, MS
12	*cis*-Linalool oxide THF[†]	1058	1059	1449	1.28	0.3	0.3	RI, MS, *^{13}C NMR*
13	*trans*-Linalool oxide THF[†]	1072	1080	1469	1.28	0.2	0.4	RI, MS
14	Linalool	1086	1085	1551	1.30	12.2	1.6	RI, MS, ^{13}C NMR
15	*trans*-Pinocarveol	1126	1125	1652	1.30	-	0.2	RI, MS, *^{13}C NMR*
16	Pinocarvone	1137	1141	1578	1.30	-	0.1	RI, MS, *^{13}C NMR*
17	Borneol	1150	1151	1708	1.30	-	0.1	RI, MS, *^{13}C NMR*
18	Cryptone	1160	1155	1664	1.30	0.4	-	RI, MS, *^{13}C NMR*
19	*p*-Cymen-8-ol	1169	1161‡	1806	1.30	-	0.1	RI, MS, *^{13}C NMR*
20	Terpinen-4-ol	1164	1164‡	1608	1.30	0.6	1.0	RI, MS, ^{13}C NMR
21	Myrtenal	1172	1172	1633	1.30	-	0.1	RI, MS, *^{13}C NMR*
22	α-Terpineol	1176	1174	1695	1.30	0.4	0.7	RI, MS, ^{13}C NMR
23	Myrtenol	1178	1180	1799	1.30	-	0.1	RI, MS, *^{13}C NMR*
24	Thymyl methyl oxide	1215	1210	1595	1.28	-	0.2	RI, MS, *^{13}C NMR*
25	Carvacryl methyl oxide	1226	1226	1610	1.28	-	0.2	RI, MS, *^{13}C NMR*
26	Bornyl acetate	1270	1271	1584	1.59	-	0.2	RI, MS, *^{13}C NMR*
27	α-Terpinyl acetate	1335	1334	1681	1.59	-	0.2	RI, MS, *^{13}C NMR*
28	α-Cubebene	1355	1351	1463	0.98	0.1	0.1	RI, MS
29	Cyclocopacamphene		1365	1479	0.98	0.2	2.0	RI, ^{13}C NMR
30	α-Copaene	1379	1373	1491	0.98	5.4	3.8	RI, MS, ^{13}C NMR
31	β-Elemene	1389	1390	1595	0.98	0.4	0.4	RI, MS, *^{13}C NMR*
32	*cis*-α-Bergamotene	1411	1411	1566	0.98	0.2	-	RI, MS
33	(*E*)-β-Caryophyllene	1421	1421	1604	0.98	4.0	0.5	RI, MS, ^{13}C NMR
34	*trans*-α-Bergamotene	1434	1432	1584	0.98	0.1	0.8	RI, MS, ^{13}C NMR
35	α-Guaiene	1440	1434	1589	0.98	-	0.3	RI, MS
36	(*E*)-β-Farnesene	1446	1447	1662	0.98	0.1	-	RI, MS
37	α-Humulene	1455	1454	1678	0.98	0.6	2.4	RI, MS, ^{13}C NMR
38	2-*epi*-(*E*)-β-Caryophyllene	1467	1458	1641	0.98	0.4	-	RI, MS
39	γ-Muurolene	1474	1473	1686	0.98	0.2	0.5	RI, MS
40	Germacrene D	1479	1476	1703	0.98	0.2	-	RI, MS
41	β-Selinene	1486	1486	1725	0.98	0.1	0.3	RI, MS
42	4-*epi*-Cubebol	1490	1490	1870	1.30	0.1	tr	RI, MS
43	α-Selinene	1494	1495	1718	0.98	-	0.4	RI, MS
44	α-Muurolene	1496	1497	1725	0.98	0.3	0.4	RI, MS, *^{13}C NMR*
45	α-Bulnesene	1503	1502	1721	0.98	-	0.5	RI, MS
46	γ-Cadinene	1507	1506	1753	0.98	0.3	-	RI, MS
47	Calamenene[+]	1517	1512	1826	0.98	-	0.1	RI, MS
48	δ-Cadinene	1520	1517	1764	0.98	3.3	1.1	RI, MS, ^{13}C NMR
49	β-Elemol	1541	1534	2074	1.30	2.7	-	RI, MS, ^{13}C NMR
50	(*E*)-Nerolidol	1553	1549	2044	1.30	0.2	0.2	RI, MS, *^{13}C NMR*
51	Spathulenol	1572	1564	2114	1.30	0.3	-	RI, MS
52	Caryophyllene oxide	1578	1574	1993	1.53	2.1	0.2	RI, MS, *^{13}C NMR*
53	Humulene oxide I	1593	1587	2009	1.53	-	0.1	RI, MS, *^{13}C NMR*
54	Humulene oxide II	1602	1597	2050	1.53	0.3	0.8	RI, MS, ^{13}C NMR
55	γ-Eudesmol	1618	1618	2159	1.30	0.7	-	RI, MS, ^{13}C NMR
56	β-Eudesmol	1641	1635	2219	1.30	1.5	-	RI, MS, ^{13}C NMR
57	α-Eudesmol	1653	1640	2210	1.30	1.2	-	RI, MS, ^{13}C NMR
	Total					96.9	96.3	

[a]Order of elution and relative percentages of individual components are given on apolar column (BP-1), excepted those with an asterisk (*), percentages on polar column (BP-20); [b]RI lit: retention indices according to Holmuscht *et al* [8], otherwise stated. [c]RI_a, RI_p = retention indices measured on apolar and polar capillary columns, respectively. [d]Response factors according to Costa *et al.* [9]. [e]g/100g. *^{13}C NMR (italic)* = compounds identified by NMR in CC fractions. tr = trace level (<0.05%). [#]Retention index according to Weyersthal *et al*. [10]. [†]Nomenclature *cis*/*trans* according to Meou *et al.* [11]. [‡]Order of elution confirmed by NMR of a CC fraction. [+]Correct isomer not determined.

In that fraction, after assignment of the signals belonging to known compounds α-copaene (16.1%), γ-muurolene (3.2%), *trans*-α-bergamotene (2.8%), and α-muurolene (1.9%), a set of 15 signals with high intensities remained unassigned (DEPT spectrum: 4 CH_3, 3 CH_2, 5 CH, and 2 C, in agreement with a mass *m/z* =204). As expected, the ^{1}H NMR spectrum of the CC fraction was complex. However, a signal at δ 0.67 ppm (d, 5.3Hz) led us to suspect a cyclopropane moiety in the framework of the molecule. This hypothesis was corroborated by the observation of specific carbon chemical shifts (CH_3 at δ 10.4 ppm, C and CH at δ 19-23 ppm). Therefore, we oriented our research towards cyclocopacamphene, an epimer of cyclosativene. Indeed, the RI_a fitted with that reported (RI = 1361) in ref [12] and ^{1}H NMR signals picked up on the spectrum fitted with those reported in ref [13]. Unfortunately, the ^{13}C NMR data were not reported. However, comparison of the ^{13}C NMR chemical shifts of our compound with those of cyclocopacamphan-12-ol [14] was informative. Indeed, 11 out of 15 carbon chemical shifts of our unidentified component were very close to those of cyclocopacamphan-12-ol; they differed by 0.0-0.2 ppm for 10 carbons, and 0.4 ppm for the last one. The expected differences in chemical shifts have been observed (Table 2): i) deshielding α-effect on C12 = +43.8 ppm; ii) deshielding β-effect

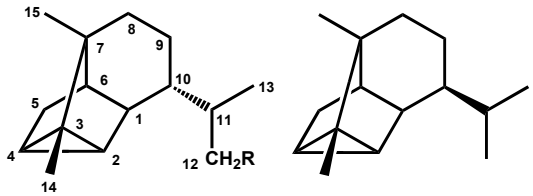

Figure 1: Structure of cyclocopacamphene (left, R=H), cyclocopacamphan-12-ol (left, R=OH) and cyclosativene (right).

Table 2: ^{13}C NMR chemical shifts (δ, ppm) of cyclocopacamphene (CCC) and cyclocopacamphan-12-ol (CCC-12-ol).

Carbons	CCC-12-ol R=OH [14]	CCC R=H	Carbons	CCC-12-ol R=OH [14]	CCC R=H
1	43.9	44.1	**9**	21.5	21.9
2	23.8	23.9	**10**	37.2	42.1
3	22.0	22.1	**11**	36.0	28.5
4	19.0	19.1	**12**	66.2	22.4
5	31.2	31.3	**13**	16.3	21.0
6	40.4	40.2	**14**	10.2	10.4
7	43.4	43.4	**15**	19.4	19.5
8	28.5	28.5			

on C11 = +7.5 ppm, iii) shielding γ-effect on C10 and C13 = -4.9 and -4.6 ppm. We concluded that the unidentified component is cyclocopacamphene.

In order to confirm the syn/anti stereochemistry of the isopropyl group *vs.* the tricyclo[2.2.1.0^{2,6}]heptane substructure, comparison of ^{13}C NMR data of cyclosativene with cyclocopacamphene should be informative. Although cyclosativene was isolated from *Abies magnifica* in 1968, and it is nowadays commercially available, its ^{13}C NMR data have not been reported. Therefore, carbon chemical shifts have been assigned using a full set of NMR sequences (Table 3). They displayed noticeable differences with those of cyclocopacamphene, obviously due to the presence of various steric γ effects of the isopropyl group. For instance, C2 of cyclosativene was shielded by -3.74 ppm. In contrast, C6, C8 and C11 of cyclocopacamphene were shielded by -5.39, -4.26 and -3.51 ppm.

Cyclocopacamphene and cyclosativene have identical RIs on the polar column (RI_p: 1479). A slight difference was observed on the apolar column. The order of elution was established by adding a small quantity of commercial cyclosativene to the hydrocarbon-fraction of CC: cyclocopacamphene (RI_a = 1365) was eluted just before cyclosativene (RI_a = 1367). Cyclocopacamphene has been obtained by either decarboxylation of cyclocamphenic acids [15] or by a multi-steps synthesis [13]. It has also been isolated [16] (without any detail) from vetiver EO. Finally, it has been identified in vetiver oil [17] and among volatiles of pineapple [12].

2.2. Composition of bark oil from Cinnamosma madagascariensis: In total, 43 components, accounting for 96.3% of the whole composition, have been identified in *C. madagascariensis* bark oil (Table 1). Twenty-two compounds have been identified by GC(RI), GC-MS and ^{13}C NMR. Analysis of fractions of chromatography permitted the confirmation of 17 other components by NMR. Identification of cyclocopacamphene has been given in detail below. The composition of the bark oil was largely dominated by β-pinene (49.9%) and α-pinene (19.5%) (Table 1). The contents of other monoterpenes varied between 0.2% and 2.1%. Various sesquiterpenes were present in appreciable amounts: α-copaene (3.8%), α-humulene (2.4%), cyclocopacamphene (2.0%) and δ-cadinene (1.1%). *C. madagascariensis* bark oil differed from the leaf oil of the same plant containing mainly myrcene, limonene, β-phellandrene and linalool. It differed also from other leaf oils with compositions dominated either by caryophyllene oxide or by 1,8-cineole [6].

Experimental

Plant material, isolation, chromatographic fractionation of EO: Leaves and bark of *C. madagascariensis* were harvested in Ambatomainty (northwestern Madagascar). The EO samples were isolated by water distillation using a Clevenger-type apparatus from leaves (213 g) and bark (405 g). Volatile hydrocarbons contained in bark oil (714 mg) were evaporated under reduced pressure at ambient temperature. The remaining mixture was subjected to CC (silica gel, 15 g, 63-200 µm). Hydrocarbons (207 mg) were eluted with *n*-pentane and oxygenated compounds (174 mg) with diethyl ether. Volatile monoterpene hydrocarbons were once again evaporated under vacuum yielding a sub-fraction (70 mg) that was used for NMR analysis.

Gas chromatography and nuclear magnetic resonance: GC-FID analyses and ^{13}C NMR spectra were recorded as previously [18]. 2D NMR spectra of cyclosativene were recorded using Bruker microprograms.

Gas chromatography-mass spectrometry: The essential oil sample was analyzed with a Perkin-Elmer TurboMass detector (quadrupole), directly coupled to a Perkin-Elmer Autosystem XL, equipped with a fused-silica capillary column (50 m x 0.22 mm i.d., film thickness 0.25 µm), BP-1 (polydimethylsiloxane). Carrier gas, helium at 0.8 mL/min; split: 1:74; injection volume: 0.5 µL; injector

Table 3: NMR data of cyclosativene.

No	^{13}C δ (ppm)	^{1}H	^{1}H δ (ppm) (HSQC)	COSY ^{1}H-^{1}H	HMBC H → C	NOESY
1	44.33	1	1.75 (s)	1, 2, 14, 10	1, 2, 3, 5, 7, 9, 11, 15	12
2	20.14	2	0.69 (dm, ^{3}J=5.3 Hz)	4	1, 5, 6, 7, 14	11
3	22.19	-	-	-	-	-
4	19.16	4	0.82 (dm, ^{3}J=5.3 Hz)	2	1, 2, 5, 6, 7	-
5	30.80	5a	1.61 (dt, ^{2}J=10.4 Hz; ^{3}J=1.6 Hz)	1, 5b	1, 2, 4, 6	5b, 15
		5b	1.03 (d, ^{2}J=10.4 Hz)	5a	1, 3, 4, 6, 7	5a
6	45.55	6	1.13 (m)	5a, 6, 8a, 9a, 8b, 9b	2, 3, 4, 8, 9, 15	-
7	43.23	-	-	-	-	-
8	32.78	8a	1.51 (m)	1, 8b, 9b, 10	1, 3, 5, 7, 9, 10, 11, 15	8b
		8b	1.27 (m)	1, 8a, 9a, 10	1, 3, 7, 9, 10, 11	8a
9	24.69	9a	1.48 (m)	1, 8b, 9b, 10	1, 3, 5, 7, 8, 10, 11, 15	9b
		9b	1.27 (m)	1, 8a, 9a, 10	1, 3, 7, 8, 10, 11	9a
10	43.61	10	1.13 (m)	6, 8a, 9a, 8b, 9b	2, 3, 4, 8, 9, 15	-
11	32.00	11	1.41 (m)	1, 10, 12, 13	2, 8, 9, 10, 12, 13	2
12	21.28*	12	0.90* (d, ^{3}J=6.7 Hz)	11	2, 4, 10, 11, 13	6, 11
13	20.88*	13	0.88* (d, ^{3}J=6.7 Hz)	11	2, 10, 11, 12	11
14	10.16	14	0.98 (s)	-	2, 3, 4, 7	-
15	19.27	15	0.76 (s)	-	1, 3, 7, 8, 9, 11	5a

* May be inversed.

temperature: 250°C; oven temperature programmed from 60°C to 220°C at 2°C/min and then held isothermal at 220°C for 20 min. Ion source temperature: 250°C; energy ionization: 70 eV; electron ionization mass spectra were acquired over the mass range 35-350 Da.

Identification and quantification of individual components: Identification of the individual components was based: i) on comparison of their GC retention indices (RI) on apolar and polar columns, with either those of authentic compounds or literature data [8] ii) on computer matching with a laboratory-made and commercial mass spectral libraries and comparison of spectra with literature data [19], iii) on comparison of the signals in the ^{13}C NMR spectra of the mixtures with those of reference spectra compiled in the laboratory spectral library, with the help of laboratory-made software [20]. Quantitative determination was according to Costa *et al.* [9]. Nonane was used as an internal reference; the percentage of each compound was calculated using response factors.

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Synergistic Activity of Essential Oils from Herbs and Spices Used on Meat Products against Food Borne Pathogens

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Essential oils (EOs) could be utilized as natural agents to improve the safety of meat products. However, the high concentration required to achieve an antimicrobial effect in foods might be incompatible with their sensory acceptance. To avoid this problem, combinations of EOs provide an effective approach reducing the odds of sensory rejection. In our study, 13 EOs of herbs and spices commonly used in the seasoning of meat products were assessed for their antimicrobial activity against *Salmonella* spp., *Listeria monocytogenes*, *Escherichia coli* and *Staphylococcus aureus*. However, only 7 of them were selected to study their synergistic effect based on their antimicrobial activity and minimum inhibitory concentration (MIC) against foodborne pathogens. EOs of thyme and cinnamon presented the largest antibacterial activity against foodborne pathogens. Combinations of selected EOs displayed a synergic effect against foodborne pathogens and also an important decrease in their individual MIC. Thyme EO presented the lowest individual MIC, but its utilization in combination decreased the MIC of the other EOs. Utilization of cinnamon EO also improved the reduction of the individual MICs of the EOs of cumin and parsley. Our results suggest the potential use of EO mixtures to control foodborne pathogens in meat products. Although the individual MIC values of selected EOs decreased, the sensory impact on meat products needs to be assessed.

Keywords: Essential oils, Foodborne pathogens, Spices, Minimum inhibitory concentration, Synergism.

Traditional dry-cured meat products are considered safe due to several factors such as reduced pH and a_w, and addition of salt, nitrites, spices and other ingredients, that make it difficult for the survival and growth of food-borne microorganisms [1]. The demand of consumers for safer foods has been leading the industry to the use of preservation methods, namely through the use of natural preservatives [2]. In addition, to increase production yield, a short drying period of meat products may occur with consequent variations of pH, a_w and increased probability of pathogen development. Also, hygiene factors such as cross-contamination, improper cleaning and disinfection procedures, incorrect food handling, time and temperature abuse, improper manufacturing processes or the absence or inadequate implementation of hazard analysis and critical control point plans, among others, imply a risk of food borne contamination [3,4]. To enhance meat products safety, essential oils [EOs] of herbs and spices commonly used in meat products as natural preservatives are receiving increased attention [5]. Herbs and spices, despite their potential use as preservatives in foods, still remain as condiments generally recognized as safe [6]. The antimicrobial effect of EOs against foodborne pathogens has been studied in fresh meat, although research of their application in dry-cured meat products is scarce [7,8]. Because differences regarding their inhibitory effect have been reported among the different EOs [9], *in vitro* inhibitory assessment must be carried out prior to their application in foods [10]. Sensory characteristics are considered a key factor in the consumers´ choice [11]. The organoleptic impact of EOs should be carefully optimized, since if high EO concentrations are required to achieve an adequate antimicrobial effect, the consumer will probably reject the product due to its odour and flavour traits [12]. To overcome that problem, the use of EO combinations to guarantee the antimicrobial activity with lower concentrations is a strategy to reduce the adverse organoleptic effects [13]. Several reports, mainly focused on fresh meat and cooked meat products, assessed the combination of EOs in the inhibition of foodborne pathogens [14,15], but little information is available regarding the use of EOs, either on their own or in combination in dry-cured meat products. The objective of this work was to evaluate the antimicrobial effect of the EOs of herbs and spices commonly used in the manufacture of dry-cured meats, alone and in combination, against *Salmonella* spp, *Escherichia coli*, *Listeria monocytogenes* and *Staphylococcus aureus*.

The main constituents of the 13 EOs studied are presented in Table 1.

The most common compounds among the EOs samples were α-pinene and ß-pinene. In contrast, garlic EO presented unique chemical compounds. The EOs of some plants were quantitatively composed of one major compound that represented more than 85% of the relative composition, such as basil (estragole), cinnamon leaf (eugenol), tarragon (anethole), lemon (limonene), orange (limonene) and thyme (thymol). However, the main chemical compound of the EOs of garlic (diallyltrisulfide), nutmeg (myristicin), bay (eucalyptol) and rosemary (camphor) EOs was under 60% of the total of their relative composition. Regarding the chemical groups, all of EOs studied presented hydrocarbon monoterpenes, 8 of 13 presented hydrocarbon sesquiterpenes and 6 of 13 presented phenylpropanoids. However, sulfur compounds were exclusively present only in garlic EO. Phenylpropanoids were

Table 1: Chemical composition (%) of essential oils of garlic, basil, cumin, tarragon, lemon, orange, cinnamon, bay, thyme, black pepper, parsley, nutmeg and rosemary.

EO	Chemical compound	%	Chemical category	CAS
Basil	Estragole	96.7	Phenylpropene	140-67-0
Bay	Eucaliptol	58.2	Hydrocarbon monoterpene	470-82-6
	α-Terpinenyl acetate	19.2	Oxygenated sesqiterpene	80-26-2
Black pepper	*trans*-Caryophyllene	57.6	Hydrocarbon sesquiterpene	87-44-5
	α-Pinene	4.6	Hydrocarbon monoterpene	80-56-8
Cinnamon	Eugenol	85.3	Phenylpropene	8015-91-6
	trans-Caryophyllene	2.4	Hydrocarbon sesquiterpene	87-44-5
Cumin	Cuminaldehyde	47.5	Aldehyde	122-03-2
	t-Terpinene	18.4	Monoterpene	586-62-9
	p-Cymene	7.5	Alkyl-benzene	99-87-6
Garlic	Diallyl Disulfide	18.9	Organosulfur	2179-57-9
	Diallyltetrasulfide	11.0	Organosulfur	2444-49-7
Lemon	Limonene	84.6	Hydrocarbon monoterpene	138-86-3
	t-Terpinene	4.71	Monoterpene	586-62-9
Nutmeg	Sabinene	23.3	Hydrocarbon monoterpene	3387-41-5
	Myristicin	43.3	Phenylpropene	607-91-0
Orange	Limonene	97.9	Hydrocarbon monoterpene	138-86-3
Parsley	α-Pinene	11.3	Hydrocarbon monoterpene	80-56-8
	Limonene	11.7	Hydrocarbon monoterpene	138-86-3
	Myristicin	44.9	Phenylpropene	607-91-0
Rosemary	α-Pinene	10.8	Hydrocarbon monoterpene	80-56-8
	Eucaliptol	13.2	Hydrocarbon monoterpene	470-82-6
	Camphor	22.4	Oxygenated monoterpene	76-22-2
Tarragon	Anethole	92.7	Phenylpropene	104-46-1
Thyme	Thymol	93.9	Monoterpene phenol	89-83-8

Table 2: Antimicrobial effect of essential oils against *Salmonella* spp., *E. coli*, *L. monocytogenes* and *S. aureus*

Essential oils	*Salmonella*spp	*E. coli*	*L. monocytogenes*	*S. aureus*
Bay	12.5 ± 1.4	NI	NI	37.8 ± 10.1
Black pepper	NI	NI	25.0 ± 4.5	NI
Cinnamon	13.7 ±1.0	12.2 ± 1.0	16.3 ± 1.9	14.8 ± 2.2
Cumin	NI	12.4 ± 1.4	10.7 ± 1.9	40.5± 10.3
Garlic	10.5 ± 1.6	NI	15.0 ± 2.6	41.5 ± 8.6
Lemon	NI	NI	24.3 ± 2.9	NI
Nutmeg	NI	NI	15.5 ± 2.4	NI
Parsley	NI	NI	20.1 ± 3.3	NI
Rosemary	13.4 ± 3.8	NI	23.8 ± 5.4	33.3 ± 3.2
Thyme	29.1 ± 9.5	32.5 ± 7.3	36.4 ± 6.8	45.1 ± 1.9

Zones of inhibition included the disk (6 mm.) diameter. NI: non-inhibitory
Values are presented as mean (mm) ± standard deviation.
Results of EOs of basil, orange and tarragon are not presented due to the absence ofinhibition for all the microorganisms.

the main chemical compounds of basil, cinnamon, nutmeg and parsley. Hydrocarbon monoterpenes were the main chemical compounds of EOs of lemon and orange while oxygenated monoterpenes were the principal chemical compounds of EOs of tarragon, basil, oregano and rosemary. Hydrocarbon sesquiterpenes were found only in 8 of 13 EOs with concentrations lower than 5% except for black pepper where *trans*-caryophyllene was present at a concentration of over 66%. In contrast, none of the EOs studied presented oxygenated sesquiterpenes.

The antibacterial activity of the EOs assessed by the disk diffusion assay (DDA) is presented in Table 2. EOs of thyme, cinnamon, rosemary, cumin, garlic, bay, black pepper, lemon, parsley and nutmeg presented an inhibition halo of over 10mm. However, EOs of orange, basil, tarragon were considered not inhibitory (halo ≤10mm).

The numbers of foodborne pathogens inhibited by the EOs were as follows: thyme and cinnamon (n=4), rosemary and garlic (n=3), cumin and bay (n=2), black pepper, lemon, parsley and nutmeg (n=1). The antimicrobial effect of the EOs was more evident against Gram-positive than Gram-negative bacteria (p<0.001). The antimicrobial effect of each EO was not statistically significant among the strains tested for each group of food borne pathogens (p>0.05)

The MICs of selected EOs are presented in Table 3. The MIC of the EOs of orange, basil and tarragon was not performed due to the absence of an inhibitory effect using the DDA (halo size <10mm).

The MIC values ranged from 62.5 ppm to >300000 ppm being, on average, higher against Gram-negative microorganisms than Gram-positive. The EOs with the highest MIC values were in accordance with the lowest inhibition activity observed in the DDA.

The EOs of thyme, garlic, cumin and cinnamon presented the lowest MIC among the 7 EOs studied. Of the Gram-negative bacteria, *Salmonella* spp. was inhibited mostly by the EOs of thyme (500 ppm), rosemary (5000 ppm) and garlic (20000 ppm), and *E. coli*by thyme (250 ppm), cinnamon (2500 ppm) and cumin (2500 ppm). Among the Gram-positive organisms, *L. monocytogenes* was mostly inhibited by thyme, garlic and cinnamon (MIC from 62.5 to 5000 ppm), while EOs of thyme, garlic and cumin (from 500 to 50000 ppm) were the most inhibitory against *S. aureus*. Moreover, the MIC values were high against *L. monocytogenes* except for bay and garlic.

To study the possibility of reducing undesirable sensory impacts of the selected EOs on dry-cured meat products, we examined the antimicrobial effect of combinations of these EOs based on the results of the DDA, MIC and potential to be used in dry-cured meat products manufacture. The combination of selected EOs against foodborne pathogens and their fractional inhibitory concentration index (FCI) are presented in Table 4.

Almost all combinations of EOs studied displayed a synergic effect against foodborne pathogens. However, the combination of the EOs of thyme/parsley against *L. monocytogenes* and thyme/cumin against *S. aureus* showed an indifferent effect. Also, the combination of thyme/garlic EOs displayed an additive effect against *S. aureus*.

When thyme EO was combined with that of cumin, about a 30-fold decrease in the MIC of cumin EO was observed against *Salmonella* spp., about 60-fold against *E. coli* and 12-fold against *S. aureus*. However, the MIC of thyme EO did not decrease. A combination of EOs of thyme/cinnamon showed a decrease of about 60-fold for the EO of cinnamon against *E. coli* and about 4-fold for that of thyme when tested against *L. monocytogenes*. The combination of thyme

Table 3: Minimal inhibitory concentration (ppm) of essential oils of black pepper (BP), bay (BP), cinnamon (CI), cumin (CU), garlic (GA), lemon (LE), nutmeg (NT), parsley (PR), rosemary (RS), thyme (THY).

Microoganisms	BP	BY	CI	CU	GA	LE	NT	PR	RS	THY
*Salmonella*spp	>300000	50000	100000	50000	20000	>300000	150000	100000	5000	500
E.coli	80000	40000	2500	2500	40000	>300000	150000	40000	2500	250
L. monocytogenes	300000	40000	5000	80000	125	300000	100000	40000	250000	62.5
S. aureus	150000	80000	2500	5000	2500	>300000	80000	40000	80000	62.5

Table 4: Fractional inhibitory concentration (FIC) indices of selected essential oils.

Microorganism	Essential oil combination					
	Thyme (THY) x Cinnamon (CI)					
	MIC_{THY}	FIC_{THY}	MIC_{CI}	FIC_{CI}	FIC	Activity
E. coli	125	0.25	39	0.02	0.27	Synergistic
L. monocytogenes	16	0.03	1250	0.06	0.09	Synergistic
	Cumin (CU) x Cinnamon (CI)					
	MIC_{CU}	FIC_{CU}	MIC_{CI}	FIC_{CI}	FIC	Activity
E. coli	78	0.03	625	0.25	0.28	Synergistic
	Thyme (THY) x Cumin (CU)					
	MIC_{THY}	FIC_{THY}	MIC_{CU}	FIC_{CU}	FIC	Activity
L. monocytogenes	125	0.25	39	0.02	0.27	Synergistic
S. aureus	500	1	156	0.03	1.03	Indifferent
Salmonella spp.	62.5	0.125	1562	0.03	0.15	Synergistic
	Cinnamon (CI) x Parsley (PR)					
	MIC_{CI}	FIC_{CI}	MIC_{PR}	FIC_{PR}	FIC	Activity
L. monocytogenes	5000	0.25	2500	0.03	0.28	Synergistic
	Thyme (THY) x Parsley (PR)					
	MIC_{THY}	FIC_{THY}	MIC_{PR}	FIC_{PR}	FIC	Activity
L. monocytogenes	500	1	2500	0.03	1.03	Indifferent
	Thyme (THY) x Garlic (GA)					
	MIC_{THY}	FIC_{THY}	MIC_{GA}	FIC_{GA}	FIC	Activity
S. aureus	250	0.5	125	0.25	0.75	Additive
	Garlic (GA) x Bay (BY)					
	MIC_{GA}	FIC_{GA}	MIC_{BY}	FIC_{BY}	FIC	Activity
Salmonella spp.	2500	0.125	1562	0.03	0.15	Synergistic
	Thyme (THY) x Rosemary (RS)					
	MIC_{THY}	FIC_{THY}	MIC_{RS}	FIC_{RS}	FIC	Activity
Salmonella spp.	125	0.25	156	0.03	0.28	Synergistic

and garlic EOs showed a decrease in MICs of about 2-fold and 10-fold respectively when tested against *S. aureus*.

Combination of cinnamon/cumin EO showed a decrease in MIC of about 4-fold and 30-fold, respectively, against *E. coli* while the reduction in MIC of cinnamon/parsley EO combination against *L. monocytogenes* was 2-fold and 16-fold, respectively.

Regarding *Samonella* spp, a combination of EOs of thyme/rosemary and garlic/bay resulted in a MIC decrease of 4-fold/30-fold in the first and 8-fold/30-fold in the second.

The application of EOs as antimicrobial agents in food is of great interest for the food industry due to their GRAS status. However, the main constrain of their application in foodstuffs is associated with changes in their organoleptic characteristics since higher concentrations of EOs are required to achieve an antimicrobial effect [16]. Therefore, the combinations of EOs to ensure the food safety without compromising the acceptance of the foodstuff could be a natural alternative [10].

In food use, a previous *in vitro* assessment is necessary to select those EOs with higher antimicrobial activity in DDA and low MIC. In our study, from a total of 13 EOs studied, only 7 of them (thyme, cinnamon, cumin, parsley, garlic, rosemary and bay) presented an inhibitory profile on foodborne pathogens that justifies further studies to evaluate their applicability in meat products manufacture. The antimicrobial effects of selected EOs from herbs and spices have been reported in the literature [5], but information available regarding their synergic effects is scarce.

The antimicrobial activity of thyme EO has been described based on its main chemical compound thymol, a phenolic monoterpenoid [2], which was the main component of our EO under study. The strong inhibitory effect was in accordance with the largest halo in the DDA and the lowest MIC value. A combination of thyme and cumin EOs displayed a synergistic effect against *Salmonella* spp and *E. coli*, although an indifferent effect was observed for *S. aureus*. In contrast, it has been reported [17] that a combination of those EOs displayed an antagonistic activity against *S. typhimurium*, an additive effect against *S. aureus* and an indifferent effect against *E. coli*. The analysis of cumin EO by GC/MS revealed that cuminaldehyde, *p*-cymene and *t*-terpinene were the main chemical compounds [18].

The monoterpene *p*-cymene is the precursor of thymol [2]. Although reports indicated its inefficient antimicrobial characteristic when used alone [19], it enhanced the effect of carvacrol and its analogue thymol [20].

p-Cymene perturbs the stability of cell membranes [20] but does not seem to have an effect on its permeability [21]. An antimicrobial effect of cuminaldehyde against *S. aureus*and *Salmonella*has been reported [22]. The mechanisms of action include inhibition of metabolism energy and interaction with the bacterial cell membrane leading to its disruption [23]. The mode of action of thymol has been associated with its interaction with the membrane proteins, modification of the membrane permeability and intracellular targets. Thus, the disruption caused by *p*-cymene on cell membranes and the change of normal physiology of the microorganisms caused by

cuminaldehyde probably enhanced the interaction with thymol, improving its action on the cell cytoplasm.

The synergistic effect observed for the combination of thyme and cinnamon EOs against *E. coli* and *L. monocytogenes* was associated with its main chemical compounds, thymol and eugenol. A combination of these components has been reported to have either a synergistic [24] or additive action [25] against *E. coli*, while other researchers [26] reported a synergistic effect against *L. innocua*. The synergistic effect of thyme and cinnamon EOs may be associated with the damage to the outer membrane caused by its main chemical compounds [24].

A combination of thyme and rosemary EOs resulted in a synergistic activity against *Salmonella* spp. The chemical components of rosemary EO, eucalyptol and camphor, observed by GC/MS have been referred to as weak antimicrobials [27], but they may enhance the antimicrobial effect of thymol [28], as previously observed for *p*-cymene.

A combination of thyme EO with either parsley EO or garlic EO showed an additive effect against *L. monocytogenes* and *S. aureus*, respectively. Although no inhibitory activity was reported for this combination against *L. innocua* [29], the presence of myristicin, cosmene or ß-pinene with inhibitory properties [30] may explain the additive effect with thyme EO. A similar situation could be addressed for the combination of cinnamon and parsley EOs against *L. monocytogenes*. It is important to note that the combination of different EOs with thyme EO resulted in a large decrease in the respective individual MICs, whereas the MIC of thyme remained equal, as observed for the combination of thyme/cumin EO or experienced a little reduction in the case of combinations of thyme/cinnamon and thyme/garlic. These results are difficult to explain because the current study was carried out with EOs that were composed of a large variety of chemical compounds. However, the interaction of phenolic compounds (thymol) with benzaldehydes (cuminaldehyde) seems to result in a better antimicrobial effect than interaction of phenolic compounds with either phenylpropanoids (eugenol) or sulfur compounds (garlic). These variations could be associated with the different cell targets of each chemical compound.

The antimicrobial effect of garlic is associated with reactions with sulfhydril groups of cellular proteins of the microorganisms disturbing the cellular metabolism [31] in contrast to terpenoids that have the cell membrane as the main target. Thus, the additive effect observed in the combination of thyme/garlic EOs against *S. aureus* and the synergic effect against *Salmonella* spp. of garlic/bay EO, with a large decrease in the MIC of bay EO, could be explained by the different antimicrobial targets of thymol, eucalyptol and sulfur compounds. A combination of cinnamon and cumin EOs displayed a synergic effect against *E. coli* [24] although an additive effect had been reported [32].

The present study showed that the EO from herbs and spices used with meat products as seasoning presented variable antimicrobial activity against *Salmonella* spp, *L. monocytogenes*, *E. coli* and *S. aureus* under *in vitro* conditions. Since these EOs have a potential to be used in meat products as ingredients, the utilization of mixtures may decrease the sensory impact while maintaining the safety and quality of meat products.

Experimental

Plant materials: The EOs [plant of origin – part of the plant which] used in our study were: basil (*Ocimum basilicum*L– leaves and flowering tops), tarragon (*Artemisa dracunculus* L. – flowers and leaves), thyme (*Thymus capitatus* Hoff. *et* Link – flowering tops), cinnamon (*Cinnamomum zeylanicum* Nees C. – leaves), parsley (*Petroselimum sativum* Hoffm. – aerial part of the plant), nugmet (*Myristica fragans* – nuts), lemon (*Citrus limonun* L.- fruit peels), orange (*Citrus sinensis* L.Osberck – fruit peels), black pepper (*Piper nigrum* L. – fruits), rosemary (*Rosmarinus officinalis* L. – entire plant, excluding woody parts), laurel (*Laurus nobilis* L. – leaves) and cumin (*Cuminun cyminum* L. – seeds). All EOs and their technical characteristics were kindly provided by Ventós Chemicals (Barcelona. Spain).

Oil analysis: Components of the EOs were analyzed by gas chromatography–mass spectrometry using a Trace GC Ultra gas chromatograph (GC/MS - Thermo Scientific) coupled to a Polaris*Q* ion trap detector mass spectrometer (Thermo Scientific) and equipped with a Zebron Inferno ZB-5HT 30m x 0.25mm x 0.25µm capillary column (Phenomenex).

The oven temperature for the gas chromatograph was initially maintained at 40°C for 5 min, then ramped at 4°C min^{-1} to 200°C and then at 10ºC min^{-1} to 260ºC, and maintained at 260ºC for 24 min. The injector temperature was 250ºC. The amount of sample injected was 1 µL in split mode (split ratio 1:52) and the carrier gas was helium at a flow rate of 0.9 mL min^{-1}. The mass spectrometer was run in electron impact (EI)] mode with electron energy at 70 eV.

The mass spectrometer was operated in full scan mode between 33 and 300 amu. The retention indices were determined in relation to a homologous series of *n*-alkanes (C_{10}–C_{30}) under the same operating conditions. Further identification was performed by comparing the mass spectra of the components of the EOs with those in the mass spectrometry library (Wiley Registry of Mass Spectra 2001 Library Data, sixth ed.) and data from the literature. Relative percentages of the components were calculated based on gas chromatography peak areas.

Microbial testing: Stock cultures (*Salmonella* spp., *E. coli*, *L. monocytogenes* and *S. aureus*) were isolated either from traditional dry cured sausages during their manufacture or from the environment of their production. For each pathogen, a strain from the culture collection was also included. All wild type isolates used in this study were identified by a species-specific PCR technique [33]. Each microorganism was maintained at -18ºC and sub-cultured twice in Brain Heart Infusion (BHI, Biokar. Beauvais, France). Incubation was made at 37ºC except for *L. monocytogenes* (30ºC). Overnight cultures in BHI were streaked on BHI agar and incubated during 18-24 h. To prepare the inoculum for a sensitivity test to EOs, a suspension of each isolate previously cultured in BHI agar wasmade in NaCl 0.85%. The turbidity of the suspension was adjusted to 2 McFarland standard (Biomerieux, Marcy-l'Etoile, France).

The antimicrobial effect of EOs against *Salmonella* spp., *L. monocytogenes* and *S. aureus* was screened by the disk diffusion assay (DDA), as described [34]. The results represented the net zone of inhibition including the diameter (6 mm) of the paper disk and are the mean of 3 determinations for each isolate tested. The antimicrobial activity of the EOs was considered as inhibitory when the halo´s size was >10 mm.

The minimum inhibitory concentration (MIC) was studied for the EOs that previously showed an inhibitory effect with the DDA (halo size >10mm). The dilutions of the EOs were established based on

the inhibitory profile observed in the DDA. The assay was based on the procedure described [35] with 96-well microtitre plates. EO dilutions were prepared directly on the Mueller-Hinton broth (MHB – Biokar, France) to the double of the desired final concentration of the EO. The inoculum of the target microorganism was prepared also in MHB to double that of the aimed concentration (ca. 5.7 log CFU/mL). In each well, 50 µL of each EO dilution in MHB was combined with 50 µL of each target microorganism suspended in MHB. The plates were covered and incubated during 24 h. The plates were then checked for visible growth in the wells.

The MIC was the lowest concentration of EO constituents at which bacteria failed to grow (no visible changes detected in the broth medium). The test was complemented with the count of appropriate serial dilutions of the culture in the well performed in MHA.

The checkerboard by broth microdilution method was performed using 96-well microtitre plates [36], with some modifications, to obtain the fractional inhibitory concentration FIC index. The microplate assay was arranged as follows: essential oil A (EOA) was diluted two-fold along the x-axis, whilst essential oil B (EOB) was diluted two-fold along the y-axis. Both EOs were diluted directly on MHB to achieve the final concentration desired. The previously determined MIC of each EO, considered as the initial concentration, was added to all wells. The plates were incubated at 37ºC for *Salmonella* spp*, E. coli* and *S. aureus* and at 30ºC for *L monocytogenes* for 24-48 h.

The FIC index was calculated by adding the FIC values of EOA and those of EOB (FICa+FICb). The FICa and FICb values represented the lowest concentrations of each EO that caused inhibition of bacterial growth in the combination tests. Calculations were performed as follows: FICa = (MIC EOA combined/MIC EOA alone); FICb=(MIC EOB/MIC EOB alone); FIC=(FICa+FICb).

The interpretation of the results [10] was as follow: FIC ≤ 0.5 assigned as a synergistic effect, $0.5<FIC\leq 4$ an additive effect, $1<FIC\leq 4$ represented as no interactive effect, and $FIC>4$ indicated an antagonistic effect between two EOs tested.

The comparison of the antimicrobial activity of EO against each microorganism was carried out by one-way analysis of variance. The Tukey-Kramer test was used to determine the significant differences ($p<0.05$) among group means. Statistical analysis was made with SPSS 19.0 software (SPSS Inc., Chicago) for Windows, considering $p< 0.05$ as statistically significant.

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Composition, *in vitro* Cytotoxicity, Anti-mildew and Anti-wood-decay Fungal Activities of the Fruit Essential Oil of *Liquidambar formosana* from Taiwan

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This study investigated the chemical composition, *in vitro* cytotoxicity, anti-mildew, and anti-wood-decay fungal activities of the essential oil isolated from the fruit of *Liquidambar formosana* from Taiwan. The essential oil from the fresh fruit was isolated using hydrodistillation in a Clevenger-type apparatus, and characterized by GC–FID and GC–MS. A total of 45 compounds were identified, representing 98.5% of the essential oil. The main components identified were α-pinene (16.8%), β-caryophyllene (10.1%), τ-muurolol (8.3%), τ-cadinol (7.6%), β-pinene (6.7%), and sabinene (5.7%). The essential oil exhibited cytotoxic activity against human oral, liver, and lung cancer cells. The active source compounds were β-caryophyllene, τ-cadinol, and τ-muurolol. The fruit essential oil was shown to have excellent anti-mildew and anti-wood-decay fungal activities, the active compounds being evaluated as τ-cadinol and τ-muurolol.

Keywords: *Liquidambar formosana*, Essential oil, Cytotoxic activity, Anti-mildew activity, Anti-wood-decay fungal activity.

Liquidambar formosana Hance (Hamamelidaceae) is a tree mainly distributed in Guangdong, Guangxi, Fugian, Jiangxi, Gueizhou, and Sichuan provinces of mainland China and Taiwan [1]. Information pertaining to the composition of this species is scanty. Chien *et al*. [2] analyzed the gum or balsam from the tree and proved that it had antifungal activity. Wang *et al*. [3] found antioxidant activity in a leaf extract, and Lin *et al.* [4] and Zhang *et al.* [5] analyzed the leaf essential oil compositions, but did not elaborate on their bioactivities. In our earlier report [6], we analyzed the leaf essential oil and proved that it had anti-inflammatory activity. However, there is practically no prior report on the essential oil and bioactivities of the fruits of this species. Therefore, in this study, the essential oil was isolated using hydrodistillation and then analyzed. Because malignant cancers have risen to the top of the list among causes of death, and also because the rainy and humid climate in Taiwan is conducive to the growth of mildew and wood decay fungi which endanger health and damage wooden furniture and building materials, the second part of the study examined the anti-cancer, anti-mildew and anti-wood-decay fungal activities of the fruit essential oil. The purpose of this study was to establish a chemical basis for the effective multipurpose utilization of the species.

Hydrodistillation of *L. formosana* fruit gave a yellow essential oil with a yield of 1.52 ± 0.03 mL/100 g, based on the dry weight of fruits. All compounds are listed in order of their elution from the DB-5 column (Table 1). A total of 45 compounds were identified, with monoterpene hydrocarbons predominant (37.6%), followed by sesquiterpene hydrocarbons (32.7%), oxygenated sesquiterpenes (24.8%), and oxygenated monoterpenes (3.4%). Among the monoterpene hydrocarbons, α-pinene (16.8%), β-pinene (6.7%) and sabinene (5.7%) were the chief compounds. Of the sesquiterpene hydrocarbons, β-caryophyllene (10.1%) was the main compound, whereas of the oxygenated sesquiterpenes, τ-muurolol (8.3%), and τ-cadinol (7.6%) were the major components.

Firstly, to evaluate the anticancer potential of *L. formosana* fruit essential oil, we tested its effect on the viability of 3 human cancer cell lines: OEC-M1 (human oral squamous) cells, J5 (human hepatocellular carcinoma) cells, and A549 (human lung adenocarcinoma) cells. Cells were incubated with various concentration of essential oils for 48 h, and then the cell viabilities were measured by the alamarBlue® proliferation assay. The results showed that treatment for 48 h reduced the viability of OEC-M1 cells, J5 cells, and A549 cells, with IC_{50} values around 38.9, 109.8, and 95.5 μg/mL, respectively. This represents the first report of cytotoxic activities of *L. formosana* fruit essential oil against human oral, liver, and lung cancer cells.

Furthermore, to ascertain the compounds responsible for the anticancer activities of the essential oil, the main components, α-pinene, sabinene, β-pinene, β-caryophyllene, τ-cadinol, and τ-muurolol, were individually evaluated for their anticancer activities. The active compounds were β-caryophyllene, τ-cadinol, and τ-muurolol. The IC_{50} values of the three compounds against the three cancer cells were 25.3, 21.6 and 15.6 μg/mL against OEC-M1 cells; 120.8, 36.3, and 32.1 μg/mL against J5 cells; 35.6, 20.8, and 19.3 μg/mL against A549 cells, respectively. These 3 compounds have been shown previously to have cytotoxic activities. For instance, β-caryophyllene has been reported to exhibit anticancer properties [7-10], while τ-cadinol was shown to destroy A-549 and HT-29 human cancer cell lines [8]. In addition, τ-muurolol was cytotoxic to A549, HT-29, and MCF-7 cancer cell lines [11-12].

We also tested the anti-mildew fungal activities of *L. formosana* fruit essential oil. Seven fungi were selected for the tests: *Aspergillus clavatus* (*A. c.*), *A. niger* (*A. n.*), *Cladosporium cladosporioides* (*Cl. c.*), *Chaetomium globosum* (*Ch. g.*), *Myrothecium verrucaria* (*M. v.*), *Penicillium citrinum* (*P. c.*), and *Trichoderma viride* (*T. v.*). These were tested in accordance with the ASTM G21, JIS Z 2911, and ATCC test method [30]. These fungi were either pathogens causing liver cancer, diseases of the genitalia, or which induced bronchitis, allergies and asthma in humans or which degraded cellulosic materials, paper, leather, and wood products [14-16]. Among the fungi tested, the growth of *A. clavatus*, *A. niger*, *Cl. cladosporioides*, *Ch. globosum*, *M. verrucaria*, *Penicillium citrinum*, and *T. viride* was completely

Table 1: Chemical composition of the fruit essential oil from *Liquidambar formosana*.

Consituents	R.I. [a]	R.I. [b]	Concentration (%)	Identification [c]
α-Thujene	923	924	0.3	MS, KI, ST
α-Pinene	937	939	16.8	MS, KI, ST
Camphene	953	954	0.4	MS, KI, ST
Sabinene	973	975	5.7	MS, KI, ST
β-Pinene	976	979	6.7	MS, KI, ST
Myrcene	989	991	0.9	MS, KI, ST
α-Phellandrene	1002	1003	1.9	MS, KI, ST
α-Terpinene	1015	1017	0.5	MS, KI, ST
p-Cymene	1026	1025	1.4	MS, KI, ST
Limonene	1028	1029	1.4	MS, KI, ST
β-Phellandrene	1029	1030	1.0	MS, KI, ST
γ-Terpinene	1058	1060	0.4	MS, KI, ST
Terpinolene	1089	1089	0.2	MS, KI, ST
Terpinen-4-ol	1175	1177	2.1	MS, KI, ST
α-Terpineol	1186	1188	1.0	MS, KI, ST
Safrole	1286	1287	0.3	MS, KI, ST
α-Copaene	1377	1377	0.4	MS, KI
β-Elemene	1390	1391	1.9	MS, KI
α-Gurjunene	1408	1410	0.3	MS, KI
β-Caryophyllene	1418	1419	10.1	MS, KI, ST
α-Humulene	1438	1439	0.8	MS, KI, ST
α-Guaiene	1440	1440	0.6	MS, KI
trans-Cadina-1(6),4-diene	1477	1477	0.7	MS, KI
γ-Muurolene	1481	1480	3.9	MS, KI
10,11-epoxy-Calemenene	1492	1492	0.3	MS, KI
Viridiflorene	1498	1497	0.3	MS, KI
Bicyclogermacrene	1500	1500	1.9	MS, KI
Germacrene A	1510	1509	0.6	MS, KI
γ-Cadinene	1515	1514	1.5	MS, KI
δ-Cadinene	1523	1523	3.9	MS, KI
trans-Calamenene	1531	1529	0.4	MS, KI
trans-Cadina-1(2),4-diene	1537	1535	0.2	MS, KI
α-Cadinene	1540	1539	0.4	MS, KI
α-Calacorene	1546	1546	1.3	MS, KI
Germacrene B	1562	1561	1.3	MS, KI
Germacrene D-4-ol	1576	1576	3.9	MS, KI, ST
Caryophyllene oxide	1583	1583	2.4	MS, KI, ST
Guaiol	1602	1601	0.7	MS, KI
1,10-di-*epi*-Cubenol	1619	1619	0.4	MS, KI
1-*epi*-Cubenol	1630	1629	0.6	MS, KI
γ-Eudesmol	1633	1632	0.2	MS, KI
τ-Cadinol	1642	1640	7.6	MS, KI, ST
τ-Muurolol	1645	1642	8.3	MS, KI, ST
Bulnesol	1673	1672	0.4	MS, KI
Cadalene	1677	1677	2.3	MS, KI
Monoterpene hydrocarbons (%)			37.6	
Oxygenated monoterpenes (%)			3.4	
Sesquiterpene hydrocarbons (%)			32.7	
Oxygenated sesquiterpenes (%)			24.8	
Essential oil yield (ml/100 g)			1.52 ± 0.03	

[a] Relative retention indices, experimental: *n*-alkanes (C9-C24) were used as reference points in the calculation of relative retention indices. [b] Retention index on a DB-5 column with reference to *n*-alkanes [13]. [c] MS, NIST and Wiley library spectra and the literature; RI, Retention index; ST, authentic standard compounds.

inhibited at concentrations of 250, 500, 125, 125, 125, 250, and 500 μg/mL, respectively. Compared with the MIC values of the essential oils from *Phoebe formosana* [17], *Machilus thunbergii* [18], *Juniperus formosana* [19], *Neolitsea narvigemma* [20], *Eucalyptus urophylla*, *E. grandis*, *E. camaldulensis*, and *E. citriodora* [21], the fruit essential oil appeared to be superior. The results verify that *L. formosana* fruit essential oil has excellent antifungal activities.

However, to ascertain the source compounds responsible for the anti-mildew activities, we also tested the major compounds of the oil (Table 2). τ-Muurolol, τ-cadinol, and β-caryophyllene exhibited the best activities among the fruit essential oil constituents, in particular τ-muurolol, which was active against all seven 7 mildew fungi with the highest antifungal indexes ranging from 80% to 100% at 100 μg/mL. Previous studies support the contention that these compounds have significant activity for suppressing microbial growth [22-23].

Table 2: Anti-mildew indices of the six main compounds (100 μg/mL) of the fruit essential oil of *Liquidambar formosana*.

Constituents	Anti-mildew fungal indices (%)						
	A. c.	*A. n.*	*Cl. c.*	*Ch. g.*	*M. v.*	*P. c.*	*T. v.*
α-Pinene	26±0.9	12±0.5	21±0.8	12±0.6	13±0.8	18±0.8	13±0.8
Sabinene	20±0.8	15±0.5	18±0.5	12±0.3	12±0.3	10±0.3	10±0.5
β-Pinene	25±0.9	15±0.8	20±0.6	18±0.5	15±0.3	20±0.5	15±0.3
β-Caryophyllene	70±1.3	60±1.3	70±1.5	48±1.3	60±1.6	38±0.8	42±0.8
τ-Cadinol	100±0	73±1.3	100±0	88±1.8	95±2.1	70±1.6	75±1.9
τ-Muurolol	100±0	80±1.3	100±0	100±0	100±0	85±1.8	96±1.8
Nystatin	100±0	100±0	100±0	100±0	100±0	100±0	100±0

A. c.: *Aspergillus clavatus*; *A. n.*: *A. niger; Ch. g.*: *Chaetonium globosum*; *Cl. c.*: *Cladosporium cladosporoides*; *M. v.*: *Myrothecium verrucaria*; *P. c.*: *Penicillium citrinum*; *T.v.*: *Trichoderma viride*. Nystatin (50 μg/mL) was used as a positive control.

Finally, in an effort to determine the anti-wood-decay fungal activities of *L. formosana* fruit essential oil, 4 fungi were tested, 2 strains of white rot (*Trametes versicolor* and *Phanerochaete chrysosporium*) and 2 strains of brown rot (*Phaeolus schweinitzii* and *Lenzites sulphureu*). The growth of *T. versicolor*, *Phane. chrysosporium*, *Phaeo. schweintizii*, and *L. sulphureu* was completely inhibited at concentrations of 25, 25, 12.5, 12.5 μg/mL, respectively. Compared with the anti-wood-decay fungal activities of essential oils such as those of *M. pseudolongifolia* [24], *M. philippinensis* [25], *Litsea mushaensis* [26], *Lit. coreana* [27], *Lit. acutivena* [28], and *Lit. linii* [26], the fruit essential oil of *L. formosana* was superior. The results verified that *L. formosana* fruit essential oil has excellent anti-wood-decay fungal activities.

Table 3: Anti-wood-decay fungal indices of the six main compounds (50 μg/mL) of the fruit essential oil of *Liquidambar formosana.*

Constituents	Anti-wood-decay fungal indices (%)			
	T. v.	*Phane. c.*	*Phaeo. s.*	*L. s.*
α-Pinene	0	0	6±0.3	12±0.4
Sabinene	0	0	10±0.3	15±0.8
β-Pinene	0	0	8±0.5	12±0.4
β-Caryophyllene	0	0	23±0.8	26±0.8
τ-Cadinol	100±0	100±0	100±0	100±0
τ-Muurolol	100±0	100±0	100±0	100±0
DDAC	100±0	100±0	100±0	100±0

T. v.: *Trametes versicolor*; *Phane. c.*: *Phanerochaete chrysosporium*; *Phaeo. s.*: *Phaeolus schweinitzii, L. s.*: *Lenzites sulphureu*. 2. DDAC (50 μg/mL) was used as a positive control.

The main ingredients of *L. formosana* fruit essential oil were individually tested for their anti-wood-decay fungal activities. The results showed that the activity was due to τ-muurolol and τ-cadinol. At a concentration of 50 μg/mL, τ-muurolol and τ-cadinol showed total growth inhibition against all the white-rot and brown-rot fungi tested (Table 3). The presence of τ-muurolol and τ-cadinol significantly contributed to the wood-decay fungal suppression activity of *L. formosana* fruit essential oil. These two compounds exhibited excellent inhibitory effects against wood decay fungi. Thus, the inhibitive activities exhibited by *L. formosana* fruit essential oil could well be due to the presence of τ-muurolol, and τ-cadinol. The results agree with those of Kondo and Imamura [29], who pointed out that the methanol extract of hinoki (*Chamaecyparis obtusa*) containing τ-cadinol, and τ-muurolol, exhibited excellent inhibitory effects against wood decaying fungi.

Experimental

Plant materials and reference compounds: Fruits of *L. formosana* were collected in September 2015 from Taipei Botanical Garden in north Taiwan (Taipei County, elevation 50 m, N 25º 01′ 48″, E 121º 30′ 35″). The samples were compared with specimen no. ou 58898 from the Herbarium of the National Chung-Hsiung University. The voucher specimen (CLH-052) was deposited in the NIU herbarium. Fruits of the species were collected for subsequent extraction and analysis. α-Pinene, sabinene, β-pinene and β-caryophyllene were purchased from Fluka Co. (Milwaukee, USA),

and τ-Cadinol and τ-muurolol were from an isolate of Ho *et al*'s study on *Machilus pseudolongifolia* [10] and *M. philippinenesis* [11] essential oils.

Isolation of the leaf essential oil: One Kg air-dried *L. formosana* fruits were distilled for 3 h using a Clevenger-type apparatus and a hydrodistillation technique. After distillation, the volume of essential oil obtained was measured, and the essential oil then stored in glass containers that were hermetically sealed with rubber lids, covered with aluminum foil to protect the contents from light, and kept refrigerated at < 4ºC until used. The yield of essential oil was 1.52 ± 0.03 mL/100 g, based on the dry mass of the fruit.

Essential oil analysis: A Hewlett-Packard HP 6890 gas chromatograph equipped with a DB-5 fused silica capillary column (30 m x 0.25 mm x 0.25 µm film thickness, J&W Scientific) and a FID detector was used for the quantitative determination of *L. formosana* fruit essential oil components. The oven temperature was programmed as follows: 50ºC for 2 min, rising to 250ºC at 5 ºC/min. Injector temperature: 270ºC. Carrier gas: He with a flow rate of 1 mL/min. Detector temperature: 250ºC, split ratio: 1:10. Diluted samples (1.0 µl, 1/100, v/v, in ethyl acetate) were injected manually in the split mode. Identification of the essential oil components was based on their retention indices and mass spectra obtained from GC/MS analysis on a Hewlett-Packard HP 6890/HP5973 equipped with a DB-5 fused silica capillary column (30 m x 0.25 mm x 0.25 µm film thickness, J&W Scientific). The GC analysis parameters listed above and the MS were obtained (full scan mode: scan time: 0.3 s, mass range was m/z 30-500) in the EI mode at 70 eV. Data are expressed as the means ± SD of 3 independent experiments.

Component identification: Identification of the fruit essential oil constituents was based on comparisons of retention index (RI) [13], retention times (RT), and mass spectra with those obtained from authentic standards and/or the NIST and Wiley libraries spectra, and literature [13,30].

Cell culture: Human oral squamous cancer OEC-M1 cells, human hepatocellular carcinoma J5, and human lung adenocarcinoma A549 were obtained from ATCC (Rockville, MD, USA) and propagated in RPMI-1640 medium supplemented with 10% heat-inactivated FCS and 2 mM L-glutamine (Life Technologies, Inc., MD), and cultured in a 37°C, 5% CO_2 incubator.

Cell viability assay: The cytotoxicity of the essential oil was assessed using the alamarBlue® proliferation assay according to a protocol from AbD Serotec. Cells (3000 cells/well) were incubated with either essential oils (dissolved in DMSO, final 0.1% DMSO in medium) or vehicle control (0.1% DMSO) for 24 h and 48 h, followed by replacing with fresh medium containing 10% alamarBlue® reagent for an additional 6 h. The absorbances at 570 nm and 600 nm were measured by a microplate reader. All values are given as means ± SD of 3 independent experiments.

Antifungal assays: The method of Su *et al.* [21] was adopted. Mold and wood decay fungi were obtained from the Culture Collection and Research Center of the Food Industry Research and Development Institute, Hsinchu City, Taiwan. References of ASTM G21, JIS Z 2911 and AATCC test method 30 were consulted for the mold fungal strains; 7 strains {*A. clavatus* (ATCC 1007), *A. niger* (ATCC 6275), *Ch. globosum* (ATCC 6205), *Cl. Cladosporioides* (ATCC 13276), *M. verrucaria* (ATCC 9095), *P. citrinum* (ATCC 9849) and *T. viride* (ATCC8678)} were tested. The wood decay fungi used were *T. versicolor* (BCRC 35253), *Phane. chrysosporium* (BCRC 36200), *Phaeo. schweinitzii* (BCRC 35365) and *L. sulphureus* (BCRC 35305). Antifungal assays were carried out in triplicate and data were averaged. Different concentrations of the essential oils (12.5-1000 µg/mL) were added to sterilized potato dextrose agar (PDA). The test plates were incubated at 27°C. When the mycelium of fungi reached the edge of the control plate, the antifungal index was calculated as follows: Anti-fungal index (%)= (1‑Da/Db) X 100, where Da is the diameter of the growth zone in the experimental dish (cm) and Db is the diameter of the growth zone in the control dish (cm). Data are expressed as the means ± SD of 5 independent experiments.

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Chemical Composition and Antibacterial Activity of the Bulgarian Endemic Species *Achillea thracica* from its Natural Habitat, and *in vitro* Propagated and *ex vitro* Established Plants

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The present study reports the chemical composition of headspace volatiles (HS) and acetone extracts of the endemic Bulgarian species *Achillea thracica* Velen. from its natural habitat (N), *in vitro* propagated (IN) and *ex vitro* established (EX) plants. Additionally, acetone extracts were tested by a disk diffusion method for antibacterial activity. Irregular monoterpenes were the most abundant HS volatile constituents, while *O*,*O*-dimethyl quercetin was the most abundant flavonoid in the acetone extracts. The secondary metabolites of *A. thracica* grown in its natural habitat (N), propagated *in vitro* (IN) and *ex vitro* established (EX) showed that the qualitative composition is mutually similar, but there are differences in the quantitative composition. Considering antibacterial activity, IN and EX samples showed moderate activity against *Pseudomonas aeruginosa* and *Escherichia coli*.

Keywords: *Achillea thracica*, *In vitro* propagated plant, *Ex vitro* established plant, Secondary metabolites, Antibacterial activity.

Achillea thracica Velen. (Asteraceae, *Achillea,* sect. *Achillea* s. lat.) is a critically endangered Bulgarian endemic species listed in the Red Data Book of the Republic of Bulgaria [1a] and in the 1997 IUCN Red List of Threatened Plants [1b]. Micropropagation is a suitable method for obtaining sufficient amount of plant material for *ex situ* conservation considering the successful organogenesis, normal histogenesis, lack of hyperhydricity and high survival rate both in *in vitro* propagation and *ex vitro* establishment [2].

According to the SciFinder search, as well as Google scholar (up until October 31 2016), there are no published data on *A. thracica* volatile components and only one paper on the composition of its flavonoids [3]. Therefore, a comparative analysis of the composition and antibacterial activity of the secondary metabolites of plants from their natural habitat (N), *in vitro* propagated (IN) and *ex vitro* established (EX) plants was undertaken.

The *A. thracica* headspace (HS) volatiles identified are listed in Table 1. The compounds consisted mainly of three types. The most abundant were irregular monoterpenes: 58.5% (N), 58.9% (IN) and 53.7% (EX), followed by terpenes: 19.5% (N), 32.6% (IN) and 21.2% (EX), and esters of either methyl butanoic or methyl butenoic acid: 12.5% (N), 2.5% (IN) and 8.4% (EX). Among the irregular monoterpenes, santolina alcohol was the most abundant component in N and EX samples (31.1% and 28.1%, respectively), while yomogi alcohol was dominant in the IN sample (21.6%). The monoterpene eucalyptol (1,8-cineole) was the second most abundant in all samples: 14.4% (N), 19.6% (IN) and 17.5% (EX). Camphor was present in approximately equal amounts in all samples: 1.0% (N), 0.6% (IN) and 0.4% (EX). Isobornyl acrylate was more prevalent in the IN sample (11.8%) than in the N (3.3%) and EX (<0.1%). There was a noticeable difference regarding the esters content; this was much lower in the IN sample than in the N and EX samples. There are no published data on *A. thracica* HS volatiles. We compared the composition of *A. thracica* HS volatiles with that of the essential oils of 23 Balkan *Achillea* taxa [4] because, to some extent, the composition of HS volatiles and essential oil monoterpenoids are comparable [6,7]. With its high content of irregular monoterpenes (19.8%), *A. nobilis* was the most similar to *A. thracica*.

Table 1: Chemical composition (%) of the HS volatiles of *Achillea thracica*.

No	RA[a]	RE[b]	Compound	Composition (%) N[c]	IN[d]	EX[c]	Class
1.	849	848.3	Ethyl 2-methylbutyrate	6.8	t[f]	0.7	E[g]
2.	906	910	Santolinatriene	1.3	3.4	2.8	IM[h]
3.	923	927	Artemisiatriene	8.3	2.3	0.3	IM
4.	999	1001	Yomogi alcohol	13.6	21.6	14.1	IM
5.	1026	1034	1,8-Cineole (*syn.* eucalyptol)	14.4	19.6	17.5	M[i]
6.	1034	1038	Santolina alcohol	31.1	14.5	28.1	IM
7.	1056	1065	Artemisia ketone	0.7	1.1	0.1	IM
8.	1080	1084	Artemisia alcohol	3.5	16.0	8.3	IM
9.	1100	1104	Isopentyl 2-methyl butanoate	2.4	0.7	2.9	E
10.	1102	1106	Isopentyl isovalerate	t	t	2.0	E
11.	1112	1114	3-Methyl-3-butenyl-3-methyl butanoate	0.8	t	0.2	E
12.	1112	1120	β-thujone	0.8	1.2	3.3	M
13.	1141	1148	Camphor	1.0	0.6	0.4	M
14.	1184	1187	(3*Z*)-Hexenyl butanoate	0.4	t	0.4	E
15.	1315	1313	(3*E*)-Hexenyl tiglate	2.1	t	2.2	E
16.	-	1382	Isobornyl acrylate	3.3	11.2	t	M
			Total identified	90.5	94.0	83.3	
			IM	58.5	58.9	53.7	
			M	19.5	32.6	21.2	
			E	12.5	2.5	8.4	

[a])RA – Adam's retention indices); [b])RE – experimental linear retention indices relative to C_8–C_{40} *n*-alkanes on an HP-5MS column; [c])N – plant grown in natural habitat); [d])IN – *in vitro* propagated plant); [e])EX – *ex vitro* established plant; [f])t – Trace (<0.1%); [g])E – esters; [h])IM – irregular monoterpenoids; [i])M – monoterpenoids.

The results of the HPLC analysis of the acetone extracts are given in Table 2. Compound **1** was 3.3 and 4.4 times less abundant in the N sample than in the IN and EX samples, respectively. The presence of components **3** and **4** was reversed: compound **3** in the N sample was 2.1 times higher than in the IN sample and 1.7 times higher than in the EX sample. Compound **2** was not detected in the N sample. Previously, the following flavonoids had been detected in *A. thracica* samples: 3-*O*-methyl quercetin, 7-*O*-methyl quercetin, 3,6-*O*-dimethyl quercetin, 3,7-*O*-dimethyl quercetin, and 3,7-*O*-dimethyl kaempferol, which differed from *A. chrysocoma* and *A. clypeolata* examined in the same study [3].

Table 2: The composition of *Achillea thracica* acetone extracts as percentages of the total absorbance of the HPLC chromatograms recorded at 350 nm.

CN [a]	Compds[b]	Rt[c]	Composition (%) N[d]	IN[e]	EX[f]
1	*O*-methyl quercetin	22.860	8.6	28.4	38.1
2	*O*-methyl kaempferol	24.978	-	3.6	3.0
3	*O,O*-dimethyl quercetin	26.907	63.7	29.7	38.3
4	*O,O*-dimethyl kaempferol	28.520	22.2	13.3	14.7

[a])CN – component number; [b]) positions of methyl groups not defined; methyl group could be in positions 3 and/or 7; [c])Rt – retention time; [d])N – plant growing in natural habitat; [e])IN – *in vitro* propagated plant; [f])EX – *ex vitro* established plant. Components whose peak area is greater than 5% in at least one sample are presented.

The acetone extracts of the *A. thracica* samples, at two different concentrations, were tested against Gram-positive and Gram-negative bacteria (Table 3). Among all the tested extracts, EX (A_{40}) could be distinguished as the most potent, while N (A_4) was the weakest. Only EX (A_{40}) showed activity against *Salmonella typhimurium*. Sample N showed a weak bacteriostatic activity against *E. coli* and *Bacillus subtilis*. IN and EX samples generally showed better activity than that of sample N.

Table 3: Results of antibacterial activity of *Achillea thricaca* extracts.

SL[c]		Bacterial strains C[a]±SD[b]/S[a]±SD[b] *P. aeruginosa*	*E. coli*	*S. typhimurium*	*S. aureus*	*B. subtilis*
EX	A_4	17.5±0.3/-	16.0±0.4/-	-/-	-/-	-/11.5±0.2
	A_{40}	17.5±0.2/-	-/11.5±0.1	10.0±0.0/-	10.0±0.1/-	11.0±0.2/-
IN	A_4	17.5±0.4/-	-/10.5±0.1	-/-	-/-	-/-
	A_{40}	11.5±0.2/-	-/-	-/-	10.1±0.0/-	-/10.7±0.1
N	A_4	-/-	-/20.0±0.5	-/-	-/-	-/-
	A_{40}	-/-	-/18.0±0.5	-/-	-/-	
PP	S1	24.0±0.5/-	17.0±0.5/23.0±0.5	18.0±0.2/20.0±0.3	23.0±0.4/-	23.0±0.4/-
	S2	17.0±0.2/-	24.0±0.6/31.0±0.7	23.0±0.4/32.0±0.8	26.0±0.4/-	30.0±0.6/-
NP	A_0	-/-	-/-	-/-	-/-	-/-

[a])C – bactericidal zone and **S**, bacteriostatic zone (mm); [b]) SD – standard deviation; [c])SL – sample label: the plant grown in natural habitat (N), cultivated *in vitro* (IN) and *ex vitro* adapted (EX); 30 µL of acetone extracts solution was applied to the disk (diameter 9 mm); PP – positive probe of commercially available antibiotics –10 µg for Streptomycin (S1) and 30 µg for Chloramphenicol (S2); NP, acetone (A_0) as negative probe. The absence of activity is marked as dash (-).

The overall results of the chemical analysis of the secondary metabolites of *A. thracica* grown in its natural habitat (N), propagated *in vitro* (IN) and *ex vitro* established (EX) show that there are differences mainly in the quantitative composition of the compounds. Since the plants are genetically the same, these differences can be explained by the different growth conditions of the samples. Morphological analyses positioned *A. thracica* between *A. clypeolata* and *A. filipendulina* [2], although *A. thracica* shows a considerably lower degree of xeromorphism [3]. According to the content of irregular monoterpenes, *A. thracica* is similar to *A. nobilis*. Acetone extracts of *in vitro* propagated and *ex vitro* established samples showed better antibacterial activity against the tested bacterial strains than the wild growing sample. The samples were more potent against Gram-negative bacteria. A possible explanation for the obtained results is the presumption that monomethyl flavanols have higher activity compared with the dimethyl compounds because the total content of monomethyl flavonols in IN and EX samples was 32.0 and 41.1% vs. 8.6% in the N sample. The abundance of dimethyl flavanols in the N sample was approximately twice that in the IN and EX samples (43.0 and 53.0%, respectively).

Experimental

Plant material: *Achillea thracica* Velen. was collected in its natural habitat near Manole village in June 2011 (Plovdiv, Bulgaria). The voucher specimen (SO107385) has been deposited in the Herbarium of Sofia University "St. Kliment Ohridski". *In vitro* shoot cultures and *ex vitro* adaptation were as previously described [3].

Dried, pulverized *A. thracica* (2 g) was extracted with 10 mL of acetone by ultrasonic extraction for 40 min and further by maceration at room temperature for 24 h. All extracts were prepared and analyzed in triplicate. The obtained masses (the mean value ± standard deviation) of extracts were 43.3±0.5 mg, 57.6±0.7 mg and 59.0±0.3 mg for N, IN and EX samples, respectively. HS volatiles were isolated from plant material (300 mg) according to a previously described procedure [5]. GC-MS, GC-FID analysis [5], and HPLC analysis were performed as previously described [5a]. The disk diffusion assay was carried out according to NCCLS [6].

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In vitro and *in vivo* Methods for the Evaluation of Natural Products against Dermatophytes

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Dermatomycoses are infections caused by fungi called dermatophytes; these affect 20-25% of the world population and the incidence continues to grow each year. Recently, an alternative for the treatment of these diseases is the use of natural products, thanks to the fact that they possess great chemical diversity and thus biological activity. However, to understand the therapeutic potential of natural products, their microbiological assessment presents certain limitations. Currently, there is no established reference method to determine the antifungal capacity *in vitro* and *in vivo* of natural products (i.e., essential oils). This review focuses on describing the various microbiological methods as well as the many adaptations used to evaluate the antifungal activity of natural products both *in vitro* and *in vivo*. In addition, the antifungal evaluation of natural products formulated in creams, gels, nanoemulsions, nanocapsules and solid lipid nanoparticles is included.

Keywords: Natural products, Dermatophytes, Susceptibility methods, Formulations.

Introduction

Dermatomycoses or superficial mycoses are infections in which a fungus invades the outer layers of the skin, hair and nails [1]. Specifically, dermatomycoses are caused by fungi called dermatophytes, which have the ability to invade keratinized tissues producing lesions popularly known as "ringworm". It is estimated that this type of disease affects 20-25% of the world population [2-3]. Conventional treatment of superficial mycoses is specifically the use of azoles. In particular, this group of antifungal drugs has a broad spectrum of action; however, the most important limitation is its poor penetration of affected tissues, in addition to the possible emergence of resistance. Recently, an alternative used in the treatment of dermatomycosis is natural products (i.e., essential oils). This is because they have great chemical diversity, and therefore, biological activity, and they are even used directly as therapeutic agents [4].There are numerous reports that indicate the antifungal activity of essential oils against a variety of dermatophytes [5]. However, to be used as antimicrobials they still have certain limitations among which are: i) the high rate of degradation and chemical reactivity of the present compounds, ii) their low solubility in water which limits their biological application, and iii) the short time of bioactivity due to their volatile nature [6]. Therefore, it is necessary to use new alternative technologies that protect the essential oil and facilitate their proper administration without losing their antifungal properties.

At the same time, to define the therapeutic potential of natural products, their microbiological assessment also has certain limitations. Currently, there is no generally established procedure to evaluate the antifungal activity of natural products against strains of dermatophyte fungi. Despite various reports, modifications and adaptations for determining the antifungal activity of natural products, it is difficult to compare the techniques or results obtained in each because of the great methodological variability applied to evaluate this type of active molecules.

In vitro susceptibility methods

Susceptibility tests are performed in order to compare the activity of the active compounds against different strains of microorganisms and detect possible resistance to them.

Microdilution technique, M38-A2 protocol of the Institute of Clinical and Laboratory Standards: For the *in vitro* evaluation of antifungal drugs there is a method approved by the Institute of Clinical and Laboratory Standards (NCCLS), known as microdilution M38-A2 for molds and filamentous fungi [7]. To perform this method it is essential to respect the recommendations established by the CLSI, such as inoculum size 0.5 x 10^3-2.5 x 10^3 CFU/mL, the culture medium that should be used (RPMI 1640), the incubation temperature and time (35°C for 96 h) and finally, the definition and assessment criteria for determining antifungal activity (ie, filamentous fungi can only be classified as sensitive or with reduced sensitivity to the antifungal agent) [8]. There are several reports that use these methodologies as a reference to evaluate the susceptibility of antifungal drugs and also naturally occurring compounds such as essential oils and fractions from plant extracts.

Oliveira *et al.*, in 2008, employed the microdilution technique, CLSI M38-A2, to determine the antifungal activity of oils that had exuded directly from trunks of trees of different species of *Copaifera*. In this study, strains of *Thrichophyton rubrum* ATCC 28189 *T. metagrophytes* ATCC 4481, *Microsporum canis* ATCC 32903 and *M. gypseum* ATCC 14683, and the culture medium

RPMI were used. Evaluation of the minimum inhibitory concentration (MIC) was performed, and a concentrations below 100 ppm was established as a criterion of antifungal activity with this level exhibiting good activity; concentrations ranging from 100 to 500 ppm had moderate activity and from 500 to 1000 ppm, weak activity. Based on their results, it was established that the oils had moderate antifungal activity against four strains of the dermatophyte fungi *T. rubrum* and *M. canis*. This also explains the difference in chemical composition between different species of *Copaifera*; likewise, the results indicate that very little is known about the relationship between chemical structures and biological activity of these compounds [9].

Cavaleiro *et al.* used the same microdilution technique, CLSI M38-A2, to evaluate the antifungal activity of essential oils of *Eryngium duriaei* against seven strains of dermatophyte fungi. Strains were used of *Epidermophyton floccosum* FF9, *M. gypseum* CECT 2905, *M. canis* FF1, *Thrichophyton verrucosum* CECT 2992, *T. rubrum* CECT 2794, *T. mentagrophytes* interdigital varieties CECT 2958 and FF7. This technique established an MIC of 0.16 uL/mL for *E. floccosum* FF9, *T. verrucosum* CECT 2992, *T. rubrum* CECT 2794 and *T. mentagrophytes* FF7. It also established an MIC of 0.32 µL/mL for *M. gypseum* CECT 2905, *T. mentagrophytes* interdigital variety CECT 2958, and *M. canis* FF1. This behavior was related to the presence of elevated concentrations of β-betulenal and 14-hydroxy-β-caryophyllene oxide [10].

Sanguinetti *et al.* evaluated the antifungal activity of *Citrus bergamia* (natural essence, distilled extract and coumarin-free extract) against clinical isolates of seven dermatophyte species: *E. floccosum, T. rubrum, T. mentagrophytes, T. interdigitale, T. tonsurans, M. canis* and *M. gypseum*. The microdilution technique, CLSI M38-A2, was modified by the addion of polysorbate 80 at a concentration of 0.001%, v/v. This technique allowed the establishment of an MIC in a concentration range of 0.16% to 2.5% for the natural essence, of 0.02% to 2.5% for the distillate extract and of 0.08% to 1.25% for the coumarin-free extract. Based on the above, it was established that the extracts (distilled and coumarin-free) had enhanced activity against the seven dermatophyte species. This activity was attributed specifically to the absence of bergapten (a phototoxic compound) [11].

Pinto *et al*. used the microdilution method CLSI M38-A2 to evaluate the antifungal activity of the essential oil of *Syzygium aromaticum* against clinical isolates of five dermatophytes: *E. floccosum*, *M. canis*, *M. gypseum, T. mentagrophytes* and *T. rubrum*. An MIC of 0.16 µL/mL for *E. floccosum, M. gypseum, T. mentagrophytes* and *T. rubrum* was established and of 0.08 uL/mL for *M. canis*. In this paper, it was concluded that the essential oil of *S. aromaticum* showed good activity against these five fungi. This activity was attributed to the high concentration of eugenol (85.3%) and not to the complex mixture of components constituting the essential oil [12]. The relationship between antimicrobial activity and the presence of phenolic hydroxyl groups in the essential oil components has been previously reported. Specifically, it was proposed that eugenol, with phenolic groups in its structure, alters the lipid bilayer of the cell and mitochondrial membrane. These modifications produce functional changes in the cell causing cell death by apoptosis [13].

Cavaleiro *et al.* performed an antifungal evaluation of the essential oil of *Angelica major* against seven species of dermatophyte fungi: *M. canis* FF1, *T. mentagrophytes* FF7, *E. floccosum* FF9, *M. gypseum* 2908, *T. rubrum* 2794, *T. interdigitale* 2968 and *T. verrucosum* 2992. They used the microdilution technique CLSI M38-A2, which consists of making a serial dilution of the essential oil of *A. major* in a concentration range 0.02 to 20 µL/mL in DMSO, at a maximum concentration of 1%, v/v. An MIC was set in a range of 0.32-0.64 µL/mL. Based on the above, Cavaleiro *et al.* found it difficult to attribute the biological activity of the essential oil to any one of its constituents, taking into account that essential oils are complex mixtures of various compounds. It also indicates that between major and minor components, complex chemical interactions could arise, which could cause a synergetic or antagonistic effect. However, the fact that major components of the essential oil are responsible for its antifungal activity cannot be ruled out. Therefore, the antifungal activity of *A. major* can be attributed to the presence, in greater proportion, of α-pinene and *cis*-β-*O*-cymene [14].

Recently Zeng *et al.* evaluated the antifungal activity of the essential oil of fennel (*Foeniculum vulgare* L.) against *T. rubrum* ATCC 40051 and five clinical isolates: *T. rubrum* 10-0982, *T. rubrum* 10-0403, *T. tonsurans* 10-0400, *T. mentagrophytes* 10-0060 and *M. gypseum* 44693-1. The microdilution technique CLSI M38-A2 was used adding polysorbate 20 (0.01%, v/v) to the culture medium (RPMI 1640). MIC values were established in a concentration range of 0.039-0.078 µL/mL for the previously mentioned strains. They demonstrated that the high antifungal activity of the essential oil of fennel can be attributed to the presence of compounds such as *trans*-anethole, pinene and fenchone [15].

It is noteworthy that the microdilution method CLSI M38-A2 has also been used to evaluate the antifungal activity of the polyphenol epigallocatechin-3-*O*-gallate (the main component of green tea) against thirty-five clinical isolates of dermatophyte fungal strains of *M. canis, T. mentagrophytes* and *T. rubrum*. With these results, it was concluded that this compound exhibited good activity, even when compared with the MIC obtained for fluconazole, the reference drug. Additionally, Park *et al.* suggest the use of polyphenol as a potential antifungal agent and its combination with other antifungal agents for the treatment of dermatophytosis [16]. When performing the test, an MIC of 2 µg/mL (p/v) was established for *M. canis* and *T. rubrum*, while for *T. mentagrophytes* an MIC of 4 µg/mL, w/v, was established. Park *et al.* observed that *T.rubrum* is the fungus most susceptible to epigallocatechin-3-*O*-gallate or fluconazole because both have similar MIC values (2 µg/mL). Although the mechanism of action of the active ingredients against dermatophytes has not yet been defined, the use of the polyphenol is suggested as an adjuvant antifungal agent to be applied in the field of antifungal therapy.

Agar dilution technique: This technique consists of making serial dilutions of the sample, which are carried out in Petri dishes and the fungus is inoculated on the surface of the plate. The inhibitory effect is established with the plate count method [17]. Despite various reports which used the reference method CLSI M38-A2, which is based on a liquid culture medium for the *in vitro* evaluation of antifungal activity, a method based on agar continues to be used to determine susceptibility of dermatophytes. The advantages of using an agar-based method include: reproducibility, precision, easy handling, and low cost [18]. There are several reports where an agar-based methodology is proposed to determine the antifungal activity against dermatophytes; however, there is only one report where this evaluation is used with natural products.

Vannini *et al.* evaluated the antifungal activity of the essential oil of *Baccharis uncinella* and *B. semiserrata* against dermatophyte fungi using the agar-dilution method. They used the strains *E. loccosum* (C114), *M. canis* (C112), *M. gypseum* (C115), *T. mentagrophytes*

(ATCC 9972) and *T. rubrum* (C137). The essential oil was added to the Petri dishes after dissolution in DMSO (40%) in a concentration range of 1000 to 7.8 µg/mL, v/v. The inoculum used was a suspension of spores adjusted between 1.0 x 10^6 to 5.0 x 10^6 spores/mL [18]. The essential oil of *B. uncinella* presented moderate antifungal activity against *M. canis* with an MIC of 500 µg/mL. In the case of the essential oil of *B. semiserrata***,** it presented moderate activity against *E. floccosum* and *T. mentagrophytes*. However, both oils showed weak activity against strains of *M. gypseum* with an MIC of 1000 µg/mL. Antifungal activity was attributed to the presence of a high content of oxygenated sesquiterpenes in *B. uncinella* oil. In the case of *B.semiserrata* oil, its activity is attributed to the presence of components such as γ-muurolol, α-cadinol, and caryophyllene oxide, which exhibit a *cis-trans* steric configuration and an axial hydroxyl group that have the ability to inhibit mycelial growth [19].

Using the same method, Rahman *et al*. determined the antifungal activity of the essential oil, the ethanolic extract, and the fractions (hexane, chloroform and ethyl acetate) of *Lonicera japonica* Thunb. For this, they performed an antifungal evaluation using the following strains of dermatophyte fungi: *M. canis* KCTC 6348, *M. canis* KCTC 6349, *M. canis* 6591, *T. rubrum* KCTC 6345, *T. rubrum* KCTC 6352, *T. rubrum* KCTC 6375, *T. mentagrophytes* KCTC 6077 and *T. mentagrophytes* KCTC 6085. The oil, the extract and its fractions were dissolved in DMSO, and added to the culture medium (Sabouraud) to obtain concentrations in the range 62.5 to 2000 µg/mL. They were then inoculated with 5 µL of a spore suspension (10^5 spores/mL). The essential oil and extract of *L. japonica* showed MICs of 62.5 to 500 µg/mL and 125 to 1000 µg/mL, respectively. Rahman *et al.* indicate that the antifungal activity of the ethanolic extract and its fractions could be attributed to the presence of some bioactive phenolic compounds. The activity of the essential oil can be attributed to the presence of phenolic compounds and mono- and oxygenated sesquiterpenes, as well as to sesquiterpene hydrocarbons. It also indicates previous reports in which this activity is specifically attributed to the components present in low amounts such as espatulenol, citrolenelil, α-cadinol and 1,8-cineol. Finally, it is mentioned that minor components are probably involved in some kind of synergism with other active compounds generating a high antifungal activity [20].

Iranshahi *et al*. evaluated the antifungal activity of the essential oil of *Ferula latisecta* fruits against dermatophyte fungi using the agar-dilution method. The essential oil presented good antifungal activity against *T. rubrum and T. verrucosom* with an MIC of 96 µg/mL. Antifungal activity was attributed to the presence of sulfur-containing components of the oil [21]. Machado *et al*. reported antifungal activity of the methanol extract, its fractions (dichloromethane and ethyl acetate) and of compounds isolated from fraction of the fruits of *Eugenia umbelliflora* against clinical isolates of five dermatophyte species: *E. floccosum, T. rubrum, T. mentagrophytes, M. canis* and *M. gypseum*. The agar-dilution method allowed the establishment of an MIC in a concentration range of 200 to 1000 µg/mL [22].

Well-diffusion technique and disk adaptation document M44-A2, 2010 CLSI: Briefly, this methodology consists of filling Petri dishes 5-12 cm in diameter with 10-20 mL of agar and then inoculating them with microorganisms. The active (i.e. essential oil) material is placed either on filter paper discs or in wells made in the agar. This procedure should be performed with active solutions of different concentrations. The effectiveness of the active solution is established by measuring the inhibition zone (diameter in mm or cm) around the disk or well [23].

Guerra-Boone *et al*. proposed use of the well diffusion and disk plate technique by adapting the method established by CLSI document M44-A2, 2010. They evaluated the antifungal activity of essential oils of *Magnolia grandiflora* (Mg), *Chrysactinia mexicana* (Cm), *Schinus molle* (Sm), *Thymus vulgaris* (Tv), *Rosmarinus officinalis* (Ro) and *Origanum majorana* (Om) against six strains of dermatophyte fungi: *T. rubrum*, *T. tonsurans*, *T. mentagrophytes*, *M. gypseum*, *M. canis* and *E. floccosum*. The culture medium in which the disk and well evaluations were performed was Muller-Hinton agar (glucose 2%, w/w). For disk diffusion, a disc of filter paper was used with 5 µL of each essential oil on a Petri plate previously inoculated with a conidial suspension equivalent to 0.5 McFarland. For well diffusion, it was necessary to remove a cylindrical portion of agar and then place 5 µL of each essential oil in the formed well. After 48 hours, it was observed that only the essential oil of Mg caused complete inhibition of *E. floccosum* [24]. However, the essential oil of Tv showed complete inhibition of the six strains of dermatophytes and the essential oil of Om presented complete inhibition only of *T. rubrum*, *T. mentagrophytes* and *E. floccosum* [25]. The antifungal activity of Tv and Om was attributed to the presence of terpenoid compounds such as thymol and carvacrol, which cause damage to the membrane of fungi by inhibiting the synthesis of ergosterol, one of its main components.

Poisoned food technique methodology: This technique consists of using cultures with five days of growth, which are perforated aseptically with a sterile bore (7 mm diameter). Mycelium discs are placed on a gel agar plate. Agar plates were prepared by impregnating these with the desired concentration of active solution at 45-50°C. Finally, the plates are incubated at 26°C and afterwards, the diameter of the colony is measured to establish inhibition of mycelial growth [26].

Marwah *et al.* evaluated the antifungal activity of the essential oil of *Plectranthus cylindraceus* against four species of dermatophytes: *T. rubrum* (ATCC 28188), *T. mentagrophytes* (ATCC 18748), *M. gypseum* (ATCC 24102) and *M. canis* (ATCC 36299). They made this assessment using the *poisoned food technique*, which consisted of mixing different amounts of essential oil and polysorbate 20, 0.01%, w/w, and subsequently adding this to the potato dextrose agar (PDA) culture medium with 0.01% polysorbate (w/w), to finally obtain essential oil concentrations in the range of 125-500 µg/mL. The plates were then inoculated with mycelial disks (5 mm diameter) from cultures grown seven days before. After three days, complete inhibition of *T. rubrum*, *M. gypseum* and *M. canis* was observed, when evaluated at a concentration of 250 µg/mL. The antifungal activity of the oil of *P. cylindraceus* was attributed to the presence of the oxygenated monoterpene carvacrol with a hydroxyl group in the meta-position. Bactericidal and a broad spectrum fungicidal activity have been attributed to this compound [27].

In vivo susceptibility methods: There are few reports that seek to demonstrate the *in vivo* effectiveness of several naturally occurring molecules. The *in vivo* technique of susceptibility consists of infecting the backs of Guinea pigs with spores of *T. mentagrophytes* on a (previously shaven) surface of 4 cm² to begin the application of treatment for six days after the infection process. The results in this trial are obtained only by direct observation of the evolution of the infected area (observation and classification of the level of inflammation and lesions present) and an agar culture of scrapings made in the same area.

Njateng *et al*. performed an antifungal *in vivo* evaluation of a dichloromethane:methanol (1:1,v/v) extract of the bark of *Polyscias fulva* Hiern (Araliaceae). Initially, they established the MIC by the

agar microdilution method in eight species of dermatophytes: *T. rubrum*, *T. mentagrophytes* (E1425), *T. ajelloi*, *T. equinum*¸ *T. terrestre*, *M. audoumii, M. gypseum* (E1420) and *M. canis* (E1423). The MICs were established in a range of 0.5 to 1 mg/mL for the crude extract of *P. fulva* for the eight dermatophyte species. To perform the *in vivo* evaluation, Guinea pigs were inoculated with 50 µL of a spore suspension adjusted to 0.6 x 10^6–1.4 x 10^6 CFU/mL of *T. mentagrophytes*. Treatments were: i) palm oil with antifungal (griseofulvin 5% w/v), and ii) palm oil extract at three different concentrations (5, 2.5, and 1.25%, w/v). After nineteen days of treatment, it was established that the activity of the extract was dose dependent and also that the topical infection was eradicated after fourteen days of daily treatment. This activity is attributed to the presence of saponins, tannins, alkaloids, anthraquinones and phenols. Finally, it was established that the bark extract of *P. fulva* 5%, w/w, had an *in vivo* activity against *T. mentagrophytes* similar to that of the antifungal griseofulvin (antifungal control), after 14 days of application. It was concluded that this *in vivo* assay is a good predictor model to evaluate topical antifungal agents [28].

Kishore *et al.* also employed the same Guinea pig model and reported *in vivo* antifungal activity of an ointment prepared from petrolatum and essential oil of *Chenopodium ambrosoides*. Initially, the MIC of the essential oil was determined by the poisoned food technique with two species of dermatophytes, *T. mentagrophytes* and *Microsporum audoumii*. With the results obtained, an MIC of 500 ppm for both species of fungi was established. For the antifungal activity of the essential oil *in vivo*, the Guinea pigs were inoculated with *T. mentagrophytes* and *M. audoumii*. After six days, the ointment with the essential oil was applied on the lesions twice daily. Finally, it was established that *C. ambrosoides* inhibits the growth of both *T. mentagrophytes* and *M. audoumii* at a concentration of 50 ppm after 15 and 13 days of treatment, respectively [29]. The observed effect is attributed to the presence of ascariodole (terpene peroxide) as a major constituent of the essential oil.

Mikaeili *et al.* determined the antifungal activity *in vitro* and *in vivo* of three extracts of *Astragalus verus* utilizing the Guinea pig model. In a first stage, the MIC was established for *T. verrucosum* by the disk diffusion method in SDA (Sabouraud Dextrose Agar). The assay was performed with aqueous, methanolic, and acetone extracts. An MIC of 160 mg/mL was established for aqueous and methanolic extracts, while for acetone an MIC of 320 mg/mL was determined. Subsequently, an *in vivo* assay was performed, where five groups of five animals each were evaluated. The treatment was administered with an aqueous extract of *A. verus* at 10, 20 and 40%, w/v. An inoculum of 0.1 mL of a suspension of conidia of *T. verrucosum*, and the formulations were applied 72 hours post-infection. The formulations were administered once a day for a week. It was shown that the formulation that completely eliminated the infection was the 20% extract; also it was shown that the extract had a dose-dependent effect [30].

The use of *in vivo* techniques to assess the biological activity of active substances, specifically natural products, is an effective tool to establish their antifungal activity for possible topical application. However, for use as antifungal agents, natural products still have certain limitations that are related to their physicochemical properties, such as solubility, volatility, chemical reactivity, and degradation. Therefore, it is necessary to search for a vehicle or carrier that protects and facilitates their administration and their subsequent release into the site of action without changing the antifungal activity. Conventional means for topical administration of active ingredients include the use of vehicles such as ointments, creams, gels, and in recent years, the use of nanoemulsions and nanoparticles [31-32].

Evaluation of topical formulations of natural products: Khosravi *et al.* developed a cream formulation adding the methanol extract of *Zataria multiflora* for the treatment of superficial mycoses. First, the antidermatophytic activity of the extract was evaluated against four different species of dermatophyte fungi. Inocula of *T. rubrum* (PTCC 5143), *M. gypseum* (PTCC 5070), *M. canis* (PTCC 5069), and *T. verrucosum* (PTCC 5056) were used. The *in vitro* tube dilution technique was applied to establish the MIC of the extract at a concentration of 0.5%, w/v. The MICs were established for dermatophytes: 2.5 mg/mL for *T. verrucosum* and 5 mg/mL for *T. rubrum*, *M. gypseum*, and *M. canis*. Subsequently, three creams were formulated with the extract of *Z. multiflora* (1, 2 and 3%). Finally, the cream containing *Z. multiflora* extract 2%, w/w was proposed as the optimum formulation since it prevented fungal growth and had good stability. This activity is related to the presence of phenolic structures such as carvacrol and thymol, the same as those found for *Z. multiflora* [33]. However, an evaluation of the permeation and *in vivo* efficacy of this formulation is still required to confirm its use for skin protection [34].

Moghimipour *et al*. developed a cream formulation with the ethanolic extract of *Eucalyptus camaldulensis* to treat dermatophytosis. Following the same procedure as Nasrin, in the first stage they determined the MIC of the extract of *E. camaldulensis* by the agar diffusion method (SDA medium). The four species of dermatophyte fungi used were *T. rubrum* (PTCC 5143), *M. gypseum* (PTCC 5070), *M. canis* (PTCC 5069) and *T. verrucosum* (PTCC 5056). MICs of 0.9 mg/mL for *M. gypseum* and *M. canis*, 0.8 mg/mL for *T. rubrum*, and 0.6 mg/mL for *T. verrucosum* were established. Subsequently, different formulations containing *E. camaldulensis* extract 1%, w/v, were evaluated. This activity was attributed to the presence of components such as saponins and phenols [35]. Moghimipour *et al.* also made a liposome-based gel formulation loaded with essential oil from leaves of *E. camaldulensis*. In the first stage, the antifungal activity of the essential oil by the plate diffusion method was determined using the same four species of dermatophytes. In a second stage, the MIC for each was established by the agar diffusion method (SDA). It was observed that the presence of the essential oil of *E.camaldulensis* in liposomes improved the stability of the formulation as well as its antifungal activity [36].

Svetlichny *et al.* evaluated the antifungal activity of solid lipid nanoparticles which contained oil from *Copaifera martii*, with and without allantoin. They determined the MIC by the microdilution technique CLSI M38-A2 for *T. rubrum* TRU31 and *M. canis* MCW3. An MIC of 1.95 µg/mL for *M. canis* MCW3 was established for both formulations of nanoparticles. While for *T. rubrum* an MIC of 500 µg/mL was found for the formulation of nanoparticles with copaiba essential oil and of 1.95 µg/mL for the formulation with allantoin. Svetlichny *et al.* concluded that nanoencapsulation technology associated with copaiba oil showed effectiveness in terms of release of active ingredients and antifungal activity [37].

Table 1 show a summary of various references with the formulations of natural products.

Conclusion: Due to their chemical diversity, natural products are a very important alternative for treatment of dermatomycoses. However, to be used as therapeutic agents, they still show certain physical and chemical limitations that hinder their administration.

Table 1: *In vitro* and *in vivo* methods for evaluating the susceptibility of dermatophytes to natural products.

Natural product	Methodology used	Results	Reference
Copaifera oils	Microdilution method CLSI M38-A2	Moderate antifungal activity against two strains of dermatophyte fungi was observed and MICs of 250 to 500 µg/mL were established.	9
Oils of *Eryngium duriaei*		The antifungal activity against seven strains of dermatophyte fungi was evaluated and an MIC of 0.16 to 0.32 $\mu g/mL^{-1}$ was established.	10
Oil of *Citrus bergamia*		The antifungal activity against clinical isolates of dermatophyte fungi was evaluated and an MIC of 0.16 to 2.5% was established. Polysorbate 80 was added to the culture medium.	11
Essential oil of *Syzygium aromaticum*		Good antifungal activity against five strains of dermatophyte fungi was observed and an MIC of 0.08 to 0.16- 32 $\mu L/mL^{-1}$ was established. Activity was attributed to the presence of eugenol.	12
Essential oil of *Angelica major*		The evaluation of a range of oil concentrations from 0.02 to 20 µL/mL was performed and an MIC of 0.32 to 0.64 µL/mL was established. The activity is attributed to the interaction between major and minor components present in the essential oil. In addition to the presence of the compounds α-pinene and *cis*-β-cymene.	14
Essential oil of *Foeniculum vulgare*		The essential oil showed a high antifungal activity since MIC values were established in a concentration range of 0.039 to 0.078 µL/mL, against six strains of dermatophyte fungi. The activity is attributed to the presence of compounds such as *trans*-anethole, pinene and fenchone.	15
Polyphenol epigalocatequin-3-*O*-gallate		Good antifungal activity against thirty-five clinical isolates of fungi and dermatophytes was observed and an MIC of 2 to 4 µg/mL was established.	16
Oil of *Copaifera martii*		An evaluation of solid lipid nanoparticles containing oil of *C martii* with and without allantoin was performed. The formulation with allantoin presented an MIC of 1.95 µg/mL against two fungi. However, the formulation without allantoin presented an MIC of 500 µg/mL for *M canis* MCW3..	37
Essential oil of *Baccharis uncinella* and *B. semiserrata*	Agar dilution technique.	The concentrations of essential oil employed were 1000 to 7.8 µg/mL (in DMSO 40%, v/v) and these only showed moderate antifungal activity against five strains of dermatophyte fungi.	18
Essential oil and ethanolic extract and its fractions (hexane, chloroform and ethyl acetate) of *Lonicera japonica*		The evaluation was performed at a concentration range of 62.5 to 2000 µg/mL. MICs of 62.5 to 500 µg/mL were established for the essential oil and an MIC of 125 to 1000 µg/mL for *L. japonica* extract. Activity is attributed to the phenolic compounds, mono-oxygenated sesquiterpenes as well as minor components.	20
Essential oil of *Boswellia sacra, Citrus bergamia, C. limon, C. medica, Cinnamomum zeylanicum, Eucalyptus globulus, Foeniculum vulgare, Helichrysum italicum, Illicium verum, Litsea cubeba, Mentha spicata, Myrtus communis, Ocimum basilicum, Origanum mejorana, O. vulgare, Pelargonium graveolens, Rosmarinus officinalis, Santalum album, Satureja montana* and *Thymus serpyllum*		The evaluation was performed at a concentration range of 10% to 0.01% (in culture medium). More effective essential oils were *T. serpyllum* with MICs of 0.025% to 0.25%, *O. vulgare* with MICs of 0.025% to 0.5% and *L. cubeba* with MICs of 0.025% to 1.5% against five dermatophytes. Activity is attributed to the presence of compounds such as thymol, carvacrole and neral, respectively.	39
Essential oil of *Thymus vulgaris* (Tv), *Origanum majorana* (Om) and *Magnolia grandiflora* (Mg)	Plate with disk technique technique with an adaptation established by CLSI-M44-A2.	It was established that the oils exhibited good antifungal activity against six strains of dermatophytes. The oil of Tv and Om showed complete inhibition and Mg showed large halos of inhibition. This activity is attributed to volatile components present in the essential oils.	24, 25
Essential oil of *Plectranthus cylandraceus*	*Poisoned food technique* methodology.	Complete inhibition of all four strains of dermatophytes employed was observed. Activity attributed to the presence of carvacrol, a major component present in the essential oil. Polysorbate 20 was added to the culture medium.	27
Methanol extract of *Zataria multiflora*	*In vitro* tube dilution technique	An MIC of 0.5% against four species of dermatophyte fungi was established. Cream is proposed as an optimal formulation (water/oil) with *Z. multiflora* extract 2% (v/v) added.	33
Ethanol extract of *Eucalyptus camaldulensis*		An MIC of 0.9 mg/mL was established against four species of dermatophyte fungi. The final product was a cream (water/oil) with an ethanol extract of *Eucalyptus camaldulensis* 1% added	35
Essential oil of *Eucalyptus camaldulensis*	Plate diffusion method Freeze thaw technique	It was established that the liposomal formulation effectively improves the stability of the essential oil and could favor its antifungal activity.	36
Essential oil of *Melaleuca alternifolia*	*In vitro* evaluation of the antifungal activity of two onychomycosis models	Evaluation of two nanostructured systems (nanoemulsions and nanocapsules containing essential oil of *M. alternifolia*) was performed. In the first model of 96-well microplates it was established that cell viability was reduced at 7 and 14 days of treatment. In the second model, infected nails were used and it was observed that both systems showed reduced fungal infection.	38
Dichloromethane-methanol extract (1:1, v/v) of the bark of *Polyscias fulva*	*In vivo dermatophyte* susceptibility technique	Preclinical results of the extract were obtained and concentrations of 1.25. 2.5 and 5 %, w/w, were used. It was established that the activity of the extract was also dose dependent and that the topical infection was eradicated after fourteen days of daily treatment.	28
Essential oil of *Chenopodium ambrosoides*		It was observed that the essential oil showed antifungal activity at a concentration of 50 ppm. Established infection caused by *T. mentagrophytes* was completely cured after thirteen days of application of the ointment of the essential oil twice a day	29

Several research groups have been given the task of obtaining sufficient evidence that allow understanding and knowing the *in vitro* and *in vivo* potential possessed by natural products. However, there are currently no standard methods for their (*in vitro* and *in vivo*) evaluation against strains of dermatophyte fungi. In this review, various alternatives are presented to evaluate essential oils as well as adaptations of methods commonly used to determine the *in vitro* and *in vivo* susceptibility of dermatophytes. Also, formulations that incorporated natural products were presented, and in some cases, it was observed that these potentiated the antifungal activity and even improved stability. In conclusion, we can say that the great potential that natural products have as one of the main alternatives for clinical use in the treatment of dermatomycosis is evident.

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Chemistry and Pharmacology of *Tinospora cordifolia*

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Tinospora cordifolia (Menispermaceae) is an Ayurvedic medicinal plant distributed throughout the Indian subcontinent and China. The whole plant is used in folk and the Ayurvedic system of medicine alone and in combination with other plants. Due to its commercial importance, *T. cordifolia* has been of intense research interest for the last four decades with the isolation of diverse compounds such as alkaloids, sesquiterpenoids, diterpenoids, phenolics, steroids, aliphatic compounds and polysaccharides, along with the discovery of a wide spectrum of pharmacological properties like immunomodulation, anticancer, hepatoprotective and hypoglycemic. Although pharmacological activities of extracts and compounds of *T. cordifolia* have been studied both *in vitro* and *in vivo*, only few mechanisms of action have been explored and need further elaboration. In the present review, the pharmacological activities of compounds and different extracts of *T. cordifolia* are highlighted, along with those of the marketed products, showing the relevance of phytochemicals and the standardization of the marketed products for medicinal use. This compilation of the extensive literature of *T. cordifolia* here will be a referral point for clinical study and the development of standardized phytomedicines in healthcare.

Keywords: *Tinospora cordifolia*, Menispermaceae, Chemical constituents, Pharmacological activity, Toxicity.

The genus *Tinospora* (Menispermaceae) possesses about 32 species of climbing shrubs that are distributed throughout tropical Africa, Madagascar, Australia and the Pacific Islands [1]. Of these species, the most medicinally and commercially important is *T. cordifolia* (Willd.) Miers ex Hook. f. & Thoms. commonly known as Guduchi or Amrita, which is distributed throughout the Indian subcontinent and some parts of China [2]. The estimated annual consumption of *T. cordifolia* in the Indian System of Medicines is approximately 1,000 tonnes [3]. *T. cordifolia* is categorized as 'Rasayana' in Ayurveda and is used as a tonic and vitalizer, and to treat diabetes, skin, heart diseases, jaundice, rheumatoid arthritis, allergies, leprosy, urinary disorders and dysentery [4,5,6]. The whole plant is reported to possess hepatoprotective, antiulcer and antioxidant properties, whereas the stems showed hepatoprotective, antipyretic, cytotoxic, antidiabetic and immunomodulatory activity [9-14]. Dried fruits are used to treat jaundice and rheumatism, whereas the leaves are used to treat diabetes [15], and the roots are employed for their powerful emetic, antistress, antioxidant, antiulcer, and hypoglycemic properties, as well as for the treatment of visceral obstructions [16-20]. *T. cordifolia* is a rich source of alkaloids, furano diterpenoids, clerodane norditerpenoids, sesquiterpenoids, phenolics, lignans, sterols, aliphatic compounds, polysaccharides, essential oil and fatty acids [7, 8]. The alkaloids (e.g. berberine), bitter compounds (tinosporin, tinosporic acid and tinosporol) and lipids have been found to exhibit medicinal effects. In its pharmacological actions, *T. cordifolia* targets body organs, mainly kidney, liver and spleen, [21]. The reviews [22-26] on *T. cordifolia* have been mostly in open access or non–SCI journals and dealt with different aspects like Ayurvedic preparation, botanical aspects with morphology, growth constrains, genetic diversity and biological activity of crude extracts. In the present review, the pharmacological activities and mechanism of actions of the phytochemicals are used as a referral point after the review by Panchabhai *et al*. in 2008 on the therapeutic evidence of different extracts of *T. cordifolia* [21].

Traditional uses of different parts of T. cordifolia: *T. cordifolia* has been used as a constituent of several folk and Ayurvedic preparations in the form of juice, decoction, paste, powder and pill to treat general debility, fever, skin diseases, chronic diarrhea, jaundice, asthma and bone fracture, which were described in ancient texts like Rasayana, Sangrahi, Balya, Agnideepana, Tridoshshamaka, Dahnashaka, Mehnashaka, Kasa-swasahara, Pandunashaka, Kamla-Kushta-Vataraktanashaka, Jwarhara, Krimihara, Prameha, Arshnashaka, and Kricch-Hridroga nashak [7]. *T. cordifolia* as a blood purifier removes defective and damaged red blood cells from peripheral blood circulation by stimulating liver and spleen. The stem of *T. cordifolia* is approved by the Ayurvedic Pharmacopoeia of India as a medicine because of its high alkaloidal content [27]. The starch from *T. cordifolia* (Guduchi satva) climbing on *Azadirachta indica* is very bitter, with more medicinal efficacy [7].

Leaves: Powdered leaves and their decoction are reported to treat gout, ulcers, jaundice, fever, and wounds, and to control blood glucose, along with cow's milk [28].
Stem: The extract of stems alone and with honey is useful as a tonic in jaundice, skin diseases [28] and fever [29]; stem starch (satva) is used as a tonic. A combination of root and stem is prescribed as an antidote to snake bite and scorpion sting [30].
Bark: In North Gujrat (India), root and stem bark of the plant is used along with milk to treat cancer [29].
Fruits: These are used in the treatment of jaundice and rheumatism [31].
Roots: Roots are used as an emetic for visceral obstructions, leprosy, diarrhea and dysentery [31, 32].

T. cordifolia in formulations: Several formulations of *T. cordifolia* are used in preclinical and clinical preparations with pharmacological properties like hepatoprotective, antioxidant, antihyperglycemic, antihyperlipidemic, antidepressant, antiamoebic, and antistress. They also have pancreatic islet superoxide dismutase, antiatherogenic, antiarthritis and immunomodulatory properties [21, 33-41]. Two formulations named as Hemoliv and HP-1 showed protective effects against CCl_4-induced hepatic damage in rats [21]. A formulation, Caps HT2, containing a methanolic extract of

T. corifolia with other herbs was evaluated for its antiatherogenic, antioxidant, anticoagulant, platelet antiaggregatory, lipoprotein lipase releasing, anti-inflammatory and hypolipidemic activities [39]. Two herbal formulations, Diasulin and Dihar, containing an ethanolic extract of roots of *T. cordifolia*, showed antihyperlipidemic, antiperoxidative and antioxidant activities [34, 42]. The therapeutic importance of *T. cordifolia* in Ayurveda and the pharmacological activities of different doses (*in vitro* and *in vivo*) have been validated [21].

Analytical study of T. cordifolia: Analytical studies of *T. cordifolia* and its marketed formulations were carried out by HPLC and RP-LCMS-DAD for its major alkaloids, berberine, palmatine and jatrorrhizine [43]. The variation of four bioactive compounds syringin, cordifolioside A, magnoflorine and tinocordiside in the stem of *T. cordifolia* was analysed by UPLC-DAD-ESI-QTOF-MS/MS and NMR [44]. Analytical studies on bioactive markers of both alkaloidal and non-alkaloidal constituents would be helpful to establish quality control measures for *T. cordifolia* [45].

Pharmacological activity of extracts: *In vivo* administration of *T. cordifolia* extract induced production of antitumor biomarkers like reactive nitrogen intermediates, tumor necrosis factor (TNF), IL-1 with significant cytotoxicity, and showed apoptosis in BMC (bone marrow cells) at high doses and leucocytosis in lower doses [46-49]. *T. cordifolia* reduced the neutropenia induced by single and multiple doses of cyclophosphamide [50]. The alcoholic extract of *T. cordifolia* activated macrophages by antigen presenting ability and phagocytosis [48]. The *in vitro* and *in vivo* activities of different parts of *T. cordifolia* are presented in Table 1.

Chemical constituents

Alkaloids: Thirteen alkaloids of isoquinoline and aporphine skeletons, amine and amide were reported [45,102,103, 117-121, 203] of which main alkaloids were protoberberine alkaloids berberine, palmatine, jatrorrhizine, magnoflorine and corydine [45, 102, 103].

Terpenoids: Thirty two diterpenoids and their glycosides of clerodane and norclerodane skeleton [103,119,123-132], one monoterpenoids [119], five sesquiterpenoids [8,120,122-123] and one triterpenoid cycloeuphordenol [104] were isolated from *T. cordifolia*. A bicyclic diterpenoid ($C_{21}H_{24}O_7$) from the whole plant was tentatively identified as tinosporin [105].

Phenolics: Four phenyl propanoids [106,119,135], two flavonoids [133,134], three lignans [110,119,136] and two benzenoid derivatives [102,122] have been isolated from *T. cordifolia*.

Steroids: Four steroids along with δ-sitosterol [128,136-138] and 2,3,14,20,22,25-hexahydroxyl-5-cholest-7-en-6-one have been reported [107].

Essential oil and aliphatic compounds: The GC-MS profile of the hydrodistilled essential oil of fresh leaves showed the presence of alcohols (32.1%), phenols (16.6%), aldehydes (16.2%), fatty acids (15.7%), alkanes (8.3%), esters (3.2%), and terpenes (1.2%), along with hydroquinone (16.6%), 2-hexenal (14.2%), palmitic acid (14.1%) and phytol (11.4%)[108]. GC-MS analysis of the hexane extract of stems identified methyl-9,12-octadecadienoate (23.2%), methyl 9-octadecenoate (19.7%), methyl hexadecanoate (16.3%) and methyl octadecanoate (5.5%) [52]. Heptacosanol, octacosanol, nonacosan-15-one and cyclohexyl-11-heneicosanone were identified from the stems and whole plant [52, 109, 110].

Polysaccharide: The polysaccharide of the stems of *T. cordifolia* was shown to be composed of glucose 98.0%, arabinose 0.5%, rhamnose 0.2%, xylose 0.8%, mannose 0.2% and galactose 0.3% units [111].

Others: Several compounds reported from *T. cordifolia* had no detailed chemical and biological study. Some of these are giloinsterol, a bitter glucoside giloin, a non-glucoside bitter substance giloinin, gilo-sterol, tinosporan acetate, tinosporic acid, tinosporal acetate, tinosporone and tinosporal [31]. Tinosporidine, cordifolone, tinosporon, tinosporic acid and tinosporol were isolated from different parts of *T. cordifolia* [112]. Two bitter compounds, tinosporide and cordifolide, were isolated from the fresh stems of *T. cordifolia* [113]. Three furanolactone diterpenoids, $C_{20}H_{20}O_6$, $C_{20}H_{22}O_8$ and $C_{26}H_{34}O_{11}$, were also reported from the stems, but with only physical properties listed [114-116]. The structures of the compounds isolated from *T. cordifolia* are presented in Figure 1.

The leaves of *T. cordifolia* are rich in protein (11.2%), calcium and phosphorus, and the stems contain an appreciable quantity of zinc [139].

Antioxidant activity: This is due to the alkaloidal constituents (choline, palmatine, tetrahydropalmatine and magnoflorine), (-)-epicatechin, and an aromatic glycoside, secoisolariciresinol [123, 136]. An arabinogalactan polysaccharide from *T. cordifolia* showed protection against iron-mediated lipid peroxidation of rat brain homogenate using lipid hydroperoxide (LOOH), possibly due to its high reactivity towards DPPH, superoxide radicals and hydroxyl radicals [140]. Our study showed that the alkaloidal fraction (mainly palmatine, jatrorrhizine and magnoflorine) had better antioxidant activity in the DPPH inhibition assay than columbin, in a dose dependent manner [141].

Hypoglycemic activity: In the Ayurvedic Pharmacopoeia of India, *T. cordifolia* is categorized as an antidiabetic herbal drug due to its alkaloids, diterpenoids and glycosidic constituents [27]. Several studies *in vitro* and *in vivo* showed that the alkaloids palmatine, jatrorrhizine and magnoflorin were synergistically responsible for the hyperglycemic effect *via* a mechanism of insulin releasing, insulin-mimicking and gluconeogenesis inhibition [143-145]. Magnoflorine was the most potent α-glucosidase inhibitor using sucrase and maltose as substrate with inhibitory values of 9.8 and 7.6 μg/mL, respectively, possibly due to blockade of glucose co-transporters Glut-2 and Glut-4, the main carriers of glucose from the intestine into circulation [144]. The antidiabetic property of palmatine was reported in myocyte L6 cells through an insulin dependent pathway and by upregulating glucose transporter-4 (Glut-4) and PPARα (peroxisome proliferator-activated receptor) expression, with berberine and palmatine having similar antidiabetic potential [145]. An isolated α-glucosidase inhibitor, apigenin-6-C-glucosyl-7-*O*-glucoside, showed mixed competitive inhibitory activities of α-glucosidase and sucrase in the range of 20-80 mg/kg compared with 100-200 mg/kg for acarbose in maltose fed rats [135]. A norclerodane diterpenonoid, tinosporaside, possessed 28% (ρ <0.01) antihyperglycemic activity, comparable with metformin 20.6 % (ρ < 0.05) in diabetic rats [146].

Antiviral activity: A diterpenoid, tinosporin, showed activity against HIV, HTLV and other viral diseases for its immunomodulatory and selective inhibition of the virus to target T helper cells [147].

Immunomodulatory activity: The immunomodulatory activity of *T. cordifolia* is due to the synergistic effects of compounds including

low molecular weight alkaloids, clerodane diterpenoids, sesquiterpenoids and phenyl propanoids (magnoflorine, *N*-formylannonain, *N*-methyl-2-pyrrolidone, 11-hydroxymuskatone, cordioside, cordifolioside A, cordifolioside B, cordial, tinocordiside and syringin), a high molecular weight arabinogalactan named G1-4A, 1,4-α-D-glucan (RR1), and the enzyme thiol amylase [30, 83, 91, 120, 148-154]. Clerodane furano diterpenoid glycosides like cordioside, cordiofolioside A and cordiol were reported for their macrophage activation, which plays an important role in specific and non-specific immune responses [148]. Cordifolioside A is an active immunostimulant used in the quality control and standardization of the plant's formulations [155]. A cadinane sesquiterpenoid glycoside, tinocordiside, possesses immunomodulatory activity [8]. The polysaccharide fraction from *T. cordifolia* effectively reduced the metastatic potential of B16F-10 melanoma cells [156] due to its antioxidant activity towards DPPH and superoxide radicals [136]. G1-4A, an arabinogalactan polysaccharide isolated from *T. cordifolia*, constitutes galactose

Table 1: Pharmacological activities of *T. cordifolia* extract

Extracts/Plant part	Pharmacological activity	Ref
	***In vitro* models**	
Hexane and MeOH (f)	Antioxidant activity	[51]
Hexane (wp)	Antimutagenic activity using *Salmonella* histidine tester strain TA 98	[52]
Dichloromethane (wp)	Alteration in radiosensitivity of HeLa cells	[53]
Alcohol (s)	Androgenic action in prostate cancer cell line LNCaP	[54]
50% Alcoholic-aqueous (s)	Antioxidant, possesses OH radical scavenging activity	[55]
MeOH, aq. & DCM (s)	Antineoplastic activity in HeLa cells	[56]
Dichloromethane (s)	Cytotoxic effect in HeLa cells due to lipid peroxidation, release of lactate dehydrogenase (LDH) and decline in glutathione-S-transferase	[12]
MeOH (b, l)	Antibacterial and anti-fungal activity	[57]
MeOH (s)	Antibacterial activity	[41]
Hexane, Chloroform, MeOH, alcohol & water (l)	Antioxidant activity	[58]
Light petroleum, EtOH & aqueous	Anti-HIV activity using reverse transcriptase (RT) inhibition assay	[59]
MeOH, MeOH-water (1:1) & water	Anti-plasmodial activity against *Plasmodium falciparum* strain FCR-3 (ATCC 30932)	[60]
Aqueous (s)	Enhanced phagocytosis using *Candida albicans* suspension	[61]
MeOH (s)	Antihyperlipidemic, in Sprague Dawley rats	62]
EtOH (s)	Tissue protective activity (P<0.05) in irradiated Swiss albino mice	[63]
Aqueous (s)	Prevention of hyperglycemia, hyperinsulinemia, hypertriglyceridemia; insulin resistance and elevated levels of hepatic total lipids in rats	[64]
Dichloromethane (s)	Alpha-glucosidase inhibitor	[65]
Alcohol (wp)	Anti-inflammatory activity on carrageenan - induced hind paw edema and cotton pellet granuloma models in male Wistar rats	[66]
Aqueous & alcohol (s)	Modulation of morphology and gluconeogenic enzymes activity in diabetic rats	[67]
Hexane extract fraction (wp)	Inducing apoptosis against Ehrlich ascites tumor (EAT) cells in mice	[68]
Dichloromethane (wp)	Antineoplastic activity in Ehrlich ascites carcinoma bearing mice	[13]
Alcohol (wp)	Retardation of tumor growth and prolonged survival of tumor-bearing mice	[69]
Alcohol (wp)	Cardioprotective activity in limiting ischemia-reperfusion induced myocardial infarction in rats	[70]
Aq., alc., light petroleum and $CHCl_3$ (wp)	Antidiabetic activity in rabbits and rats	[14]
Light petroleum (wp)	Antidepressant like activity in mice	[71]
Aqueous (s, l)	Protective effects against lead intoxication on hematological values	[72]
Water soluble fraction (l)	Immunomodulatory activity and disease resistance in *Oreochromis mossambicus*	[46]
Dichloromethane (wp)	Radiosensitizing activity in tumor bearing rats by elevating levels of lipid peroxidation and DNA damage of tumor cells	[73]
EtOH (s)	Antiosteoporosis activity in female rats	[74]
EtOH & Light petrolelum (l)	Immunostimulatory activity in *Oreochromis mossambicus*	[75]
Alcohol (wp)	Proliferation and myeloid differentiation of bone marrow hematopoietic precursor cells in mice	[76]
80% Hydroalcoholic (r)	Inhibited lipid peroxidation, induces enzymes of carcinogen metabolism and antioxidant system in mice	[77]
Aqueous & alcoholic (wp)	Antihyperglycemic effect in diabetic mice and rats	[78]
Alcohol (r)	Antistress activity in albino rats of the Charles-Foster strain	[79]
Aqueous (r)	Antidiabetic activity in albino rats of Wistar strain	[20,80]
Aqueous (wp)	Radioprotective effect in Swiss albino mice	[81]
Aqueous (s)	Hepatoprotective and immunomodulatory activity in albino rats of Wistar strain	[82]
MeOH (s)	Antioxidant activity in C3H mice	[83]
Alcohol (r)	Antidiabetic activity in albino Wistar rats	[84]
50% Aqua-alcoholic	Radioprotective activity in albino mice	[85,86]
Ether, alcohol & aqueous (l, r, s)	Hepatoprotective activity in Wistar strain albino rats	[87]
MeOH (s)	Antitumor and immunomodulatory activity	[88]
Aqueous (l)	Larvicidal activity against *Pediculus humanus capitis*, *Anopheles subpictus* and *Culex quinquefasciatus*	[89]
Alcohol (r)	Antiulcer activity in albino rats of Charles Foster strain	[90]
50% Aqueous-alcoholic	Enhancement of mammary gland immunity and therapeutic potential in cows	[91]
Alcohol (s)	Immunomodulatory activity in Wistar rats	[92]
Alcohol-water (7:3) (s)	Antidiabetic activity using type-2 diabetes in Sprague-Dawley rats	[93]
EtOH (r)	Chemopreventive potential against aflatoxin B_1-induced toxicity in mice	[94]
50% Aqueous-alcoholic (s)	Antipsychotic activity in amphetamine challenged mice	[95]
Light petroleum (s)	Antiobesity activity in rats	[96]
Aqueous (s)	Antiinflammatory and immunosuppressive effect in albino rabbits	[97]
Alcohol (r)	Protective effect induced by aflatoxin –B_1 in Swiss albino mice	[98]
70%MeOH (s)	Antifertility effect in rats	[99]
Aqueous (r)	Antioxidant activity in albino rats of Wistar strain	[18]
Aqueous (r)	Hypolipidemic action in alloxan diabetic albino rats of Wistar strain	[19]
Aqueous (r)	Antioxidant activity in albino rats of Wistar strain	[100]
Aqueous (s)	Immunotherapeutic modification *Eschercihia coli* peritonitis in mice	[101]

b bark; f flower; l leaves; r root; s stem; wp whole plant.

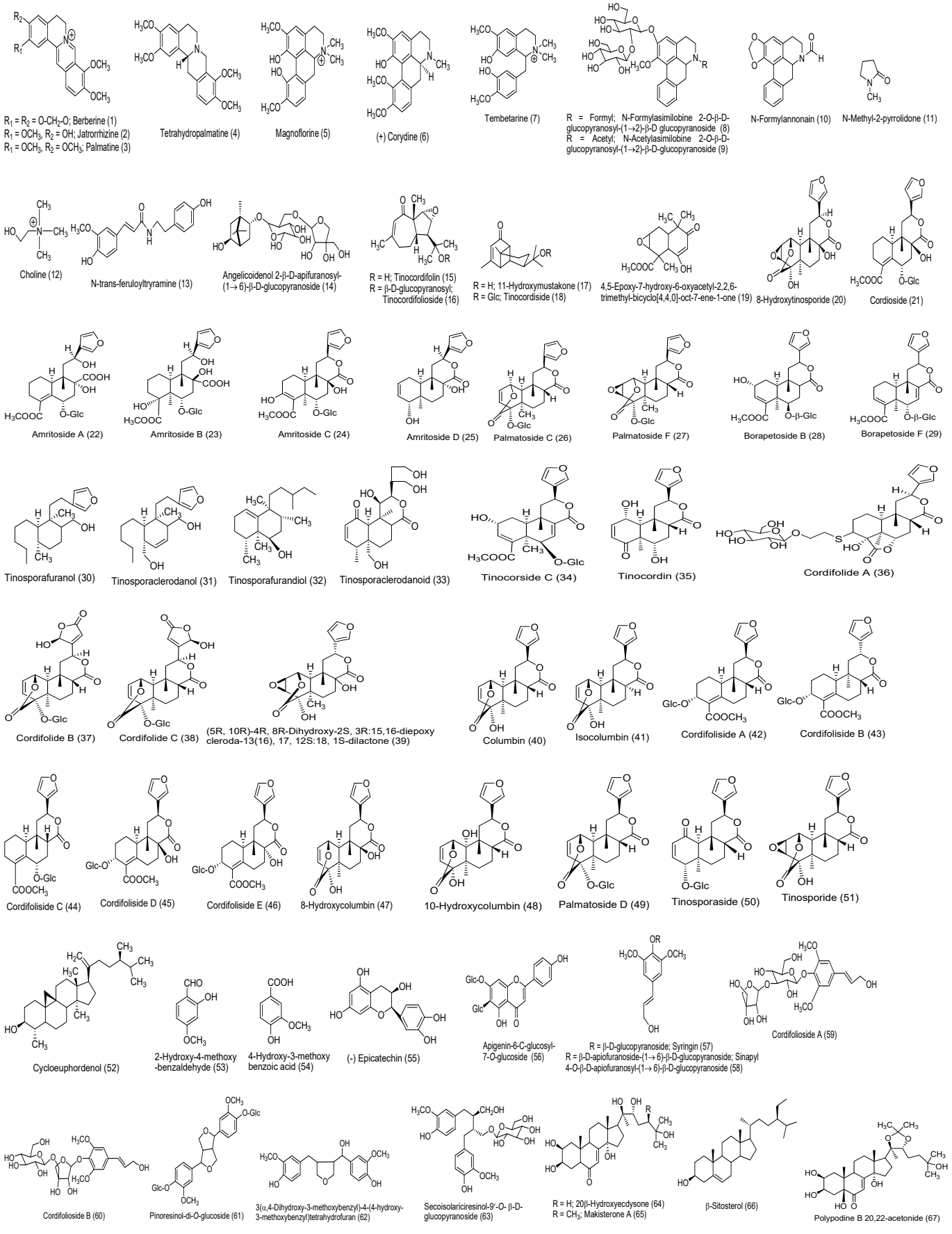

Figure 1: Structures of chemical constituents of *Tinospora cordifolia*.

(32%), arabinose (31%), galacturonic acid (35%) and rhamnose (1.4 %) by GC-MS, and induced maturation of immature dendrite cells of bone marrow and spleen by modulation of cytokines, co-stimulatory molecules and others which consequently lead to adaptive immunity by clonal expression and differentiation through antigen specific T cells [157]. G1-4A also induces phenotype of bone marrow dendritic cells (BMDC) after maturation which releases nitric oxide to generate peroxynitrate to kill tumor cells by phagocytosis [158]. Another polysaccharide (1→4)-α-D-glucan (named RR1) isolated from *T. cordifolia* with a molecular weight >550 KDa has (1→4)-α-glycosidic linked back bone and (1→6)-α-glycosidic linked branches which exhibited unique immune stimulating properties as a non-cytotoxic and non-proliferating to normal as well as to tumor cell lines in the concentration range of 0-1000 μg/mL [152]. RR1(α-D-glucan) activates different subsets of lymphocytes like natural killer (NK) cells (333%), T cells (102%) and B cells (39%) at 100 μg/mL concentration and immunomodulation in normal lymphocytes by increasing the synthesis of IL-1β, IL-6, IL-12 p70, IL-12 p40, IL-18, IFN-γ, tumor necrosis factor (TNF-α) and monocyte chemoattractant protein (MCP)-1 without affecting IL-2, IL-4, IL-10, (INF)-α and TNF-β [151]. Water solubility of α-D-glucan has the advantage over the β-glucan which forms granuloma [151]. The immune system activation by α-D-glucan was shown to be associated with signal transduction pathway in mouse macrophages [152] whereas intravenous administration of 10 mg/kg α-D-glucan in anaesthetized rats leads to significant tachycardia without hypotension [153]. An immunomodulatory protein named guduchi ImP, with a molecular weight 25 kDa, showed lymphoproliferative and macrophage-activating properties [154].

Antilarvicidal activity: A chalcone, cordifolin [1-(2',3',4'-trihydroxyphenyl)-3-(4"-methoxyphenyl)-propen-1-one], exhibited insect growth regulatory activity against larvae of *Spodoptera litura* [159].

Anticancer activity: Berberine possesses antineoplastic activity on mice bearing Ehrlich ascites carcinoma at a dose of 10 mg/kg body weight and acts as a topoisomerase II inhibitor [13, 160, 161], whereas columbin, a furanolactone diterpenoid, showed chemopreventive ability against human colon cancer [162]. A long-chain aliphatic alcohol, octacosanol, acts as an antiangiogenic compound (*in vivo*) that inhibits the secretion of vascular endothelial growth factor into ascites fluid by tumor cells at the molecular level, as well as inhibiting the activity of matrix metalloproteinases (MMPs) and translocation of transcription factor NF kappa B to the nucleus [68]. A clerodane diterpenoid, (5*R*,10*R*)-4*R*,8*R*-dihydroxy-2*S*,3*R*:15,16-diepoxycleroda-13(16),17,12*S*:18, 1*S* dilactone, was found to prevent chemically- induced hepatocellular carcinoma in rats, and inhibit tumor growth through an antioxidant and detoxification mechanism. The dual action of the diterpenoid was transmitted by blocking carcinogen metabolic activation and enhancing carcinogen detoxification [163].

Antiosteoporotic activity: The steroidal constituent, 20-hydroxy-β-ecdysone, showed an antiosteoporotic effect in the treatment of osteoporosis and osteoarthritis without activating the estrogen receptor [164]. Ecdysone derivatives have been well studied in the treatment of different estrogenic and androgenic disorders as well as age-related skin conditions [165]. *T. cordifolia* has potential for the treatment of osteoporosis, osteoarthritis and bone related problems.

Platelet aggregation activity: A phenolic amide, *N-trans*-feruloyl tyramine was shown to possess platelet aggregation activity [166].

Pharmacological activity of chemical constituents of T. cordifolia: Several compounds were found to be pharmacologically active in different assay systems, although clerodane diterpenoids and their glycosides were claimed as the major non-alkaloidal constituents, and their variable abundance in poor yield, are limiting factors for determining biological study.

Toxicity of T. cordifolia: In Ayurveda, *T. cordifolia* is reported as a safe drug, whereas regular use of high doses can cause constipation. No report is available on its toxicity [167]. A toxicity study of *T. cordifolia* on Swiss albino mice using a high dose level of 9 mL/kg for decoction and 8 g/kg for the whole plant powder showed no mortality and an LD_{50} value was found to be higher than 1g/kg in oral administration without affecting the GI motility of normal animals [6]. Another toxicity study by Agarwal *et al*. showed that a dose of up to 3 g/kg of *T. cordifolia* had no adverse effect on animals [168]. Upadhyay *et al*. carried out a toxicity study on *T. cordifolia* and found that a 500 mg/day dose for a period of 21 days is safe in healthy volunteers [7, 169]. Several other studies have also shown a lack of toxicity [12, 170]. *T. cordifolia* administration to normal volunteers has been found to be safe in a phase I study [6]. Considering toxicity studies in different animal models and routine use by practitioners, *T. cordifolia* can be used as a safe herbal drug.

Conclusion and future prospects: Literature documentation across different disciplines of study reveals that *T. cordifolia* has been considerably explored in the area of biological activity of different extracts, fractions and compounds with the quantification of some bioactive compounds. The plausible mechanisms of action in *in vitro* and *in vivo* models were also explored for crude extracts, formulations, and compounds along with clinical and toxicological studies. The pharmacological data for *T. cordifolia* and its several isolated compounds undisputedly support its use as an ingredient in herbal drugs and formulations. Furthermore, the compounds isolated from *T. cordifolia* having antidiabetic, anti-inflammatory, anticancer and immunomodulatory activity can be used as therapeutic agents against these diseases either alone or in combination in a standardized form. The abundant nature of *T. cordifolia* in subtropical Asian countries makes it a commercially low cost and effective herbal complementary medicine. The biological study and clinical trials of *T. cordifolia* are indicative of its safety and potent therapeutic value as a health supplement of commercial importance, as well as a store house for future drug development in critical diseases where modern medicines have limited therapeutic potential.

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Perspectives of the *Apiaceae* Hepatoprotective Effects – A Review

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The liver has the crucial role in the regulation of various physiological processes and in the excretion of endogenous waste metabolites and xenobiotics. Liver structure impairment can be caused by various factors including microorganisms, autoimmune diseases, chemicals, alcohol and drugs. The plant kingdom is full of liver protective chemicals such as phenols, coumarins, lignans, essential oils, monoterpenes, carotenoids, glycosides, flavonoids, organic acids, lipids, alkaloids and xanthenes. *Apiaceae* plants are usually used as a vegetable or as a spice, but their other functional properties are also very important. This review highlights the significance of caraway, dill, cumin, aniseed, fennel, coriander, celery, lovage, angelica, parsley and carrot, which are popular vegetables and spices, but possess hepatoprotective potential. These plants can be used for medicinal applications to patients who suffer from liver damage.

Keywords: Apiaceae, Liver, Hepatoprotective, Phytotherapy.

Introduction

The liver as a vital organ has a crucial role in the regulation of various physiological processes and in the excretion of endogenous waste metabolites and xenobiotics. It has a superior role in the maintenance, performance and regulation of body homeostasis: it is involved in carbohydrate and fat metabolism, bile secretion, vitamin storage and synthesis of useful biologically active compounds. Liver injury or dysfunction is a major health problem that challenges not only the health care professionals, but also the pharmaceutical industry and drug regulatory agencies [1]. Liver structure impairment can be caused by various factors including microorganisms, autoimmune diseases, chemicals, alcohol and drugs. Hence, the modern lifestyle, insufficient physical activity, environmental impact, medication and food additives cause a change in oxidative balance and consequently in oxidative stress and thus influence both life quality and disease development, including liver function impairment.

Despite the fact that the advances in modern medicine are significant, there is no drug which is completely effective and safe in the treatment of liver diseases and which regenerates the hepatic tissue, stimulates the renewal of the hepatic function or completely protects the liver from damage. Herbal drugs, which have been traditionally used in the treatment of various medical conditions for centuries, have gained popularity in recent years because of their safety, efficacy and cost effectiveness, cultural acceptability and minimal side effects [2, 3]. Herbal drugs are used worldwide, mainly in developing countries as the primary health care [2].

The use of natural remedies for the treatment of liver diseases has a long history. Medicinal plants are a significant source of hepatoprotective drugs. It has been claimed by Pharmacopeia Foundation that about 170 phytoconstituents isolated from 110 plants belonging to 55 families possess hepatoprotective activities [2]. Liver protective plants contain a variety of chemical constituents such as phenols, coumarins, lignans, essential oils, monoterpenes, carotenoids, glycosides, flavonoids, organic acids, lipids, alkaloids and xanthenes [2].

Plants from the *Apiaceae* (or *Umbelliferae*) family are widespread throughout the world; however, they are most common in temperate regions. The representatives of this family are annual and perennial herbs characterized by a well-developed secretory system in all plant parts, such as schizogeneus secretory cavities in the root, phloem in the stem and leaves and clearly-delimited tissue known as vittae in the fruit [4]. These structures are important for depositing essential oils, which give the specific odor and flavor to each plant. Due to their flavor, a large number of plants from this family are used as vegetables or spices. Lately, the focus has been directed to the great potential of these plants as functional food [5, 6]. Their use in folk medicine, phytotherapy, as health-promoting agents, and homeopathy medicaments has also been on the rise.

A large number of the members of this family are cultivated for various purposes. Some plants, such as celery, lovage, angelica, parsley and carrot include a taproot, which is used as a vegetable to flavor dishes or to prepare alcoholic and non-alcoholic drinks. Some of them, such as parsley, dill and coriander, having aromatic leaves, are used for flavoring food or as a garnish. Celery and fennel are used for their luscious leaf stalks. Also, the seeds from *Apiaceae* plants are used. Caraway, aniseed and cumin are used exclusively as seed spices. On the other hand, most of these plants are used for essential oil distillation, which has a wide range of application in food preparation and the food industry (Table 1). Apart from the essential oils, there are a lot of constituents determined in these plants, such as fatty oils, vitamins, flavonoids, carotenoids, chlorophylls, and polyphenols (Table 1). Their complex chemical composition has a high biological activity. *Apiaceae* plants are reported to possess a lot of nutraceutical properties, such as antioxidant, antimicrobial, antidiabetic, hypolipidemic, antispasmodic and carminative, anticarcinogenic/antimutagenic, anti-inflammatory, antistress, antiulcerogenic and many others [7-17].

This review highlights the significance of the *Apiaceae* family, the source of many popular vegetables and spices, and potential hepatoprotective sources and their possible medicinal applications.

Table 1: *Apiaceae* plants, their usage and chemical composition.

Herb	Used part	Chemical composition
Caraway	**Seed** is used in cakes, cheese, confections, fresh cabbage, meat dishes, rye bread, salads **Seed essential oil** is used to flavor chewing gum, candy, liqueurs.	essential oil, fatty oil, vitamins (A, B6, B12, C, D, E), flavonoids, proteins [18, 19]
Dill	**Seed** is used in pickled cucumbers, bread, processed meats, sausages, cheese, condiments **Leaf** is used in pickles, while fresh is used for garnish or to flavor salads, vegetable dishes, sea food, soups, yogurt, mayonnaise.	essential oil, fatty oil, vitamin C, carotenoids, flavonoids, coumarins, chlorophylls [6, 20]
Cumin	**Seed** is used as a flavor component in beverages, confectioneries, baked goods, meat and meat products, condiments and relishes, gravies, snack foods, gelatines and puddings.	essential oil, fatty oil, flavonoids, anthocyanins [21-23]
Aniseed	**Seed** is used in beverages, baked goods, condiments, relishes, oils and fats, frozen dairy, gravies, meat products, soft candy **Seed essential oil** is used in chewing gums, gelatines, puddings, soft and hard candies.	essential oil, fatty oil, flavonoids, coumarins [6, 24]
Fennel	**Seed** is used in meat dishes, in curries, spice blends, soups, vegetables, breads. **Leaf** fresh and chopped can be used as garnish for fish dishes, sauces, salads, stews and curries. **Leaf stalk (pseudobulb)** is used raw in salads, stuffing or blanched as vegetable, in soups and sauces and baked. **Seed and herb essential oil** is used in beverages, condiments, relishes, baked goods, frozen dairy, gelatines, puddings, meat products, candies.	essential oil, fatty oil, vitamin C, flavonoids, coumarins and minerals (K, Na, Ca, Mg), nitrates [6, 25-27]
Coriander	**Leaf** is used to make chutneys and sauces, green salsas, dips, snacks, soups **Seed** is used in couscous, stews and salads **Seed essential oil** is used in beverages, baked goods, condiments, relishes, meat products	essential oil, fatty oil, vitamins (A and C), flavonoids, coumarins [6, 28]
Celery	**Root, leaf and leaf stalk** are used as a vegetable, fresh for salads, for the preparation of juices, in meat dishes, snacks, gravies, sauces **Seed** is used in pickling vegetables, salad dressings, breads, biscuits, soups, celery salt, bouquet garni	essential oil, fatty oil, flavonoids, coumarins, minerals (Na, P, K, Ca, Mg, Fe, Cu, Mn, Zn) [6, 29, 30]
Lovage	**Leaf** is used for seasoning soups, sauces, meat dishes **Root** is used for producing soup seasonings, finished flavorings in liqueurs and tobacco **Seed** is used as spice, for flavoring cakes, soups, salads, for pickled vegetables (especially cabbage and cucumbers).	essential oil, flavonoids, coumarins, phenolic acids, saponins, alkaloids [31, 32]
Angelica	**Root** is used in herbal liqueurs and bitter spirits, in flavoring meat and canned vegetables. **Herb** is used for decorating cakes and pastry, and to flavor jams and jellies, confectionaries and liqueurs. **Leaf** chopped is added to fruit salads, fish dishes, and cottage cheese. **Seed** is used in alcoholic distillates.	essential oil, coumarins, phenolic compounds [*33]*
Parsley	**Leaf** is used as a garnish (for salads, soups, boiled potatoes and egg dishes), blended in dips, cooked sauces and stews **Root** is used as a vegetable to enhance soups flavor , stews and condiments **Essential oil** is used to flavor meat sauces, pickles, spice blends, baked goods, oils and fats, processed vegetables, soups, gelatines and puddings	essential oil, vitamin C, tocopherol, carotenoids, flavonoids, coumarins, sterols, triterpenes [6, 34, 35]
Carrot	**Root,** raw, is used for juices, salads, cakes, for pickling, while cooked it is used in casseroles, soups and stews **Seed essential oil** is used in beverages, baked goods, condiments, relishes, meat products	essential oil, carotenoids (β-carotene, α-carotene, γ-carotene, lycopene, cryptoxanthin, leutein, violaxanthin) [36-38]

Caraway (*Carum carvi* L.): Caraway seed (Carvi fructus) and its essential oil (Carvi aetheroleum) are official drugs in the European Pharmacopoeia (Ph.Eur.2011). Caraway is traditionally used as a remedy for a range of health problems, especially stomach ache, flatulence, intestinal spasms and for treating obesity [43].Caraway seed contains 2-7% essential oil with carvone and limonene constituting more than 90% [39-41]. Caraway seed essential oil has been reported to have potential therapeutic effects, mainly due to its high antioxidant activity [18]. Considering the radical scavenging [42] and good antioxidant profile of caraway essential oil, it has been proposed for its multifaceted pharmacological properties [14].

Examination of the antioxidant properties of Carvi aetheroleum conducted *in vitro* by both free radical scavenging capacity (RSC) and protective effect on lipid peroxidation (LP) showed that it could be used as a safe antioxidant and antiseptic supplement. Further research conducted *in vivo* with carbon tetrachloride (CCl_4) induced hepatotoxicity in mice showed that Carvi aetheroleum was able to reduce the 2.2-diphenyl-1-picrylhydrazyl (DPPH) levels in a dose-dependent manner and neutralize H_2O_2 [44]. Other *in vivo* assays also showed the potent hepatoprotective properties of Carvi aetheroleum, which modulated the antioxidant defense system by changing the oxidative stress parameters, namely LP, myeloperoxidase (MPO) and glutathione (GSH) [45].

Dill (*Anethum graveolens* L.): Dill, also known as European dill, is a native of the Mediterranean region, but it is also cultivated across Europe and America. Seed and leaf, named dill weed, are the mainly used parts. Dill seed essential oil has similar composition to caraway seed, with carvone and limonene as the dominant compounds [46], while the main compounds in the essential oil are α-phellandrene, apiole, dill ether, limonene, geraniol and *p*-cymene [47]. Both oils possess a high antioxidant capacity [48, 49]. There is also Indian dill (*A. sowa*), which has a slightly different chemical composition in comparison with European dill, and is mainly grown in Africa [50]. Even though these plants are not official drugs, they are widely used in traditional medicine, as well as in modern phytotherapy and in everyday nutrition as a spice [51, 52]. Their stimulant, carminative, antibacterial, antispasmodic, antiulcer, and antidiabetic activities, as well as their antioxidant, hypolipidemic, and diuretic effects have been reported [11, 13].

The hepatoprotective effect of dill seed oil against CCl_4 induced hepatotoxicity in rats was found as it decreased the serum aspartate transaminase (AST) and alanine aminotransferase (ALT) levels and significantly increased the level of serum total protein and albumin. Furthermore, by supplementing dill oil, suppression of the increased alkaline phosphatase (ALP) activity was achieved with the simultaneous decrease of raised bilirubin. This fact suggests that this oil could have the ability to stabilize biliary dysfunction in rat liver during the hepatic injury caused by CCl_4. Additionally, dill seed oil treatment significantly reversed CCl_4 induced effects like enhanced LP. Consequently, it is plausible that the hepatoprotection mechanism of dill seed oil is due to its antioxidant effect [53]. Hepatoprotective and antioxidant efficacy of dill herb ethanol extract was performed in CCl_4 induced hepatotoxicity in rats where the results were comparable with the normal and standard hepatoprotective drug silymarin [54]. The powder of dill herb and its hydro-alcoholic extract significantly increased the activity of antioxidant and liver function enzymes in paracetamol induced liver injury [55].

Pre-treatment with a dill ethanol extract showed an antioxidant activity and hepatoprotective effect (decreased the AST and ALT levels) on paracetamol-induced hepatic damage in rats by

decreasing hepatic steatosis and hepatic necrosis [56]. The investigations showed that the dill leaf water extract could either increase glutathione biosynthesis or reduce the extent of oxidative stress leading to less glutathione degradation or that it could have both effects [57].

Nowadays a commercial medicinal form of this plant formulated as dill tablet (DT) is available. DTs contain mainly dill (68%), and other herbs such as chicory, fine leaf fumitory and lime that display a potential hepatoprotective effect against CCl_4 induced liver damage based on both biochemical markers and antioxidant status [58].

Cumin (*Cuminum cyminum* L.): Cumin is a native of Egypt and is extensively cultivated for seed in Asia. The distinctive flavor and aroma are due to essential oil, the dominant compounds of which are γ-terpinene-7-al, cumin-aldehyde, β-pinene and γ-terpinene [21-23, 59, 60]. This plant, as well as dill, is not an official drug; it is widely used in Indian traditional medicine and as a spice [14]. In medicine, cumin is important as an antispasmodic agent, carminative and as an appetizer. It also has good antimicrobial and antioxidative properties [9, 61, 62].

Normal serum glutamic-pyruvic transaminase (SGPT) and serum glutamic oxaloacetic transaminase (SGOT) levels were restored when cumin was administered in low doses to profenofos intoxicated mice. This fact suggests that cumin has a vital role in reducing hepatotoxicity at the cellular and biochemical levels [63]. The aqueous ethanol extract of cumin seeds, in a dose-dependent manner, significantly reduced the SGPT, SGOT, ALP and serum total bilirubin (STB) levels in nimesulide induced hepatotoxicity in rats. Additionally, histopathological examination of the rat liver tissue showed a reduction in ballooning degeneration, fibrosis, inflammation and apoptosis of the hepatocytes, which all supported hepatoprotection [64].

Cumin seed powder demonstrated strong hepatoprotective activity upon CCl_4 induced hepatic damage in rats. The levels of urea, bilirubin and creatinine were normalized in cumin powder treated rats and a significant reduction of ALP and STB confirmed the hepatoprotective effect. Additionally, the significantly lower LP suggests that cumin seed powder is efficient against free radical injury [65].

Aniseed (*Pimpinella anisum* L.): Aniseed (Anisi fructus), as well as its essential oil (Anisi aetheroleum), are official drugs in the European Pharmacopoeia (Ph.Eur.2011). Aniseed contains 1.5-6% essential oil with *trans*-anethole as the major compound, which comprises more than 90% [66, 67]. Anise has been traditionally used as an analgesic, anti-inflammatory, appetizer, hypnotic, expectorant, antibacterial and hepatoprotective agent and to increase milk secretion [7, 8, 68].

Recent investigations have shown a potential protective effect of Anisi aetheroleum against CCl_4 induced fibrosis in rats. It was demonstrated that the rats treated with Anisi aetheroleum orally for 7 successive weeks showed a significant protection against the induced increase in serum liver enzymes (AST, ALT, ALP), restored the total protein level and improved the increased triglycerides (TG), total cholesterol, and low density lipoprotein cholesterol (LDL-C), and decreased the high density lipoprotein cholesterol (HDL-C). A significant curative effect of Anisi aetheroleum on biochemical parameters was supported by histopathological examination [69].

The protective properties against CCl_4 was confirmed with *n*-hexane aniseed extract both *in vitro* and *in vivo* as revealed by reduction in cell death, serum transaminase levels, LDH activity, and liver histopathological changes. Moreover, liver hepatocellular carcinoma (HepG2 cells) and rat liver treated with *n*-hexane aniseed extract showed higher levels of glutathione (GSH) and lower levels of thiobarbituric acid reactive substances (TBARs). In this study, hydroalcoholic aniseed extract and Anisi aetheroleum showed no significant protective properties against CCl_4 induced injury, presuming that hydrophobic substances, such as anethole, were the major components for the hepatoprotective effect of aniseed, but further investigations are necessary for confirmation [70]. An aniseed extract decreased serum AST and ALT levels compared with those in the CCl_4 group, although these were not as low as those in the silibinin control group [29].

Fennel (*Foeniculum vulgare* Mill.): Fennel seed (Foeniculi fructus) and essential oil (Foeniculi aethroleum), obtained from seed or from herb, are official drugs in the European Pharmacopoeia (Ph.Eur.2011). Both essential oils have a similar chemical composition to aniseed with *trans*-anethole as the main compound, but at a lower range [25, 71-73]. Fennel oil, like aniseed, possesses a good antioxidative activity and could contribute to the daily antioxidative diet [15]. Antibacterial, antiviral, antifungal, insecticidal, anti-inflamatory and antidiabetic activities of fennel essential oil, along with vasorelaxant, antiplatelet and antithrombotic properties were established [72, 74-77].

Decreased levels of serum AST, ALT, ALP and bilirubin were registered after administration of Foeniculi aetheroleum to CCl_4 induced liver injury model rats [78]. Histopathological findings also suggested that Foeniculi aetheroleum prevented the development of chronic liver injuries and acted as a strong hepatoprotective agent against hepatic induced fibrosis [79, 80].

It was reported that fennel essential oil increased the activity of superoxide dismutase (SOD) and catalase (CAT) [81]. Conversely, diglucoside stilbene trimers and a benzoisofuranone derivative present in fennel did not show strong antioxidant activity [82]. Another study showed that the administration of Foeniculi aetheroleum significantly decreased the levels of serum AST and ALT and significantly increased the level of serum total protein and albumin in rats with induced CCl_4 hepatotoxicity [53]. Furthermore, Foeniculi aetheroleum supplementation induced significant reverse changes in alkaline phosphatase (ALP), raised bilirubin, malondialdehyde (MDA), and SOD values, improving the antioxidant defense mechanism and preventing the formation of excessive free radicals. In addition, rats with CCl_4 induced fibrosis treated orally with Foeniculi aetheroleum showed significant protection against induced increase in serum liver enzyme (AST, ALT, ALP), and the improvement of other biochemical parameters of liver function [69].

The co-administration of Foeniculi aetheroleum with emamectin benzoate (EB) mitigated the hemotoxicity, immunotoxicity and hepatotoxicity induced by sub-chronic treatment of EB in male rats. This may be attributed to antioxidant, anti-inflammatory and hepatoprotective activities of Foeniculi aetheroleum. Therefore, this study indicates that supplementation of this oil may ameliorate toxic effects in individuals who are at risk of prolonged EB exposure [83]. The findings with *trans*-anethole, a major component of *F. vulgare*, suggest that it protects the liver against ischemia /reperfusion (I/R) injury by suppressing interferon regulatory factor

(IRF-1) and by high mobility group box (HMGB1) release and subsequent toll-like receptor (TLR) activation [84].

Coriander (*Coriandrum sativum* L.): Coriander seed (Coriandri fructus), as well as its essential oil (Coriandri aetheroleum), are official drugs in the European Pharmacopoeia (Ph.Eur.2011). Coriandri aetheroleum contains mainly linalool [85], while coriander herb oil has a significantly different composition with decanal, *trans*-2-decenal, 2-decen-1-ol, cyclodecane and *cis*-2-dodecenal as the main compounds [86]. However, both oils possess a good antioxidative activity [87, 88]. The seed is mainly used for medicinal purposes and has been used as a drug for indigestion, against worms, rheumatism, and pain in the joints, showing a potent antioxidant effect [89]. Coriander seed oil extract showed antibacterial and antifungal properties, a cardiovascular protective effect as well as gut modulation activities. Additionally, it lowers blood pressure and has a diuretic effect [90-96].

The oral administration of both aqueous and ethanol coriander seed extracts attenuated, to some extent, the histopathological changes to lead treated mice and significantly reduced the adverse effects related to most of the altered biochemical parameters and hepatic and renal oxidative stress [97]. Treatment with an aqueous extract of coriander leaf normalized enzymatic and nonenzymatic parameters in cadmium treated animals [98]. In addition, both coriander leaf and seed helped improve the adverse effects of thioacetamide induced hepatotoxicity which was confirmed by the histological study [99].

The ethanol-water extract of coriander leaf in a dose dependent manner protected the liver from the oxidative stress induced by CCl_4, significantly lowering serum transaminases (SGOT, SGPT), and TBARs levels. Hepatic enzymes like SOD, CAT, and glutathione peroxidase (GPx) were significantly increased with the plant extract treatment against CCl_4 treated rats. The activity of the leaf extract was comparable with that of the standard drug, silymarin [100].

Significant improvement in all biochemical parameters, which were close to the control, was found in rat liver supplemented with aqueous coriander seed extract and then administrated with paracetamol. The results were confirmed by histopathological examination of the liver tissue [101]. Another study pointed out that ethanolic extract of *C. sativum* displays a hepatoprotective effect by reducing liver weight, activities of SGOT, SGPT, and ALP, and direct bilirubin in CCl_4 intoxicated animals. Administration of coriander extract resulted in the fatty deposit disappearing, ballooning degeneration and necrosis, which all indicated antihepatotoxic activity [102].

A study which aimed to investigate the antioxidant activity of *C. sativum* leaf extract on CCl_4 treated Wistar albino rats showed that the serum from pre-treated animals had significant increases in SOD, CAT, and GPx activity, when compared with CCl_4 treated rats. Oral administration of the leaf extract significantly reduced the toxic effects of CCl_4 and, at a dose of 200 mg/kg, was comparable with the standard drug, silymarin. Based on these results, it was observed that *C. sativum* extract protected the liver from oxidative stress induced by CCl_4 and thus helped in the evaluation of the traditional claim for this plant [103].

Celery (*Apium graveolens* L.): Celery is not an official drug, but is among the favorite vegetables. It has a characteristic flavor similar to that of fennel and aniseed. The flavor is grassy and hay like, rather bitter [104] which originates from essential oil with limonene as the dominant compound, while phthalides are present in small amounts that give the characteristic fragrance [105, 106]. Celery is used in the pharmaceutical industry and traditional medicine because of its numerous beneficial properties important for preventing cardiovascular diseases, lowering blood glucose and serum lipid levels, decreasing blood pressure and strengthening the heart. This herb has antimicrobial, anti-inflammatory and antioxidant effects [107].

A significant hepatoprotective activity of the celery seed methanol extract was reported on rat liver damage induced by a single dose of paracetamol or thioacetamide [108]. Several liver function tests, such as SGOT and SGPT, ALP, sorbitol dehydrogenase (SDH), glutamate dehydrogenase (GLDH) and SBR were performed which showed a global ameliorated hepatic status.

The pre-treatment of celery seed extract to rats with induced hepatocarcinogenesis resulted in the elevation of quinonereductase (QR), glutathione-S-transferase (GST) and serum gamma glutamyl transferase (GGT) activities and the reduction of GSH in tissues after i.p. injection of 2-acetylaminofluorine [109].

Juices from celery root and leaf influenced the biochemical parameters and showed antioxidative effects when applied with doxorubicine to Wistar laboratory rats. The serum lipid peroxidases (LPx) activity was decreased only by administering celery leaf juice (alone or with doxorubicin), while the activities of xanthine oxidase (XOD), CAT, and GPx were increased with celery root and leaf juices [110].

The potential of celery juice in the prevention of oxidative stress and hepatotoxic effects induced by lead-acetate and an exposure to gamma-radiation (4Gy) was examined on an animal model. The results showed that celery juice normalized the levels of SBR, total protein and albumin. The intake of celery juice promoted a significant decrease in TBARs and protein carbonyl content (PCC), as well as an increase of GSH, CAT and SOD activitie, which confirmed the enhancement of the antioxidant defense mechanisms in rats [16]. The supplemented diet with a dry powder of celery leaf lowered the elevated serum level of liver enzymes and blood lipids in hypercholesterolemic rats. The histopathological lesions seen in the liver of these rats were also ameliorated. This study suggests that the dietary intake of celery can be beneficial to patients suffering from hypercholesterolemia and liver diseases [111].

The hepatoprotective potential of celery was also examined on fish. The study on *Pangasius sutchi* revealed that a short-term treatment with celery leaf powder could protect the liver from paracetamol induced liver injury [112].

Horse celery (*Smyrnium olusatrum* L.): Horse celery is a wild culinary plant all parts of which are edible. Its use was diminished after the domestication of celery. The essential oils of the root, herb and flower are characterized by a high content of oxygenated sesquiterpenoids, most of them furanosesquiterpenoids, while the green and ripe fruit are dominated by monoterpene hydrocarbons with β-phellandrene and α-pinene as major constituents [113]. The main component of the aboveground parts and the root is isofuranodiene, which possesses cytotoxic activity against the human colon cancer cell line and has hepatoprotective activity [114, 115]. It was proven that isofuranodiene protected the liver against D-galactosamine/lipopolysacchride (GalN/LPS)-induced injury in rats. According to the obtained results, it can be suggested that this plant may be a potential functional food ingredient for the prevention and treatment of liver diseases [115].

Lovage (*Levisticum officinale* Koch.): Lovage root (Levistici radix) is an official drug in the European Pharmacopoeia (Ph.Eur.2011). However, leaf, herb and seed are also used. The lovage flavor, like the celery one, originates from essential oil where the dominant compound is β–phellandrene, while phthalides are present in small amounts and give the characteristic fragrance [105]. Although lovage is used in traditional medicine as an emmenagogue, carminative, diuretic and remedy for various skin ailments, it possesses proven anti-inflammatory, antioxidant and anticancer properties [116].

The results of biochemical tests, in which rats were treated with lovage essential oil, a lovage fruit infusion and lovage herb infusion after subacute intoxication with acrylamide (hepatic cytolysis and proteosynthesis indicators) highlighted the high antitoxic potential of the lovage volatile oil [117]. Moreover, the essential oil of *L. officinale* showed antiproliferative activity *in vitro* [31].

Angelica (*Angelica archangelica* L.)*:* Angelica root (Angelicae radix) is an official drug in the European Pharmacopoeia (Ph.Eur.2011). However, the leaf and the seed are also used. The whole plant contains essential oil with α-and β-phellandrenes, as well as phthalides that considerably influence the oil flavor [118]. Angelica has traditionally been used as a carminative, diaphoretic and diuretic [119]. However, angelica also possesses antianxiety, antimicrobial and antioxidant properties [120-122].

The dietary supplementation of New Zealand albino rabbits with angelica improved the body burden of lead and therefore protected liver function against lead toxicity. However, the exact mechanism of the angelica protection effect was unclear [123].

Treating mice with angelica ameliorated the chronic ethanol induced hepatotoxicity effects. It was found that angelica inhibited malondialdehyde formation in mouse liver homogenates both *in vitro* and *in vivo*. Angelica is a cytoprotective agent efficient against chronic ethanol induced hepatotoxicity, possibly by inhibiting the production of oxygen free radicals that cause LP, and therefore, indirectly protecting the liver from oxidative stress [124].

Parsley (*Petroselinum crispum* L.): Parsley is not an official drug, but it is among the favorite vegetables. The root and leaf are used as well as seed, which are usually used for essential oil extraction. All parts contain essential oil rich in myristicin, apiol, and α- and β-pinene that give its characteristic fragrance [125]. Parsley leaf is used for treating various problems such as constipation, colic, edema, rheumatism, and prostate and liver diseases. Parsley is mostly used because of its antimicrobial, anti-anemic, hemorrhagic, antiplatelet, anticoagulant, antihyperlipidemic and laxative properties [126-129].

Parsley showed a hepatoprotective effect against an acute liver injury induced by CCl_4, significantly decreasing AST, ALT and GGT. Also, SOD and CAT were decreased in the group treated with parsley. The expression of tumor necrosis factor-alpha (TNF-α) was improved in the group treated with CCl_4 and parsley when compared with the group treated with CCl_4 only. Moreover, parsley reduced fatty degeneration, cytoplasmic vascularization and necrosis of the liver in the CCl_4 treated group. Although CCl_4 caused a decrease in non-protein sulfhydryl (NP-SH) level, the extract of parsley significantly renewed the NP-SH concentration [34, 130]. The hepatoprotective effect of parsley leaf was proven in a few studies made on liver injury caused by either paracetamol [131] or sodium valproate [132], as well as injuries due to the complication of diabetes [133]. Vitamin C and flavonoids are probably responsible for the hepatoprotective role of parsley [134]. Additionally, myristicin from the essential oil induced the activity of GST enzyme in the liver [135].

P. crispum extract administrated to rats fed with a fructose-enriched diet, which caused dyslipidemia, hepatic steatosis and infiltration of inflammatory cells in the liver and higher plasma hepatic markers, reversed the metabolic changes, and attenuated the chronic changes induced in non-alcoholic fatty liver disease (NAFLD) [136].

Carrot (*Daucus carota* L.): The cultivated carrot (*D. carota* ssp. *sativus*) is mainly consumed as a root vegetable, while its seed oil is sometimes used as a flavoring agent in food products and in the cosmetics industry [36, 137]. Wild carrot seed (*D carota* ssp. *carota*) has been used for medicinal purposes since ancient times. Nowadays, it is established that the oil of this seed possesses antinociceptive, anti-inflammatory, hypoglycemic, antidiabetic, antioxidative and anticancer activities [138].

Pre-treatment with a cultivated carrot seed extract significantly decreased the SGPT, SGOT and ALP levels in animals with thioacetamide induced oxidative stress. There was also a significant increase observed in SOD, CAT, GRD, GPx and GST, while the levels of LPO were significantly reduced, which showed a great antioxidant potential of the carrot extract [139]. The carrot extract could provide a significant protection against paracetamol, isoniazid and alcohol induced hepatocellular injury in animal models [140].

It was reported that a carrot seed extract could lower the plasma levels of AST, ALT and bilirubin in a dose-dependent manner in CCl_4 induced hepatotoxicity [141] and lindane induced hepatotoxicity [142] where the carrot extract also restored the depressed antioxidant and HDL cholesterol levels to near normal values. Another study showed that oral treatment with kaempferol isolated from carrot seed reversed all the serum and liver parameters in the paracetamol treated rats [143].

Methanol-acetone extract of wild carrot umbels demonstrated significant DPPH activity and high ferric reducing antioxidant power (FRAP) values. The sesquiterpene-rich fraction had the highest ferrous ion chelating (FIC) ability. Pretreatment with this extract reversed the CCl_4 decrease in SOD, CAT, and GST levels and significantly reduced the hepatic damage. The current results suggest that wild carrot oil fractions exhibit a unique chemical composition and possess significant antioxidant activities as well as hepatoprotective effects against CCl_4-induced hepatotoxicity [144].

Conclusion: Medicinal plants can serve as a vital source of potentially useful new compounds for the development of effective therapies to combat a variety of liver problems. The members of the *Apiaceae* family have demonstrated their heptatoprotective effects by both ameliorating hepatic functions and regenerating hepatic tissue on animal studies. The possible mechanism of their activities against chemically induced hepatotoxicity is their rich natural antioxidant content which inhibits undesirable oxidation processes occurring in the liver. The hepatoprotective potential of the *Apiaceae* family herbs should not be underestimated, and in the light of the presented evidence of their efficacy in animal studies, further detailed research is required. The reported studies have used various extracts of the same plant which were obtained from different parts of the given plant, or if the same parts were used, different extraction methods have been applied and the extracts differed in the composition and content of the active compounds. Even if the same extracts have been used in various studies, the doses applied were inconsistent. Well-designed human studies with

standardized extracts of the defined plant parts where the hepatoprotective activity of the *Apiaceae* herbs should be monitored are urgent and welcomed. Further studies should define the parts of the herbs which are the most potent in liver damage protection, as well as the safest and the most effective applied dose.

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