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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
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.

Prof. Vassya Bankova
Guest Editor
Head of Lab Natural Product Chemistry
Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences
Sofia, Bulgaria
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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 CHCl3 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-secoeudesmanolides – 14-(3-methylpentanoyl)-6-deoxybritannilactone, 14-(3-methylbuta-noyl)-6-deoxybritannilactone, and 14-(2-methylpropanoyl)-6-deoxybritannin, as well as 4H-tomentosin have been isolated. Varieties of lactones with secoeudesmane and secoaguaiane 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 (R² = 0.9954 and R² = 0.9910 for ABTS and DPPH, respectively).
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 CHCl₃ and MeOH. The corresponding crude extracts were compared by TLC (Silica gel 60 and RP-18, F₂₅₄ ᵃᵣ (Merck), CHCl₃-diethyl ether, 50:1; CHCl₃-acetone, 10:1 and 1:1; MeOH-H₂O, 1:1) and used for determination of the total phenolics, flavonoids and antioxidant capacity. Further, the CHCl₃ and MeOH extracts from flower heads (30 g) of the plant were worked up for isolation of the individual compounds. The CHCl₃ extract (1.5 g) was fractionated by column chromatography (CC) on silica gel using CHCl₃-acetone mixtures with increasing polarity to give 11 fractions (F₁₋₁₁). Further separation by CC and prep. TLC (silica gel, n-hexane-diethyl ether, 5:1) of Fr. F₂ (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 F₁ (silica gel, n-hexane-diethyl ether, 1:1) using these compounds as standards. Prep. TLC (silica gel, CHCl₃-acetone, 5:1) of F₁ (47 mg) afforded ivalin (7 mg) and britannin (14 mg). Gaillardin (33 mg) and pulchellin C (18 mg) were obtained from F₅ (65 mg) and F₁₁ (92 mg), resp. after recrystallization (CHCl₃). Prep. TLC (silica gel, CHCl₃-acetone, 5:1) of F₉ (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-H₂O–H₂O, 3:1–7:5 (50 mg) yielded luteolin (9 mg), quercetin (1.8 mg), luteolin-7-O-glucoside (2.0 mg), and 1,5-dicaffeoylquinic acid (6.4 mg). All isolated compounds were proved by comparison of their ¹H NMR spectral data with those in the literature.

**Determination of the content of total phenolic compounds (TPC) and total flavonoids (FC) and antioxidant capacity:** 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.

**Statistical analysis:** Correlation coefficients (R²) 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.

**References**


Production of Δ⁷-Sterols from *In Vitro* Root Cultures of Endangered *Gypsophila trichotoma*

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Species of 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. Δ⁷-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 Δ⁷-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. Δ⁷-Sterols, which are unusual and rare in the plant kingdom, are present in the roots of this species. 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 Δ⁷-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 Mg²⁺ and Ca²⁺ 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, Mg²⁺ and Ca²⁺ should be present in considerable concentration in order for Δ⁷-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–Liun modified media (MS–Li) for 1 L medium, pH 5.6 (before autoclaving).

<table>
<thead>
<tr>
<th>Compound</th>
<th>MS-Li</th>
<th>MS-Li+Mg²⁺</th>
<th>MS-Li+Ca²⁺</th>
<th>MS-Li+Ca²⁺</th>
<th>MS-Li+NaCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH₄NO₃</td>
<td>1650</td>
<td>1650</td>
<td>1650</td>
<td>1650</td>
<td>1650</td>
</tr>
<tr>
<td>KNO₃</td>
<td>1900</td>
<td>1900</td>
<td>1900</td>
<td>1900</td>
<td>1900</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>370</td>
<td>0</td>
<td>370</td>
<td>370</td>
<td>370</td>
</tr>
<tr>
<td>CaC₂H₃O₂</td>
<td>440</td>
<td>0</td>
<td>440</td>
<td>440</td>
<td>440</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>170</td>
<td>170</td>
<td>170</td>
<td>170</td>
<td>170</td>
</tr>
<tr>
<td>NaCl</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H₂BO₃</td>
<td>8.60</td>
<td>8.60</td>
<td>8.60</td>
<td>8.60</td>
<td>8.60</td>
</tr>
<tr>
<td>Ca(NO₃)₂·4H₂O</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>KCl</td>
<td>0.025</td>
<td>0.025</td>
<td>0.025</td>
<td>0.025</td>
<td>0.025</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>Na2MoO₄·2H₂O</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>CuSO₄·5H₂O</td>
<td>0.025</td>
<td>0.025</td>
<td>0.025</td>
<td>0.025</td>
<td>0.025</td>
</tr>
<tr>
<td>Na₂EDTA</td>
<td>0.025</td>
<td>0.025</td>
<td>0.025</td>
<td>0.025</td>
<td>0.025</td>
</tr>
<tr>
<td>FeSO₄·7H₂O</td>
<td>27.8</td>
<td>27.8</td>
<td>27.8</td>
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<td>27.8</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>Vitamin B₁</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>Vitamin B₃</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>Mio-inositol</td>
<td>0.100</td>
<td>0.100</td>
<td>0.100</td>
<td>0.100</td>
<td>0.100</td>
</tr>
<tr>
<td>Glucose</td>
<td>2.00</td>
<td>2.00</td>
<td>2.00</td>
<td>2.00</td>
<td>2.00</td>
</tr>
<tr>
<td>Sucrose</td>
<td>30000</td>
<td>30000</td>
<td>30000</td>
<td>30000</td>
<td>30000</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Root Culture</th>
<th>Stigmast-7-ene-3-O-β-D-glucopyranoside, % ± SD</th>
<th>Stigmast-7-ene-3-one, % ± SD</th>
<th>Stigmast-7-ene-3-ol, % ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS - Li</td>
<td>0.61 ± 0.01</td>
<td>2.11 ± 0.01</td>
<td>2.11 ± 0.01</td>
</tr>
<tr>
<td>MS - Li + NaCl</td>
<td>1.65 ± 0.01</td>
<td>6.03 ± 0.04</td>
<td>6.03 ± 0.04</td>
</tr>
<tr>
<td>MS - Li + Mg²⁺</td>
<td>0.24 ± 0.02</td>
<td>2.73 ± 0.02</td>
<td>2.73 ± 0.02</td>
</tr>
<tr>
<td>MS - Li + Ca²⁺</td>
<td>0.05 ± 0.04</td>
<td>0.59 ± 0.04</td>
<td>0.59 ± 0.04</td>
</tr>
<tr>
<td>MS - Li + Ca²⁺</td>
<td>0.78 ± 0.02</td>
<td>1.14 ± 0.03</td>
<td>1.14 ± 0.03</td>
</tr>
</tbody>
</table>

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
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 (tR = 16.312 min); Stigmast-7-ene-3-one (tR = 18.143 min); Stigmast-7-ene-3-ol (tR = 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 CH2Cl2. The dichlormethane 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 13C 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 Kruskhal-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 ≤ 0.05 was used as significance criteria.

**References**


Evaluation of Glaucine Content in Bulgarian Black Sea Coast
Localities of Glaucium flavum Crantz. (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 morphinan alkaloids and the principal of them are glaucine, isocorydine, corydine, protopine, isoboldine, corunine, chelidonine, sanguinarine, cataline, 7-oxoglausine, thaliporphone [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].

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.

<table>
<thead>
<tr>
<th>Locality, GPS coordinates, altitude</th>
<th>Glaucine in the dry plant material (%)</th>
<th>Glaucine in CAM (%)</th>
<th>CAM in the dry plant material (%)</th>
<th>Sample dry weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Durankulak N 43.69110, E 28.56325, 1 m</td>
<td>1.0±0.1</td>
<td>1.4±0.04</td>
<td>43.6±2.9</td>
<td>72.5±2.1</td>
</tr>
<tr>
<td>Shabla N 43.54282, E 28.60612, 3 m</td>
<td>2.1±0.1</td>
<td>1.5±0.03</td>
<td>76.0±3.1</td>
<td>68.1±1.4</td>
</tr>
<tr>
<td>Skorpilovtsi N 42.93834, E 27.89775, 5 m</td>
<td>0.9±0.1</td>
<td>0.6±0.1</td>
<td>47.8±2.8</td>
<td>36.5±4.8</td>
</tr>
<tr>
<td>Pomorie N 42.58634, E 27.63191, 6 m</td>
<td>2.3±0.1</td>
<td>2.0±0.04</td>
<td>74.2±1.7</td>
<td>59.4±1.3</td>
</tr>
<tr>
<td>Varvara N 42.13460, E 27.89718, 0 m</td>
<td>2.0±0.04</td>
<td>0.8±0.02</td>
<td>68.4±1.1</td>
<td>47.2±1.4</td>
</tr>
<tr>
<td>Ahtopol N 42.10403, E 27.93655, 4 m</td>
<td>2.0±0.04</td>
<td>2.3±0.1</td>
<td>68.7±1.2</td>
<td>58.1±2.3</td>
</tr>
<tr>
<td>Sinemorets N 42.06564, E 27.97379, 6 m</td>
<td>2.1±0.1</td>
<td>1.6±0.1</td>
<td>60.6±4.1</td>
<td>50.5±1.5</td>
</tr>
</tbody>
</table>
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 NH4OH (pH 9-10) and then extracted five times with CH2Cl2. The combined CH2Cl2 extracts were dried over anhydrous Na2SO4, 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 CHCl3 and 0.1 ml MeOH. 3 µl from sample were applied three times to DC Alufolien Kieselgel 60 F254 (Merck) and the sheets were developed with solvent system petroleum:CHCl3:Me2CO: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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References

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 *Crataegus 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 ssp.*, 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]. Hydroalcoholics extracts of *Crataegus* species have been found to possess hypcholesterolemic 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 Crataegus 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 92.4 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...
C. orientalis leaves and berries. 

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, isoorciquitin 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-glycosylflavonones, hyperoside and isoorciquitin. 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 isoorciquitin 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 (IC50 = 29.7 µg/g) than that of the berries (IC50 = 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 isoorciquitin. The berries extract exhibited lower antioxidant activity probably due to considerably lower amounts of hyperoside, isoorciquitin, 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 Fe2+ 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 Fe2+ 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 metabolites formed by them. Explicitly, 12-HHT (12(S)-hydroxy(5Z,8E,10E)-heptadecatrienoic acid), TXB2 (thromboxane B2) and PGE2 (prostaglandin E2) are inflammation mediators derived from arachidonic acid metabolism, which is catalyzed by COX-1 enzyme, while 12-HETE (12(S)-hydroxy-(5Z,8Z,10E,14Z)-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. Some C. orientalis leaves extract showed concentration-dependent inhibition of COX-1 pathway products 12-HHT and TXB2, reaching IC50 values below the lowest applied concentration (68.9% and 55.2% of 12-HHT and TXB2 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. oxyacantha tincture and its isolated flavonoids inhibit the formation of potent inflammatory and platelet aggregation mediator TXA2 an unstable metabolite which is hydrolyzed within about 30 seconds to TXB2 [23]. Taking into account the TXB2 inhibition potential of C. orientalis extracts determined in our study, some attention should be drawn to Crataegus species as a possible inhibitor of TXA2 synthase. Since TXA2 synthase is an enzyme that follows COX-1 in the formation of TXA2, a possible target for moderating inflammation by Crataegus species could be this point of arachidonic acid metabolism. Inhibition of PG E2 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

### 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.

<table>
<thead>
<tr>
<th>#</th>
<th>Compound</th>
<th>Leaves (mg/g DW)</th>
<th>Berries (µg/g)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Chlorogenic acid</td>
<td>11.8 ± 0.5</td>
<td>0.9 ± 0.0</td>
<td>0.000002</td>
</tr>
<tr>
<td>2</td>
<td>Epicatechin</td>
<td>7.2 ± 0.4</td>
<td>n.d.</td>
<td>0.000006</td>
</tr>
<tr>
<td>3</td>
<td>Vitexin</td>
<td>2.7 ± 0.0</td>
<td>0.5 ± 0.0</td>
<td>0.000000</td>
</tr>
<tr>
<td>4</td>
<td>Rutin</td>
<td>2.5 ± 0.0</td>
<td>0.6 ± 0.0</td>
<td>0.000000</td>
</tr>
<tr>
<td>5</td>
<td>Hyperoside</td>
<td>12.9 ± 0.1</td>
<td>1.4 ± 0.0</td>
<td>0.000000</td>
</tr>
<tr>
<td>6</td>
<td>Isoorciquitin</td>
<td>10.4 ± 0.1</td>
<td>0.9 ± 0.0</td>
<td>0.000000</td>
</tr>
<tr>
<td>7</td>
<td>Quercetin</td>
<td>0.5 ± 0.0</td>
<td>1.0 ± 0.0</td>
<td>0.000000</td>
</tr>
<tr>
<td>Total phenolic</td>
<td>94.2 ± 0.1</td>
<td>77.6 ± 0.8</td>
<td>0.000003</td>
<td></td>
</tr>
<tr>
<td>IC50</td>
<td>29.7 ± 0.1</td>
<td>111.9 ± 0.2</td>
<td>0.000001</td>
<td></td>
</tr>
</tbody>
</table>

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

### Table 2: IC50 values for COX-1 and 12-LOX assay of examined C. orientalis extracts.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>COX-1 pathway</th>
<th>12-LOX pathway</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC50 (mg/mL)</td>
<td>IC50 (µg/mL)</td>
</tr>
<tr>
<td>12-HHT</td>
<td>TXB2</td>
<td>PGE2</td>
</tr>
<tr>
<td>L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

[C. orientalis leaves extract showed concentration-dependent inhibition of COX-1 pathway products 12-HHT and TXB2, reaching IC50 values below the lowest applied concentration (68.9% and 55.2% of 12-HHT and TXB2 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. oxyacantha tincture and its isolated flavonoids inhibit the formation of potent inflammatory and platelet aggregation mediator TXA2 an unstable metabolite which is hydrolyzed within about 30 seconds to TXB2 [23]. Taking into account the TXB2 inhibition potential of C. orientalis extracts determined in our study, some attention should be drawn to Crataegus species as a possible inhibitor of TXA2 synthase. Since TXA2 synthase is an enzyme that follows COX-1 in the formation of TXA2, a possible target for moderating inflammation by Crataegus species could be this point of arachidonic acid metabolism. Inhibition of PGE2 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

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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, isoorientin 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-Ciocalteu 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 0-30 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 x 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 fomric 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\(_5\) (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×(R−R)/R0, 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-(5Z,8Z,10E,14Z)-eicosatetraenoic acid; 12-HHT: 12(S)-hydroxy(5Z,8E,10E)-heptadecatrienoic acid; 12-LOX: 12-lipoxygenase; COX-1: cyclooxygenase-1; PGB2: prostaglandin B2; PGE2: prostaglandin E2; TXA2: thromboxane A2; TXB2: thromboxane B2.

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References

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

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\textsuperscript{b}Department of Pharmacognosy and Molecular Basis of Phytotherapy, Medical University, ul. S. Banacha 1, Warsaw, Poland
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Leaves of \textit{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. \textit{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 (Table 1). 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: \textit{Eriobotrya japonica}, Cinchonain, Antioxidant, Polyphenols.

\textit{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 \textit{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 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'-diphenylpicrylhydrayl radical, as well as the highest concentration 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\textsubscript{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.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>EC\textsubscript{50} DPPH (μg/mL)</th>
<th>Reducing power AAE (%)</th>
<th>LA-Ion reduction</th>
<th>TPC Total Polyphenols (GAE) mg/g fraction</th>
<th>Tannins content (GAE) mg/g fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td>39.4</td>
<td>1.7</td>
<td>37.3</td>
<td>23.7</td>
<td>78.4</td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>60.2</td>
<td>1.9</td>
<td>43.5</td>
<td>24.8</td>
<td>22.4</td>
</tr>
<tr>
<td>Diethyl ether</td>
<td>47.3</td>
<td>2.4</td>
<td>18.7</td>
<td>20.9</td>
<td>39.4</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>22.3</td>
<td>4.5</td>
<td>57.4</td>
<td>69.5</td>
<td>177.4</td>
</tr>
<tr>
<td>n-Butanol</td>
<td>35.4</td>
<td>3.1</td>
<td>57.5</td>
<td>66.5</td>
<td>89.4</td>
</tr>
</tbody>
</table>

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

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...
The negative ionization parent prediction by HR-TOF-MS we identified compound 1 tentatively as a protocatechuic acid hexoside. The negative ionization parent prediction by HR-TOF-MS we identified compound 1 tentatively as a protocatechuic acid hexoside. However, based on their MS-MS spectra and molecular formula (compounds 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 Ib. 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 Ib, where it achieved an EC50 of 5.05 μmol/L compared to compound 1, where it achieved an EC50 of 5.05 μmol/L compared to compound 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.

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

<table>
<thead>
<tr>
<th>Variable</th>
<th>Reducing power</th>
<th>LA-Peroxid</th>
<th>TPC Total Polyphenols</th>
<th>Tannins content</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DPPH EC50 mg/mL</td>
<td>AAE (%)</td>
<td>(GAE) mg/g fraction</td>
<td>(GAE) mg/g fraction</td>
</tr>
<tr>
<td>DPPH EC50</td>
<td>1.00</td>
<td>-0.83</td>
<td>-0.83</td>
<td>-0.83</td>
</tr>
<tr>
<td>AAE 37°C</td>
<td>1.00</td>
<td>-0.83</td>
<td>-0.83</td>
<td>-0.83</td>
</tr>
<tr>
<td>AAE 90°C</td>
<td>-0.43</td>
<td>1.00</td>
<td>0.48</td>
<td>0.66</td>
</tr>
<tr>
<td>LA-Peroxid</td>
<td>-0.83</td>
<td>-0.83</td>
<td>0.60</td>
<td>0.60</td>
</tr>
<tr>
<td>TPC</td>
<td>-0.94</td>
<td>0.71</td>
<td>0.66</td>
<td>0.84</td>
</tr>
<tr>
<td>Tannins</td>
<td>-0.83</td>
<td>0.54</td>
<td>0.66</td>
<td>1.00</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention time [min]</th>
<th>UV [nm]</th>
<th>[M-H]+ m/z</th>
<th>MS2 [M-H]+ m/z</th>
<th>MS2 [M-H]+ m/z</th>
<th>qTOF n/z</th>
<th>Error (ppm)</th>
<th>formula**</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 *Protocatechuic acid O-hexoside</td>
<td>1.9</td>
<td>285</td>
<td>857</td>
<td>739</td>
<td>37.8</td>
<td>739.1668</td>
<td>-2.5</td>
<td>C21H20O12</td>
</tr>
<tr>
<td>2 *putative Protocatechuic acid O-hexoside derivative</td>
<td>3.5</td>
<td>260, 294</td>
<td>507</td>
<td>153, 505</td>
<td>131,513, 109</td>
<td>504.9900</td>
<td>-0.7</td>
<td>C21H20O11</td>
</tr>
<tr>
<td>3 Chlorogenic acid</td>
<td>17.7</td>
<td>241, 295</td>
<td>615</td>
<td>308</td>
<td>306</td>
<td>613.1621</td>
<td>-9.5</td>
<td>C13H16O9</td>
</tr>
<tr>
<td>4 Cinchonain Id 7-O-flavonopyranoside</td>
<td>20.7</td>
<td>239, 280</td>
<td>291</td>
<td>289</td>
<td>245,205</td>
<td>289.0726</td>
<td>-2.9</td>
<td>C13H16O9</td>
</tr>
<tr>
<td>5 (±)-Epicatechin</td>
<td>23.1</td>
<td>785</td>
<td>873</td>
<td>783.1691</td>
<td>783.1953</td>
<td>-2.5</td>
<td>C13H16O9</td>
<td></td>
</tr>
<tr>
<td>6 Kaempferol-3-O-(4&quot;-O)-L-(2&quot;,4&quot;)-di-E-feruloyl)-rhamnoside</td>
<td>26.7</td>
<td>239</td>
<td>865</td>
<td>739</td>
<td>739,695,577</td>
<td>739.2536</td>
<td>-23.5</td>
<td>C21H20O15</td>
</tr>
<tr>
<td>7 Procyasin C1</td>
<td>27.9</td>
<td>281</td>
<td>739</td>
<td>587</td>
<td>739.1668</td>
<td>-2.5</td>
<td>C21H20O15</td>
<td></td>
</tr>
<tr>
<td>8 Cinchonain Ib</td>
<td>28.9</td>
<td>220, 283</td>
<td>611</td>
<td>453</td>
<td>609</td>
<td>609.1475</td>
<td>-2.3</td>
<td>C21H20O15</td>
</tr>
<tr>
<td>9 Rutin</td>
<td>30.4</td>
<td>255, 352</td>
<td>465</td>
<td>303</td>
<td>431</td>
<td>431.0999</td>
<td>-3.6</td>
<td>C21H20O15</td>
</tr>
<tr>
<td>10 Hyperoside</td>
<td>30.9</td>
<td>256, 352</td>
<td>465</td>
<td>303</td>
<td>465</td>
<td>465.0885</td>
<td>-0.8</td>
<td>C21H20O15</td>
</tr>
<tr>
<td>11 Isoquercetin</td>
<td>31.4</td>
<td>266, 344</td>
<td>597</td>
<td>465</td>
<td>465</td>
<td>465.1518</td>
<td>-1.1</td>
<td>C21H20O15</td>
</tr>
<tr>
<td>12 Kaempferol pentosyl-hexoside</td>
<td>32.7</td>
<td>449</td>
<td>287</td>
<td>447</td>
<td>285</td>
<td>447.0950</td>
<td>-3.9</td>
<td>C21H20O15</td>
</tr>
<tr>
<td>13 Kaempferol-3-glucoside</td>
<td>34.1</td>
<td>257, 345</td>
<td>449</td>
<td>303</td>
<td>407</td>
<td>447.0944</td>
<td>-2.6</td>
<td>C21H20O15</td>
</tr>
<tr>
<td>14 Quercetin rhamnoside</td>
<td>37.8</td>
<td>220, 264</td>
<td>433</td>
<td>287</td>
<td>431</td>
<td>431.0999</td>
<td>-3.6</td>
<td>C21H20O15</td>
</tr>
<tr>
<td>15 Kaempferol glycoside</td>
<td>42.6</td>
<td>220, 711</td>
<td>485</td>
<td>709</td>
<td>709.3805</td>
<td>-9.9</td>
<td>C21H20O15</td>
<td></td>
</tr>
<tr>
<td>16 unknown compound</td>
<td>54.3</td>
<td>221, 321</td>
<td>999</td>
<td>823</td>
<td>997</td>
<td>997.4646</td>
<td>0.4</td>
<td>C6H12O3</td>
</tr>
<tr>
<td>17 Nerolidol-3-O-L-rhamnopyranosyl-(1-4)-O-L-rhamnopyranosyl-(1-2)-O-L-(4&quot;-E- feruloyl)-rhamnopyranosyl-(1-6)-O-L-glucopyranoside</td>
<td>61.8</td>
<td>221, 311</td>
<td>635</td>
<td>633</td>
<td>no data</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* 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 “Rosc_Eriobotrya_japonica_CN_Nawrot2015-1”). The dried leaves (7.0 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 x 100 mL), diethyl ether (4 x 100 mL), ethyl acetate (5 x 100 mL) and finally n-butanol (5 x 100 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:

\[
%\text{DPPH} = \frac{(A_{\text{abt}}-A_{\text{abr}})}{A_{\text{abt}}} \times 100
\]

where Abt is the absorbance of DPPH solution with the test extracts, Abr 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 EC50 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 FeSO₄. 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 MS¹ and HR-MS we used the qTOF mass spectrometer, whereas for MS³ 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 EC50 were calculated using Statistica 12 (Statsoft, Poland).

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References


LC/DAD/MS® 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), prolinospermic 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 emphasised 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 Ronniger 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, antispasmodic, 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 intracellular metal 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®). 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 MS² to m/z 179 and 161, characteristic for caffeic acid. The loss of 162 amu indicates the presence of a sugar moiety.
 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 MS2 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 MS2 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 MS2 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-hexose. In the literature there are data about the presence of 5′-hydroxyjasmonic acid 5′-hexoside in Thymus species [9]. Compound 6, proliothospermic acid, had a deprotonated molecular ion at m/z 357 and characteristic MS2 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].

Flavonols: Five flavonols were identified according to the UV–Vis absorption maxima and MS data (Table 1). The [M]+ ions at m/z 301 and 285 indicated two aglycones with the corresponding molecular ions: quercetin (aglycone of 11) 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].

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]−, luteolin pentosyl-hexoside (14) ([M−H−132-162]−), luteolin acetyl-pentosyl-hexoside (16) ([M−H−42-132-132]−), luteolin acetyl hexoside (17) ([M−H−42-132]−), 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.

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

<table>
<thead>
<tr>
<th>Compounds</th>
<th>t&lt;sub&gt;R&lt;/sub&gt; (min)</th>
<th>UV (nm)</th>
<th>[M−H]− (m/z)</th>
<th>[M−H−198−162]− (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fлавоноиды</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 Quinic acid</td>
<td>7.9</td>
<td>238, 284</td>
<td>191</td>
<td>173, 111</td>
</tr>
<tr>
<td>2 Caffeic acid hexoside</td>
<td>8.5</td>
<td>238, 288sh, 328</td>
<td>359</td>
<td>179, 163</td>
</tr>
<tr>
<td>3 Salviaflaside derivative</td>
<td>20.2</td>
<td>238, 290sh, 330</td>
<td>701</td>
<td>521, 359</td>
</tr>
<tr>
<td>4 Rosmarinic acid</td>
<td>22.6</td>
<td>238, 288sh, 328</td>
<td>359</td>
<td>179, 163</td>
</tr>
<tr>
<td>5 Hydroxojasmonic acid O-hexoside</td>
<td>22.8</td>
<td>252, 300sh, 332</td>
<td>359</td>
<td>179, 163</td>
</tr>
<tr>
<td>6 Prolithospermic acid</td>
<td>22.9</td>
<td></td>
<td>357</td>
<td>313, 269</td>
</tr>
<tr>
<td>7 Salvianolic acid B</td>
<td>23.8</td>
<td>238, 290sh, 330</td>
<td>717</td>
<td>519, 475</td>
</tr>
<tr>
<td>8 Salvianolic acid A</td>
<td>28.0</td>
<td>240, 298sh, 328</td>
<td>493</td>
<td>359, 161</td>
</tr>
<tr>
<td>9 Monomethyl lithospermate</td>
<td>34.8</td>
<td>290, 326</td>
<td>551</td>
<td>519, 359</td>
</tr>
<tr>
<td>Fлавон</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 Apigenin C-hexoside-C-hexoside</td>
<td>22.9</td>
<td>238, 272, 336</td>
<td>593</td>
<td>473, 353</td>
</tr>
<tr>
<td>11 Apigenin 7′-O-glucoside</td>
<td>29.6</td>
<td>286, 332</td>
<td>431</td>
<td>269</td>
</tr>
<tr>
<td>12 Luteolin dihexoside</td>
<td>24.7</td>
<td>254, 286sh, 308, 334</td>
<td>609</td>
<td>447, 285</td>
</tr>
<tr>
<td>13 Luteolin pentosyl-hexoside</td>
<td>25.5</td>
<td>234, 256, 328</td>
<td>579</td>
<td>447, 285</td>
</tr>
<tr>
<td>14 Luteolin 7′-O-glucoside</td>
<td>26.4</td>
<td>234, 256, 328</td>
<td>447</td>
<td>285</td>
</tr>
<tr>
<td>15 Luteolin acetyl pentosyl-hexoside</td>
<td>29.1</td>
<td>234, 292sh, 326</td>
<td>621</td>
<td>579, 561, 285</td>
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<tr>
<td>16 Luteolin acetyl hexoside</td>
<td>29.2</td>
<td>264, 344</td>
<td>489</td>
<td>285</td>
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<tr>
<td>17 Luteolin pentoside</td>
<td>30.7</td>
<td>240, 288, 328</td>
<td>593</td>
<td>285</td>
</tr>
<tr>
<td>18 Luteolin dipentoside</td>
<td>31.0</td>
<td>292, 334</td>
<td>549</td>
<td>417, 285</td>
</tr>
<tr>
<td>19 Luteolin pentoside</td>
<td>32.6</td>
<td>232, 288</td>
<td>417</td>
<td>371, 285</td>
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<tr>
<td>20 Luteolin dipentoside</td>
<td>36.4</td>
<td>256, 350</td>
<td>591</td>
<td>549, 531, 285</td>
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<tr>
<td>Fлаванон</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>11 Quercetin 3′-O-glucoside</td>
<td>24.5</td>
<td>236, 282, 348</td>
<td>463</td>
<td>301</td>
</tr>
<tr>
<td>12 Quercetin 3′-rutinoside</td>
<td>31.9</td>
<td>236, 282, 348</td>
<td>609</td>
<td>301</td>
</tr>
<tr>
<td>13 Kaempferol pentosyl-hexoside</td>
<td>29.9</td>
<td>288, 334</td>
<td>579</td>
<td>417, 285</td>
</tr>
<tr>
<td>14 Kaempferol acetyl pentosyl-hexoside</td>
<td>31.3</td>
<td>256, 350</td>
<td>621</td>
<td>417, 285</td>
</tr>
<tr>
<td>15 Kaempferol</td>
<td>43.3</td>
<td>256, 352</td>
<td>285</td>
<td>241, 216</td>
</tr>
<tr>
<td>Fлаван</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>16 Hesperetin glucoside</td>
<td>25.0</td>
<td>290</td>
<td>463</td>
<td>301</td>
</tr>
<tr>
<td>17 Naringenin</td>
<td>38.9</td>
<td>290</td>
<td>271</td>
<td>177, 151</td>
</tr>
</tbody>
</table>

Flavonoids: To the best of our knowledge, flavones are the dominant group of flavonoids present in Thymus species, followed by flavanon and flavanols. From the MS data, eleven flavones were detected. The [M]+ 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 citriodius [9]. Compound 10 had a deprotonated molecular ion at m/z 593, and MS2 fragments at 473 and 353. Both subsequent losses of 120 amu indicate C-glycosylation [10c]. So, compound 10 was tentatively identified as apigenin C-hexosid-C-hexoside, which was previously reported in T. vulgaris [11].

Flavanones: From the literature it can be found that flavanones, especially naringenin, eriodictiol and hesperetin, are present in different forms (as aglycones, glucosides and rutinosides) in...
Thymus species [9, 10a]. In the extracts of *T. alsarensis* only two flavonanes 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 flowers, followed by leaf, root and stem extracts. It was in the range from 8.15 to 314 mg/kg dry herb for flowers, 20.6 to 224 mg/kg 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 6.89; 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 flavonoids 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 Thymus species [9, 10a].

**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 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 TI in different parts of the plant indicated that the higher content of TI 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 TI content does not suggest any correlation (positive or negative) between the total phenolic content and the content of TI 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 (TI 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), proanthocyanidin 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 *Thymes* 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.
Table 3: Collection data for *Thymus alloadsensis*.

<table>
<thead>
<tr>
<th>Sample</th>
<th>N</th>
<th>Altitude/m</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>41.16459</td>
<td>21.94654</td>
<td>740 R*</td>
</tr>
<tr>
<td>2</td>
<td>41.15731</td>
<td>21.94211</td>
<td>772 R</td>
</tr>
<tr>
<td>3</td>
<td>41.15699</td>
<td>21.9448</td>
<td>753 A</td>
</tr>
<tr>
<td>4</td>
<td>41.13479</td>
<td>21.94189</td>
<td>1035 R</td>
</tr>
<tr>
<td>5</td>
<td>41.15734</td>
<td>21.94783</td>
<td>875 A</td>
</tr>
<tr>
<td>6</td>
<td>41.16172</td>
<td>21.94944</td>
<td>736 A</td>
</tr>
<tr>
<td>7</td>
<td>41.15731</td>
<td>21.94211</td>
<td>772 R</td>
</tr>
<tr>
<td>8</td>
<td>41.15758</td>
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</tr>
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<td>9</td>
<td>41.13708</td>
<td>21.94469</td>
<td>986 R</td>
</tr>
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<td>41.14761</td>
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<td>895 A</td>
</tr>
<tr>
<td>11</td>
<td>41.14612</td>
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<td>966 R</td>
</tr>
<tr>
<td>14</td>
<td>41.15959</td>
<td>21.94511</td>
<td>757 A</td>
</tr>
</tbody>
</table>

* 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 HNO₃ (69%, m/v) and 2 mL H₂O₂ (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-MS-MS 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 (R² ≥ 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. alloadsensis* are given in Table S1-S4, and photographs of *T. alloadsensis* in Figure S1.

**References**


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 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.

Ultrasound- and microwave-assisted extraction of elecampane (Inula helenium L.) roots.

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 (Rf = 0.50), sucrose (Rf = 0.44), fructooligosaccharides (FOSs), including 1-kestose (Rf = 0.37), nystose Rf = (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=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.

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.

The data from spectrophotometric and HPLC-RID analyses are summarized in Table 1.
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, fructose and glucose (Table 1). The low molecular weight carbohydrates were easily extracted by methanol, 70% (v/v) ethanol and distilled H2O. 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).

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

<table>
<thead>
<tr>
<th>Carbohydrate</th>
<th>UAE</th>
<th>UAE 70%EtOH</th>
<th>H2O</th>
<th>UAE</th>
<th>MAE</th>
<th>MAE 70%EtOH</th>
<th>MAE H2O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fructose</td>
<td>2.3±0.2</td>
<td>2.5±0.2</td>
<td>2.5±0.2</td>
<td>2.2±0.1</td>
<td>2.4±0.2</td>
<td>2.5±0.3</td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>0.3±0.1</td>
<td>0.2±0.1</td>
<td>0.3±0.1</td>
<td>0.3±0.1</td>
<td>0.2±0.1</td>
<td>0.2±0.1</td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>0.3±0.1</td>
<td>0.8±0.1</td>
<td>0.6±0.2</td>
<td>0.3±0.1</td>
<td>0.8±0.1</td>
<td>0.8±0.1</td>
<td></td>
</tr>
<tr>
<td>1-Kestose</td>
<td>0.4±0.1</td>
<td>0.5±0.1</td>
<td>0.5±0.1</td>
<td>0.4±0.1</td>
<td>0.5±0.1</td>
<td>0.5±0.1</td>
<td></td>
</tr>
<tr>
<td>Nystose</td>
<td>0.3±0.1</td>
<td>0.4±0.1</td>
<td>0.4±0.1</td>
<td>0.3±0.1</td>
<td>0.4±0.1</td>
<td>0.3±0.1</td>
<td></td>
</tr>
<tr>
<td>Inulin</td>
<td>2.5±0.1</td>
<td>21.2±0.3</td>
<td>19.3±1.1</td>
<td>1.8±0.2</td>
<td>38.0±1.1</td>
<td>38.4±0.4</td>
<td></td>
</tr>
<tr>
<td>Total fructans</td>
<td>5.8±0.3</td>
<td>25.0±0.6</td>
<td>42.0±1.3</td>
<td>5.0±0.1</td>
<td>23.7±0.8</td>
<td>33.5±1.1</td>
<td></td>
</tr>
<tr>
<td>Yield (%)</td>
<td>30</td>
<td>41</td>
<td>58</td>
<td>28</td>
<td>35</td>
<td>48</td>
<td></td>
</tr>
</tbody>
</table>

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

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].

### 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.

<table>
<thead>
<tr>
<th>No Compound</th>
<th>UAE</th>
<th>UAE 70%EtOH</th>
<th>H2O</th>
<th>UAE</th>
<th>MAE</th>
<th>MAE 70%EtOH</th>
<th>MAE H2O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenolic acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1  Gallic</td>
<td>nf</td>
<td>nf</td>
<td>0.2*</td>
<td>nf</td>
<td>0.1*</td>
<td>0.1*</td>
<td></td>
</tr>
<tr>
<td>2  Hydroxybenzoic</td>
<td>0.1*</td>
<td>0.1*</td>
<td>0.1*</td>
<td>0.1*</td>
<td>0.1*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3  Chlorogenic</td>
<td>1.3±0.2</td>
<td>1.8*</td>
<td>0.1*</td>
<td>0.2*</td>
<td>1.0*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4  Neocatecholic</td>
<td>0.2±0.1</td>
<td>0.6*</td>
<td>0.2*</td>
<td>0.2*</td>
<td>0.2*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5  Caffeic</td>
<td>0.4*</td>
<td>0.2*</td>
<td>0.2*</td>
<td>0.2*</td>
<td>0.2*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6  p-Coumaric</td>
<td>0.1*</td>
<td>0.1*</td>
<td>0.1*</td>
<td>0.1*</td>
<td>0.1*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7  Syringic</td>
<td>0.2±0.1</td>
<td>0.3±0.1</td>
<td>nf</td>
<td>trace</td>
<td>0.1*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8  Ferulic</td>
<td>0.6±0.1</td>
<td>1.0±0.1</td>
<td>nf</td>
<td>nf</td>
<td>nf</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9  3,4-Dihydroxybenzoic</td>
<td>nf</td>
<td>nf</td>
<td>trace</td>
<td>0.1*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 Vanillic</td>
<td>nf</td>
<td>nf</td>
<td>trace</td>
<td>0.4±0.1</td>
<td>trace</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11 Cinnamic</td>
<td>nf</td>
<td>nf</td>
<td>trace</td>
<td>0.4±0.1</td>
<td>trace</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flavonoids</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 Quercetin</td>
<td>nf</td>
<td>0.6*</td>
<td>0.2*</td>
<td>0.2±0.1</td>
<td>0.3*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13 Kaempferol</td>
<td>0.5*</td>
<td>0.1*</td>
<td>0.2*</td>
<td>0.2±0.1</td>
<td>0.1*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14 Myricetin</td>
<td>0.7±0.1</td>
<td>0.2*</td>
<td>trace</td>
<td>0.3±0.1</td>
<td>0.1*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15 Catechin</td>
<td>10.5±0.1</td>
<td>1.0±0.1</td>
<td>2.2±0.1</td>
<td>6.5±0.1</td>
<td>0.6*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16 Epicatechin</td>
<td>nf</td>
<td>nf</td>
<td>nf</td>
<td>nf</td>
<td>nf</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17 Quercetin-3-O-beta-glucopyranoside</td>
<td>0.1*</td>
<td>nf</td>
<td>nf</td>
<td>nf</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ndf – 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 acid.
The current research revealed the efficiency of UAE for extraction of phytochemicals from *I. helenium* roots. The 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, ferulic, and caffeic acids), and also inulin with potential immunostimulating and other health beneficial properties.

Experiment

**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 H₂O 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 45°C [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 F₂₅₄ 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:HO₂Ch:COOH (7:5:4.2) (v/v/v/v) that was developed over 4 and 8 cm, respectively. The TLC plates were dipped in diphenylamine-amlene-H₃PO₄-acetone (1:1:5:50) (w/v/w/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 H₂O, 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 H₂O 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 (r² = 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(NO₃)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 25°C 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, epicatechin, 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.

Acknowledgments - The authors thank Assist. Prof. Manol Ognyanov PhD and Irina Yanakieva (LBAS, Plovdiv) for HPLC analysis of some phenolic compounds.

References


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 quasiequimolar 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 medicine [5]. It has also gained attention due to its silydianin, which are located predominantly in the fruit and seeds.

The active component of this herb is silymarin, which is a mixture of phenolic compounds, namely 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 quasiequimolar 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 chemopreventative 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](image1.png)

![Silybin B](image2.png)

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

\[
\log Pm = -2.945(\pm0.228) + 0.600(\pm0.046)\log D - 7.655(\pm0.811)\text{TPSA/MW}
\]

\[
n = 196, F = 1.108, F = 338.9
\]

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Predicted logPm (cm/s)</th>
<th>logD at pH=7.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silybin A</td>
<td>-4.345</td>
<td>1.77</td>
</tr>
<tr>
<td>Silybin B</td>
<td>-4.345</td>
<td>1.77</td>
</tr>
<tr>
<td>Dehydrodihydroxyperil A</td>
<td>-4.822</td>
<td>1.93</td>
</tr>
<tr>
<td>Dehydrodihydroxyperil B</td>
<td>-4.862</td>
<td>1.93</td>
</tr>
<tr>
<td>Isohydroxyperil A</td>
<td>-4.315</td>
<td>1.82</td>
</tr>
<tr>
<td>Isohydroxyperil B</td>
<td>-4.315</td>
<td>1.82</td>
</tr>
<tr>
<td>Dehydrodihydroxyperil A</td>
<td>-4.832</td>
<td>1.93</td>
</tr>
<tr>
<td>Silychristin ABl</td>
<td>-4.667</td>
<td>1.78</td>
</tr>
<tr>
<td>Silychristin ABl</td>
<td>-4.667</td>
<td>1.78</td>
</tr>
<tr>
<td>Dehydrodihydroxyperil A</td>
<td>-4.822</td>
<td>1.93</td>
</tr>
<tr>
<td>Dehydrodihydroxyperil A</td>
<td>-4.822</td>
<td>1.93</td>
</tr>
</tbody>
</table>

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

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).

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...
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 Asp351 (Figure 5).

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.

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 10R/11R and 10S/11S 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, 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-hydroxytamoxifen). 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.

Acknowledgments - The Support of “Program for career development of young scientists, BAS” DFNP-141/2016 and ESF COST Action CM1407 is gratefully acknowledged.

References
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, 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'-4(S)-o-methylbutrylalkannin (2), isobutyrylalkannin (3), propionylalkannin (4), acetylalkannin (5), β-hydroxyisovalerylalkannin (6), and β,β-dimethylacrylalkannin (7). All structures were identified by 1D 1H-/13C- 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, Isohexenynaphthazarins, 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 [3]. 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 Boraginaceae 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

<table>
<thead>
<tr>
<th>Naphthoquinones</th>
<th>O. kaheirei</th>
<th>O. graeca</th>
<th>O. erecta</th>
<th>A. sfikasiana</th>
<th>C. columnae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deoxyalkannin (1)</td>
<td>√</td>
<td>√</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2'-4(S)-o-methylbutrylalkannin (2)</td>
<td>√</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isobutyrylalkannin (3)</td>
<td>√</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Propionylalkannin (4)</td>
<td>√</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetylalkannin (5)</td>
<td>√</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Hydroxyisovalerylalkannin (6)</td>
<td>√</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mixture (2'-4(S)-o-methylbutrylalkannin, isobutyrylalkannin, β,β-Dimethylacrylalkannin (7)</td>
<td>√</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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 tested 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 $2.0 \times 10^{-2}$- $4.90 \times 10^{-3}$ 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 [α]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 CDCl₃ 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 nm, Merck). TLC plates (Kieselgel 60 F254) 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* (Crette, Chania), *O. graeca* and *A. sfikasiana* (Parnon Mountain, Peloponnesse) 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/CH₂Cl₂ 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 1H- (400 MHz, CDCl₃) and 13C-NMR (50 MHz, CDCl₃) 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 22785) 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)

<table>
<thead>
<tr>
<th>Samples</th>
<th>S. aureus</th>
<th>S. epidermidis</th>
<th>P. aeruginosa</th>
<th>E. coli</th>
<th>E. cloacae</th>
<th>C. albicans</th>
<th>C. propionic</th>
<th>C. glabrata</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>O. kaheirei</em></td>
<td>3.00</td>
<td>2.76</td>
<td>3.00</td>
<td>3.78</td>
<td>3.13</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>A. sfikasiana</em></td>
<td>0.0029</td>
<td>0.0029</td>
<td>0.0050</td>
<td>0.0057</td>
<td>0.0047</td>
<td>0.0047</td>
<td>0.0060</td>
<td>0.0056</td>
</tr>
<tr>
<td><em>O. graeca</em></td>
<td>0.0021</td>
<td>0.0020</td>
<td>0.0033</td>
<td>0.0037</td>
<td>0.0039</td>
<td>0.0040</td>
<td>0.0051</td>
<td>0.0048</td>
</tr>
<tr>
<td><em>O. erecta</em></td>
<td>3.50</td>
<td>2.95</td>
<td>3.75</td>
<td>4.22</td>
<td>3.87</td>
<td>3.96</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>C. columnae</em></td>
<td>3.70</td>
<td>3.10</td>
<td>3.97</td>
<td>4.57</td>
<td>4.10</td>
<td>4.56</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Deoxyalkannin (1)</td>
<td>0.0029</td>
<td>0.0027</td>
<td>0.0034</td>
<td>0.0035</td>
<td>0.0038</td>
<td>0.0040</td>
<td>0.0057</td>
<td>0.0055</td>
</tr>
<tr>
<td><strong>2''-(S)-α-Methylbutyrylalkannin (2)</strong></td>
<td>0.0024</td>
<td>0.0022</td>
<td>0.0033</td>
<td>0.0037</td>
<td>0.0039</td>
<td>0.0053</td>
<td>0.0051</td>
<td>0.0048</td>
</tr>
<tr>
<td>Isobutyrylalkannin (3)</td>
<td>0.0022</td>
<td>0.0020</td>
<td>0.0029</td>
<td>0.0032</td>
<td>0.0034</td>
<td>0.0036</td>
<td>0.0055</td>
<td>0.0051</td>
</tr>
<tr>
<td>Propionylalkannin (4)</td>
<td>0.0022</td>
<td>0.0022</td>
<td>0.0028</td>
<td>0.0032</td>
<td>0.0035</td>
<td>0.0038</td>
<td>0.0057</td>
<td>0.0052</td>
</tr>
<tr>
<td>Acetylalkannin (5)</td>
<td>0.0026</td>
<td>0.0024</td>
<td>0.0034</td>
<td>0.0035</td>
<td>0.0038</td>
<td>0.0039</td>
<td>0.0056</td>
<td>0.0055</td>
</tr>
<tr>
<td>β-Hydroxyisovalerylalkannin (6)</td>
<td>0.0020</td>
<td>0.0019</td>
<td>0.0027</td>
<td>0.0032</td>
<td>0.0033</td>
<td>0.0036</td>
<td>0.0049</td>
<td>0.0048</td>
</tr>
<tr>
<td>Mix of β,β-dimethylbutyrylalkannin (7) + (3)</td>
<td>0.0020</td>
<td>0.0019</td>
<td>0.0024</td>
<td>0.0030</td>
<td>0.0033</td>
<td>0.0035</td>
<td>0.0048</td>
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</tr>
</tbody>
</table>

### References


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

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\textsuperscript{a}Department of Chemical and Physical Properties of Food, Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, Olsztyn, Poland
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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 \textit{(Sideritis scardica)}, Mashterka tea \textit{(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\textsuperscript{•} and ABTS\textsuperscript{•+} 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, anti-allergic and immune response activities, as well as anti-allergic and anti-diabetic properties [1]. The antioxidant potential of herbal teas has been confirmed by several authors [2-5] using several chemical methods (ABTS, FRAP, and 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-irradiation. During this process, OH\textsuperscript{•} free radicals are created. These radicals interact with the DNA of microorganisms, leading to their death [7]. It is worth emphasizing that gamma-irradiation 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 Stujner 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.

<table>
<thead>
<tr>
<th>Herbal tea</th>
<th>Total phenolic contents (mg/g extract)</th>
<th>Total phenolic contents (mg/g tea)</th>
<th>Tannin contents (mg/g extract)</th>
<th>Tannin contents (mg/g tea)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Irradiated</td>
<td>Control</td>
<td>Irradiated</td>
</tr>
<tr>
<td>Mursalski</td>
<td>268±5</td>
<td>272±10</td>
<td>15.1±0.3</td>
<td>16.8±0.6*</td>
</tr>
<tr>
<td>Mashterka</td>
<td>104±7</td>
<td>121±5*</td>
<td>35.5±3</td>
<td>36.4±1.6</td>
</tr>
<tr>
<td>Good night</td>
<td>215±6</td>
<td>226±4</td>
<td>14.9±0.6</td>
<td>15.8±0.3</td>
</tr>
<tr>
<td>Staroplaninski</td>
<td>209±6</td>
<td>241±5**</td>
<td>12.4±0.4</td>
<td>14.8±0.3***</td>
</tr>
<tr>
<td>Trakia</td>
<td>176±3</td>
<td>174±4</td>
<td>15.3±0.2</td>
<td>17.0±0.4***</td>
</tr>
<tr>
<td>Planinski</td>
<td>257±5</td>
<td>273±10</td>
<td>27.7±0.6</td>
<td>28.5±1.01</td>
</tr>
</tbody>
</table>

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

Figure 1: Antiradical activity against ABTS•⁺ in 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.

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); Staroplanski tea (Balkan mix: wild thyme, peppermint, marjoram, blackberry leaves and elderflowers); Traika 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•+ 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 EC50, 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 Prism - Sowfware.

**References**


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 ICP 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).
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 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 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 Gleysoi [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 gleicy 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 homogenity 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 HNO₃ and redox reaction with H₂O₂ 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.

<table>
<thead>
<tr>
<th>Trace elements</th>
<th>LOD for soil (mg kg⁻¹)</th>
<th>LOD for plant material (mg kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cd</td>
<td>0.0111</td>
<td>0.013</td>
</tr>
<tr>
<td>Co</td>
<td>0.0201</td>
<td>0.041</td>
</tr>
<tr>
<td>Cr</td>
<td>0.0931</td>
<td>0.053</td>
</tr>
<tr>
<td>Cu</td>
<td>0.1483</td>
<td>0.088</td>
</tr>
<tr>
<td>Fe</td>
<td>3.4387</td>
<td>0.430</td>
</tr>
<tr>
<td>Mn</td>
<td>0.1902</td>
<td>0.025</td>
</tr>
<tr>
<td>Ni</td>
<td>0.1493</td>
<td>0.029</td>
</tr>
<tr>
<td>Pb</td>
<td>0.0731</td>
<td>0.062</td>
</tr>
<tr>
<td>Zn</td>
<td>0.1987</td>
<td>0.060</td>
</tr>
<tr>
<td>As</td>
<td>0.0499</td>
<td>0.055</td>
</tr>
</tbody>
</table>

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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References


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 possess 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-borisi* 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).
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*\textsubscript{ST} is 0.585 (P < 0.001). Based on the estimated *F*\textsubscript{ST} value, a low level of gene flow (*N*\textsubscript{m} = 0.250; *N*\textsubscript{m} < 1) was found. The AMOVA analysis points to higher levels of variation among populations (58%) than within populations (Table 2).

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

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 1](image1.png)

**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](image2.png)

**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 palearctic 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. Konyavskata 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](image3.png)

**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.

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

<table>
<thead>
<tr>
<th>Population name</th>
<th>N</th>
<th>P (%)</th>
<th>SI</th>
<th>Private bands</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tsarvenyano village</td>
<td>20</td>
<td>60.21</td>
<td>0.354 (0.027)</td>
<td>12</td>
</tr>
<tr>
<td>Vukovo village</td>
<td>20</td>
<td>56.87</td>
<td>0.211 (0.029)</td>
<td>4</td>
</tr>
<tr>
<td>Mean (SE)</td>
<td>58.54</td>
<td>0.283 (0.026)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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

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

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>df</th>
<th>Sum of squares</th>
<th>Mean sum of squares</th>
<th>Variance components</th>
<th>Variation (%)</th>
<th><em>F</em>\textsubscript{ST}</th>
<th><em>P</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Among populations</td>
<td>1</td>
<td>45.071</td>
<td>45.071</td>
<td>3.064</td>
<td>58</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Within populations</td>
<td>26</td>
<td>56.571</td>
<td>2.176</td>
<td>2.176</td>
<td>42</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>27</td>
<td>101.643</td>
<td>5.240</td>
<td>100</td>
<td>0.585</td>
<td>&lt;0.001</td>
<td></td>
</tr>
</tbody>
</table>
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 (Ta) for ISSR primers used in this study.

<table>
<thead>
<tr>
<th>Primer sequence</th>
<th>Total number of bands</th>
<th>Number of polymorphic bands</th>
<th>Polymorphisms (%)</th>
<th>Ta (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(CA)8G</td>
<td>15</td>
<td>15</td>
<td>100</td>
<td>60</td>
</tr>
<tr>
<td>(CA)8A</td>
<td>13</td>
<td>13</td>
<td>100</td>
<td>60</td>
</tr>
<tr>
<td>(CA)8T</td>
<td>15</td>
<td>15</td>
<td>100</td>
<td>60</td>
</tr>
<tr>
<td>(AC)8C</td>
<td>14</td>
<td>14</td>
<td>100</td>
<td>60</td>
</tr>
<tr>
<td>(AC)8YT</td>
<td>17</td>
<td>17</td>
<td>100</td>
<td>55</td>
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<tr>
<td>(AC)8C</td>
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<td>14</td>
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<td>60</td>
</tr>
<tr>
<td>(AC)8G</td>
<td>15</td>
<td>15</td>
<td>100</td>
<td>60</td>
</tr>
<tr>
<td>(AG)8YT</td>
<td>17</td>
<td>17</td>
<td>100</td>
<td>55</td>
</tr>
<tr>
<td>(AG)8YC</td>
<td>16</td>
<td>16</td>
<td>100</td>
<td>55</td>
</tr>
<tr>
<td>(AG)8G</td>
<td>15</td>
<td>15</td>
<td>100</td>
<td>55</td>
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<tr>
<td>(AC)8YT</td>
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<tr>
<td></td>
<td>156</td>
<td>156</td>
<td></td>
<td></td>
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</tbody>
</table>

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 GenAIEx v.6.5 [14a,14b] and ARLEQUIN v.3.5.1 [14c]. GenAIEx 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].

Acknowledgments – This work was supported by the Bulgarian National Science Fund under Grant DFNI-BO2/18. The authors wish to thank Mr. Stoyan Stoyanov (IBER, BAS) and Dr. Bryoya Sidjimova (IBER, BAS) for providing us with plant material. The sample collection was made with permission from the MOEW, according to the legislation № 634/24.04.2015.

References

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 monoterpenes 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 sweet 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 linallyl- 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.

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 1A, 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...
oxidative stress. 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). Prolineline 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].

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≤0.01) and salinity (r=0.899 and 0.945, P≤0.05), 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-

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 nerylacetates (Table 3). Generally, SWD reduced significantly the total amount of monoterpenic 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-f-limonene. 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 CO2 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 glycerol 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 against oxidative stress. 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-
mechanism against stress factors as suggested [32], although this hypothesis needs further investigation. Generally, 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 Scalfert 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 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 Na₂SO₄ and stored at 4-6 °C 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 C₉-C₂₂ 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 11.70 (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.

Acknowledgments - This work was supported financially by the Aristotle University of Thessaloniki and Hellenic Agricultural Organization DEMETER.
References


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 cytoxic 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% 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 cytoxic 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%), 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%).

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.
Table 1: Chemical composition of Brazilian red propolis essential oil

<table>
<thead>
<tr>
<th>No</th>
<th>Compounds</th>
<th>RI&lt;sup&gt;a&lt;/sup&gt;</th>
<th>RI&lt;sup&gt;b&lt;/sup&gt;</th>
<th>1-R</th>
<th>2-R</th>
<th>3-R</th>
<th>% of TIC&lt;sup&gt;c&lt;/sup&gt;</th>
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<tbody>
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<td>1200</td>
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<tr>
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</tr>
</tbody>
</table>

*Retention indices on apolar column (HP-5MS).<sup>1</sup> Retention indices on apolar column of literature ([15] and NIST).<sup>2</sup> **The total ion current (TIC) generated depends on characteristics of the compound concerned and is not a true quantification.<sup>3</sup> 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

<table>
<thead>
<tr>
<th>No</th>
<th>Compounds</th>
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</table>

*Retention indices on apolar column (HP-5MS).<sup>1</sup> Retention indices on apolar column of literature ([15] and NIST).<sup>2</sup> **The total ion current (TIC) generated depends on characteristics of the compound concerned and is not a true quantification.<sup>3</sup> Traces of compound identified. In quantification of different classes of compounds the amounts of traces are not included.

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Oxygenated mono- and sesquiterpenes predominate over terpene hydrocarbons and sesquiterpenes are more abundant than monoterpenes. The oxygenated monoterpenes represent 12.2% of the oil, with geraniol 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 as 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 their 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 Na2SO4, 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 El-mass spectra with NIST library spectra and literature [15].

Acknowledgments - The authors wish to thank “Pulolenn” France for providing the propolis samples.

References


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 (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.

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 sabine (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-terpinenol and 8-α-acetoxyelemol were greater in the oil of J. horizontalis, 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.
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 method (58.7, 59.5, and 57.2 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*, *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 sabine was the major oil constituent of *J. horizontalis* and *J. scopulorum* essential oils. The results of this study agree with the previous work by Adams et al. [7]. They reported that the oils of several varieties of *J. communis* from the USA 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 sabine 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. Butkine 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 α-pinene (8.7%). Ehsani et al. [27] reported other main compounds in the essential oil as sabine (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 sabine 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, Saskatchewan, Canada, with the major oil constituents sabine (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 sabine (57-61%), higher α-pinene, and similar limonene concentrations compared with the results reported by Adams [17].

### J. scopulorum

Adams and Hagerman [29] reported sabine as the major oil constituent of greenhouse grown *J. scopulorum* ranging from 33 to 39% of the oil. Sabine was the major constituent in the essential oil of *J. scopulorum* collected in the Bighorn Mountains in Wyoming. Adams [30] reported sabine (46.3%) as the major oil constituent of *J. scopulorum* sampled from Durango, CO. Sabine concentration in the essential oil obtained from most of the *J. scopulorum* accessions ranged from 41 to 55%, whereas in one of the accessions, sabine 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 sabine 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, sabine, 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. sabine varied from 51 to 77% of the oil of a single female tree [14], while sabine 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, sabine, myrcene, limonene, and 8-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 subsamples, 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 x 0.32 mm x 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 (ORACool) 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 μmol 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 Staphylococcus 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, sabinine, 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% 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 microtitre-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 antioxidant, 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 Angelica 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 sabine, 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 were α-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.

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<td>9.318</td>
<td>1046</td>
<td>3.6</td>
</tr>
<tr>
<td>17</td>
<td>γ-Terpine</td>
<td>9.729</td>
<td>1058</td>
<td>1.0</td>
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<tr>
<td>18</td>
<td>Terpinolene</td>
<td>10.858</td>
<td>1086</td>
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<tr>
<td>24</td>
<td>4-Menth-1,5-dien-8-ol</td>
<td>14.081</td>
<td>1165</td>
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<tr>
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<td>Terpinen-4-ol</td>
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<td>1175</td>
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<tr>
<td>27</td>
<td>Cryptone</td>
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<tr>
<td>30</td>
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<td>19.299</td>
<td>1283</td>
<td>0.4</td>
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<tr>
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<td>22.935</td>
<td>1364</td>
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<tr>
<td>33</td>
<td>Cubenene</td>
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<td>1374</td>
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<tr>
<td>34</td>
<td>α-Copaene</td>
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<tr>
<td>35</td>
<td>β-Elemene</td>
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<td>36</td>
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<tr>
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<tr>
<td>39</td>
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<tr>
<td>40</td>
<td>trans-Muurol-4(14),5-diene</td>
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<tr>
<td>41</td>
<td>α-Muurolene</td>
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<tr>
<td>42</td>
<td>Cuparene</td>
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<td>1506</td>
<td>0.1</td>
</tr>
<tr>
<td>43</td>
<td>β-Bisabolene</td>
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<td>1510</td>
<td>0.3</td>
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<tr>
<td>44</td>
<td>α-Copaene-11-ol</td>
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<td>1.3</td>
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<tr>
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<tr>
<td>50</td>
<td>δ-Cadinene</td>
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<tr>
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<tr>
<td>52</td>
<td>Cuparene</td>
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<tr>
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<td>55</td>
<td>Spathulenol</td>
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<td>0.5</td>
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<tr>
<td>56</td>
<td>Oxacyclotetradecane-2-one + NI</td>
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<td>1626</td>
<td>0.5</td>
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<tr>
<td>57</td>
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<td>Oxathie</td>
<td>35.715</td>
<td>1680</td>
<td>0.3</td>
</tr>
</tbody>
</table>

*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.

Keywords: Angelica, Staphylococcus aureus, Escherichia coli, MIC, MBC.
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 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 H2; 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 106 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-3H-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.

**References**


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, 1E,3R,4S,5E,7Z-1-bromo-3,4,8-(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\(_2\)Cl\(_2\), C\(_{10}\)H\(_{11}\)BrCl\(_4\), C\(_{10}\)H\(_{9}\)BrCl\(_6\) 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 1E,3R,4S,5E,7Z-1-bromo-3,4,8-trichloro-7-(dichloromethyl)-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] 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 Henkies 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.

![Photograph of a Namibian Plocamium sample](image)

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 1E,3R,4S,5E,7Z-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 1E,3R,4S,5E,7Z-1-bromo-3,4,8-trichloro-7-(dichloro-methyl)-3-methylocta-1,5,7-triene. With the 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 C₆H₁₃BrCl⁺ 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 3R*,4R* diastereomer of compound 8, while the other is the 1Z isomer (but with 3R*,4S*). The remaining two isomers detected in the current study could possibly be diastereomers of the 7E 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: C₁₀H₁₂BrCl₆. Although the mass spectrum of compound 12 also displayed some similarities with that of compound 8 (including the
Rapid ID of halogenated monoterpenes using GC-MS and RI

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

<table>
<thead>
<tr>
<th>R1</th>
<th>Predicted R1</th>
<th>RI</th>
<th>Diagnostic ions (m/z)</th>
<th>Proposed ion formula</th>
<th>Proposed molecular structure and/or molecular formula</th>
<th>Identification</th>
<th>Relative amounts (n = 5)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>28.06</td>
<td>1698</td>
<td>1674</td>
<td>133</td>
<td>C5H5Cl-</td>
<td>MS, RI</td>
<td>0.52 ± 0.40</td>
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<td>2</td>
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<td>-</td>
<td>1706</td>
<td>162, 164 (3:1)</td>
<td>unknown</td>
<td>MS</td>
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<tr>
<td>3</td>
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<td>1766</td>
<td>89, 91 (3:2)</td>
<td>C6H7Cl-</td>
<td>MS, RI</td>
<td>0.64 ± 0.14</td>
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<tr>
<td>4</td>
<td>31.70</td>
<td>1788</td>
<td>1840</td>
<td>114, 116 (3:3)</td>
<td>C6H7Cl-</td>
<td>MS, RI</td>
<td>2.34 ± 0.51</td>
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<tr>
<td>5</td>
<td>32.23</td>
<td>1788</td>
<td>1867</td>
<td>114, 116 (3:3)</td>
<td>C6H7Cl-</td>
<td>MS, RI</td>
<td>1.74 ± 0.46</td>
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<tr>
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<td>32.95</td>
<td>1860</td>
<td>1902</td>
<td>167, 169, 171 (3:4:1)</td>
<td>C6H10BrCl-</td>
<td>MS, RI</td>
<td>1.17 ± 0.23</td>
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<tr>
<td>7</td>
<td>35.16</td>
<td>2053</td>
<td>2015</td>
<td>167, 169, 171 (3:4:1)</td>
<td>C6H10BrCl-</td>
<td>MS, RI</td>
<td>0.58 ± 0.07</td>
</tr>
<tr>
<td>8</td>
<td>35.98</td>
<td>2053</td>
<td>2053</td>
<td>167, 169, 171 (3:4:1)</td>
<td>C6H10BrCl-</td>
<td>MS, RI</td>
<td>88.79 ± 1.61</td>
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<tr>
<td>9</td>
<td>36.34</td>
<td>2053</td>
<td>2077</td>
<td>167, 169, 171 (3:4:1)</td>
<td>C6H10BrCl-</td>
<td>MS, RI</td>
<td>0.54 ± 0.11</td>
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<tr>
<td>10</td>
<td>37.46</td>
<td>2053</td>
<td>2138</td>
<td>167, 169, 171 (3:4:1)</td>
<td>C6H10BrCl-</td>
<td>MS, RI</td>
<td>0.78 ± 0.30</td>
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<tr>
<td>11</td>
<td>37.54</td>
<td>2053</td>
<td>2142</td>
<td>167, 169, 171 (3:4:1)</td>
<td>C6H10BrCl-</td>
<td>MS, RI</td>
<td>1.28 ± 0.09</td>
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<tr>
<td>12</td>
<td>39.13</td>
<td>2207</td>
<td>2227</td>
<td>167, 169, 171 (3:4:1)</td>
<td>C6H10BrCl-</td>
<td>MS, RI</td>
<td>1.23 ± 0.23</td>
</tr>
</tbody>
</table>

Retention time of the compounds in the GC-MS total ion chromatogram; Predicted retention indices, calculated according to the procedure described by Stein and co-workers [11], using the formula RI = \( \sum_{n=1}^{3} l_n d_n + h \) with \( h = -23 \); Kováts retention indices determined relative to the retention times of a series of alkanes analysed under the same conditions; Relative amounts calculated as a percentage of the total compounds detected by GC-FID. The mean ± standard deviation is reported. 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; C6H4BrCl2-). 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: C10H10Br2Cl2 (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 C6H5Cl- due to cleavage of the 3–4 bond. 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 C4H5Cl+ due to cleavage of the 3–4 bond. The fact that this compound has been identified in *P. suhrii* before [8] provides 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.

![Figure 3 Chemical structures of compounds 13 - 15](image)

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...
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\(_{14}\)Cl\(_2\) 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 \((C_\text{n}-C_\text{40} \text{ n-alkanes, all with an even number of carbons})\) was purchased from Sigma-Aldrich (Taufkirchen, Germany). The authentic reference material \(1E,3R,4S,5E,7Z-1\)-bromo-3,4,8-trichloro-7-(dichloro-methyl)-3-methylocta-1,5,7-triene \(1E,3R,4S,5E,7Z-1\)-bromo-3,4,8-trichloro-7-(dichloro-methyl)-3-methylocta-1,5,7-triene was obtained 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 methanic
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

\[ RI = \sum_{n} f_n g_n + h \]

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- (+122), -CH\(_2\) (+102), -CH\(_3\) (+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.

Acknowledgements - We would like to thank the Ministry of Fisheries and Marine Resources for the Research/Collection permit that was used in collecting the samples used in this study. We also would like to thank the Department of Chemistry and Biochemistry and School of Pharmacy of the University of Namibia.

Supplementary data – Mass spectra and chromatograms.

References
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. 1H NMR spectroscopy was used as a complementary analytical tool to monitor the residual presence of sesquiterpene 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 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 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 4E-S:** 48 hours of maceration with shaking, plus 30 minutes in a sonication bath
- **Method 4E-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.

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

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), 2S (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.

<table>
<thead>
<tr>
<th>Sample Auck2</th>
<th>% costunolide</th>
<th>% dehydrocostus lactone</th>
<th>% costunolide plus % dehydrocostus lactone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Method 2S (30)</td>
<td>0.91±0.08</td>
<td>1.36±0.06</td>
<td>2.27±0.07</td>
</tr>
<tr>
<td>Method 3S (30)</td>
<td>1.01±0.06</td>
<td>1.47±0.04</td>
<td>2.47±0.05</td>
</tr>
<tr>
<td>Method 1S (30)</td>
<td>0.83±0.02</td>
<td>1.18±0.01</td>
<td>2.01±0.01</td>
</tr>
</tbody>
</table>

Table 3: Repeated assays with sample Auck3.

<table>
<thead>
<tr>
<th>Sample Auck3</th>
<th>% costunolide</th>
<th>% dehydrocostus lactone</th>
<th>% costunolide plus % dehydrocostus lactone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Method 2S (30)</td>
<td>1.85±0.07</td>
<td>1.76±0.08</td>
<td>3.61±0.05</td>
</tr>
<tr>
<td>Method 3S (30)</td>
<td>1.78±0.09</td>
<td>1.68±0.07</td>
<td>3.46±0.06</td>
</tr>
<tr>
<td>Method 3S (30)</td>
<td>2.17±0.03</td>
<td>2.03±0.02</td>
<td>4.21±0.02</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Sample Auck4</th>
<th>% costunolide</th>
<th>% dehydrocostus lactone</th>
<th>% costunolide plus % dehydrocostus lactone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Method 2S (30)</td>
<td>0.82±0.09</td>
<td>1.31±0.08</td>
<td>2.13±0.07</td>
</tr>
<tr>
<td>Method 2U (30)</td>
<td>0.82±0.03</td>
<td>1.28±0.06</td>
<td>2.09±0.04</td>
</tr>
<tr>
<td>Method 1S (30)</td>
<td>0.89±0.05</td>
<td>1.37±0.04</td>
<td>2.26±0.05</td>
</tr>
<tr>
<td>Method 1U (30)</td>
<td>0.86±0.08</td>
<td>1.32±0.07</td>
<td>2.18±0.04</td>
</tr>
</tbody>
</table>

1H 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 6.8 and 5.5, after extraction of the sample using the method 3S (30).

In conclusion, sonication is less invasive than 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 sesquiterpenes 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.

<table>
<thead>
<tr>
<th>Sample</th>
<th>% costunolide</th>
<th>% dehydrocostus lactone</th>
<th>% costunolide plus % dehydrocostus lactone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Auck5</td>
<td>0.65±0.02</td>
<td>0.95±0.03</td>
<td>1.61±0.02</td>
</tr>
<tr>
<td>Auck6</td>
<td>1.06±0.05</td>
<td>1.30±0.06</td>
<td>2.36±0.05</td>
</tr>
<tr>
<td>Auck7</td>
<td>0.81±0.03</td>
<td>0.68±0.04</td>
<td>1.49±0.02</td>
</tr>
<tr>
<td>Auck8</td>
<td>0.02±0.04</td>
<td>2.15±0.06</td>
<td>2.17±0.05</td>
</tr>
<tr>
<td>Auck9</td>
<td>1.31±0.06</td>
<td>1.35±0.03</td>
<td>2.66±0.05</td>
</tr>
<tr>
<td>Auck10</td>
<td>1.53±0.08</td>
<td>1.46±0.07</td>
<td>2.99±0.04</td>
</tr>
</tbody>
</table>

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 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: Ex extractions were performed using an electronic Sonorex RH 100 SH ultrasonic bath (Bandelin, Berlin, Germany) and a Bandelin electronic Sonopuls, 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-Q Plus 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 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 then 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 1H 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.

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.

References


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 45.9% 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-aminonucleoines, 8-aminooquinolines, arylamino-alcohols, artemisins, 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-glucopyranosyl ester (2a), 15α-O- acetyl-16,17-epoxy-ent-kauran-19-oic acid α-D-glucopyranosyl ester (2b), 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 methyl ester (3d), 16,17-epoxy-ent-kauran-19-oic acid α-D-glucopyranosyl ester (4a), 15α-O-acetyl-16,17-epoxy-ent-kauran-19-oic acid α-D-glucopyranosyl ester (4b).

Ent-kaur-16-en-19-oic acid (kaurenic acid, 1a) and ent-kaur-16-en-19-αl (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. schultsii. Both are Venezuelan species of the Espelettiaceae subclass (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 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 gluco-pyranosyl 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 (% IBF) 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-gluco-pyranosyl 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-gluco-pyranosyl 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-αl (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 (% IBF) produced by the tested kaurenes using chloroquine as a positive control.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>% IBF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroquine</td>
<td>95.7±0.01</td>
</tr>
<tr>
<td>1a</td>
<td>73.5±0.04</td>
</tr>
<tr>
<td>1c</td>
<td>48.4±0.1</td>
</tr>
<tr>
<td>1d</td>
<td>23.7±0.07</td>
</tr>
<tr>
<td>2a</td>
<td>57.6±0.03</td>
</tr>
<tr>
<td>2b</td>
<td>92.6±0.04</td>
</tr>
<tr>
<td>3a</td>
<td>53.3±0.07</td>
</tr>
<tr>
<td>3b</td>
<td>50.2±0.09</td>
</tr>
<tr>
<td>3e</td>
<td>52.6±0.03</td>
</tr>
<tr>
<td>3c</td>
<td>63.5±0.01</td>
</tr>
<tr>
<td>3b</td>
<td>58.0±0.01</td>
</tr>
<tr>
<td>3c (10%) + 70% (97%)</td>
<td>57.8±0.07</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Compound</th>
<th>% P</th>
<th>SDPI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroquine</td>
<td>0.2±0.06</td>
<td>29.1±1.7</td>
</tr>
<tr>
<td>2a</td>
<td>4.5±0.02***</td>
<td>25.5±1.3***</td>
</tr>
<tr>
<td>1a</td>
<td>8.5±0.05***</td>
<td>18.0±0.8**</td>
</tr>
<tr>
<td>3b</td>
<td>12.7±1.2***</td>
<td>12.7±1.7</td>
</tr>
<tr>
<td>4b</td>
<td>15.2±1.7***</td>
<td>10.3±1.3</td>
</tr>
<tr>
<td>Saline</td>
<td>27.6±2.8</td>
<td>9.0±1.9</td>
</tr>
</tbody>
</table>

The results are expressed as the mean ± SEM. %P: percentage of parasitemia at 48h 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-kaur-19-oic acid α-D-gluco-pyranosyl 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, 1H NMR).

Preparation of ent-kaur-16-en-19-oic acid α-D-gluco-pyranosyl ester (1b): This compound was obtained as described by Visbal et al. [2e]. Kaurenic acid silver salt was obtained by treating a solution of sodium kaurenate with an aqueous solution of AgNO3. 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-D-glucopyranosyl alcohol. Upon evaporation of the solvent, 1b, obtained as a white solid, (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. schultsii, as described by Brieskorn and Poehlmann [3b], and compared with an authentic sample (mmp, TLC, 1H 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 schultzi [3b], was used as starting material. To obtain the gluco-pyranosyl 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, 1H and 13C 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-
removed by distillation. The reaction product was submitted to a silica gel column (80 g) containing 20% AgNO₃. Fractions (50 mL each) were inspected by TLC and GC-MS. Fractions 1-3 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-kauren-16-19-α-0-acetyl-d-glucopyranosyl methyl ester, identical to the methyl ester of the O-acetyl derivative of grandifloric acid obtained from *E. schulzii* resin. Fractions 47-60 yielded a mixture. Finally, fractions 61-81 yielded 472 mg of 3e, identical to that obtained by Rojas et al. [4b].

17-Hydroxy-ent-kauren-15-19-19-α-0-acetyl-d-glucopyranosyl methyl ester (3b): Three hundred mg (0.8 mmol) of 3c was dissolved in dry MeOH and treated overnight at room temp with anhydrous NH₄. 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-kauren-16-19-19-α-0-acetyl-d-glucopyranosyl ester (4a): One g (3.1 mmol) of 15α-hydroxy-ent-kauren-16-19-19-α-0-acetyl-d-glucopyranosyl ester (4c) was dissolved in 20 mL of pyridine and treated overnight with 1.25 g (1.25 mmol) of CrO₃-pyridine complex (Saret's reagent). The following day, 100 mL of H₂O was added to the reaction mixture and the oxidation product was extracted with Et₂O. The Et₂O 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-kauren-16-19-19-α-0-acetyl-d-glucopyranosyl ester, 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-kauren-16-19-19-α-0-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.

1H and 13C NMR: Table 3.

HRMS m/z: [M⁺] 501.2457 (C₉H₁₈O₅Na⁺).

15-Oxo-ent-kauren-16-19-19-α-0-acetyl-d-glucopyranosyl ester (4a): A solution (250 mg, 0.506 mmol) of 4a in acetone was treated with a solution of dimethylether. Crystalization from acetone yielded 252 mg of 4b.
MP: 142-145°C. IR (KBr): 3369 (OH), 2930 (CH), 1733 (CO) cm\(^{-1}\). \(^1\)H and \(^13\)C NMR: Table 4.

HRMS m/z: [M\(^+\)] 517.2457 (C\(_2\)H\(_9\)O\(_2\)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.

**Ent-kaur-16-en-19-ol (6a):** This compound 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\(^{-1}\). \(^1\)H and \(^13\)C NMR: Table 4.

**Ent-kaur-16-en-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 dioxygen and stirred with 46 mg of SeO\(_2\) and 0.41 mL of 30% H\(_2\)O\(_2\) 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 114-116°C; [M\(^+\)] 348 (C\(_{21}\)H\(_{28}\)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). ß-Hematin synthesis was initiated by the addition of acetate buffer (100 μL, 0.2M, pH 4.4). Plates were incubated at 37°C 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\(^5\) 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\(^5\) 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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**References**


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 Briaridae) 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*.

Briarenol B (1) was obtained as a white amorphous powder. From the HRESIMS, the molecular formula of I was determined to be C_{24}H_{32}O_{9} from the ion at *m/z* 487.1936 (calcd for C_{24}H_{32}O_{9} + Na, 487.1944), indicating nine degrees of unsaturation. IR spectrum analysis showed that I had absorption peaks at 3479, 1766 and 1732 cm⁻¹, suggesting that the structure of I included hydroxy, γ-lactone and ester groups. In the ¹³C and DEPT NMR spectra (Table 1), the signals of four carbons at δC 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 I; this result was further supported by the ¹H NMR spectrum of I, which showed three olefin proton signals at δH 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 δC 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 (δH 2.07, 1.98, each 3H × s) were also noted in I. Based on the aforementioned unsaturation data, the structure of I was determined to be that of a diterpenoid with four rings. Furthermore, the signals of two oxygenated quaternary carbons at δC 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 δH 1.49 (3H, s, H-18) further supported this observation.

The ¹H–¹H COSY spectrum of I 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-13/H-14 and H-11/H-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 I, such as H-2, H-9, H-10, H-15/C-1; H-3, H-4, H-7, H-16/C-5; H-6, H-9, H-10, H-18/C-8; H-9, H-18/C-17; and H-18/C-19, which provided confirmation of the carbon skeleton (Table 1). An allylic coupling between H-6/H-16 in the ¹H–¹H COSY spectrum and

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**Figure 1:** The structures of briarenol B (1) and briarenolide ZII (2).
correlations between H$_2$-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$_2$-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$_2$-15/C-1, -2, -10, -14, the C-15 methyl group was positioned at C-1. In addition, the carbon signals at δC 170.0 and 170.3 were correlated with the signals of the methyl protons at δH 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 carbonyl (δC 170.0) of the acetate was confirmed by the connectivity associated with H-9 (δH 4.42, 1H, d, J = 9.2 Hz) was associated with H-9 (δH 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-10 and C-9/C-10, indicating that the methyl group at C-10 was β-oriented; a 16.0 Hz coupling constant between H-3 (δH 5.80) and H-4 (δH 5.13) indicated the trans geometry of the C-3/4 double bond; and a correlation between the C-6 olefin proton (δH 5.43) and the C-16 vinyl methyl (δH 1.88) suggested a Z-configuration of the C-5/6 double bond. Moreover, H-3 was found to be associated with H$_2$-15, but not with H-2 and H-4, and H-4 was associated with H-2, but not with H$_2$-15; these results demonstrated the 3E,5Z-configuration of Δ3,5. Therefore, based on the above findings, the s-trans diene moiety in I was elucidated. The NMR data of I were found to be similar to those of briarane derivatives with a 3E,5Z-diene system, such as xeevaxcavatin M [9], briaranolides I and J [10], excavatolide F [11] and briarenolide ZII (Figure 1) [12]. Thus, briarane I was identified as the 12-O-debutyryl derivative of 2.

### Table 1: 1H and 13C NMR spectroscopic data, and 1H–1H COSY and HMBC correlations for 1.

<table>
<thead>
<tr>
<th>C/H</th>
<th>δH ($^a$)</th>
<th>δC ($^b$)</th>
<th>1H–1H COSY</th>
<th>HMBC (H→C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.37 d (9.6)</td>
<td>45.4 (C)$^d$</td>
<td>H-3, -14, -15</td>
<td>acetate carbonyl</td>
</tr>
<tr>
<td>2</td>
<td>5.80 dd (16.0, 9.6)</td>
<td>126.3 (CH)</td>
<td>H-2, -4</td>
<td>C-5</td>
</tr>
<tr>
<td>3</td>
<td>6.76 d (16.0)</td>
<td>138.6 (CH)</td>
<td>H-3</td>
<td>C-2, -3, -5, -6</td>
</tr>
<tr>
<td>4</td>
<td>140.5 (C)</td>
<td>45.4 (C)</td>
<td>75.9 (CH)</td>
<td>H-3, -4, -5, -6</td>
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<tr>
<td>5</td>
<td>5.71 d (4.4)</td>
<td>118.4 (CH)</td>
<td>H-7, -9</td>
<td>C-1, -2, -8, -11, -14, -16</td>
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<td>6</td>
<td>7.01 (C)</td>
<td>6.76 d (16.0)</td>
<td>138.6 (CH)</td>
<td>H-3, -4, -5, -6</td>
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<td>7</td>
<td>1.42 s</td>
<td>16.2 (CH$_3$)</td>
<td>H-11, -20</td>
<td>C-1, -2, -8, -11, -14, -15, -20</td>
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<tr>
<td>8</td>
<td>1.99 m</td>
<td>38.8 (CH)</td>
<td>H-9, -11</td>
<td>C-1, -2, -8, -11, -14, -15, -20</td>
</tr>
<tr>
<td>9</td>
<td>4.42 d (9.2)</td>
<td>74.6 (CH)</td>
<td>H-10, -OH-9</td>
<td>C-1, -2, -8, -11, -14, -15, -20</td>
</tr>
<tr>
<td>10</td>
<td>1.99 m</td>
<td>42.8 (CH)</td>
<td>H-10, -12, -20</td>
<td>C-10, -20</td>
</tr>
<tr>
<td>11</td>
<td>3.90 m</td>
<td>67.5 (CH)</td>
<td>H-11, -12, -13</td>
<td>n.o.$^a$</td>
</tr>
<tr>
<td>12</td>
<td>1.82–1.94 m</td>
<td>29.6 (CH$_2$)</td>
<td>H-12, -14</td>
<td>C-11, -12</td>
</tr>
<tr>
<td>13</td>
<td>4.90 dd (3.2, 2.8)</td>
<td>74.8 (CH)</td>
<td>H$_2$-13, -14</td>
<td>C-10, -11</td>
</tr>
<tr>
<td>14</td>
<td>1.42 s</td>
<td>16.2 (CH$_2$)</td>
<td>H-12</td>
<td>C-1, -2, -10, -14</td>
</tr>
<tr>
<td>15</td>
<td>1.88 s (1.2)</td>
<td>23.5 (CH)</td>
<td>H-6</td>
<td>C-4, -5, -6</td>
</tr>
<tr>
<td>16</td>
<td>6.34 (C)</td>
<td>1.49 s</td>
<td>10.2 (CH$_2$)</td>
<td>C-8, -17, -19</td>
</tr>
<tr>
<td>17</td>
<td>171.2 (C)</td>
<td>20.12 d (6.8)</td>
<td>9.9 (CH$_3$)</td>
<td>H-11</td>
</tr>
<tr>
<td>18</td>
<td>170.0 (C)</td>
<td>1.98 s</td>
<td>21.3 (CH$_2$)</td>
<td>Acetate carbonyl</td>
</tr>
<tr>
<td>19</td>
<td>170.3 (C)</td>
<td>20.7 s</td>
<td>21.2 (CH$_3$)</td>
<td>Acetate carbonyl</td>
</tr>
</tbody>
</table>

$^a$ 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 13C, DEPT and HSQC experiments. n.o. = not observed.

### 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 I were assigned as 1R, 2S, 7S, 8R, 9S, 10S, 11R, 12S, 14S and 17R.

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

<table>
<thead>
<tr>
<th>Compounds</th>
<th>COX-2</th>
<th>iNOS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100.0 ± 0.02</td>
<td>0.79 ± 0.01</td>
</tr>
<tr>
<td>LPS</td>
<td>100.0 ± 18.4</td>
<td>100.0 ± 7.50</td>
</tr>
<tr>
<td>1</td>
<td>58.2 ± 33.5</td>
<td>158.1 ± 13.7</td>
</tr>
<tr>
<td>2</td>
<td>89.5 ± 4.00</td>
<td>47.2 ± 7.20</td>
</tr>
</tbody>
</table>

*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 (I), 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 Fushanch 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 i55 spectrophotometer; peaks are reported in cm⁻¹. NMR spectra were recorded on a Varian Mercury Plus 400 spectrometer using the residual CHCl₃ signal (δH 7.26 ppm) as the internal standard for ¹H NMR and CDCl₃ (δC 77.1 ppm) for ¹³C 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 F254 (0.25 mm); the plates were sprayed with 10% H₂SO₄ solution followed by heating to visualize the compounds. Normal phase HPLC (NP-HPLC) was performed using a HPLC system equipped with a Rhodyne 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 Rhodyne 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 NMN MBA (Specimen number: NMN MBA-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 H₂O. 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 subfractions, 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 subfractions, V₁–V₁₄. Fraction V₉ was further purified by RP-HPLC, using MeOH:H₂O (45:55, flow rate: 4.0 mL/min) as the mobile phase to afford 1 (3.0 mg, δₗ = 87 min).

**Briarenol B (1)**

White amorphous powder. 

MP: 185–186°C. [α]D²⁰ = −12 (c 0.2, CHCl₃).

IR (neat): νmax 3479, 1766, 1732 cm⁻¹.

¹H NMR (400 MHz, CDCl₃): Table 1.

¹³C NMR (100 MHz, CDCl₃): Table 1.

ESIMS: m/z (%): 487 [M + Na]⁺.


**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 trypsin 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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**References**


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. Furthermore, we examined the inhibitory effects of the constituents on the invasion of HT 1080 cells. Interestingly, 3β-hydroxylanosta-8,24-dien-21-αl [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 sclerotic of this fungus (Kabanoanatake in Japanese, Chaga 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 metastasis of cancer was left uncharacterized. In addition, anti-metastastic experiment in vivo of the extract was also uncharacterized.

Metastasis 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.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>I. obliquus</td>
<td></td>
</tr>
<tr>
<td>G. lucidum</td>
<td></td>
</tr>
<tr>
<td>P. umbellatus</td>
<td></td>
</tr>
<tr>
<td>W. extensa</td>
<td></td>
</tr>
<tr>
<td>L. edodes</td>
<td></td>
</tr>
<tr>
<td>A. subrufescens</td>
<td></td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Dose (mg/kg/d, p.o.)</th>
<th>Numbers of Nodules</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>13</td>
<td>92.4±15.2</td>
</tr>
<tr>
<td>MeOH extract</td>
<td>250</td>
<td>66.3±18.0</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>31.4±11.8</td>
</tr>
</tbody>
</table>

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–H₂O mixture to furnish an EtOAc-soluble fraction and aqueous layer. The aqueous layer was further extracted with n-BuOH to give n-BuOH and H₂O-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 H₂O- [inhibition (%): 19.0 ± 18.8] soluble fractions did not show significant inhibition.

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) [9], 3β,25-dihydroxylanosta-8,23-diene (2) [1,10], inotodiol (3) [1,11], trametenolic acid (4) [1,11], 3β-hydroxylanosta-8,24-dien-21-ol (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 collected in Russia. As a result, compounds 1-6 were isolated with those of Japan. Inotodiol (3) and lanosta-8,24-diene-3-one were isolated as well as those of Japan. Obliquus *I. obliquus* collected in Russia. Furthermore, compounds 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.

The effects of the principal constituents from the sclerotia of *I. obliquus* collected in Japan and Russia, we examined the constituents from the sclerotia of *I. obliquus* collected in Japan with those of Russia, 3β-hydroxylanosta-8,24-dien-21-ol (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 on proliferation of HT1080 cells by WST-8 and calcein-AM assay, significantly different from the control, *p* < 0.05, **p* < 0.01.

![Figure 1: Structures of Constituents 1-9.](Image)

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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Conc. (μg/mL)</th>
<th>0</th>
<th>30</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>EtOAc-soluble fraction</td>
<td>0.0±4.0</td>
<td>10.1±9.6</td>
<td>82.5±1.7**</td>
<td></td>
</tr>
<tr>
<td>n-BuOH-soluble fraction</td>
<td>0.0±11.1</td>
<td>18.8±18.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H₂O-soluble fraction</td>
<td>0.0±13.3</td>
<td>--</td>
<td>19.0±18.8</td>
<td></td>
</tr>
</tbody>
</table>

Values represent the means±S.E.M. (n=4).

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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Conc. (μM)</th>
<th>0</th>
<th>10</th>
<th>30</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.0±9.1</td>
<td>12.1±5.3</td>
<td>52.7±3.1**</td>
<td>79.6±3.0**</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.0±4.2</td>
<td>–12.2±7.0</td>
<td>–0.9±6.2</td>
<td>28.8±5.2**</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.0±7.6</td>
<td>2.5±10.4</td>
<td>31.4±10.3*</td>
<td>50.9±4.9**</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.0±9.9</td>
<td>0.8±22.9</td>
<td>–9.3±17.8</td>
<td>19.0±14.3</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.0±6.0</td>
<td>5.9±7.6</td>
<td>37.9±3.0*</td>
<td>62.3±1.3**</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0.0±13.2</td>
<td>–22.2±6.1</td>
<td>17.4±10.2</td>
<td>44.4±9.6*</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>0.0±5.7</td>
<td>–2.2±6.4</td>
<td>13.1±10.8</td>
<td>28.4±5.6</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>0.0±5.0</td>
<td>–1.9±7.3</td>
<td>12.6±3.2</td>
<td>24.1±3.2</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>0.0±4.7</td>
<td>4.5±9.0</td>
<td>–6.3±12.9</td>
<td>15.5±6.7</td>
<td></td>
</tr>
</tbody>
</table>

Values represent the means±S.E.M. (n=4).

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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Conc. (μM)</th>
<th>0</th>
<th>10</th>
<th>30</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>WST-8 assay</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.0±1.0</td>
<td>10.3±0.9**</td>
<td>33.1±9.9**</td>
<td>98.6±0.3**</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>0.0±0.9</td>
<td>13.4±1.1**</td>
<td>33.1±7.7**</td>
<td>98.5±3.3**</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>0.0±1.5</td>
<td>11.8±1.6**</td>
<td>35.5±9.0*</td>
<td>97.0±1.5**</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td>0.0±0.6</td>
<td>3.5±1.3</td>
<td>9.2±1.9</td>
<td>87.5±1.5**</td>
<td></td>
</tr>
<tr>
<td>48 h</td>
<td>0.0±1.3</td>
<td>4.5±0.7</td>
<td>13.9±1.6*</td>
<td>90.5±1.3**</td>
<td></td>
</tr>
<tr>
<td>72 h</td>
<td>0.0±0.5</td>
<td>10.4±0.5**</td>
<td>16.6±1.4**</td>
<td>88.4±3.1**</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td>0.0±1.6</td>
<td>–0.7±0.2</td>
<td>–1.1±1.5</td>
<td>25.2±1.1**</td>
<td></td>
</tr>
<tr>
<td>48 h</td>
<td>0.0±0.9</td>
<td>0.3±1.7</td>
<td>–2.7±0.8</td>
<td>6.6±0.9**</td>
<td></td>
</tr>
<tr>
<td>72 h</td>
<td>0.0±0.6</td>
<td>–0.9±1.9</td>
<td>–2.0±0.6</td>
<td>24.5±4.8**</td>
<td></td>
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<tr>
<td>Calcein-AM assay</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>24 h</td>
<td>0.0±5.6</td>
<td>20.5±1.7**</td>
<td>34.6±3.3**</td>
<td>84.7±2.9**</td>
</tr>
<tr>
<td>48 h</td>
<td>0.0±2.6</td>
<td>16.2±1.0**</td>
<td>40.4±0.7**</td>
<td>94.6±0.7**</td>
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</tr>
<tr>
<td>72 h</td>
<td>0.0±1.1</td>
<td>14.8±1.8**</td>
<td>48.5±1.0*</td>
<td>90.9±2.5**</td>
<td></td>
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<tr>
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<tr>
<td>24 h</td>
<td>0.0±6.1</td>
<td>6.1±3.7</td>
<td>–6.6±4.2</td>
<td>63.7±3.6**</td>
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<tr>
<td>48 h</td>
<td>0.0±2.4</td>
<td>12.2±1.1</td>
<td>17.0±1.1</td>
<td>66.9±12.6**</td>
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<tr>
<td>72 h</td>
<td>0.0±1.6</td>
<td>9.7±1.6</td>
<td>27.8±2.0**</td>
<td>85.4±7.7**</td>
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<td></td>
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<tr>
<td>24 h</td>
<td>0.0±5.4</td>
<td>–11.5±2.4</td>
<td>–4.2±4.1</td>
<td>22.4±11.0</td>
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<tr>
<td>48 h</td>
<td>0.0±3.0</td>
<td>0.2±3.0</td>
<td>2.4±1.8</td>
<td>47.6±4.5**</td>
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</tr>
<tr>
<td>72 h</td>
<td>0.0±1.2</td>
<td>–2.7±0.8</td>
<td>2.6±2.3</td>
<td>42.1±6.8**</td>
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</tr>
<tr>
<td>Deguelin*</td>
<td>24 h</td>
<td>0.0±1.4</td>
<td>–1.8±5.1</td>
<td>17.0±1.4</td>
<td>–</td>
</tr>
<tr>
<td>48 h</td>
<td>0.0±1.6</td>
<td>51.0±2.1**</td>
<td>40.4±2.7**</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>72 h</td>
<td>0.0±0.6</td>
<td>98.5±0.1**</td>
<td>98.5±0.1**</td>
<td>–</td>
<td></td>
</tr>
</tbody>
</table>

Values represent the means±S.E.M. (n=4).

Significantly different from the control, *p* < 0.05, **p* < 0.01.

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 addition, 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-ol (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; 1H-NMR spectra, JEOL JNM-ECS400 (400 MHz), JEOL JNM-LA500 (500 MHz), and JEOL JNM-ECA600 (600 MHz) spectrometers; 13C-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 SC18-MS-II (250 x 4.6 mm i.d. and 250 x 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 RP-18 F 254S (Merck, 0.25 mm) reversed-phase silica gel column chromatography, Chromatorex; normal-phase silica gel column chromatography, preparative purposes. The following materials were used for standard; HPLC, a Shimadzu RID-6A refractive index and SPD-10A vp UV-VIS detectors. COSMOSIL SC18-MS-II (250 x 4.6 mm i.d. and 250 x 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 60F254 (Merck, 0.25 mm) (ordinary phase) and Silica gel RP-18 F254 (Merck, 0.25 mm) (reversed phase); reversed-phase HPTLC, precoated TLC plates with Silica gel RP-18 WF254S (Merck, 0.25 mm). Detection was achieved by spraying with 1% Ce(SO4)2–10% aqueous H2SO4 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 (H. M.) 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. subrubescens 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 MatrigelTM) was from BD Biosciences (NJ, USA); Cell Culture InsertTM was from BD Falcon (NJ, USA); Cell Counting Kit-8TM and Cell Counting Kit-FTM 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 g) from the extract was partitioned into an EtOAc–H2O (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 (54.9 g, 4.4%) and an H2O-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, v/v) → EtOAc → CHCl3–MeOH (50:1 → 20:1 → 10:1 → 5:1, v/v) → MeOH] to give 14 fractions (Fr. I–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–H2O (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–H2O (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–H2O (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–H2O (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–EtOAc (90:10, v/v)] to furnish ergosteryl peroxide (8, 30 mg, 0.0035%). Fraction 12 (18.9 g) was subjected to reversed-phase silica gel CC [950 g, MeOH–H2O (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–H2O (2080, v/v)] to furnish 3,4-dihydroxybenzaldehyde (9, 59 mg, 0.022%). The isolation method of compounds 1–6 was reported in the previous 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–H2O (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 H2O-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-hexene-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-hexene-EtOAc (10:1 → 2:1, v/v) → EtOAc] and HPLC [MeOH–H2O (85:15, v/v)] to give betulin (7, 0.3 mg, 0.0002%)]

Cell Culture: Human fibrosarcoma HT1080 cells (Cell No. JCRB9113) were obtained from Health Science Research Resources 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 InsertTM and 24-well multiplates as reported previously [7,8]. The upper side of each filter of Cell Culture InsertTM 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 InsertTM 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 10^5 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™ 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™) 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-F™) 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.

\[
\text{Inhibition} \% = \frac{(A - B)}{A} \times 100
\]

**References**


**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.

**Acknowledgments** - This research was supported in part by a Ministry of Education, Culture, Sports, Science and Technology (MEXT)-Supported Program for the Strategic Research Foundation at Private Universities 2015-2019. The authors thank KSA International Inc. (Kyoto, Japan) for providing of Russian *I. obliquus*. 

A and B indicate optical density or fluorescence intensity of vehicle and test compound-treated groups (n=4).
A New Cytotoxic Cyclolanostane Triterpenoid Xyloside from *Souliea vaginata*

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‡ These two authors contributed equally to this work.

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*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 CCl4-induced injury of human HepG2 cells, respectively.

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

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

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

$J = 10.2, 9.6 \text{ Hz}$, and $H-17 (\delta_H 2.48, d, J = 10.2 \text{ Hz})$. In addition, the $^1$H NMR spectrum also showed one anomeric doublet proton signal at $\delta_H 4.84 (J = 7.8 \text{ Hz})$ in the downfield region indicating the presence of a $\beta$-linked sugar and other signals for a pentose at $\delta_C 1.03$ ($t, J = 8.4 \text{ Hz, H-2'}$), 4.17 ($t, J = 8.4 \text{ Hz, H-3'}$), 4.22 (m, H-4’), 3.73 ($t, J = 10.8 \text{ Hz, H-5'}$), and 4.35 (dd, $J = 10.8, 4.8 \text{ Hz, H-5'}$). 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 $\delta_C 30.9$ (C-19), one oxymethylene carbon at $\delta_C 74.3$ (C-18), three oxygenated methine carbons at $\delta_C 87.7$ (C-3), 80.1 (C-15), and 78.3 (C-16), two oxysteriary carbons at $\delta_C 86.6$ (C-20) and 77.2 (C-25), one keto carbonyl group at $\delta_C 216.5$ (C-24) and two ester carbonyls at $\delta_C 171.1$ and 171.4. All carbon-bound protons were assigned based on HSQC and $^1$H–$^1$H correlation spectra. The $^1$H 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
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 CCl₄-induced injury of human HepG2 cells using MTT assay, respectively. Compound 1 showed significant inhibitory effects with IC₅₀ 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.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>R₅₀(μM)</th>
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<tr>
<td>HT-29</td>
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<tr>
<td>A549</td>
<td></td>
</tr>
<tr>
<td>MDA-MB231</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>11.2 ± 2.9</td>
</tr>
<tr>
<td>5-FU²</td>
<td>57.6 ± 5.3</td>
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</table>

Values present mean ± SD of triplicate experiments. ²Positive 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 C₁₈ reversed-phase silica gel (40–60 μm, Merck, Darmstadt, Germany) were used for column chromatography. Precoated silica gel GF₂₅₄ 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 CH₂Cl₂-MeOH-formic acid (15:1) to yield I (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 CHCl3. 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 μL. The injection and detector temperature were set at 290°C, and the split ratio was 1/10. The presence of α-xylene in the acid hydrolysate of 1 was confirmed by comparison of its retention time with that of a standard sample. The retention times (τH) of α-xylene were 8.17 and 9.11 min.

Soulieoside P

Colorless amorphous powder. [α]D: +23 (c 0.10, MeOH).

IR (KBr) νmax: 3400, 2920, 1720, 1650, 1238, 1065 cm⁻¹.

1H (600 MHz, pyridine-d5) and 13C NMR (150 MHz, pyridine-d5): Table 1.

HRESIMS m/z: [M + Na]+ 743.3948 (calcd. for C39H60O12Na 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% FBS (fetal bovine serum), 100 μg/mL penicillin, and 100 μg/mL streptomycin. Cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO2. 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 (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% CO2. HepG2 cells (5 × 10⁴/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]. Cytotoxic activity of test samples on CCl4-induced cell injury was investigated. In total, 5 × 10⁴ 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% CCl4 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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References


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-methylenecholesterol-5-ene-1α,3β,5α,6β,11α-triol (1), 24-methylenecholesterol-5-ene-1α,3β,5α,6β,11α-triol (2), 24-methylenecholesterol-5-ene-1α,3β,5α,6β,11α-triol (3), 24-methylenecholesterol-5-ene-1α,3β,5α,6β,11α-triol (4), 24-methylenecholesterol-5-ene-1α,3β,5α,6β,11α-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], anti-inflammatory [12, 13] 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. crassum 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), 472.4126 (M\(^+\)-H\(_{2}\)O), 412 (M\(^+\)-OAc-H\(_{2}\)O) and 394 (M\(^+\)-COO-H\(_{2}\)O) confirmed the presence of a terminal methylene group at \(\delta\) 4.62 and \(\delta\) 1.22 (s), and an acetoxy at \(\delta\) 2.02 (s). Signals at \(\delta\) 0.86 (3H, s), 0.98 (2H, d, \(J\) = 6.8 Hz) and a doublet of doublet (1H, \(J\) = 6.8, 1.2 Hz), and an acetoxy at \(\delta\) 2.02 (s). Signals at \(\delta\) 0.66 (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. The IR spectrum demonstrated the presence of hydroxyl (3500 cm\(^{-1}\)), unsaturated hydroxyl at 3454 combined with carbonyl \(1725 \text{ cm}^{-1}\) and a terminal methylene double bond \(1646, 1410, 1250 \text{ and } 880 \text{ cm}^{-1}\). The \(^1\)H NMR spectrum (CDCl\(_3\), 600 MHz) confirmed the presence of a terminal methylene group at \(\delta\) 4.62 and 4.68 [each a singlet], an olefinic proton at \(\delta\) 5.56 (d, \(J\) = 6.2), five methyl signals at \(\delta\) 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 \(\delta\) 2.02 (s). Signals at \(\delta\) 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. The above data suggested that this was a tri-hydroxylated 24-methylenecholesterol-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 3β-carbonyl proton in a steroid skeleton [16]. The oxygenated methine signals at δC 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-3, C-11; 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-9/H-1, H-11; Me-16/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 dissection [17] and Polythoa tuberculosa [15], while 24-methylenecholestan-1α,3β,5α,6β,11α-pentol (4) was isolated from both the Formosan soft coral Simularia giberrosa [18] and the South China Sea soft coral L. crassum [19]. Compound 3 was only recently isolated from Sinularia polyactyla collected from the Red Sea [20], while 24-methylenecholestan-3β,5α,6β,11β-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 IC50 values of 1.90, 5.82 and 6.46 µM, respectively. HepG2 cells lines on the other hand showed a greater sensitivity towards compounds 1, 5 and 6 than other cell lines (IC50 1.90, 3.00 and 3.77 µM respectively).

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

<table>
<thead>
<tr>
<th>Compound/extract</th>
<th>Cancer cell line (IC50, µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HepG2</td>
</tr>
<tr>
<td>EtOAc extract*</td>
<td>2.1</td>
</tr>
<tr>
<td>1</td>
<td>1.90</td>
</tr>
<tr>
<td>2</td>
<td>19.52</td>
</tr>
<tr>
<td>3</td>
<td>1.94</td>
</tr>
<tr>
<td>4</td>
<td>6.74</td>
</tr>
<tr>
<td>5</td>
<td>3.00</td>
</tr>
<tr>
<td>6</td>
<td>3.77</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>2.21</td>
</tr>
<tr>
<td>Vinblastine</td>
<td>3.20</td>
</tr>
</tbody>
</table>

*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 cambrenane 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 Δ5 sterol 4 together with 5,6-diol derivative 5 supports the assumption that the unoxidized Δ5 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 1H and 150 MHz for 13C using CDCl3 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 Quadrapole TQD mass spectrometer (Waters, Milford, MA, USA) for ESI-MS. Perkin-Elmer model 343 plus polariometer 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 F254, 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 (ABB-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 H2O. 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. [α]D25 25.8 (c 0.28, MeOH).

IR (KBr) cm−1: 3500, 1725, 1646, 1600, 1250, 880.

1H NMR (CDCl3, 600 MHz) δH: 5.66, (br s H-1); 1.75, 2.08 (m, each, H-2); 3.96 (m, H-3); 2.22, 2.37 (each, H-4); 5.56, (d, J = 6.2 Hz, H-6); 1.99, 1.65 (each, H-7); 1.53 (m, H-8); 1.63 (m, H-9); 3.88 (m, H-11); 1.24, 2.31 (each, H-12); 1.13 (m, H-14); 1.24, 1.63 (m, each, H-15); 1.24, 2.00 (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 (each, H-22); 1.24, 1.39 (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 (each, H-28); 2.02 (s, OAc).

13C NMR (150 MHz) δC: 78.3 (CH, C-1); 35.6 (CH2, C-2); 66.7 (CH, C-3); 41.8 (CH2, C-4); 137.0 (C, C-5); 124.5 (CH, C-6); 31.3 (CH2, C-7); 31.5 (CH, C-8); 48.9 (CH, C-9); 41.6 (C, C-10); 68.9 (CH, C-11); 51.1 (CH2, C-12); 43.1 (C, C-13); 56.3 (CH, C-14); 24.0 (CH2, C-15); 28.3 (CH2, C-16); 55.7 (CH, C-17); 13.1 (CH3, C-18)
Steroids from Lobophytum crassum

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18); 18.6 (CH3, C-19); 35.6 (CH, C-20); 18.7 (CH3, C-21); 34.5 (CH2, C-22); 30.8 (CH2, C-23); 156.8 C, C-24); 33.8 (CH, C-25); 21.9 (CH3, C-26); 22.0 (CH2, C-27); 106.0 (CH2, C-28); 21.6 171.9 (CH, C respectively, OAc).

EI MS 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% CO2. 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% (IC50) was determined. This work was carried at the Regional Center for Mycology and Biotechnology, Al-Azhar University, Cairo, Egypt.

References
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. \textit{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 \textit{R. sativus} sprout exhibits significant but variable cytotoxic effects on human lung adenocarcinoma cells depending on their \textit{p53} status. The MeOH extract decreased the viability of \textit{p53}-deleted human lung cancer cells (H1299 and Calu-6) by inducing apoptosis; this effect, however, did not occur for wild-type \textit{p53} cancer cells (A549), for cells expressing a \textit{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 \(\beta\)-sitosterol as a major component of the MeOH extract. Direct treatment with \(\beta\)-sitosterol significantly reduced the viability of Calu-6 cells, suggesting that it may, in part, contribute to \textit{R. sativus} sprout’s anti-tumor activity. This work provides experimental evidence for a novel biological application of \textit{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:** \textit{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.

\textit{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 \textit{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 \textit{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-butyln 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 \textit{R. sativus} sprouts on human lung adenocarcinoma cells.

\textit{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 \textit{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 \textit{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 \textit{in vitro}, suggesting that it has excellent potential use in cancer management [15]. The anti-cancer activity of \textit{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 \textit{R. sativus} sprout on human lung adenocarcinoma cells and found that it dramatically reduced cell viability by inducing apoptosis depending on \textit{p53} status. Additionally, we phytochemically investigated the MeOH extract, which led us to isolate a major component, \(\beta\)-sitosterol. To the best of our knowledge, \textit{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 \textit{R. sativus} sprout on human lung cancer cells and isolated the main component that is the likely causative agent.
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 (IC₅₀ 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 IC₅₀ 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 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 CH₂Cl₂ soluble and EtOAc fractions. Phytochemical investigation of the consolidated CH₂Cl₂ 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 ¹H and ¹³C 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 IC₅₀ 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.
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 (H) and 175 MHz (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 Prominance HPLC System with SPD-20A/20AV Series Prominance 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 deposited at the herbarium at the School of Pharmacy, Sungkyunkwan University, Suwon, Korea. The air-dried sprouts of *R. sativus* were fractionated by sequential liquid-liquid partitioning of H2O (200:1, 100:1, 30:1, 10:1, 5:1 and 2:1) to yield 6 fractions (A1-A6).

**Isolation of main constituents:** The crude extract (2 g) was fractionated by sequential liquid-liquid partitioning of H2O (200 mL) with dichloromethane (CH2Cl2, 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 CH2Cl2-MeOH (200:1, 100:1, 50:1, 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, tR = 30 min). Compound 1, β-sitosterol, was then dissolved in EtOH 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 supplemented 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% CO2.

**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 (IC50) 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 deoxynucleotidyl 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 fluorescein-conjugated dNTPs 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.
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 CHCl₃-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 CHCl₃-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 hepatocyte- and prostate-carcinoma cell lines (IC₅₀ 14-37 µM), while primary human fibroblasts were less vulnerable (IC₅₀ 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], galactagogue [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 CHCl₃-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*.
sensitive (IC \(_{50}\), 13.6 and 36.6 μM) than the fibroblasts, i.e., the HepG2 and LNCaP cell lines were consistently more selectivity index (SI) is the IC\(_{50}\) 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\(_{50}\), 21.4 to 66.5 μM), the HepG2 and LNCaP cell lines were consistently more sensitive (IC\(_{50}\), 13.6 and 36.6 μM) than the fibroblasts, i.e., the selectivity indices were consistently >1. Asparagocisde 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 \(A. \) racenosus 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 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 PBH 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\(_2\)Cl\(_2\)-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.

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

<table>
<thead>
<tr>
<th>Compound</th>
<th>R(_{\alpha})</th>
<th>R(_{\beta})</th>
<th>R(_{\epsilon})</th>
<th>[M-H](^-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Shatavarin IX</td>
<td>β-D-glucose</td>
<td>β-D-glucose</td>
<td>H</td>
<td>901.47</td>
</tr>
<tr>
<td>2 Asparagocisde</td>
<td>β-D-glucose</td>
<td>α-L-arabinose</td>
<td>α-L-arabinose</td>
<td>1003.51</td>
</tr>
<tr>
<td>3 Asparanin A</td>
<td>β-D-glucose</td>
<td>H</td>
<td>H</td>
<td>739.42</td>
</tr>
<tr>
<td>4 Shatavarin V</td>
<td>α-L-rhamnose</td>
<td>β-D-glucose</td>
<td>H</td>
<td>885.47</td>
</tr>
</tbody>
</table>

- Shatavarin IV: β-D-glucose α-L-rhamnose H | 885.47

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

<table>
<thead>
<tr>
<th>Samples</th>
<th>HepG2 SI</th>
<th>LNCaP SI</th>
<th>Fibroblasts SI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Defatted E7OH extract (μg/mL)</td>
<td>798 ± 54.6</td>
<td>1.44 ± 0.22</td>
<td>469 ± 7.30</td>
</tr>
<tr>
<td>Saponin enriched extract (μg/mL)</td>
<td>207 ± 15.4</td>
<td>1.47 ± 0.15</td>
<td>87.3 ± 1.58</td>
</tr>
<tr>
<td>Shatavarin IX (μM)</td>
<td>13.7 ± 1.62</td>
<td>15.8 ± 1.09</td>
<td>163.2 ± 2.43</td>
</tr>
<tr>
<td>Asparagocisde (μM)</td>
<td>15.6 ± 2.43</td>
<td>5.01 ± 1.12</td>
<td>29.3 ± 0.57</td>
</tr>
<tr>
<td>Asparanin A (μM)</td>
<td>14.1 ± 0.96</td>
<td>3.61 ± 0.20</td>
<td>36.6 ± 1.77</td>
</tr>
<tr>
<td>Shatavarin V (μM)</td>
<td>4.08d</td>
<td>1.24 ± 0.37</td>
<td>11.40 ± 1.08</td>
</tr>
<tr>
<td>Shatavarin IV (μM)</td>
<td>1.79 ± 0.01</td>
<td>1.18d</td>
<td>1.18e</td>
</tr>
<tr>
<td>Palmitic acid (μM)</td>
<td>0.33c</td>
<td>2.57 ± 0.21</td>
<td>42.4 ± 3.25</td>
</tr>
<tr>
<td>Saponin enriched extract (46 ± 2% of saponins)</td>
<td>2.52c</td>
<td>3.25e</td>
<td>42.4 ± 3.25</td>
</tr>
</tbody>
</table>
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 45°C under vacuum.

The HPCPC separation of *Asparagus racemosus* saponin enriched extract (2.0 g) using CHCl₃-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 CH₂Cl₂-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 R₀ 0.51. The TLC band was cut-out and extracted using CH₂Cl₂-MeOH (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 shatavari V by comparing their NMR spectroscopic and ESI/MS data with those previously reported [12-13,16a,16b].

**Human cell lines and cell cultures:** The human hepatocarcinoma cell line HepG2 (ATCC® HB8065™), prostatic carcinoma 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% CO₂ 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 μL well⁻¹ of 1:1 DMSO:MeOH. Absorbance was measured at 570 nm using a microplate reader. The IC₅₀ values were calculated using GraphPad Prism 5 software. The selectivity index (SI) was calculated from the IC₅₀ 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).

**Disclosure statement** - No potential conflict of interest was reported by the authors.

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**References**


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 IC50 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, epimartidine); crinine- (ambelline, 11-O-acetylambelline, crinine, undulatine, buphanamine, buphanidrine, buphanisine, 1-O-acetylbulbisine), galanthamine- (galanthamine, chlidanthine), lycorine- (caranine, acetylcaranine, lycorine, 1-O-acetyllycorine, galantine, 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 (IC50 = 15.8 ± 1.2 µM) compared with the known AKR1C3 inhibitor, indomethacin (IC50 = 3.7 µM) [8]; eleven alkaloids showed weak inhibitory potency and the others were considered as inactive (Figure 1).
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 *Zephyrantes 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 MgCl₂), either 20 µM (for screening purposes) or 4–100 µM (for IC₅₀ 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% NH₄OH 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 tubes 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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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 CH2Cl2 [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.

Table 1: Various conditions for selective esterification using TMSCl.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Solvent (5 mL)</th>
<th>TMSCl (eq.)</th>
<th>Monooester (Yield %)</th>
<th>Diester (Yield %)</th>
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<tbody>
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<tr>
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<td>2a</td>
<td>24</td>
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<td>10</td>
<td>n.d.</td>
<td>99</td>
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<td>68</td>
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<td>6</td>
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<td>19</td>
<td>t-BuOH</td>
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<td>n.d.</td>
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</table>

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

The mono and diesters could be easily separated by pH-controlled extraction. Fortunately, pure products were obtained as indicated by 1H-NMR and TLC, without the need for purification by silica-gel column chromatography. The yield of 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 esterification proceeded with regard to 1H-NMR data. Although excess TMSCl was required for the completion of esterification, the resulting alkyl TMS ether could be azeotropically removed by simple evaporation with alcohol.

Regarding acidic amino acids (aspatic acid and glutamic acid), various alcohols were used 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 NaHCO3 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 CHCl3 and aqueous HCl (Table 1) [5].

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 NaHCO3 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 CHCl3 and aqueous HCl (Table 1) [5].

The mono and diesters could be easily separated by pH-controlled extraction. Fortunately, pure products were obtained as indicated by 1H-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 12-19).

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 propargylxoxy carbonyl 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 alkyn-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.](image)

<table>
<thead>
<tr>
<th>Entry</th>
<th>Solvent (5 mL)</th>
<th>TMSCl (eq.)</th>
<th>Yield (%)</th>
<th>Monester (b)</th>
<th>Diester (b)</th>
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<tbody>
<tr>
<td>1</td>
<td>MeOH</td>
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<td>71 (only γ)</td>
<td>3a</td>
<td>8</td>
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<td>MeOH</td>
<td>3</td>
<td>3a</td>
<td>41 (αγ = 3:1)</td>
<td>59</td>
</tr>
<tr>
<td>3</td>
<td>MeOH</td>
<td>5</td>
<td>3a</td>
<td>24 (αγ = 5:1)</td>
<td>75</td>
</tr>
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<td>4</td>
<td>MeOH</td>
<td>10</td>
<td>3a</td>
<td>1 (αγ = 5:3)</td>
<td>92</td>
</tr>
<tr>
<td>5</td>
<td>EtOH</td>
<td>1</td>
<td>84 (only γ)</td>
<td>6b</td>
<td>10</td>
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<tr>
<td>6</td>
<td>EtOH</td>
<td>5</td>
<td>18 (only γ)</td>
<td>6b</td>
<td>69</td>
</tr>
</tbody>
</table>

*a*The product did not need purification by silica-gel column chromatography, because no by-product was detected by 'H-NMR and TLC for each reaction. *b*Estimated by '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, byproduct 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-methyl ester as a colorless liquid (3a, 0.20 g, 0.75 mmol, 75%).

Methyl N-Boc-l-aspartate (2a)

1H 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)

1H 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%)

1H 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%)

1H 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_2CH3), 4.20 (t, 1 H, J = 5.5 Hz, Hα).

### Acknowledgments

The authors thank Dr. Christopher Vavricka for his critical reading of the manuscript. The authors are also grateful to Dr. Kei Matsumoto at Division of Instrumental Analysis, Okayama University for the NMR spectral measurements. Financial support from KAKENHI (No. 25405114 and 30170145) was acknowledged.

### References


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

Thanh Tra Nguyen\textsuperscript{ab}, Bich Ngan Truong\textsuperscript{a}, Huong Doan Thi Mai\textsuperscript{b}, Marc Litaudon\textsuperscript{c}, Van Hung Nguyen\textsuperscript{a}, Thao Do Thi\textsuperscript{a}, Van Hieu Tran\textsuperscript{a}, Dang Thach Tran\textsuperscript{a}, Van Minh Chau\textsuperscript{a} and Van Cuong Pham\textsuperscript{a,}\textsuperscript{*}

\textsuperscript{a}Advanced Center for Bisorganic Chemistry, Institute of Marine Biochemistry, VAST, 18 Hoang Quoc Viet, Cau Giay, Hanoi, Vietnam
\textsuperscript{b}Institute of Chemistry, VAST, 18 Hoang Quoc Viet, Cau Giay, Hanoi, Vietnam
\textsuperscript{c}Institut de Chimie des Substances Naturelles, CNRS-ICSN, UPR 2301, Univ. Paris-Sud, 91198, Gif-sur-Yvette, France
\textsuperscript{d}Institute of Biotechnology, VAST, 18 Hoang Quoc Viet, Cau Giay, Hanoi, Vietnam
\textsuperscript{e}Industrial University of Vinh, 26 Nguyen Thai Hoc, Vinh, Nghe-An, Vietnam

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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\textsubscript{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, Hydnocarpin, 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 \cite{1}. The *Hydnocarpus* genus consists of about 40 species. Many *Hydnocarpus* species have been used in folk medicine \cite{2}. A literature overview showed that the bioactive compounds of *Hydnocarpus* were assigned as flavonolignan \cite{3a-e} and cyclopentyl fatty acids \cite{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 \cite{2}. Also, hydnocarpin, a flavonolignan isolated from *Hydnocarpus*, is reported to exhibit antimicrobial and anticancer activity \cite{2.5a-b}. Biological screening of other species of *Hydnocarpus* revealed that plants of this genus could be a rich source of bioactive molecules \cite{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]\textsuperscript{+} at m/z 267.1960 was observed. The \textsuperscript{1}H NMR spectrum of 1 showed a singlet olefinic proton at δ\textsubscript{H} 5.95 (H-2), aliphatic protons at δ\textsubscript{H} 2.57 (2H, m, CH\textsubscript{2}-4), 2.41 (2H, m, CH\textsubscript{2}-5), 2.39 (2H, t, J = 7.5 Hz, CH\textsubscript{2}-6), 2.34 (2H, t, J = 7.5 Hz, CH\textsubscript{2}-15), 1.63 (2H, quint, J = 7.5 Hz, CH\textsubscript{2}-14), and 1.57 (2H, quint, CH\textsubscript{2}-7), and overlapped protons at δ\textsubscript{H} 1.27-1.28. Analysis of the \textsuperscript{13}C NMR spectrum, with the aid of the HSQC spectrum, revealed the presence of a ketone at δ\textsubscript{C} 210.6 (C-3), three sp\textsuperscript{3} carbons at δ\textsubscript{C} 183.7 (C-1), 179.3 (C-16) and 129.4 (C-2), and twelve methylenes. The COSY spectrum of 1 indicated correlations between protons of CH\textsubscript{2}-4 and CH\textsubscript{2}-5, and those of the side chain starting from CH\textsubscript{2}-6 to CH\textsubscript{2}-15. Analysis of the HMBC spectrum revealed the presence of a cyclopentenyl ring by cross-peaks of ketone carbon C-3 with H-2, CH\textsubscript{2}-4 and CH\textsubscript{2}-5, and those of protons of CH\textsubscript{2}-5 with C-1 and C-2. In addition, the HMBC correlations of C-1 with the protons of CH\textsubscript{2}-6 and CH\textsubscript{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\textsubscript{2}-14.

Compound 2, obtained as an amorphous solid, was optically active \{[\alpha]\textsubscript{D} -68 (c 0.16, MeOH)\}. Its HRESI mass spectrum showed the proton adduct molecular ion [M+H]\textsuperscript{+} at m/z 288.1091, consistent with the molecular formula C\textsubscript{12}H\textsubscript{17}NO\textsubscript{7}.

Figure 1: Structures of compounds 1 – 3.

Figure 2: Selected HMBC correlations of 1.
The presence was observed in the $^1$H NMR spectrum of 2 of two olefinic protons at $\delta_H 16.16 (H-2)$ and 6.24 (H-3), an anomic proton at $\delta_H 5.06$, and seven protons in the carbohydrate region. The $^{13}$C NMR and DEPT spectra of 1 were presented [7a-c]. These two compounds are reported here for the first time and named hydnohainanin A and B, respectively. These two compounds are similar in structure to the $1,4$-dihydroxy-2-cyclopenten-1-carbonitrile [8]. As $\beta$-D-allopyranose was present in the structures of both 2 and 3, their aglycones were thus enantiomers. Compounds 2 and 3 had chemical shifts and proton coupling constants (Table 2) close to those of the $1,4$-cis isomers (taratophylin 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 Flavocurtaeae species predominantly produce $1,4$-cis-dihydroxy-2-cyclopenten-1-carbonitrile [8].

Table 1: NMR data of compound 1 (CDCl₃).

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<th>Position</th>
<th>$\delta_C$</th>
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Table 2: NMR data of compounds 2 and 3 (CD$_2$OD).

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</tbody>
</table>

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 these four isomers. However, the proton data are almost similar for these four isomers. Moreover, 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 (4S) and positive for 3 (4R) [7e,9c]. Additionally, the molecular rotation of 2 [(M)-195] is more levorotatory in comparison with 3 ([(M)-63]). The observed rotations of 2 (negative) and 3 (positive) could hence confirm the 4S and 4R configuration for 2 and 3, respectively. As 2 had a negative rotation (same effect with allylic hydroxy for 4S) and a positive one (same effect with allylic hydroxy for 4R), 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 taratophylin and epivolkenin [7e]. The absolute configurations of 2 and 3 are thus suggested as (1R,4S for 2 and 1S,4R for 3). These two compounds are reported here for the first time and named hydnohainanin A and B, respectively.

The known compounds, hydnoacarpic acid [10], taratophylin [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 IC₅₀ 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 CDCl₃ and CD$_2$OD at $\delta$ 7.27 and 3.31 ppm, respectively, while the $^{13}$C chemical shifts were referenced to the central peak at $\delta$ 77.0 (CDCl₃), and 49.0 (CD$_2$OD). For HMBC experiments the delay

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

Hydnocarpus hainanensis

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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 CH2Cl2) to furnish 9 fractions. Fraction 4 (2.2 g) was subjected to CC on Sephadex LH-20 (MeOH/CH2Cl2: 9/1), yielding 2 sub-fractions. Sub-fraction 1 (1.1 g) was purified by preparative TLC. 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 U/mL), streptomycin (100 µg/mL) and gentamicin (10 µg/mL). Stock solutions of compounds were prepared in DMSO/H2O (1/9), and the cytotoxicity assays were carried out in 96-well microtiter plates against either cancer or normal cells (3 x 10^4 cells/mL) using a modification of the published method [12]. After 72 h incubation at 37°C in air/CO2 (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 IC50 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.

Hydnocarpanic acid (1)

Colorless oil.

Hydnocarpanic acid A (2)

White amorphous solid.

Hydnocarpanic B (3)

White amorphous solid.

Hydnocarpanic acid B (3)

White amorphous solid.

Hydnocarpanic acid C (4)

White amorphous solid.

Hydnocarpanic acid D (5)

White amorphous solid.

Hydnocarpanic acid E (6)

White amorphous solid.

Hydnocarpanic acid F (7)

White amorphous solid.

Hydnocarpanic acid G (8)

White amorphous solid.

Hydnocarpanic acid H (9)

White amorphous solid.

Hydnocarpanic acid I (10)

White amorphous solid.

Hydnocarpanic acid J (11)

White amorphous solid.

Hydnocarpanic acid K (12)

White amorphous solid.

Hydnocarpanic acid L (13)

White amorphous solid.

Hydnocarpanic acid M (14)

White amorphous solid.

Hydnocarpanic acid N (15)

White amorphous solid.

Hydnocarpanic acid O (16)

White amorphous solid.

Hydnocarpanic acid P (17)

White amorphous solid.

Hydnocarpanic acid Q (18)

White amorphous solid.

Hydnocarpanic acid R (19)

White amorphous solid.

Hydnocarpanic acid S (20)

White amorphous solid.

Hydnocarpanic acid T (21)

White amorphous solid.

Hydnocarpanic acid U (22)

White amorphous solid.

Hydnocarpanic acid V (23)

White amorphous solid.

Hydnocarpanic acid W (24)

White amorphous solid.

Hydnocarpanic acid X (25)

White amorphous solid.

Hydnocarpanic acid Y (26)

White amorphous solid.

Hydnocarpanic acid Z (27)

White amorphous solid.

Hydnocarpanic acid AA (28)

White amorphous solid.

Hydnocarpanic acid BB (29)

White amorphous solid.

Hydnocarpanic acid CC (30)

White amorphous solid.

Hydnocarpanic acid DD (31)

White amorphous solid.

Hydnocarpanic acid EE (32)

White amorphous solid.

Hydnocarpanic acid FF (33)

White amorphous solid.

Hydnocarpanic acid GG (34)

White amorphous solid.

Hydnocarpanic acid HH (35)

White amorphous solid.

Hydnocarpanic acid II (36)

White amorphous solid.

Hydnocarpanic acid JJ (37)

White amorphous solid.

Hydnocarpanic acid KK (38)

White amorphous solid.

Hydnocarpanic acid LL (39)

White amorphous solid.

Hydnocarpanic acid MM (40)

White amorphous solid.

Hydnocarpanic acid NN (41)

White amorphous solid.

Hydnocarpanic acid OO (42)

White amorphous solid.

Hydnocarpanic acid PP (43)

White amorphous solid.

Hydnocarpanic acid QQ (44)

White amorphous solid.

Hydnocarpanic acid RR (45)

White amorphous solid.

Hydnocarpanic acid SS (46)

White amorphous solid.

Hydnocarpanic acid TT (47)

White amorphous solid.

Hydnocarpanic acid UU (48)

White amorphous solid.

Hydnocarpanic acidVV (49)

White amorphous solid.

Hydnocarpanic acid WW (50)

White amorphous solid.

Hydnocarpanic acid XX (51)

White amorphous solid.

Hydnocarpanic acid YY (52)

White amorphous solid.

Hydnocarpanic acid ZZ (53)

White amorphous solid.

Hydnocarpanic acid AAA (54)

White amorphous solid.

Hydnocarpanic acid BBB (55)

White amorphous solid.

Hydnocarpanic acid CCC (56)

White amorphous solid.

Hydnocarpanic acid DDD (57)

White amorphous solid.

Hydnocarpanic acid EEE (58)

White amorphous solid.

Hydnocarpanic acid FFF (59)

White amorphous solid.

Hydnocarpanic acid GGG (60)

White amorphous solid.

Hydnocarpanic acid HHH (61)

White amorphous solid.

Hydnocarpanic acid IJJ (62)

White amorphous solid.

Hydnocarpanic acid KKK (63)

White amorphous solid.

Hydnocarpanic acid LLL (64)

White amorphous solid.

Hydnocarpanic acid MMM (65)

White amorphous solid.

Hydnocarpanic acid NNN (66)

White amorphous solid.

Hydnocarpanic acid OOO (67)

White amorphous solid.

Hydnocarpanic acid PPP (68)

White amorphous solid.

Hydnocarpanic acid QQQ (69)

White amorphous solid.

Hydnocarpanic acid RR (70)

White amorphous solid.

Hydnocarpanic acid SS (71)

White amorphous solid.

Hydnocarpanic acid TT (72)

White amorphous solid.

Hydnocarpanic acid UU (73)

White amorphous solid.

Hydnocarpanic acid VV (74)

White amorphous solid.

Hydnocarpanic acid WW (75)

White amorphous solid.

Hydnocarpanic acid XX (76)

White amorphous solid.

Hydnocarpanic acid YY (77)

White amorphous solid.

Hydnocarpanic acid ZZ (78)

White amorphous solid.

Hydnocarpanic acid AAAA (79)

White amorphous solid.

Hydnocarpanic acid BBBB (80)

White amorphous solid.

Hydnocarpanic acid CCCC (81)

White amorphous solid.

Hydnocarpanic acid DDDD (82)

White amorphous solid.

Hydnocarpanic acid EEEE (83)

White amorphous solid.

Hydnocarpanic acid FFFF (84)

White amorphous solid.

Hydnocarpanic acid GGGG (85)

White amorphous solid.

Hydnocarpanic acid HHHH (86)

White amorphous solid.

Hydnocarpanic acid IJJJ (87)

White amorphous solid.

Hydnocarpanic acid KKKK (88)

White amorphous solid.

Hydnocarpanic acid LLLL (89)

White amorphous solid.

Hydnocarpanic acid MMMM (90)

White amorphous solid.

Hydnocarpanic acid NNNN (91)

White amorphous solid.

Hydnocarpanic acid OOOO (92)

White amorphous solid.

Hydnocarpanic acid PPPP (93)

White amorphous solid.

Hydnocarpanic acid QQQQ (94)

White amorphous solid.

Hydnocarpanic acid RR (95)

White amorphous solid.

Hydnocarpanic acid SS (96)

White amorphous solid.

Hydnocarpanic acid TT (97)

White amorphous solid.

Hydnocarpanic acid UU (98)

White amorphous solid.

Hydnocarpanic acid VV (99)

White amorphous solid.

Hydnocarpanic acid WW (100)

White amorphous solid.

Hydnocarpanic acid XX (101)

White amorphous solid.

Hydnocarpanic acid YY (102)

White amorphous solid.

Hydnocarpanic acid ZZ (103)

White amorphous solid.


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, chromone, 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 of S. divaricata showed cytotoxic activity with an IC50 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, Cytotoxicity, Apoptosis.

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 IC50 values of 4.41 and 5.21 μM, respectively. Etoposide and cisplatin were used as the positive controls, and had IC50 values of 0.41 μM and 1.61 μM, respectively.

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

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC50 (μM)</th>
<th>Compound</th>
<th>IC50 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.41±0.31</td>
<td>5</td>
<td>&gt;100</td>
</tr>
<tr>
<td>2</td>
<td>5.21±0.62</td>
<td>6</td>
<td>&gt;100</td>
</tr>
<tr>
<td>3</td>
<td>17.4±1.77</td>
<td>etoposide</td>
<td>0.41±0.15</td>
</tr>
<tr>
<td>4</td>
<td>&gt;100</td>
<td>cisplatin</td>
<td>1.61±0.22</td>
</tr>
</tbody>
</table>

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

Compound 1 and the 11-hydroxy derivative (2) of 1 are chromone derivatives whose side-chain moiety forms a six-membered ring bearing an angelyol group at the C-3' hydroxy group; they showed potent cytotoxic activity against HL-60 cells. Displacing the angelyol group with an acetyl or glucosyl group diminished cytotoxic potency, suggesting that the presence of the angelyol moiety enhances cytotoxic activity. The furanochromone 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 I-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.](image)

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 chromatin.

![Figure 3: Induction of DNA fragmentation by either 1 or etoposide in HL-60 cells.](image)

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 (ΔΨm) is one of the intracellular events that occur during the induction of apoptosis. The ΔΨm 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.](image)

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 I-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.](image)

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.](image)

![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.](image)

![Figure 8: Caspase-3 activity in the lysates of cells treated with either 1 or etoposide.](image)

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-angeloyllhmaudol (1).

![Figure 9: Mitochondria-dependent apoptosis signaling pathway.](image)

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 1H 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 Sily sia 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% \( \text{H}_2\text{SO}_4 \) 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, etosipide, 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 (395 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\(_2\)O (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\(_2\)O (190:10:1) and using ODS silica gel CC eluted with MeCN/MeOH/ H\(_2\)O (1:1:1) to give 4 (145 mg) and 5 (50.0 mg). Fraction C was separated using silica gel CC eluted with MeCN/MeOH/H\(_2\)O (1:3:1, 1:2) and using silica gel CC eluted with EtOAc/MeOH/H\(_2\)O (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 x 10\(^5\) 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\(_2\)O (1:1) solution containing the sample was added to give the final concentrations of 0.1-100 μM, and 4 μL of ETOH/H\(_2\)O (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 x 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 I 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 I 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 x 10\(^6\)) were centrifuged for 5 min at 10,000 g. The cell pellet was suspended in 600 μL of 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\(_2\)O (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-EDETA 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 (ΔΨ\(_m\)):** The mitochondrial membrane potential (ΔΨ\(_m\)) was investigated using the MitoCaptureTM Apoptosis Detection Kit (BioVision, CA, U.S.A.), according to the manufacturer’s procedure. HL-60 cells (5 x 10\(^5\)) were treated with either 50 μM of I 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 MitoCaptureTM 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 x 10\(^6\)) were treated with either 50 μM of I 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 I 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 I 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-pNA (p-nitroanilide)]. After incubation for 2 h at 37°C, the absorbance of the liberated chromophore pNA was measured using a microplate reader at 405 nm. The activity of caspase-3 was evaluated in triplicate.

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References
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, Benzo furan 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, benzo furans, benzo furan 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°-hydroxyegonol gentiobioside (1), egonol glucoside (2), syringin (3), (+)-1-hydroxypinoresinol-4°-β-D-glucoside (4), pinoresinol-4°-O-β-D-glucoside (5), (+)-lari cine resinol 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).

![Figure 1: Chemical structures of isolated compounds (1−7) from *S. obassia*.](image)

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-diphenyl tetrazolium bromide (MTT) assay according to Mosmann [3a] with adriamycin as the positive control, compound 2 revealed cytotoxic activity 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 benzo furan lignan and the flavonoid skeletons are essential for cytotoxic activity [3b,3c]. In addition, compound 1 is a benzo furan 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.

**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, CHCl₃, EtOAc, and H₂O, successively, to afford n-hexane (53 g), CHCl₃ (35 g), EtOAc (6 g), and H₂O (35 g) soluble fractions, respectively. The EtOAc-soluble fraction (6.0 g) was subjected to a silica gel column and eluted with CHCl₃-MeOH (30:1) to obtain compound E₁₂.3 (25.0 mg) was chromatographed over a silica gel column and eluted with MeOH-H₂O (1:3 → 1:1) to afford compound E₁ (15.6 mg). Fraction E₁₀ (521.7 mg) was applied to an RP-C₁₈ silica gel column and eluted with MeOH-H₂O (55:45 → 65:35) over 60 min; flow rate: 3 mL/min; UV detection at 210 nm; an YMC-Pack ODS-A, 250 × 20 mm column eluted with a gradient solvent system of MeOH-H₂O (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, Rₜ = 56 min). Compound 7 (13.5 mg, Rₜ = 30 min) was obtained by HPLC [eluted with MeOH-H₂O (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-C₁₈ silica gel column and eluted with MeOH-H₂O (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 MeOH-H₂O (1:3 → 1:1) to afford compound 3 (40.0 mg) and 8 sub-fractions (E₁₂.1 ~ E₁₂.8). Sub-fraction E₁₂.3 (25.0 mg) was chromatographed over a silica gel column and eluted with CHCl₃-MeOH (30:1) 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, t-glutamine, and pyridoxine hydrochloride supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin in a 96-well plate at a density of 6 × 10⁴ cells/mL. After reaching confluence (2 × 10⁵ 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 as a control. The experiment was allowed to proceed for 48 h at 37°C in a humidified 5% CO₂ 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 IC₅₀ was estimated visually. Values are expressed as mean ± SD.

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**References**


Isolation and Characterization of Antiangiogenesis Compounds from the Fungus Aspergillus terreus Associated with Apostichopus japonicus Using Zebrafish Assay

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Three compounds, (+)-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 C24H24O7. Its UV spectrum showed absorption of butyrolactones at 230 and 310 nm. Combined with 1H and 13C 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 C24H26O8. Its UV spectrum showed characteristic absorption of butyrolactones at 230 and 310 nm. Combined with 1H and 13C NMR spectral data, compound 3 was identified as terrelactone A [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 (IC50 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.

Table 1: Antiangiogenesis activity of compounds 1 and 2 using zebrafish assay.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Cone (μg/mL)</th>
<th>Number of vessels in zebrafish (Y ± SD)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>25.8 ± 1.3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PTK 787</td>
<td>0.5</td>
<td>0.20 ± 0.45**</td>
<td>1.24508E-10</td>
</tr>
<tr>
<td>100</td>
<td>14.6 ± 3.7**</td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>19.2 ± 2.6**</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>22.8 ± 3.8*</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>18.4 ± 2.5**</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>21.6 ± 3.4*</td>
<td>0.03</td>
<td></td>
</tr>
</tbody>
</table>
| **P < 0.01 significantly different from the control. *P < 0.05 significantly different from the control. −, all of the zebra fish died, and the vessels were not calculated.**

** Table 1: Antiangiogenesis activity of compounds 1 and 2 using zebrafish assay.

Figure 1: Structures of compounds 1, 2 and 3.

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, Antion Paar MCP 300 (Antion Paar) polarimeter; NMR, Varian INOVA-600 MHz spectrometer; HRESIMS, Agilent QTOF-6530; CC, silica gel (200-300 mesh, Qingdao Haiyang Chemicals), octadecylsilica 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 zebra fish captured using an Olympus DP72 imaging system. The length of embryos was captured using the DP72 imaging system. The length of embryos was captured using the DP72 imaging system. The length of embryos was captured using the DP72 imaging system.

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 differentiated, 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 GAG TAC CAC-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-CHCl₃-MeOH (2:1:1), which yielded 3 (1.0 mg). Fr. (5-8)-4 was subjected to semi-preparative reverse-phase HPLC (octadecylsilane (ODS) (YMC, 250 x 10 mm) (30% MeOH-H₂O, v/v, 5 min; 30%–100% MeOH-H₂O, 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 CaCl₂, 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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References

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 molecular weight) also showed adsorption potency to Cs in that order. For Sr adsorption, kelp, CMC-Na, MC, AP (high molecular 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 (ALANa): 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,
reported that apple pectin reduced the 137Cs whole body count in 3.2 (Chs), 1.68 (CMC-Na), 4.47×10⁻¹ (MC) and 1.20×10⁻¹ (AP) adsorption of Cs and Sr to the equivalent of ALA-Na. It has been 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 adsorbing 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 Wako Pure Chemical Industries, Ltd. (Osaka, Japan); and SUN FIBER (guar gum degradation product) from Taiyo Kagaku Co. Ltd. (Mie, Japan).

**Pholisota microspera** (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; 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)) and strontium chloride (SrCl₂; 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 monuclear 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, Sr 0.05 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:

\[
\log V = \frac{1}{n} \log C + \log K(1)
\]

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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Adsorptive potency of samples to Cs and Sr

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References


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. \textit{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 \textit{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, \textit{Ex vitro} rooting, Germination treatment, Gibberellic acid, Seed dormancy, Seed viability.

Corema album (L.) D. Don (Ericaceae subfam. Ericoideae tribu Empetreae) is a dioecious woody shrub endemic to the Atlantic coast of the Iberian Peninsula, commonly known as Camarina or Camarinha. \textit{C. album} is an important species in sand dune ecosystems, being the dominant species in the areas where it grows. \textit{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 \cite{1}. \textit{C. album} berries were traditionally used in popular medicine as an anti-inflammatory \cite{1, 2} and recent studies have discovered important pharmacological properties of its fruits and leaves \cite{3-5}. According to León-González \textit{et al.} \cite{3}, extracts from \textit{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 \textit{C. album} fruit and leaf extracts showed an outstanding protection against challenge-induced damage. These findings support the traditional use of \textit{C. album} as a medicinal plant. Recently, the species has been proposed as a suitable crop for berry production for the food industry \cite{6}.

Several factors make the natural regeneration of \textit{C. album} difficult. Seeds show low germination under natural conditions and are endozoochorous, presenting physiological dormancy, which is broken after consumption by vertebrates like seagulls \cite{7, 8}, rabbits, and foxes \cite{7, 9-11}. \textit{C. album} seedling mortality rate in natural conditions is high, reaching 99\% during the summer season \cite{10}. Moreover, the habitat of \textit{C. album} is dwindling with its populations being fragmented \cite{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 \textit{Retama monosperma} \cite{12}, and the invasive shrub \textit{Acacia longiforme} \cite{13}. \textit{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 \cite{14}.

Facing the ongoing habitat loss and disturbance in \textit{C. album} communities, regeneration under natural conditions is really low, both in the northern \cite{7, 10} and southern limits of its biogeographical distribution area \cite{9, 10, 15}. Under this scenario it is necessary to gain knowledge on the mechanisms underlying \textit{C. album} propagation, not only for maintaining and regenerating degraded \textit{C. album} populations, but also for future feasible agricultural and pharmacological use \cite{3-6}. So far there is very limited information on the germination responses of \textit{C. album} \cite{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 \textit{C. album} such as the American endemism Ceratiola ericoides \cite{17}, but studies involving Ericaceae propagation in the Iberian Peninsula or Mediterranean species are scarce \cite{18}. In cuttings propagation, several phytohormones and growth factors such as indolebutyric acid (IBA) are described as effective rooting triggers \cite{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 \cite{20}. Various methods can be used for cuttings propagation of shrub species, but the one needed for \textit{C. album} has not yet been described.

In this study we aimed to assess germination and clonal propagation requirements for \textit{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 \textit{in vitro} treatments (physical and physiological). We further evaluated the effectiveness of clonal propagation by cuttings hardening procedures in \textit{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 \textit{C. album} clonal propagation under greenhouse conditions.
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 (GA₃) 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 GA₃ treatments (29-30 days), with the exception of GA₃ 25 ppm, which showed a longer time for t₀ (45 days). The time of first germination (t₀) 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: t₀, t₅₀, ttotal, 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).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>t₀</th>
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<th>ttotal</th>
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<td>GA₃ 25 ppm</td>
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<td>57</td>
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<td>48</td>
<td>71</td>
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<td>48</td>
<td>71</td>
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<td>30</td>
<td>35</td>
<td>59</td>
<td>47.9</td>
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<tr>
<td>Fox</td>
<td>63</td>
<td>67</td>
<td>71</td>
<td>66.5</td>
</tr>
<tr>
<td>pH 1.6</td>
<td>128</td>
<td>131</td>
<td>143</td>
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<td>pH 2</td>
<td>131</td>
<td>131</td>
<td>144</td>
<td>136.3</td>
</tr>
</tbody>
</table>

**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).

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).
Dispersers of Delibes [13] described foxes and rabbits to be the most common natural dispersers such as rabbits and foxes (Figure 1). Fedriani and Delibes [10], which under natural conditions is broken by ingestion by endozoocorous species and unspecialized vertebrate dispersers such as rabbits and foxes play a key role in distribution, the same as our study site, while Calviño-Cancela et al. [16], who recently 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 endozoochorous species and unspecialized vertebrate dispersers such as rabbits and foxes play a key role in C. album regeneration in its 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 GA₃ 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 GA₃ 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.

GA₃ 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 GA₃ 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 GA₃ 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 ericoidea, 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-treated 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 4°C 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.

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 triphenyl 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 naphthaleneaceticamide (Rootone, Compo®).
4) Rooting dust growth regulator mix of IBA 0.1% and NAA 0.1, w/w (Inabaprot, 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 Inabaprot) 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 Ceratioda 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°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).

**Acknowledgments** - This research was funded by Universidad de Sevilla. We thank Junta de Andalucia for providing permits to access Doñana Natural Park.

**References**

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Table 1: Chemical constituents of the essential oil of Geranium kikianum.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Class/Type</th>
<th>RI</th>
<th>RI’</th>
<th>MO/M*</th>
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</thead>
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<td>β-Elemene</td>
<td>SH</td>
<td>1366</td>
<td>t</td>
<td>A/VPN</td>
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<td>Oil yield (%)</td>
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**Total identified (%)** 98.3

1) The abbreviations of the compound classes are given at the end of the table;
2) RI: Linear retention indices determined experimentally on the HP-5MS column relative to a series of n-alkanes (C15-C30);
3) RA: Relative area, t, traces (<0.1%);
4) MO: Mode of identification;
5) A: Reference [28];
6) MF: Mass FINDER database;
7) MS: Mass spectra from our own laboratory database.

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 monoterprenoids, 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 germacrene content of *G. macrorrhizum* (49.7%) [3a], and *G. kikianum* (45.6%), their essential oils significantly differ in the amount of monoterprenoids. Unlike *G. kikianum* essential oil, which is characterized by a complete absence of monoterprenoids, *G. macrorrhizum* oil contains a relatively notable amount of this chemical class (9.9%) [3a]. Similarly, according to Stoeva [9], germacrene comprised 50-55%, as a notable amount of this chemical class (9.9%) [3a]. Similarly, this acyclic diterpene alcohol has not frequently been reported as an unique alcohol and the third most abundant constituent was the prominent alcohol and the third most abundant constituent was the tertiary alcohol elemo (49.7%) [3a], and terpinolene, p-cymene, α-pinene, δ-3-carene, α-phellandrene, limonene and borneol. Moreover, some taxa of *Geranium* contain monoterprenoids 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-terpinel (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.

<table>
<thead>
<tr>
<th>Compound</th>
<th>GAE [mg/g] extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total phenolics</td>
<td>100.2±1.7*</td>
</tr>
<tr>
<td>Non-flavonoids</td>
<td>88.2±2.4</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>11.9±1.2</td>
</tr>
</tbody>
</table>

*Gallic acid equivalents; 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 (IC50) were calculated. IC50 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-costa (3.6%) and 2(7Z)-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 (IC50=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 (IC50=0.21±0.01 mg/mL), and even almost tenfold higher than that of thymol (IC50=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. 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 Peloponnesse, 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 (% 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 C10-C26 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 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:

$$IC(\%) = \frac{[A_0 - A_1]}{A_0} \times 100,$$

where A₀ and A₁ 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 IC₅₀ value, a concentration required to quench 50% of DPPH radical. Experiments were carried out in triplicate.

References


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%), $\beta$-phellandrene (15.3%) and linalool (12.2%). The bark oil, investigated for the first time, contained $\beta$-pinene (49.9%) and $\alpha$-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 Tsaramandrosoro contained mainly linalool (72.5%) [5]. Leaf oil samples from Katsepy and Tsaramandrosoro belonged to four chemotypes characterized by (i) linalool (ii) 1,8-cineole (iii) geraniol/neral and (iv) geranic acid (48.8%) [6]. An EtOAc extract of *C. macrocarpa* led to the identification of ten components, including cinnamarcins A-D, cinnamodial, cinnamolide, bemadienolide, and isopolypedial [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%), $\beta$-phellandrene (15.3%) and linalool (12.2%). $\alpha$-Copaene (5.4%), (E)-$\beta$-caryophyllene (4.0%), $\delta$-cadinene (3.3%) $\beta$-elemol (2.7%), caryophyllene oxide (2.1%), $\beta$-eudesmol (1.5%) and $\alpha$-eudesmol (1.2%) were the main sesquiterpenes. 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 $\beta$-pinene (49.9%) and $\alpha$-pinene (19.5%). However, a component (2.0% of the whole composition) was not identified and its identification is detailed below.

2.1. Identification of cyclosativene: Retention indices of the unidentified component (RI<sub>\text{EI} </sub> = 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<sub>\text{EI} </sub> = 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 monoterpenic hydrocarbons ($\beta$-pinene, $\alpha$-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.
However, a signal at δ 0.67 ppm (d, 5.3 Hz) led us to suspect a cyclopropane moiety in the framework of the molecule. This signal was observed in the DEPT spectrum, indicating two methyl groups.

In the 1H NMR spectrum, the compound showed a complex pattern with high intensities. Two sets of signals were observed, corresponding to a trans-2,5-dimethyl-1,3-dihydro-2H-pyran-2-one (RI = 1503) and a trans-2,5-dimethyl-1,3-dihydro-2H-pyran-2-one (RI = 1504). These signals were assigned to compound (C. madagascariensis).

The 13C NMR spectrum showed several peaks, indicating the presence of a cyclopropane ring. The chemical shifts were in agreement with those reported in ref [12] and [13]. Unfortunately, the 13C NMR data were not reported. However, comparison of the 13C NMR chemical shifts of our compound with those of cyclocopacamphan-12-ol [14] was informative. Indeed, 11 out of 15 chemical shifts matched those of cyclocopacamphan-12-ol, and the remaining four differences were within the expected limits (RI = 1361) in ref [12].

The compound was identified as cyclocopacamphan-12-ol, an epimer of cyclosativene. The 1H NMR spectrum fitted with those reported in ref [13]. Unfortunately, the 13C NMR data were not reported. However, comparison of the 13C NMR chemical shifts of our compound with those of cyclocopacamphan-12-ol was informative. Indeed, 11 out of 15 chemical shifts matched those of cyclocopacamphan-12-ol, and the remaining four differences were within the expected limits (RI = 1361) in ref [12].

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In order to confirm the syn/anti stereochemistry of the isopropyl group.

We concluded that the unidentified component is cyclocopacamphene.

They displayed noticeable differences with those of cyclocopacamphene.

In contrast, C6, C8 and C11 of cyclocopacamphene were shielded by -3.74 ppm. In contrast, C2 of cyclosativene was shielded by -5.39, -4.26 and -3.51 ppm.

In order to confirm the syn/anti stereochemistry of the isopropyl group.

Cyclocopacamphene and cyclosativene have identical RIs on the capillary column.

Cyclocopacamphene and cyclosativene were isolated from Abies magnifica in 1968, and it is nowadays commercially available, its 13C NMR data have not been reported.

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).

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 13C NMR.

<table>
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<th>No</th>
<th>¹³C δ (ppm)</th>
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<td>2, 3, 4, 8, 9, 15</td>
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<td>32.00</td>
<td>11</td>
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<tr>
<td>12</td>
<td>21.28*</td>
<td>12</td>
<td>0.90 (d, J=6.7 Hz)</td>
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<tr>
<td>13</td>
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<tr>
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<td>-</td>
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<tr>
<td>15</td>
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<td>0.76 (s)</td>
<td>-</td>
<td>1, 3, 7, 8, 9, 11</td>
<td>5a</td>
</tr>
</tbody>
</table>

* 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 spectrometric libraries and comparison of spectra with literature data [19], iii) on comparison of the signals in the 13C 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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References
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 lowest antibacterial activity against foodborne pathogens. Combinations of selected EOs displayed a synergetic 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 aw, and addition of salt, nitrates, 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 reduction of 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 lowest antibacterial activity against foodborne pathogens. Combinations of selected EOs displayed a synergetic 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.

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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 β-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 (eugenol), 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...
The antibacterial activity of the EOs assessed by the disk diffusion assay (DDA) is presented in Table 2. EOs of thyme, cinnamon, nutmeg, parsley, and bay were considered not inhibitory (halo size <10mm). However, EOs of rosemary, cumin, garlic, bay, black pepper, parsley, nutmeg and parsley presented an inhibition halo of over 10mm. 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 MIC values 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 EOs of thyme, garlic, cumin, cinnamon, and parsley 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 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 6.25 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.

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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 *Salmonella* 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. 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 monoterpane *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 compounds 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, cosmeone or *β*-piene 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 sulphydryl groups of cellular proteins of the microorganisms disturbing the cellular metabolism [31] in contrast to terpenoids that have 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* 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*– leaves and flowers), thyme (*Thymus capitatus* Hoff. et Link – flowering tops), thyme (*Thymus capitatus* Hoff. et Link – flowering tops), thyme (*Thymus capitatus* Hoff. et Link – flowering tops), thyme (*Thymus capitatus* Hoff. et Link – flowering tops), cinnamon (*Cinnamomum zeylanicum* Nees C. – leaves), parsley (*Petroselinum sativum* Hoffm. – aerial part of the plant), nutmeg (*Myristica fragans* – nuts), lemon (*Citrus limon* L. - fruit peels), orange (*Citrus sinensis* L. Osbeck – fruit peels), black pepper (*Piper nigrum* L. – fruits), rosemary (*Rosmarinus officinalis* L. – entire plant, excluding woody parts), laurel (*Laurus nobilis* L. – leaves) and cumin (*Cuminum 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 PolarisQ 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 10°C min⁻¹ to 260°C, and maintained at 260°C for 24 min. The injector temperature was 250°C. 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 (C₁₀–C₃₀) 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 collection culture 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 -18°C and subcultured twice in Brain Heart Infusion (BHI, Biokar. Beauvais, France). Incubation was made at 37°C except for *L. monocytogenes* (30°C). 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 was made 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 microtiter 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 microtiter 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 37°C for Salmonella spp., E. coli and S. aureus and at 30°C 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≤4 an additive effect, 1<FIC≤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%), 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, t-cadinol, and t-muurolol. The fruit essential oil was shown to have excellent anti-mildew and anti-wood-decay fungal activities, the active compounds being evaluated as t-cadinol and t-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, Fujian, Jiangxi, Guizhou, 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 and 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 monoterpenes 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, t-muurolol (8.3%), and t-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 concentrations 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 IC50 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, t-cadinol, and t-muurolol, were individually evaluated for their anticancer activities. The active compounds were β-caryophyllene, t-cadinol, and t-muurolol. The IC50 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 t-cadinol was shown to destroy A-549 and HT-29 human cancer cell lines [8]. In addition, t-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 suppressed by the essential oil.
inhibited at concentrations of 250, 500, 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], Eucaalyptus 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), t-Muurolol, t-cadinol, and β-caryophyllene exhibited the best activities among the fruit essential oil constituents, in particular t-muurolol, which was active against all seven 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 microbical growth [22-23].

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 (Tremetem versicolor and Phanerochaete chrysosporium) and 2 strains of brown rot (Phaeolus schweinitzii and Lenzites sulphureus). The growth of T. versicolor, Phanerochaete chrysosporium, Phaeolus schweinitzii, and L. sulphureus 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. pseudologifolia [24], M. philippinensis [25], Litsea mushaensis [26], Lit. coreana [27], Lit. acutivina [28], and Lit. lini [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.

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 t-muurolol and t-cadinol. At a concentration of 50 μg/mL, t-muurolol and t-cadinol showed total growth inhibition against all the white-rot and brown-rot fungi tested (Table 3). The presence of t-muurolol and t-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 t-muurolol, and t-cadinol. The results agree with those of Kondo and Imamura [29], who pointed out that the methanol extract of hinoki (Chamaecyparis obtusa) containing t-cadinol, and t-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 25° 01´ 48˝, E 121º 30´ 35˝). The samples were compared with specimen no. ou 58898 from the Herbarium of the National Chung-Hsing 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, sabine, β-pinene and β-caryophyllene were purchased from Fluka Co. (Milwaukee, USA),

Isolation of the leaf essential oil: One Kg air-dried L. formosana fruits were distilled for 3 h using a Cleverenger-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 ≤ 4°C 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: 50°C for 2 min, rising to 250°C at 5 °C/min. Injector temperature: 270°C. Carrier gas: He with a flow rate of 1 mL/min. Detector temperature: 250°C, 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% CO2 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), Phaeno. 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.

References


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 O₂-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.

**Table 1:** Chemical composition (%) of the HS volatiles of *Achillea thracica*.

<table>
<thead>
<tr>
<th>No.</th>
<th>RA²</th>
<th>RE³</th>
<th>Compound</th>
<th>Composition (%)</th>
<th>Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>849</td>
<td>848.3</td>
<td>Ethyl 2-methylbutyrate</td>
<td>6.8</td>
<td>E²</td>
</tr>
<tr>
<td>2.</td>
<td>906</td>
<td>910</td>
<td>Santolinalatriene</td>
<td>1.3</td>
<td>E³</td>
</tr>
<tr>
<td>3.</td>
<td>923</td>
<td>927</td>
<td>Artemisialatriene</td>
<td>8.3</td>
<td>E³</td>
</tr>
<tr>
<td>4.</td>
<td>999</td>
<td>1001</td>
<td>Yomogi alcohol</td>
<td>13.6</td>
<td>E³</td>
</tr>
<tr>
<td>5.</td>
<td>1026</td>
<td>1034</td>
<td>1,8-Cineole (trans-eucalyptol)</td>
<td>14.4</td>
<td>E³</td>
</tr>
<tr>
<td>6.</td>
<td>1034</td>
<td>1038</td>
<td>Santolina alcohol</td>
<td>31.1</td>
<td>E³</td>
</tr>
<tr>
<td>7.</td>
<td>1056</td>
<td>1065</td>
<td>Artemisia ketone</td>
<td>0.7</td>
<td>E³</td>
</tr>
<tr>
<td>8.</td>
<td>1080</td>
<td>1084</td>
<td>Artemisia alcohol</td>
<td>3.5</td>
<td>E³</td>
</tr>
<tr>
<td>9.</td>
<td>1100</td>
<td>1104</td>
<td>Isopentyl 2-methyl butanoate</td>
<td>2.4</td>
<td>E³</td>
</tr>
<tr>
<td>10.</td>
<td>1102</td>
<td>1106</td>
<td>Isopentyl isovalerate</td>
<td>1</td>
<td>E³</td>
</tr>
<tr>
<td>11.</td>
<td>1112</td>
<td>1114</td>
<td>3-Methyl-3-butenyl-3-methyl butanoate</td>
<td>0.8</td>
<td>E³</td>
</tr>
<tr>
<td>12.</td>
<td>1112</td>
<td>1120</td>
<td>β-thujone</td>
<td>0.8</td>
<td>M³</td>
</tr>
<tr>
<td>13.</td>
<td>1141</td>
<td>1148</td>
<td>Camphor</td>
<td>1.0</td>
<td>M³</td>
</tr>
<tr>
<td>14.</td>
<td>1184</td>
<td>1187</td>
<td>(3Z)-Hexenyl butanoate</td>
<td>0.4</td>
<td>E³</td>
</tr>
<tr>
<td>15.</td>
<td>1315</td>
<td>1313</td>
<td>(3E)-Hexenyl tiglate</td>
<td>2.1</td>
<td>E³</td>
</tr>
<tr>
<td>16.</td>
<td>-</td>
<td>1382</td>
<td>Isobornyl acrylate</td>
<td>3.3</td>
<td>E³</td>
</tr>
</tbody>
</table>

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 monoterpe 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*. 
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, 3,6,0-dimethyl quercetin, 3,7,0-dimethyl quercetin, and 3,7-O-dimethyl kaempferol, which differed from *A. chrysocaoma* and *A. clypeolata* examined in the same study [3].

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 (A0) could be distinguished as the most potent, while N (A2) was the weakest. Only EX (A0) 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.

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 flavonols 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 flavonols in the N sample was approximately twice that in the IN and EX samples (43.0 and 53.0%, respectively).

### Table 2: The composition of *Achillea thracica* acetone extracts as percentages of the total absorbance of the HPLC chromatograms recorded at 350 nm.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Rf</th>
<th>IN&lt;sup&gt;a&lt;/sup&gt;</th>
<th>EX&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Composition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>O-methyl quercetin</td>
<td>22.860</td>
<td>8.6</td>
<td>28.4</td>
</tr>
<tr>
<td>2</td>
<td>O-methyl kaempferol</td>
<td>24.978</td>
<td>-</td>
<td>3.6</td>
</tr>
<tr>
<td>3</td>
<td>O,0-dimethyl quercetin</td>
<td>26.907</td>
<td>63.7</td>
<td>29.7</td>
</tr>
<tr>
<td>4</td>
<td>O,0-dimethyl kaempferol</td>
<td>28.520</td>
<td>-</td>
<td>22.2</td>
</tr>
</tbody>
</table>

<sup>a</sup>JCN – component number; <sup>b</sup> positions of methyl groups not defined; methyl group could be in positions 3 and/or 7; <sup>c</sup>N – plant growing in natural habitat; <sup>d</sup>IN – *in vitro* propagated plant; <sup>e</sup>EX – *ex vitro* established plant. Components whose peak area is greater than 5% 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 (A0) could be distinguished as the most potent, while N (A2) was the weakest. Only EX (A0) 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 thracica* extracts.

<table>
<thead>
<tr>
<th>SL&lt;sup&gt;f&lt;/sup&gt;</th>
<th>P. aeruginosa</th>
<th>E. coli</th>
<th>S. typhimurium</th>
<th>S. aureus</th>
<th>B. subtilis</th>
</tr>
</thead>
<tbody>
<tr>
<td>EX A&lt;sub&gt;0&lt;/sub&gt;</td>
<td>17.5±0.2&lt;sup&gt;f&lt;/sup&gt;</td>
<td>16.0±0.4&lt;sup&gt;f&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>A&lt;sub&gt;1&lt;/sub&gt;</td>
<td>17.5±0.2&lt;sup&gt;f&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IN A&lt;sub&gt;1&lt;/sub&gt;</td>
<td>17.5±0.2&lt;sup&gt;f&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>A&lt;sub&gt;2&lt;/sub&gt;</td>
<td>11.5±0.2&lt;sup&gt;f&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>N A&lt;sub&gt;1&lt;/sub&gt;</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>A&lt;sub&gt;2&lt;/sub&gt;</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PP SI</td>
<td>24.0±0.5&lt;sup&gt;f&lt;/sup&gt;</td>
<td>17.0±0.52/0.05</td>
<td>18.0±0.2/20.0±0.3</td>
<td>23.0±0.4&lt;sup&gt;f&lt;/sup&gt;</td>
<td>23.0±0.4&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>S2</td>
<td>17.0±0.2&lt;sup&gt;f&lt;/sup&gt;</td>
<td>24.0±0.61/0.07</td>
<td>23.0±0.4/32.0±0.8</td>
<td>26.0±0.4&lt;sup&gt;f&lt;/sup&gt;</td>
<td>30.0±0.6&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>NP A&lt;sub&gt;1&lt;/sub&gt;</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>f</sup>JCN – bacterialic zone and S, bactericidal zone (mm); <sup>g</sup>SD – standard deviation; <sup>h</sup>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<sub>0</sub>) as negative probe. The absence of activity is marked as dash (-).
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 Trichophyton 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 duriae* 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 µL/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 addition 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 *Syzgium 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 µL/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 synergistic 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.

Yamini *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. laccosum* (C114), *M. canis* (C112), *M. gypseum* (C115), *T. mentagrophytes* (C116) and *T. interdigitale* (C117). They then performed an antifungal evaluation of the essential oil of *Baccharis uncinella* against seven strains of dermatophyte fungi: *E. laccosum* FF3, *M. canis* FF9, *M. gypseum* 2908, *T. mentagrophytes* 2958, *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 *Baccharis uncinella* 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 synergistic 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 *Baccharis uncinella* can be attributed to the presence, in greater proportion, of α-pinene and cis-β-O-cymene [14].

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Evaluation of natural products against dermatophytes

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(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 10⁵ to 5.0 x 10⁶ 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. gyrophorum 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 Loniceria 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 (10⁵ 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 espantoleno, citrilenell, α-cadinol and 1,8-cineole. 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. verrucosum 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. gyrophorum. The agar-dilution method allowed the determination 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. gyrophorum, 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 terpeneol 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 ciliatus against four species of dermatophytes: T. rubrum (ATCC 28188), T. mentagrophytes (ATCC 18748), M. gyrophorum (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. gyrophorum and M. canis was observed, when evaluated at a concentration of 250 µg/mL. The antifungal activity of the oil of P. ciliatus 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.

Njenteg 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
ag agar microdilution method in eight species of dermatophytes: *T. rubrum*, *T. mentagrophytes* (E1425), *T. ajelloi*, *T. equinum*, *T. terrestrae*, *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 an *in vivo* antifungal activity of an ointment prepared from petrolatum and essential oil of *Chenopodium ambrosioides*. 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. ambrosioides* 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:** Moghimipour 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* 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.
in vitro and in vivo methods for evaluating the susceptibility of dermatophytes to natural products.

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<td>Freeze thaw technique</td>
<td>It was established that the liposomal formulation effectively improves the stability of the essential oil and could favor its antifungal activity.</td>
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<td>Essential oil of Melaleuca alternifolia</td>
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<td>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.</td>
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<tr>
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<td></td>
<td>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</td>
<td>29</td>
</tr>
</tbody>
</table>

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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References


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 vitality givers, 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, antitumor and antioxidant properties, whereas the stems showed hepatoprotective, antipryeric, cytotoxic, antiadiabetic and immunomodulatory activity [9-14]. Dried fruits are used to treat jaundice and rheumatism, whereas the fruits are used to treat diabetes [15], and the roots are employed for their powerful emetic, antistress, antioxidant, antitumor, 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 nor diterpenoids, 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 preparations, 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, Dahashaka, Mehnashaka, Kasa-swahasara, 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 stanch 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, antiinfluenza, 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 CCl4-induced hepatic damage in rats [21]. A formulation, Caps HT2, containing a methanolic extract of...
**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 cyclooxygenidone [104] were isolated from *T. cordifolia*. A bicyclic diterpenoid (C$_{21}$H$_{22}$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 6-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 (15.7%), alkanes (8.3%), esters (3.2%), and terpenes (1.2%). Several compounds reported from *T. cordifolia* were identified by using lipid hydroperoxide (LOOH), possibly due to its high reactivity towards DPPH, superoxide radicals and hydroxyl radicals [140]. Our study showed that the alkaloid fraction (mainly palmitine, jatrorrhizine and magnoflorine) had better antioxidant activity in the DPPH inhibition assay than cumarin, in a dose dependent manner [141].

**Hypoglycemic activity:** In the Ayurvedic Pharmacopeia of India, *T. cordifolia* is categorized as an antidiabetic herbal drug due to its potential [145]. An isolated α-glucosidase inhibitor, apigenin-6-C-β-D-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 diterpenoid, C$_{20}$H$_{20}$O$_{6}$, C$_{20}$H$_{22}$O$_{6}$ and C$_{20}$H$_{24}$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, palmitine, tetrahydropalmatine and magnoflorine), (-)-epicatechin, and an aromatic glycoside, secosolaricoserin [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 palmitine, jatrorrhizine and magnoflorine) had better antioxidant activity in the DPPH inhibition assay than cumin, in a dose dependent manner [141].

**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 (magnoflorone, N-formylnammon, 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, 84]. Magnoflorine, cordioside, cordifolioside 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 B16F10 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.

<p>| Table 1: Pharmacological activities of T. cordifolia extract |
|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Extracts/Plant part</th>
<th>Pharmacological activity</th>
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<tr>
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<td>MeOH, aq. &amp; DCM (s)</td>
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</tr>
<tr>
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<td>[12]</td>
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<td>[57]</td>
</tr>
<tr>
<td>MeOH (s)</td>
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<td>Hexane, Chloroform, MeOH, alcohol &amp; water (l)</td>
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<td>Light petroleum, EtOH &amp; aqueous (s)</td>
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<td>[59]</td>
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<tr>
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<td>Tissue protective activity (P&lt;0.05) in irradiated Swiss albino mice</td>
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<tr>
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<td>Alpha-glucosidase inhibitor</td>
<td>[65]</td>
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<tr>
<td>Alcohol (wp)</td>
<td>Anti-inflammatory activity on carrageenan - induced hind paw edema and cotton pellet granuloma models in male Wistar rats</td>
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<tr>
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<tr>
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<tr>
<td>Alcohol (wp)</td>
<td>Proliferation and myeloid differentiation of bone marrow hematopoietic precursor cells in mice</td>
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<tr>
<td>80% Hydroalcoholic (r)</td>
<td>Inhibited lipid peroxidation, induces enzymes of carcinogen metabolism and antioxidant system in mice</td>
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<td>Antihyperglycemic effect in diabetic mice and rats</td>
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<tr>
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<td>Antistress activity in albinus rats of the Charles-Foster strain</td>
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<td>Aqueous (r)</td>
<td>Antidiabetic activity in albinus rats of Wistar strain</td>
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<tr>
<td>Aqueous (wp)</td>
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<td>Aqueous (s)</td>
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<tr>
<td>MeOH (s)</td>
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<td>Alcohol (r)</td>
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<td>50% Aqua-alcoholic</td>
<td>Radioprotective activity in albino mice</td>
<td>[85,86]</td>
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<td>Ether, alcohol &amp; aqueous (1, r, s)</td>
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<td>[87]</td>
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<td>MeOH (s)</td>
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<td>50% Aqueous-alcoholic (s)</td>
<td>Antipsychotic activity in amphetamine challenged mice</td>
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<td>Protective effect induced by aflatoxin -B1 in Swiss albino mice</td>
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<td>[18]</td>
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<td>Hypolipidemic action in alloxan diabetic albino rats of Wistar strain</td>
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<td>Antioxidant activity in albino rats of Wistar strain</td>
<td>[100]</td>
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<tr>
<td>Aqueous (s)</td>
<td>Immunotherapeutic modification Excherchia coli peritonitis in mice</td>
<td>[101]</td>
</tr>
</tbody>
</table>

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 dendritic 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 peroxynitrite to kill tumor cells by phagocytosis [158]. Another polyaccharide (1→4)-α-D-glucan (named RR1) isolated from T. cordifolia with a molecular weight >550 KD 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 antiinflammatory 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 alphatic 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, (5R,10R)-4R,8R-dihydroxy-2S,3R:15,16-diepoxycleroda-13(16),17,12S,18,1S dilactone, was found to prevent chemically-induced hepaticellular 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 LD₅₀ 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 anti diabetic, anti-inflamatory, antitumor 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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**References**


Chemistry and pharmacology of *Tinospora cordifolia*  

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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. 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 schizogenous 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, antidepressant, antibiotic 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.

<table>
<thead>
<tr>
<th>Herb</th>
<th>Used part</th>
<th>Chemical composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caraway</td>
<td>Seed used in cakes, cheese, confections, fresh cabbage, meat dishes, rye bread, salads</td>
<td>essential oil, fatty oil, vitamins (A, B6, B12, C, D, E), flavonoids, proteins [18, 19]</td>
</tr>
<tr>
<td>Seed essential oil</td>
<td>is used to flavor chewing gum, candy, liqueurs.</td>
<td></td>
</tr>
<tr>
<td>Dill</td>
<td>Seed used in pickled cucumbers, bread, processed meats, sausages, cheese, condiments</td>
<td>essential oil, fatty oil, vitamin C, carotenoids, flavonoids, coumarins, chlorophylls [6, 20]</td>
</tr>
<tr>
<td>Leaf</td>
<td>Leaf is in pickled, while fresh is used for garnish or to flavor salads, vegetable dishes, sea fish, soups, yogurt, mayonnaise.</td>
<td></td>
</tr>
<tr>
<td>Cumin</td>
<td>Seed used as a flavor component in beverages, confectioneries, baked goods, meat and meat products, condiments and relishes, gravies, snack foods, gelatines and puddings.</td>
<td>essential oil, fatty oil, flavonoids, coumarins [21-23]</td>
</tr>
<tr>
<td>Aniseed</td>
<td>Seed is used in beverages, baked goods, condiments, relishes, oils and fats, frozen dairy, gravies, meat products, soft candy</td>
<td>essential oil, fatty oil, flavonoids, coumarins [6, 24]</td>
</tr>
<tr>
<td>Fennel</td>
<td>Seed is in meat dishes, in curries, space blends, soups, vegetables, breads, lush seeds, stews and curries.</td>
<td>essential oil, fatty oil, vitamin C, flavonoids, coumarins and minerals (K, Na, Ca, Mg), nitrates [6, 25-27]</td>
</tr>
<tr>
<td>Leaf</td>
<td>Leaf fresh and chopped can be used as garnish for fish dishes, sauces, salads, stewed, stews and curries.</td>
<td></td>
</tr>
<tr>
<td>Lovage</td>
<td>Seed used in pickling vegetables, salad dressings, breads, biscuits, soups, celery salt, bouquet garni.</td>
<td></td>
</tr>
<tr>
<td>Celery</td>
<td>Root, leaf and leaf stalk are used as a vegetable, fresh for salads, for the preparation of juices, in meat dishes, snacks, gravies, sauces</td>
<td>essential oil, fatty oil, minerals (Na, P, K, Ca, Mg, Fe, Cu, Mn, Zn) [6, 29, 30]</td>
</tr>
<tr>
<td>Lovage</td>
<td>Seed is used in pickling vegetables, salad dressings, breads, biscuits, soups, celery salt, bouquet garni.</td>
<td></td>
</tr>
<tr>
<td>Coriander</td>
<td>Leaf is used to make chutneys and sauces, green salads, dips, snacks, soups</td>
<td>essential oil, fatty oil, vitamins (A and C), flavonoids, coumarins [6, 28]</td>
</tr>
<tr>
<td>Seed used in couscous, stews and salads</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Seed essential oil</td>
<td>is used in beverages, baked goods, condiments, relishes, meat products</td>
<td></td>
</tr>
<tr>
<td>Angelica</td>
<td>Root is used in herbal liqueurs and bitter spirits, in flavoring meat and canned vegetables.</td>
<td>essential oil, coumarins, phenolic compounds [33]</td>
</tr>
<tr>
<td>Herb</td>
<td>Herb is used for decorating cakes and pastry, and to flavor jams and jellies, confectionaries and liqueurs.</td>
<td></td>
</tr>
<tr>
<td>Leaf</td>
<td>Leaf is chopped to add fruit salads, fish dishes, and cottage cheese.</td>
<td></td>
</tr>
<tr>
<td>Parsley</td>
<td>Seed is used in alcoholic distillates.</td>
<td>essential oil, vitamin C, tocopherol, carotenoids, flavonoids, coumarins, sterols, triterpenes [6, 34, 35]</td>
</tr>
<tr>
<td>Seed used in as a garnish (for salads, soups, boiled potatoes and egg dishes), blended in dips, cooked sauces and stews</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Root</td>
<td>Root is used as a vegetable to enhance soups flavor, stews and condiments</td>
<td>onium, phenolic acids, naproxen, alkaloids [31, 32]</td>
</tr>
<tr>
<td>Essential oil</td>
<td>Essential oil is used to flavor meat sauces, pickles, spice blends, baked goods, oils and fats, processed vegetables, soups, gelatines and puddings.</td>
<td></td>
</tr>
<tr>
<td>Carrot</td>
<td>Root, raw, is used for juices, salads, cakes, for pickling, while cooked it is used in casseroles, soups and stews</td>
<td>essential oil, carotenoids (β-carotene, α-carotene, y-carotene), tocopherol, cryptoxanthin, lutein, violaxanthin) [36-38]</td>
</tr>
<tr>
<td>Seed essential oil</td>
<td>is used in beverages, baked goods, condiments, relishes, meat products</td>
<td></td>
</tr>
</tbody>
</table>

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 anti-septic supplement. Further research conducted in vivo with carbon tetrachloride (CCl₄) 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 H₂O₂ [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, antiseptic, 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 CCl₄ induced hepatotoxicity in rats was found as it decreased the serum aspartate transaminase (AST) and alanine transaminase (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 CCl₄. Additionally, dill seed oil treatment significantly reversed CCl₄ 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 CCl₄ 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 CCl₄ 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 prophenofos 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 CCl₄ 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 CCl₄ 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 CCl₄ was confirmed with n-hexane aniseed extract both in vitro and in vivo as revealed by reduction in cell death, serum transaminase levels, LDL 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 CCl₄ 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 CCl₄ group, although these were not as low as those in the silybin control group [29].

**Fennel (Foeniculum vulgare Mill.):** Fennel seed (Foeniculi fructus) and essential oil (Foeniculi aetheroleum), 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-inflammatory 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 CCl₄ 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 CCl₄ 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 CCl₄ 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
paracetamol. The results were confirmed by histopathological studies and then administrated with silymarin, which showed a significant hepatoprotective activity against worms, rheumatism, and pain in the joints, showing a potent antioxidant effect [89]. Coriander seed oil extract showed antibacterial and antifungal properties, a cardioprotective 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 antihapatotoxic 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 (GDH) 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 doxorubicin 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 activity, 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 olsustrum 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 isofuranodienone, which possesses cytotoxic activity against the human colon cancer cell line and has hepatoprotective activity [114, 115]. It was proven that isofuranodienone protected the liver against D-galactosamine/lipopolysaccharide (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].

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 analool [85], while coriander herb oil has a significantly different composition with decanal, trans-2-decenal, 2-decen-1-ol, cyclodecane and cis-2,3-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, a cardiovascular protective effect as well as gut modulation activities. Additionally, it lowers blood pressure and has a diuretic effect [90-96].
Lovage (Levisticum officinale Koch.): Lovage root (Levisticum 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 [125]. 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 antiinflammatory, 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 CCl4, 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 CCl4 and parsley when compared with the group treated with CCl4 only. Moreover, parsley reduced fatty degeneration, cytoplasmic vascularization and necrosis of the liver in the CCl4 treated group. Although CCl4 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, antiadipetic, antioxidative and antitumor 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, GPs 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 CCl4 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 CCl4 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 CCl4-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 hepatoprotective 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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