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Volatile constituents of selected Parmeliaceae lichens

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Abstract: The acetone soluble fraction of the methanol extracts of Parmeliaceae lichens: *Hypogymnia physodes*, *Evernia prunastri* and *Parmelia sulcata*, growing on the same host tree (*Prunus domestica*) and at the same locality was analyzed for the first time by GC and GC–MS. The major identified components were olivetol (33.5 % of the *H. physodes* extract), atraric acid (30.1 and 30.3 % of the *E. prunastri* and *P. sulcata* extracts, respectively), orcinol (25.0 % of the *E. prunastri* extract), vitamin E (24.7 % of the *P. sulcata* extract) and olivetonide (15.7 % of the *H. physodes* extract). Even though all the identified compounds are known, a number of them were found for the first time in the examined lichens, *i.e*., orcinol monomethyl ether (*H. physodes*), orcinol, atranol, lichesterol, ergosterol (*H. physodes* and *P. sulcata*), methyl haematommate, atraric acid, olivetol, vitamin E (*H. physodes* and *P. sulcata*) and *β*-sitosterol (*P. sulcata*).

Keywords: *Hypogymnia physodes*; *Evernia prunastri*; *Parmelia sulcata*; volatile constituents.

INTRODUCTION

Lichens are probably the earliest colonizers of terrestrial habitats on Earth, with fossil records tracing back to 400–600 million years ago.[1,2] These fossils indicate that fungi developed symbiotic partnerships with photoautotrophs before the evolution of vascular plants. There are about 300 genera and 18000 species of presently recognized lichens. They produce more than 800 unique secondary metabolites as adaptations for growing in harsh living conditions. Most of the secondary metabolites present in lichens are produced through the polyketide pathway and consist mainly of monocyclic phenols, bicyclic phenols joined by an

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ester bond (depsides), both ester and ether bonds (depsidones) or a furan heterocycle (dibenzofurans and usnic acid), anthraquinones, xanthones, chromones and secondary aliphatic acids and esters.[3]

The production of secondary metabolites is costly to the organisms in terms of nutrient and energy; hence, one would expect that the plethora of metabolites produced by lichens would have biological significance to the organisms. Some of the possible biological functions of lichen metabolites, as summarized by Huneck and Yoshimura,[4] are:

– Antibiotic activities – provide protection against microorganisms.

– Photoprotective activities – aromatic substances that absorb UV light to protect photobionts from intensive irradiation.

– Promote symbiotic equilibrium by affecting the cell wall permeability of photobionts.

– Chelating agents – capture and supply of important minerals from the substrate.

– Antifeedant/antiherbivory activities – protect the lichens from insect and animal feedings.

To date, high-pressure liquid chromatography (HPLC) methods have been used most frequently for the analysis of the secondary metabolites in lichens.[5,6] Gupta *et al*.[5] developed an HPLC method with photodiode array detection for the determination of methyl β-orcinolcarboxylate and ethyl haematommate in the thalii of various lichen species.

Identification of atranorin, chloratranorin, atranol, chloratranol and esters of haematommic acid by HPLC–MS using negative ion atmospheric pressure chemical ionisation in oakmoss absolute was reported by Hiserdodt *et al*.[6] HPLC methods, however, need standards for compound identification or isolation of extract constituents and their structure elucidation. The economic factor of very expensive HPLC grade solvents and long analysis time should not be neglected. In addition, the volatile part of the extracts might represent a minor proportion of the constituents and consequently identification by HPLC could be difficult or even impossible. On the other hand, good software (such as Amdis[7]) for searching various MS libraries (Adams[8], Wiley[9], and Massfinder[10]) enables constituent identification by GC–MS for many known compounds without isolation and standards.

Direct GC–MS analysis of a solvent extract is not straightforward because of the non-volatile residue, which remains undetected and stuck in the chromatographic system. However, GC–MS gave good results with mono-aromatic compounds in an oak moss absolute fraction,[11] identification and quantification of atraric acid,[12] and the fatty acids of the genus *Collema* lichens.[13]

Only HPLC fingerprints of various lichen species[5] have been exploited to date, whereas, to the best of our knowledge, there have been no attempts at

creating a database of GC–MS profiles of lichen volatiles. Herein for the first time, the composition of the volatiles from the acetone soluble fractions of the methanol extracts of the lichen species: *Hypogymnia physodes*, *Evernia prunastri* and *Parmelia sulcata*, growing on the same host tree, collected at the same time and locality, are reported. The presented results might help in the enlightenment of relationships between lichens and their host and indicate the possibilities of GC–MS for the analyses of secondary metabolites in lichens.

EXPERIMENTAL

Lichen material

Lichens species: *Hypogymnia physodes* (L.) Nyl., (syn: *Parmelia duplicata* var. *Douglasicola* Gyelnik, *Parmelia physodes* (L.) Ach., *Parmelia oregana* Gyelnik; common names: monk's-hood lichen, hooded tube lichen, puffed lichen), *Evernia prunastri* (L.) Ach. (common name: oakmoss), and *Parmelia sulcata* Taylor (common name: shield lichen) growing on a *Prunus domestica* tree were collected on the locality: Serbia, Niš, Suva Planina (meaning "dry mountain" in Serbian, Bojanine vode, altitude of 860 m above sea level, coordinates 43° 10' N, 22° 10' E in April 2009). The lichen material was air-dried for 10 days and stored at ambient temperature (25±2 °C) without exposure to direct sunlight. Voucher specimens were deposited in the Herbarium collection at the Department of Biology and Ecology, Faculty of Science and Mathematics, University of Niš under the acquisition numbers: 17 (*H. physodes*), 18 (*E. prunastri*), and 20 (*P. sulcata*). Taxonomical identifications were performed by Dr. Slaviša Stamenković, Department of Biology and Ecology, Faculty of Science and Mathematics, University of Niš.

Extraction

Powdered, air-dried lichen material (10 g) was extracted with methanol (250 mL) in the dark at room temperature for 24 h. The methanol was evaporated under reduced pressure. The extract yields were 14.4 % (*H. physodes*), 11.5 % (*E. prunastri*) and 11.0 % (*P. sulcata*). The dry residues were then extracted with acetone (3 x 20 mL). The acetone was evaporated under reduced pressure. The percentages of the acetone-soluble fractions calculated based on the dry methanol extract were: 53.9 % (*H. physodes*), 57.6 % (*E. prunastri*) and 32.1 % (*P. sulcata*).

GC–MS and GC analyses

The GC–MS analyses were realised using a Hewlett-Packard 6890N gas chromatograph equipped with a fused silica capillary column DB-5MS (5 % phenylmethylsiloxane, 30 m×0.25 mm, film thickness 0.25 μm, Agilent Technologies, USA) and coupled to a 5975B mass selective detector from the same company. The injector and interface were operated at 250 and 380 °C, respectively. The oven temperature was raised from 70–290 °C at a heating rate of 5 °C min^{-1} and then held isothermally for 10 min. Helium at a flow rate of 1.0 mL min^{-1} was used as the carrier gas. The samples, 1 μL of the acetone solutions (1:100), were injected in a pulsed split mode (the flow was 1.5 mL min^{-1} for the first 0.5 min and then set to 1.0 mL min^{-1} throughout the remainder of the analysis; split ratio 40:1). The MS conditions were as follows: ionization voltage of 70 eV, acquisition mass range 35–500 and scan time 0.32 s. The extract constituents were identified based on their linear retention indices (relative to C12––C33 alkanes on the DB-5MS column) and by the application of the AMDIS software (automated Mass Spectral Deconvolution and Identification System, Ver. 2.1, DTRA/NIST, 2002).

The GC (FID) analyses were performed under the same experimental conditions using the same column as described for the GC–MS. The relative proportions of the constituents were percentages from the GC peak areas without any corrections.

RESULTS AND DISCUSSION

Qualitative composition and relative abundances of the volatiles of acetone fractions are presented in Table I. Structures of main aromatic constituents are shown in Table II and in Fig. 1.

TABLE I. Volatiles (%) of the acetone soluble fraction of the methanol extract of the examined Parmeliaceae lichens

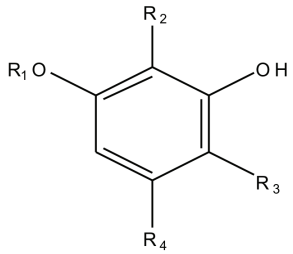
Compound	*RI*[a]	*H. physodes*	*E. prunastri*	*P. sulcata*
3-Methoxy-5-methylphenol (*syn.* orcinol monomethyl ether)	1317	t[b]	5.7	–[b]
5-Methylbenzene-1,3-diol (*syn.* orcinol)	1369	0.6[b]	25.0	t[b]
3-Chloro-2,6-dihydroxy-4-methylbenzaldehyde (*syn.* chloroatranol)	1494	0.5[b]	–	–
2,6-Dihydroxy-4-methylbenzaldehyde (*syn.* atranol)	1546	5.1[b]	2.1	5.2[b]
Methyl 2-hydroxy-4-methoxy-6-methylbenzoate (*syn.* sparassol)	1580	–	1.6	–
Methyl 2,4-dihydroxy-6-methylbenzoate (*syn.* methyl orsellinate)	1661	–	10.2	–
Methyl 3-formyl-2,4-dihydroxy-6-methylbenzoate (*syn.* methyl haematommate)	1669	1.5[b]	3.4	1.9[b]
Methyl 2,4-dihydroxy-3,6-dimethylbenzoate (*syn.* methyl *β*-orcinolcarboxylate; atraric acid)	1703	17.2[b]	30.1	30.3[b]
2,4-Dihydroxy-6-methylbenzoic acid (*syn.* orsellinic acid)	1745	–	1.1	–
5-Pentylbenzene-1,3-diol (*syn.* 5-pentylresorcinol; olivetol)	1755	33.5[b]	–	1.6[b]
Hexadecanoic acid (*syn.* palmitic acid)	1958	0.9	t	2.1
(9*Z*,12*Z*)-9,12-octadecadienoic acid (*syn.* linoleic acid)	2128	0.6	0.9	2.1
(9*Z*,12*Z*,15*Z*)-9,12,15- Octadecatrienoic acid (*syn.* *α*-linolenic acid)	2134	–	–	3.3
(9*Z*)-9-Octadecenoic acid (*syn.* oleic acid)	2141	3.2	t	3.5
Octadecanoic acid (*syn.* stearic acid)	2159	0.9	t	0.7
6,8-Dihydroxy-3-pentylisochromen-1-one (*syn.* olivetonide)	2290	15.7	–	–
2,4-Dihydroxy-6-(2-oxoheptyl)benzoic acid (*syn.* olivetonic acid)	2337	7.7	–	–
2,6-Diacetyl-3,7,9-trihydroxy-8,9*b*-dimethyl-9b*H*-dibenzofuran-1-one (*syn.* usnic acid)	2706	–	11.4	–
Nonacosane	2900	–	–	2.4
α-Tocopherol (*syn.* vitamin E)	3112	0.6[b]	–	24.7[b]
Ergosta-5,8,22-trien-3*β*-ol (*syn.* lichesterol)	3147	1.3[b]	t	1.8[b]

TABLE I. Continued

Compound	*RI*[a]	*H. physodes*	*E. prunastri*	*P. sulcata*
Ergosta-5,7,22-trien-3*β*-ol (*syn.* ergosterol)	3177	2.1[b]	t	4.5[b]
Stigmast-5-en-3*β*-ol (*syn. β*-sitosterol)	3297	–	–	10.6[b]
Total		91.4	91.5	91.4

[a]Experimental retention indices; [b]found for the first time in the lichen taxon, t: trace amount (<0.05 %), –: not detected

TABLE II. Structures of some of the compounds identified in the examined lichen extracts



Compound	R_1	R_2	R_3	R_4
Orcinol monomethyl ether	CH_3	H	H	CH_3
Orcinol	H	H	H	CH_3
Chloroatranol	H	CHO	Cl	CH_3
Atranol	H	CHO	H	CH_3
Sparassol	CH_3	H	$COOCH_3$	CH_3
Methyl orsellinate	H	H	$COOCH_3$	CH_3
Methyl haematommate	H	CHO	$COOCH_3$	CH_3
Atraric acid	H	CH_3	$COOCH_3$	CH_3
Orsellinic acid	H	H	COOH	CH_3
Olivetol	H	H	H	C_5H_{11}

All the obtained chromatograms of the examined extracts showed only a few prominent peaks corresponding to components present in significant amounts (*i.e.*, more than 10 %): *E. prunastri* 4 and *H. physodes* and *P. sulcata* both 3 %.

Orcinol and atraric acid were the major components of the *E. prunastri* extract, detected in approximately equal quantities and comprising together 55.1 %. Orcinol derivatives with the aldehyde group in the vicinity of two phenolic groups, atranol and methyl haematommate, were also present but in smaller quantities, as well as orcinol monomethyl ether. Among coupled phenolic compounds, only the dibenzofuran derivative usnic acid was identified. It seems possible that the volatility of other dibenzofurans, depsides and depsidones, was not sufficient for GC analysis.

Alongside atraric acid, that was present in all the investigated samples in considerable amounts, the *H. physodes* extract was composed of olivetol (33.5 %), olivetonide (15.7 %) and olivetonic acid (7.7 %).

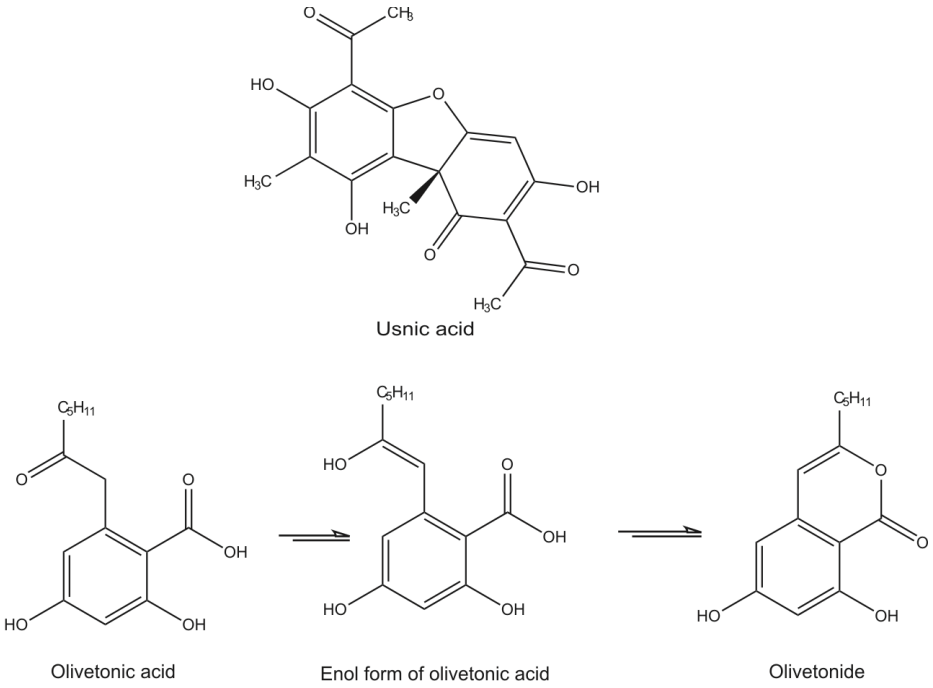


Fig. 1. Structures of usnic acid, olivetonic acid, its enol form and the lactone olivetonide.

The *P. sulcata* extract differed from the others in that it contained a high content of vitamin E (24.7 %) and three sterols comprising together 16.9 %. The methyl ester of *β*-orinolcarboxylic acid (atraric acid) was the most abundant mono-aromatic compound, followed by atranol.

Previously published results showed that *E. prunastri* extracts are characterized by the presence of evernic acid, evernin and usnic acid[14] while physodic, isophysodic, physodalic and 3-hydroxyphysodic acids dominated the *H. physodes* extracts.[15] Atranorin, usnic and salazinic acid[16] were detected in *P. sulcata* extracts.

The observed mono-aryl compounds in the lichen extracts could be both considered as authentic lichen metabolites but also as possible products of hydrolysis, transesterification and decarboxylation of depsides during the extraction and analysis processes.[17]

Hydrolysis of both evernin and atranorin produces methyl *β*-orcinolcarboxylate. Haematommic acid is also a hydrolytic product of atranorin and its decarboxylation yields atranol. Additionally, methyl *β*-orcinolcarboxylate hydrolysis of evernin leads also to 4-*O*-methylorsellinic acid, which upon decarboxylation gives orcinol monomethyl ether as the product that was identified in the *E. prunastri* and *H. physodes* extracts. The product of olivetoric acid hydrolysis is olivetonic acid, enol form of which in turn can be lactonized to olivetonide,

which was previously identified in an extract of treemoss (*Pseudoevernia furfuracea*) growing on cedar trees.[14] Olivetol was also found as one of *P. furfuracea* constituents.[18]

It is believed that the sterols detected in lichen extracts are true lichen metabolites but these could also have migrated from the host tree to the lichen.[14] In the present work, β-sitosterol was not detected in the *E. prunastri* and *H. physodes* extracts, while it was the third most abundant component (10.6 %) in the *P. sulcata* extract. Lichesterol and ergosterol were found in all examined extracts but in different quantities. The obtained results suggested that if sterols do migrate to the lichen from the host tree, the migration is dependent on both the nature of sterols and the lichen species.

The high contents of 5-alkyl resorcinols (orcinol and olivetol) in the examined extracts indicate that the related carboxylic acids (orsellinic and olivetonic acid) readily decarboxylate, contrary to methyl β-orcinolcarboxylate, which is stable under the above-specified experimental condition. Namely, β-orcinol (2,5--dimethylresorcinol) was not detected in any of the samples examined in this study.

CONCLUSIONS

The GC–MS profiles of the acetone-soluble fraction of the methanol extracts of Parmeliaceae lichens: *H. physodes*, *E. prunastri* and *P. sulcata*, growing on the same host tree (*Prunus domestica*) and at the same locality are relatively simple with a few dominant components. Olivetol (33.5 %), atraric acid (17.2 %) and olivetonide (15.7 %), were the major components of the *H. physodes* extract. Atraric acid constituted around one third of the *E. prunastri* and *P. sulcata* extracts. An additional characteristic of the *E. prunastri* extract was the high content of orcinol (25.0 %). The *P. sulcata* extract contained 24.7 % vitamin E. Even though all the identified compounds are known, a number of them were evidenced for the first time in the examined lichens: orcinol monomethyl ether (*H. physodes*), orcinol, atranol, lichesterol, ergosterol (*H. physodes*, *F. caperata* and *P. sulcata*), methyl haematommate, atraric acid, olivetol, vitamin E (*H. physodes* and *P. sulcata*) and β-sitosterol (*F. caperata* and *P. sulcata*).

Bearing in mind that all the investigated lichen species grew under the same conditions (host tree and locality) and were harvested at the same time, it could be concluded that the production of secondary metabolites of the lichens was not affected by the host tree or environmental factors and that observed differences in the extract compositions were genetically predetermined.

The obtained GC–MS profiles of the studied lichen volatiles could be considered as a useful complementary tool to HPLC–MS analysis for the differentiation of lichen specimens due to the availability and straightforwardness of the cheaper GC–MS analyses compared to HPLC–MS.

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ИЗВОД

ИСПАРЉИВИ САСТОЈЦИ ОДАБРАНИХ PARMELIACEAE ЛИШАЈЕВА

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Ацетонска фракција метанолних екстраката Parmeliaceae лишајева: *Hypogymnia physodes*, *Evernia prunastri* и *Parmelia sulcata*, сакупљених са истог супстрата (*Prunus domestica*) и локалитета, анализирана је методама гасне хроматографије и гасне хроматографије са масеним детектором (GC и GC–MS) по први пут. Главне идентификоване компоненте екстраката су: оливетол (33,5 %, *H. physodes*), атрарна киселина (30,1 и 30,3 %, *E. prunastri* и *P. sulcata*, редом), орцинол (25,0 %, *E. prunastri*), витамин E (24,7 % *P. sulcata*) и оливетонид (15,7 % *H. physodes*). Све идентификоване компоненте су познате, али су неке од њих по први пут идентификоване у испитиваним лишајевима: орцинол-монометил-етар (*H. physodes*), орцинол, атранол, личестерол, ергостерол (*H. physodes* и *P. sulcata*), метил-хематомат, атрарна киселина, оливетол, витамин E (*H. physodes* и *P. sulcata*) и *β*-ситостерол (*P. sulcata*).

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