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ISOLATION AND IDENTIFICATION OF SECONDARY METABOLITES OF *UMBILICARIA CRUSTULOSA* (ACH.) FREY†

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Abstract. *Herein, we have studied secondary metabolites of* Umbilicaria crustulosa *(Ach.) Frey. By using preparative HPLC, four compounds were isolated from the* U. crustulosa *methanol extract. The structure of isolated lichen substances was determined on the basis of their* ^{1}H, ^{13}C *and 2D NMR spectra as follows: methyl orsellinate, lecanoric acid, methyl lecanorate and gyrophoric acid. In addition to methanol, the composition of acetone and ethanol extracts were also studied (analytical HPLC). Relative distributions (%) of the detected constituents were as follows (in methanol/acetone/ethanol extracts): methyl orsellinate (5.7/1.5/0.9), lecanoric acid (17.9/5.7/6.7), crustinic acid (8.0/2.8/2.5), methyl lecanorate (4.8/0/0) and gyrophoric acid (59.2/78.0/85.7). A significant difference in the chemical profiles of the studied extracts was in the presence/absence of methyl esters of lichen acids. Nonetheless, the chemical composition of the ethanol extracts (no ethyl esters were detected) and the fact that treatment of acetone and ethanol extracts with methanol does not lead to changes in their composition suggests that methyl esters were not artifacts of the isolation procedure. The lower content of methyl orsellinate and the absence of methyl lecanorate from acetone and ethanol extracts may be the result of different solubilities of these compounds in methanol, ethanol and acetone.*

Key words: Umbilicaria crustulosa *(Ach.) Frey, extracts, HPLC, NMR assignment*

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Dedicated to Professor Radosav Palić on the occasion of his 70th birthday.
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1. Introduction

A lichen is a composite organism that emerges from algae or cyanobacteria (photobionts) living among the filaments (hyphae) of a fungus (mycobiont) in a mutually beneficial (symbiotic) relationship. Lichens produce different types of secondary metabolites (aliphatic, cycloaliphatic, aromatic and terpenic compounds), some of which have important ecological roles (Huneck and Yoshimura, 1996). These substances have shown relevant antimicrobial activity and may be used as a valuable source of antibiotics (Lawrey, 1989). The diverse biological potential (antibiotic, antiviral, antitumor, anti-allergic, antiherbivore) of lichen extracts/metabolites have been scientifically proven (Manojlovic et al., 2012). Secondary metabolites are also important in lichen taxonomy. Due to their comparable polarities, some of lichen substances have similar retention times even with different combination of stationary and mobile phases. For this reason, isolation of pure constituents from the complex matrix of lichen solvent extracts can still be a laborious and challenging task.

Extracellular products of relatively low molecular weight, unique to lichens – these are produced by the fungal partner – are called lichen acids; structures of some of these are given in Fig.1. They are usually crystalline compounds deposited on the surface of hiphes and can be isolated from the lichen matrix using solvent extraction (Buçukoglu et al., 2012). Depsides, tridepsides and tetradepsides, which are the most numerous lichens substances, consist of two, three or four hydroxybenzoic acid residues linked by ester groups. The second most important class of lichens compounds are depsidones which have an additional ether bond between aromatic rings and are reported to have stronger antioxidant activity than depsides (Stojanovic et al., 2011).

Some lichen species, for example, those belonging to genus *Umbilicaria* (they have many common names, e.g., *rock tripe* is a collective name for different *Umbilicaria* species that grow on the rocks), are commonly used by people. Some of them can be eaten if they are properly prepared. In the past, Native Americans used lichens in the ancient medicine and in China people are still using some species of *Umbilicaria* in traditional medicine. Because almost all species of *Umbilicaria* contain a large amount of gyrophoric acid, rock tripes have been favored by North Americans as a source of fermentation dyes. *Umbilicaria* species are characterized by the presence of lecanoric acid, gyrophoric acid, hiascic, ovoic, crustinic, umbilicaric and norstictic acid (Serina and Arroyo, 1996; Fig. 1).

Having in mind all the aforementioned (significance of *Umbilicaria* taxa and potential problems related to their analysis), the aims of this study were to a) screen methanol, acetone and ethanol (solvents regularly used for the extraction of lichen material) extracts of *Umbilicaria crustulosa* (Ach.) Frey for lichen acids; b) compare chemical compositions of these extracts; c) find optimal and reproducible method for isolation of lichen acids using preparative HPLC; and d) determine the structure of the isolated compounds using 1D and 2D NMR.

2. Matherials and Methods

2.1. Lichen material

The lichen species *Umbilicaria crustulosa* (Ach.) Frey was collected from Mt. Stara planina (Babin zub) in Serbia on May 21, 2013 (for preparative HPLC) and on May 30,

2015 for the preparation of extracts with different solvents). A voucher specimen has been deposited in the Herbarium Moesiacum Niš (HMN), Department of Biology and Ecology, Faculty of Sciences and Mathematics, University of Nis, Serbia, under voucher number 9373.

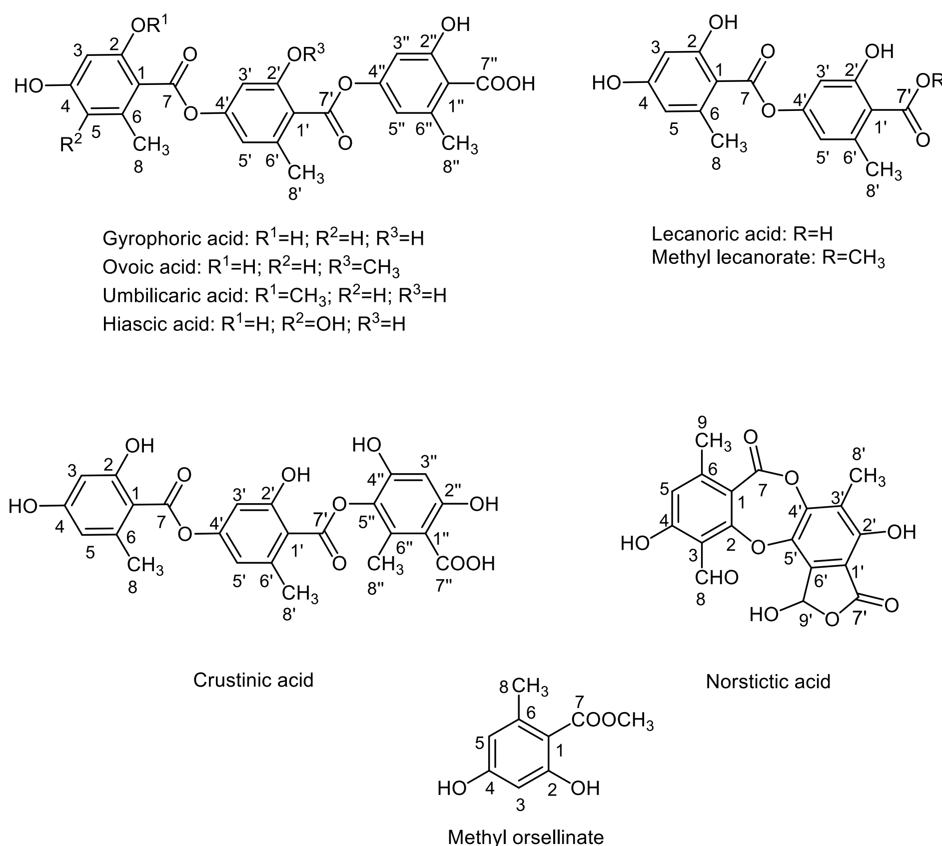

Fig. 1 Structures of some of the *U. crustulosa* constituents

2.2. Extraction

All extracts were prepared in triplicate according to the following procedure: the mixture of powdered air-dried lichen material (10 g) and 100 mL of solvent (methanol, ethanol or acetone) was sonicated for 30 min, and then left at room temperature overnight and filtered. The dry residues of extracts were obtained using a rotary evaporator with the water bath set at 40 °C. The yield of methanol and acetone extracts was 7.4±0.4 and 3.4±0.3% (respectively) in the case of lichen samples collected in 2013. For the lichen samples collected in 2015, the yields of methanol, ethanol and acetone extracts were: 6.6±0.3 %, 4.8±0.2 % and 2.7±0.4 %, respectively. Dry acetone or ethanol extract (10 mg) mixed with 1 ml of methanol were subjected to the same procedure as the lichens during the extraction.

2.3. HPLC analysis

HPLC analysis was performed isocratic (mobile phase – methanol : water : formic acid = 80 : 20 : 0.2 (v:v:v)) by a liquid chromatograph (Agilent 1200 series), equipped with a diode array detector (DAD), Chemstation Software (Agilent Technologies), a quaternary pump, an online vacuum degasser, an auto sampler and a thermostatted column compartment, on an Agilent, Zorbax Eclipse XDB-C18, 5 μm, 4.6×150 mm column, at a flow-rate of 0.5 mL min^{-1}. The column temperature was 25 °C. The injected volume of samples was 2 μL and it was done automatically using an auto sampler. The spectra were acquired in the range 190 – 400 nm and chromatograms plotted at 254. Identification was based on retention times, UV spectra and co-injection of compounds isolated by preparative HPLC.

Preparative HPLC was carried out using the same HPLC system 1200 series with a semi-preparative diode array detector, and a Zorbax Eclipse XDB-C18 Semi-Prep, 5μm, 9.4x250 mm column. The mobile phase, methanol : water : formic acid = 80 : 20 : 0.2 (v:v:v), was pumped at 2.5 $mLmin^{-1}$ flow rate, while the injection volume was 30 μL (100 mg of the dry extract dissolved in 1 mL of methanol), at 25 °C.

2.4. NMR experimental conditions

NMR spectra were recorded at 25 °C in $CDCl_3$ with TMS as an internal standard for methyl orsellinate (**1**) while for lecanoric acid (**2**), methyl lecanorate (**3**), and gyrophoric acid (**4**) (Fig. 1) spectra were recorded in DMSO-d_6. Chemical shifts are reported in ppm (δ) and referenced to TMS (δ_H = 0 ppm) in ^{1}H NMR spectra and/or to $^{13}CDCl_3$ (δ_C = 77.16 ppm) in heteronuclear 2D spectra. Scalar couplings are reported in Hertz. 10 mg of the compound was dissolved in 1 ml of the deuterated solvent, and 0.7 ml of the solution transferred into a 5 mm Wilmad, 528-TR-7 NMR tube.

The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance III 400 MHz NMR spectrometer (^{1}H at 400 MHz, ^{13}C at 101 MHz), equipped with a 5-mm dual ^{13}C/^{1}H probe head. The ^{1}H spectra were recorded with 16 scans, 1 s relaxation delay, 4 s acquisition time, 51 280 FID size, 51 280 FID size, with 6410 Hz spectral width, and an overall data point resolution of 0.0003 ppm. The ^{13}C spectra were recorded with Waltz 16^{1}H broadband decoupling, 12 000 scans, 0.5 s relaxation delay, 1 s acquisition time, 65 536 FID size, 31 850 Hz spectral width, and an overall data point resolution of 0.005 ppm.

Standard pulse sequences were used for 2D spectra. ^{1}H–^{1}H gDQCOSY and NOESY spectra were recorded at spectral widths of 5 kHz in both $F2$ and $F1$ domains; 1 K × 512 data points were acquired with 32 scans per increment and the relaxation delays of 2.0 s. The mixing time in NOESY experiments was 1 s. Data processing was performed on a 1K × 1K data matrix. Inverse-detected 2D heteronuclear correlated spectra were measured over 512 complex points in $F2$ and 256 increments in $F1$, collecting 128 (gHMQC) or 256 (^{1}H–^{13}C gHMBC) scans per increment with a relaxation delay of 1.0 s. The spectral widths were 5 and 27 kHz in $F2$ and $F1$ dimensions, respectively. The gHMQC experiments were optimized for C–H couplings of 165 Hz; the ^{1}H–^{13}C gHMBC experiments were optimized for long-range C–H couplings of 10 Hz. Fourier transforms were performed on a 512 × 512 data matrix. $\pi/2$ Shifted sine-squared window functions were used along $F1$ and $F2$ axes for all 2D spectra.

2.5. Identification of extracts constituents

Methyl orsellinate (**1**), lecanoric acid (**2**), methyl lecanorate (**3**), and gyrophoric acid (**4**) were identified based on the results of NMR analyses (Tables 1 and 2). Crustinic acid (**5**) was identified comparing its UV spectra and retention index (RI) with published data (Serina et al. 1996.) RI was calculated by the formula: RI = (Rt of compound – Rt of acetone)/ (Rt of gyrophoric acid – Rt of acetone)

3. Results and Discussion

Using preparative HPLC, four compounds (methyl orsellinate (**1**), lecanoric acid (**2**), methyl lecanorate (**3**), and gyrophoric acid (**4**); Fig. 1) were isolated from methanol extract of *U. crustulosa*. Their structures were determined on the basis of NMR spectra (Tables 1 and 2). ^{1}H and ^{13}C NMR Chemical shifts for **1-4** (Tables 1 and 2) are in a good agreement with the previously published data (Huneck et al., 1996; Narui et al., 1998).

Table 1 ^{1}H chemical shifts (ppm) of methyl orsellinate (**1**), lecanoric acid (**2**), methyl lecanorate (**3**), and gyrophoric acid (**4**) isolated from methanol extract of *U. crustulosa*

Compound	1	2	3	4
H-3	6.28 (d, J=1.8Hz)	6.22 (bs)	6.22 (bs)	6.22 (bs)
H-5	6.24 (d, J=1.8Hz)	6.21 (bs)	6.21 (bs)	6.20 (bs)
H-3’/H-3’’		6.44 (bs)	6.60 (bs)	6.54 (bs), 6.67 (bs)
H-5’/ H-5’’		6.39 (bs)	6.58 (bs)	6.50 (bs), 6.67 (bs)
H-8	2.49 (s)	2.36 (s)	2.34 (s)	2.35 (s)
H-8’		2.46 (s)	2.23 (s)	2.36 (s)
H-8’’				2.46 (s)
Exchangeable protons	11.72 (s)	10.39 (s), 10.03 (s), 11.62 (s)	10.32 (s)	10.01 (s), 10.31 (s), 10.47 (s)
-COOC$\underline{H_3}$	3.92 (s)		3.81 (s)	

d-doublet, bs-brod signal, s-singlet; polar protons: 2-, 4-, 2’-, 4’- and/or 2’’- O$\underline{H}$ or –COO$\underline{H}$.

Table 2 ^{13}C chemical shifts (ppm) methyl orsellinate (**1**), lecanoric acid (**2**), methyl lecanorate (**3**), and gyrophoric acid (**4**) isolated from methanol extract of *U. crustulosa*

Compound	1	2	3	4
C1	105.76	108.09	109.36	108.41
C2	165.57	160.26	157.80	159.82
C3	101.28	100.39	100.98	100.39
C4	160.16	161.03	160.54	160.95
C5	111.25	109.84	110.75	109.69
C6	144.00	140.29	139.20	140.07
C7	172.00	167.32	167.70	167.02
C8	24.24	21.40	21.8	21.15
-COO$\underline{CH_3}$	51.87			
C1’		116.35	119.22	118.06
C2’		162.95	156.68	156.14
C3’		107.20	107.55	107.08*
C4’		151.58	152.14	151.95
C5’		113.25	114.58	114.11**
C6’		140.92	138.54	137.82
C7’		171.13	169.73	165.62
C8’		22.08	19.91	19.21
-COO$\underline{CH_3}$			52.44	
C1’’				116.98
C2’’				162.93
C3’’				107.03*
C4’’				151.74
C5’’				113.51**
C6’’				140.25
C7’’				170.65
C8’’				21.47

*; ** – Assignments may be interchanged

In addition to the correspondence of the herein with the previous data on ^{1}H and ^{13}C chemical shifts of compounds **1**-**4** (Huneck et al., 1996; Narui et al., 1998), the identification of methyl orsellinate and methyl lecanorate was further corroborated by the interactions observable from NOESY and HMBC spectra (Fig. 2). nOe Interaction of protons at 3.94 ppm with protons of methyl group (C8-H) at 2.43 ppm indicates that these groups were spatially close to each other. The same was true for C6 methyl group and H5 atom (δ 6.28 ppm). As expected, there was a HMBC interaction between protons at 3.94 ppm with C atom at 172.13 ppm of methyl orsellinate. In the case of methyl lecanorate, the interaction between the protons at 3.81 ppm and C atom at 169.73 ppm indicates the ester bonded methyl group in the methyl lecanorate. A difference is noticeable in the shifts of carbonyl C depending on the substituents. Namely, ArCOOH and $ArCOOCH_3$ have higher δ values than ArCOOAr (C7, C7’ and C7’’). The differences in the chemical shifts of the H3 and H5

protons were large enough to appear as separate signals only in methyl orsellinate while in compounds 2, 3 and 4 they appear as broad singlets. Also, H3' and H5' protons in 2, 3 and 4, as well as H3” and H5” in 4, have the form of broad singlets. Consequently, coupling constants could be determined only for H3 and H5 in 1. The values of the constant were 1.8 Hz, which is characteristic for *meta* coupling.

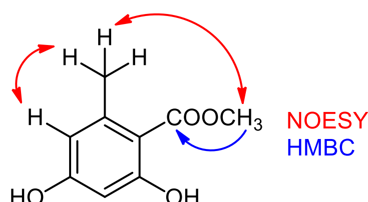

Fig. 2 Selected NOESY and HMBC correlations observable from the corresponding 2D NMR spectra of methyl orsellinate

Serina and Arroyo (1996) studied 56 *U. crustulosa* samples from Spain and reported two chemotypes: the gyrophoric acid type, with gyrophoric acid as the main component (89.8±5.4%) followed by a smaller amount of lecanoric acid (4.5±1.5%) and crustinic acid (4.2±3.3%); the crustinic acid type, with crustinic acid as the main component (78.6±6.9%) followed by lecanoric acid (7.0±2.2%) and gyrophoric acid (13.9±5.8%). Crustinic acid was the major secondary metabolite in 46% of the *U.crustulosa* analyzed specimens. The percentage composition of the herein studied methanol extract was as follows: methyl orsellinate 17.5 %, gyrophoric acid 31.2 %, lecanoric acid 18.6 %, methyl lecanorate 2.5 % and crustinic acid 2.1 % (Table 3). Methyl esters were not detected in acetone extracts studied by Serina and Arroyo (1996). To test if the differences in the herein and previously applied experimental procedures (i.e. type of solvent used for the extraction of the lichen material) was responsible for the observed differences in the chemical composition (presence/absence of methyl esters of lichen acids) of the different *U. crustulosa* population, we decided to extract additional 10 g of *U. crustulosa* using acetone as a solvent. The following compounds were detected in the resulted extract (HPLC analysis): methyl orsellinate 1.0 %, gyrophoric acid 88.4 %, lecanoric acid 3.3 % and crustinic acid 3.8 % (Table 3).

Table 3 Retention times and the relative amounts (% of the peak area in the HPLC chromatogram) of the compounds detected in the *U. crustulosa* solvent extracts

Compound	Rt (min)	A_0	B_0	A	B	C
Methyl orsellinate (1)	4.12	17.5	1.0	5.7	1.5	0.9
Lecanoric acid (2)	4.89	18.6	3.3	17.9	5.7	6.7
Crustinic acid* (5)	5.17	2.1	3.8	8.0	2.8	2.5
Methyl lecanorate (3)	6.74	8.2	/	4.8	/	/
Gyrophoric acid (4)	7.89	31.2	88.4	59.2	78.0	85.7

*experimental RI 0.43, reference RI 0.44 (Serina et al. 1996); Rt (Retention time); A_0 (methanol extract, lichen material collected in 2013); B_0 (acetone, lichen material collected in 2013); A (methanol extract, material collected in 2015); B (acetone extract, material collected in 2015); C (ethanol extract, material collected in 2015)

Interestingly, methyl lecanorate was not detected in the *U. crustulosa* acetone extract, and the amount of methyl orsenillate was lower compared to the same in the methanol extract. In order to probe if methyl esters detected in the methanol extract were in fact the artifacts of the isolation procedure (formed from the corresponding acids), we decided to repeat the extraction experiment using ethanol as a solvent. For that purpose, we collected a new batch of lichens (May 2015). The extraction was performed in parallel with acetone, methanol and ethanol. The yields of methanol and acetone extracts were slightly lower than those observed for the lichen material harvested in 2013 (7.4±0.4 and 3.4±0.3%, respectively, for the 2013 samples; 6.6±0.3 % and 2.7±0.4, respectively, for the 2015 samples). Nonetheless, the chemical compositions of the both studied methanol extracts (2013 and 2015 samples) were comparable with respect to the content of lecanoric acid, while the content of other compounds was (slightly) different: the 2013 sample contained more methyl orsellinate and methyl lecanorate and less crustinic and gyrophoric acid than the 2015 sample. Differences in the composition of the 2013 and 2015 acetone extracts were not prominent to the same extent as in the methanol extracts. The compositions of the ethanol and acetone extract of the 2015 samples were similar to each other and different from the composition of the methanol extract of the 2015 sample. No ethyl esters of lichen acids were detected in the ethanol extract. When acetone and ethanol extracts were subjected to the same procedures as lichen material for methanol extraction, no changes in their composition were noticed. The above data show that methyl esters were not produced during extraction of the lichen material with methanol, i.e. that they are not artifacts.

4. Conclusion

Herein analyzed samples of *U. crustulosa* can be categorized as a gyrophoric chemotype. The yields and the composition of the resulting extracts are susceptible to the type of solvent used for the extraction. Herein presented results suggest that methyl esters of lecanoric and orsellinic acid were not formed from gyrophoric/ lecanoric/orsellinic acid during extraction with methanol. The used preparative HPLC conditions have enabled a satisfactory separation of the constituents of the methanol extract.

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IZOLOVANJE I IDENTIFIKACIJA SEKUNDARNIH METABOLITA VRSTE *UMBILICARIA CRUSTULOSA* (ACH.) FREY

U ovom radu, ispitivani su sekundarni metabolite vrste lišaja Umbilicaria crustulosa (*Ach.) Frey. Primenom preparativne HPLC metode izolovana su 4 jedinjenja iz metanolnog ekstrakta* U. crustulosa. *Na osnovu rezultata ^{1}H, ^{13}C i 2D-NMR eksperimenata, utvrđeno je das u ova jedinjenja: metil-orselinat, lekanorna kiselina, metil-lekanorat i giroforna kiselina. Pored metanolnog, analiziran je i hemijski sastav acetonskog i etanolnog ekstrakta (analitička HPLC analiza). Procentna zastupljenost (%) detektovanih sastojaka bila je sledeća (u metanolnom/acetonskom/etanolnom ekstraktu): metil-orselinat* 5.7/1.5/0.9)*, lekanorna kiselina* (17.9/5.7/6.7)*, krustinska kiselina* (8.0/2.8/2.5), *metil-lekanorat* (4.8/0/0) *i giroforna kiselina* (59.2/78.0/85.7). *Značajnu razliku u hemijskom profilu proučavanih ekstrakata činilo je pisustvo/otsustvo estara lišajevskih kiselina. Ipak, na osnovu hemijskog sastava etanolnog ekstrakta (nisu detektovani odgovarajući etil-estri) i činjence da tretiranje acetonskog i etanolnog ekstrakta metanolom ne dovodi do promena u njihovom sastavu, izgleda da detektovani metil-estri nisu artefakti nastali u toku ekstrakcije. Manji sadržaj metil-orsenilata i odsustvo metil-lekanorata u acetonskom i etanolnom ekstraktu u odnosu na metanolni je verovatno posledica različite rastvorljivosti ovih jedinjenja u metanolu, etanolu i acetonu.*

Ključne reči: Umbilicaria crustulosa *(Ach.) Frey, ekstrakti, preparativna HPLC, NMR*