HPLC/UV profile and determination of total phenolic and flavonoid contents of lichen *Umbilicaria crustulosa* growing in Serbia

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Abstract: The lichens synthesize a large number of secondary metabolites and most of these compounds are unique to lichen. The present study provides data concerning the chemical characterization and determination of total phenolic and flavonoid contents of lichen extracts of *Umbilicaria crustulosa*. Chemical profiling of the extracts was done by high-performance liquid chromatography coupled with a UV detector (HPLC/UV), while the determination of total phenolic and flavonoid contents was done by the spectrophotometric method. HPLC analysis of the acetone and methanol extracts of *U. crustulosa* lichen revealed the presence of the methyl-orselinate, lecanoric acid, crustinic acid, haematommic acid, gyrophoric acid, methyl lecanorate, physodic acid, atranorin and chloroatranorin as the main compounds. The most abundant compounds of the acetone and methanol extracts were the tri-depside gyrophoric acid (59.27 % and 58.32 %) and didepside lecanoric acid (7.41 % and 11.43 %). The results of the total phenolic content (TPC) and total flavonoid content (TFC) show that the acetone extract had higher values of TPC (205.46 mg GA/g**)** and TFC (290.18 mg RE/g; 160.50 mg QE/g). The investigated extracts of the lichen *U. crustulosa* can be used as a significant source of biologically active compounds.

Keywords: Lichens, Phenols, HPLC, *Umbilicaria*

1. Introduction

Lichens are a group of organisms that are numerous and represented, but very little known. The lichens synthesize a large number of secondary metabolites and most of these metabolites are unique to lichen [1]. These are mainly monoaromatics, depsides, depsidones, depsones, pulvinates, dibenzofurans, anthraquinones, and xanthones. These compounds are generally insoluble in water and can be extracted with organic solvents. [2]. The genus *Umbilicaria* includes foliose lichens that are used in folk medicine as purgatives (laxatives). Due to its specific chemical composition, the genus *Umbilicharia*

has shown significant biological activity [3]. For this reason and due to the lack of data on the *Umbilicaria crustulosa* species, we performed a chemical analysis and examination of the total polyphenols and flavonoid contents of the methanolic and acetone extracts.

2. Materials and Methods

Lichen was collected at the site of the mountain Stara Planina. Specimen of the lichen *U. crustulosa* was determined using the relevant key and monographs. The dried lichen material is crushed to a fine powder using a mill. Thereafter, a separate extraction was performed with the acetone and methanol using maceration.

HPLC analyses were performed on the Agilent 1200 Series (Agilent Technologies, Santa Clara, CA, USA) using the C18 column (ZORBAX Eclipse XDB-C18; 25cm×4.6mm; 5 μm). Separate dot detection was performed using a Diode Array Detector (DAD). Identification of individual constituents of the extracts was made by comparing the retention times (t_R) and UV spectra of the compounds with standards (λ =200-400 nm).

The total content of polyphenols in the two extracts was determined with the Folin-Ciocalte reagent, by the spectrophotometric method [4]. The content of the total polyphenols was calculated using an equation obtained from a standard gallic acid calibration curve, and the results were expressed in mg equivalents of gallic acid per g of dry extract (mg GA/g).

The content of total flavonoids in the extracts was determined according to the method described by Sushant et al. [5]. Rutin and quercetin were used as standards, and the results are expressed in milligrams of quercetin equivalents and milligrams of rutin equivalents per gram of dry extract (mg QE/g dry extract or mg RE/g dry extract).

3. Results and Discussion

The HPLC chromatograms of the acetone and methanol extracts of *U. crustulosa* were recorded at 254 nm (Figure 1). HPLC analysis of the acetone and methanol extracts of *U. crustulosa* lichen revealed the presence of the monoaromatic compounds methyl-orselinate, lecanoric acid (didepside), crustinic acid (tridepside), haematommic acid (monoaromatic component), gyrophoric acid (tridepside), methyl lecanorate (didepside), physodic acid (depsidone) and β -orcinol depside atranorin and chloroatranorin as the main components. The most abundant compound of the acetone and methanol extract of *U. crustulosa* is the tri-depside gyrophoric acid (59.27 % and 58.32 %). Apart from gyrophoric acid, lecanoric acid was also identified in high abundance in the acetone and methanol extract (7.41 % and 11.43 %, respectively). The combined occurrence of gyrophoric and lecanoric acids could indicate that lecanoric acid may be a hydrolysis product of gyrophoric acid [6,7]. Crustinic acid is a very common component of *Umbilicaria* species [6], but in our sample, it was found only in the acetone extract (3.23 %). The reason for this may be the greater presence of methyl orsellinate in the methanol extract (5.24 %) than in the acetone extract (2.89 %) due to the hydrolysis process. Physodic acids have previously been reported from the lichen *Hypogymnia physodes* and other lichens genus [8], but this is the first time to confirm the presence and isolate of this metabolite in the lichen *U. crustulosa.* The composition of the acetone extract is

different due to the presence of atranorin and chloratranorin, which were not identified in the methanol extract.

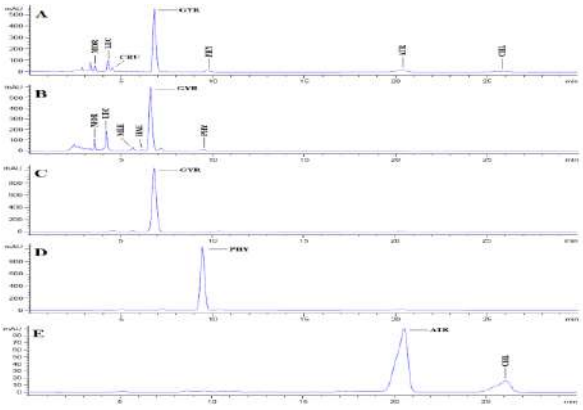

Figure 1. HPLC chromatograms of the acetone and methanol extracts of the lichen *Umbilicaria crustulosa* obtained at 254 nm. (A) the chromatogram of *Umbilicaria crustulosa* acetone extract; (B) the chromatogram of *Umbilicaria crustulosa* methanolic extract; (MOR-methyl orsellinate; LEC-lecanoric acid; CRU-crustinic acid; GYR-gyrophoric acid; PHY-physodic acid; ATR-atranorin; CHL-chloroatranorin; MLE-methyl lecanorate; HAE-haematommic acid.

In *Umbilicaria* species atranorin and chloratranorin could be found in small amounts [7]. Haematommic acid was also found in a very small amount in our methanolic extract (0.49 %). Detailed data on the retention time, absorption maxima, and content of the identified secondary metabolites are presented in Table 1.

Table 1. Retention time, absorbance maxima, and relative abundance of the examined substances.

Compound	Retention time (t_R±SD)*(min)	Absorbance maxima (nm)	Relative abundance %	
			Acetone	Methanol
Methyl orsellinate	3.54±0.02	218, 270,308	2.89	5.24
Lecanoric acid	4.22±0.04	220, 270, 312	7.41	11.43
Crustinic acid	4.51±0.01	220,268, 308	3.23	ND
Methyl lecanorate	5.62±0.01	228, 270, 308	ND	2.63
Haematommic acid	6.13±0.01	202, 236, 258, 280, 344	ND	0.49
Gyrophoric acid	6.70±0.10	214, 270, 304	59.27	58.32
Physodic acid	9.61±0.18	212, 263, 314	1.95	1.70
Atranorin	20.43±0.01	210,252, 321	6.65	ND
Chloroatranorin	25.90±0.01	213, 252, 315, 350	3.34	ND

*Values are the means of three determinations±SD, ND-not detected.

The results of the total phenolic content (TPC) and total flavonoid content (TFC) are presented in Table 2. It can be observed that the acetone extract had higher values of TPC (205.46 mg GA/g**)** and TFC (290.18 mg RE/g; 160.50 mg QE/g;), both when quercetin

was used as the standard and when rutin was used as the standard. The examined extracts showed a higher amount of total phenols and flavonoids than the extracts of *Umbilicaria* species by other researchers [3]. The reason for this may be the influence of the extraction method or environmental factors.

Table 2. The total polyphenols and flavonoids content of the extracts of the lichen *U. crustulosa*.

Lichen extracts	Phenolics content (mg GA/g)	Flavonoids content (mg RE/g)	Flavonoids content (mg QE/g)
Acetone	205.46±0.39	290.18± 0.91	160.50 ± 0,56
Methanol	156.36±0.59	253.52± 0.53	138.09±0.32

*Values are expressed as mean ± SD of triplicate measurements; GA – gallic acid equivalents; RE - rutin equivalents; QE - quercetin equivalents.

3. Conclusions

This is the first report of the chemical analysis of the lichen *U. crustulosa* collected from S tara Planina and the identification of physodic acid from this lichen. Both investigated extracts contain a high amount of phenolic and flavonoid compounds. Future investigations will be focused on isolation compounds and the determination of their biological activities.

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