Lichenochemical analysis and *in vitro* antioxidant activity of extracts and gyrophoric acid from lichen *Umbilicaria grisea*

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Abstract: This research investigates the chemical composition, total phenolic and flavonoid content, and antioxidant activity of acetone, methanol, and ethanol extracts of the lichen *Umbilicaria grisea*, and its major secondary metabolite, gyrophoric acid. The extracts were analyzed using high-performance liquid chromatography (HPLC-UV) and spectrophotometric assays. The results showed significant levels of phenolic compounds and flavonoids, which contribute to the lichen's antioxidant potential. The antioxidant activity was evaluated using *in vitro* assays such as DPPH and ABTS radical scavenging activity. The extracts demonstrated potent antioxidant activity, suggesting their potential as natural antioxidants. The identification of bioactive compounds, high phenolic and flavonoid content, and significant antioxidant activity support the potential use of *U. grisea* as a natural source of therapeutic agents. Further studies are needed to elucidate the underlying mechanisms responsible for these biological activities and evaluate the efficacy and safety of these extracts for potential pharmaceutical applications.
Keywords: *Umbilicaria grisea*, gyrophoric acid, lichen bioactive compounds, antioxidant activity, natural product discovery.

1. Introduction

Lichens, symbiotic organisms with fungus and photosynthetic partners like algae or cyanobacteria, are gaining attention for their chemical diversity, biological activities, and potential therapeutic applications. They are rich sources of secondary metabolites with diverse structures and properties [1]. Due to more synonyms and misidentifications, the number of species from the lichen genus *Umbilicaria* varies between authors, who suggest about 70-80 species [2]. *Umbilicaria grisea* Hoffm. is a temperately distributed species found on siliceous, granite, and steep rock surfaces. The thallus is foliose, with a diameter of 1-5 cm and is attached to the substrate via the umbilicus. Gyrophoric acid is a tridepside, while lecanoric acid, a biosynthetic-related

depside, occurs in a lower concentration (90:1) [3]. Umbilicaric, hiascic, crustinic and ovoic acids are also present *Umbilicaria* tridepsides as well as depsidone norstictic acid [4]. *U. grisea* is understudied in its chemical composition and potential bioactivities. Understanding its antioxidant activity can provide valuable insights into its potential applications in pharmaceutical and biomedical fields.

The aim of this research was to identify the main bioactive secondary metabolites of the acetonic, methanolic and ethanolic extracts of the lichen *Umbilicaria grisea* Hoffm. by the HPLC–UV analysis and to determine total phenolic and flavonoid content, and antioxidant (DPPH and ABTS assays) activity of these three extracts.

2. Materials and methods

U. grisea lichen is collected from Mt. Kopaonik in Serbia. The lichen was identified using standard methods. The lichen material was ground in a laboratory mill and extracted using acetone, methanol, and ethanol as solvents. The extracts were filtered and evaporated to dryness in a vacuum evaporator. The dry extracts were poured into opaque glass bottles and stored in a refrigerator for future analyses.

Gyrophoric acid, obtained from the acetone extract of *U.grisea*, was isolated through a series of steps which included recrystallization, extraction with benzene and centrifugation. Identification was performed by comparing chromatographic and spectroscopic data with the standard.

The extracts were analyzed using an Agilent 1200 Series Gradient HPLC System coupled with a photodiode array detector. The mobile phase consisted of methanol, water, and phosphoric acid (80:20:0,9 %). The analysis time was 30 minutes, with a 1.0 ml/min flow, and 10 μl injected sample amount [5]. Standard compounds were isolated from various sources, and their structures were confirmed through mass spectrometry, ^{1}H, and ^{13}C-NMR data. The *in-vitro* antioxidant activity of the lichen extracts was determined spectrophotometrically using DPPH and ABTS methods [6, 7]. The antioxidant capacity of the tested extracts was expressed as an inhibitory concentration (IC50). The total phenolic content was determined using the Folin-Ciocalteu method, with gallic acid (GA) as a standard and total flavonoid content was determined using a colorimetric method based on the reaction of flavonoids with $AlCl_3$, and rutin and quercetin were used as standards [5].

3. Results and discussion

The extraction yield and occurrence of individual compounds are shown in Table 1, and the total phenolic content and the total flavonoid content in each extract are presented in Table 2. The results indicate that different solvents used for lichen extraction had varying effects on the flavonoid content. Acetone extraction yielded the highest flavonoid content. Additionally, the results highlight the importance of solvent selection in the lichen extraction protocols to maximize the recovery of specific compounds of interest. The presence of secondary metabolites in the acetone, methanol and ethanol extracts was analyzed using the HPLC-UV method. Lecanoric acid, umbilicaric acid and gyrophoric acid were identified in all three extracts along with

depsidone norstinic acid and the depside atranorin which were reported for the first time in this species. The retention time of the examined lichen substances and their absorbance maxima are presented in Table 3.

Table 1. Yield of lichen extracts and presence of secondary metabolites in the *U. grisea* extracts.

Extracts	Used Eluent	Yields (%)	NOR	LEC	UMB	GYR	ATR
UGA	acetone	12,37 ± 1.33	+	+	+	+	+
UGM	methanol	14,2 ± 2.74	+	+	+	+	-
UGE	ethanol	8,75 ± 1.26	-	+	+	+	-

UGA- *U. grisea* acetonic extract; UGM- *U. grisea* methanolic extract; UGE- *U. grisea* ethanolic extract

Table 2. Total phenolic and flavonoid contents of *U. grisea* extracts (mean ± SD, n=3).

Sample	Total Phenolic Content (mg GAE/g Dry Extract)	Total Flavonoid Content (mg QE/g Dry Extract)	Total Flavonoid Content (mg RU/g Dry Extract)
UGA	56.615 ± 0.385	62.657 ± 0.160	116.303 ± 0.262
UGM	67.128 ± 0.222	54.324 ± 0.893	102.667 ± 1.461
UGE	44.821 ± 0.801	28.213 ± 0.424	59.939 ± 0.694

GAE- Gallic acid equivalent; QE- quercetin equivalent; RU- rutin equivalent.

Table 3. Retention time of the examined lichen substances and their absorbance maxima (nm)

Secondary metabolite	Substance class	Retention time (t_R ± SD)* (min)	UV spectrum Absorbance maxima (nm)
Norstictic acid (NOR)	depsidone	3.21± 0.10	212, 239, 320
Lecanoric acid (LEC)	depside	4.06±0.20	212, 270, 304
Umbilicaric acid (UMB)	tridepside	6.29± 0.20	210, 254, 292
Gyrophoric acid (GYR)	tridepside	6.34± 0.10	212, 270, 304
Atranorin (ATR)	depside	18.97± 0.10	212, 278, 312^m

* Values are the means of three determinations ± SD, m – minor absorbance maximum.

These compounds were identified based on the retention times and UV spectra compared with standard substances previously isolated from lichens and identified by spectroscopic methods in our laboratory. Depside lecanoric acid, tridepsides umbilicaric acid and gyrophoric acid have been previously detected in *U.grisea*, while depsidone norstictic acid and depside atranorin were found for the first time in this species [6].

The chromatographic analysis showed that atranorin was not found in the methanol and the ethanol extracts, while norstictic acid was not identified in the ethanol extract. In addition, gyrophoric acid was isolated from the acetone extract of the lichen *U.grysea*. The gyrophoric acid purity was confirmed by HPLC (98.77 %). The different chemical compositions of these extracts and isolated gyrophoric acid made it possible to further use them in the investigation of the antioxidant activity (presented in Table 4). The acetone extract showed slightly better antioxidant activity compared to the other two extracts in all tests used. This may be related to the higher content of the flavonoid compounds in this extract. Also, gyrophoric acid shows significantly better antioxidant activity compared to all extracts, but weaker compared to ascorbic acid and Trolox.

Table 4. Results of *in vitro* antioxidant activity test.

Sample	DPPH (IC50 μg/ml)	ABTS (IC50 μg/ml)
UGA	3261.00 ± 141.38	2441.53 ± 142.71
UGM	3298.00 ± 50.24	2103.58 ± 156.69
UGE	3419.33 ± 108.58	2689.24 ± 197.78
GYR	354.17 ± 12.15	246.99 ± 11.88
AA	10.53 ± 1.57	8.28 ± 0.24
Trolox	15.59 ± 3.31	12.40 ± 0.40

3. Conclusions

The lichen *U. grisea* species studied in this research can be attractive as a source of bioactive substances with potential health benefits. This research contributes to general knowledge about this lichen species and the genus *Umbilicaria*. Furthermore, this research can provide valuable insights into the potential use of *U. grisea* extracts in the development of new therapeutic agents for combating oxidative stress-related disorders.

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