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Bioactive Components Analysis and Pharmacological Properties of Extracts and Metabolites of Lichen *Umbilicaria crustulosa*

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Abstract: Lichens, a diverse group of organisms, have a unique structure consisting of fungal filaments and photosynthetic partner cells. This research conducted a comprehensive chemical analysis and evaluation of the anti-inflammatory and antioxidant properties of methanolic and acetone extracts from *Umbilicaria crustulosa* lichen, along with its isolated metabolites. The process involved separating atranorin and chloratranorin fractions, physodic acid, and gyrophoric acid. Secondary metabolites were identified using chromatographic and spectroscopic data. The total polyphenols content was determined spectrophotometrically. This study examined the antioxidant activity of extracts of the lichen *U. crustulosa* and the isolated fractions using three methods: DPPH scavenging activity, ABTS scavenging activity, and reducing power. This study also evaluated the acute oral toxicity and the anti-inflammatory activity of the extracts in Wistar albino rats. A higher content of the total phenolic compounds was found in the acetone extract, but antioxidant and anti-inflammatory activities were more prominent in the methanolic extract. The isolated atranorin and chloratranorin fractions and compound physodic acid showed the highest antioxidant activity. No toxic effects were noted in the acute oral toxicity study. This study highlights the potential of the investigated lichen as a valuable source of novel biological agents.

Keywords: lichen; *Umbilicaria crustulosa*; chemical analysis; antioxidant activity; anti-inflammatory activity

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1. Introduction

Lichens have a unique dual structure consisting of fungal filaments intertwined with the cells of the photosynthetic partner (microalga or cyanobacteria). This structure forms thalli, which can vary in shape, size, and color [1]. Lichens represent a diverse and abundant group of organisms that remain relatively understudied. Within the scientific community, they are recognized as a distinct phylogenetic group within the broader biodiversity of the planet, with their nomenclature and taxonomy governed by the regulations outlined in the International Code of Botanical Nomenclature [2]. The morphological features of lichen thalli exhibit considerable variability, posing challenges for identification. Therefore, studying chemotaxonomy becomes crucial, as it aids in the identification of chemical

constituents and potential pharmaceutical applications. The primary organic compounds extracted from lichens are secondary metabolites, predominantly sourced from the fungal component residing on the hyphae surface rather than within the cell wall [3]. These compounds typically exhibit low solubility in water and are often extracted using organic solvents. Lichens biosynthesize a diverse array of secondary metabolites, many of which are unique to this symbiotic organism. These metabolites encompass monoaromatics, depsides, depsidones, depsones, pulvinates, dibenzofurans, anthraquinones, and xanthones [3–5]. However, their structural resemblance poses challenges in their identification process [5]. Lichens often contain notable levels of secondary metabolites, typically ranging from 0.1 to 10% of their dry weight, though in certain instances, concentrations may extend up to 30% [6].

Lichens exhibit a broad spectrum of biological potential; however, they have historically been overlooked by mycologists and pharmaceutical industries due to their slow growth and challenges associated with artificial cultivation. The primary agents responsible for their biological activity are their unique metabolites [3,7]. Lichens are recognized as a significant source of natural antioxidants, particularly phenols renowned for their potent antioxidative and antiradical properties. As a result, lichens are attributed with various therapeutic effects, including antibacterial, anti-inflammatory, antiallergic, antimutagenic, antiviral, and anticancer activities [3,7,8].

The *Umbilicaria* genus encompasses foliose lichens traditionally employed in folk medicine for their purgative (laxative) properties. The US National Park Service, Anchorage, AK, in 1987, referenced them in the literature as "rock tripe" [9]. The *Umbilicaria* genus is widely distributed across the northern hemisphere, including North America, Europe, and Antarctica. Considering that almost all species of *Umbilicaria* contain gyrophoric acid, in the past the Indians used them as a coloring agent. Some species are used as food or form part of traditional medicine in China [10].

Given its unique chemical composition, the *Umbilicaria* genus has demonstrated significant biological activity in our research and that of others [11,12]. Due to the limited data available on *Umbilicaria crustulosa* species, we conducted a comprehensive chemical analysis and evaluation of the anti-inflammatory and antioxidant properties of the methanolic and the acetone extracts from *U. crustulosa* lichen, along with its isolated metabolites. This research holds particular significance as there is scarce information regarding the relationship between the chemical composition and antioxidant/anti-inflammatory effects of *U. crustulosa* lichen. Additionally, this research identified previously unidentified compounds in the *U. crustulosa* species through isolation and characterization processes. This is the first time that the anti-inflammatory activity of *U. crustulosa* lichen extracts has been investigated.

2. Materials and Methods

2.1. The Process of Collecting and Identifying Lichen Samples

Lichen specimens of the type *U. crustulosa* were collected from the eastern slope of the Stara Planina mountain in the Republic of Serbia. The Department of Biology and Ecology at the Faculty of Natural Sciences and Mathematics, University of Niš, identified the specimens using relevant keys and monographs [13], and archived them in the herbarium (Voucher number: 9373).

2.2. The Process of Preparing the Lichen Extracts

The dried *U. crustulosa* lichen material was ground into a powder (2–6 mm). Then, separate extractions were conducted using acetone and methanol, employing conventional techniques such as maceration. The material was placed in each solvent for 3 days in a dark place at room temperature, with occasional shaking. The extraction process utilized 50 g of the lichen sample with 500 milliliters of the solvents. Evaporation of the solvents was performed under reduced pressure on the rotary vacuum evaporator. Dry extracts (acetone: 1.57 g; methanol: 1.01 g) were obtained and stored in dark glass bottles for further testing.

2.3. Isolation Process of Lichen Metabolites and Fractions

The fraction containing atranorin and chloratranorin was isolated according to the procedure described in the work of Lukač and Onderk [14]. The dry acetone extract was washed three times with toluene, the resulting solution was allowed to stand overnight (5–7 °C), and the crystallized fraction was filtered off and allowed to dry. The obtained fraction was recrystallized from acetone. The crystals were then dissolved in a mixture of chloroform/methanol, and by standing in the cold, white crystals appeared which were separated, determined, and the presence of the atranorin and chloratranorin fraction was determined.

The isolation of physodic acid was performed by dissolving the dry acetone extract of *U. crustulosa* in benzene, the residue that did not dissolve was washed again with benzene and did not dissolve, it separated in the form of a white precipitate. The precipitate was dissolved in the acetone and then it was subjected to centrifugation at 12,000 rpm for 10 min. The supernatant was separated, evaporated, and dissolved in a mixture of methanol/water, left to stand in the cold overnight (5–7 °C) and white crystals appeared in the form of needles, which were separated and determined.

The isolation of gyrophoric acid was performed using the dry acetone of *U. crustulosa* which was extracted with the benzene (3 times). To maximize the yield, the benzene phase was centrifuged at 12,000 rpm for 10 min. The resulting supernatant was then mixed with the remaining precipitate from the benzene extraction. The mixture was then dried at room temperature and dissolved again in fresh acetone, after which it underwent recrystallization.

The identification of secondary metabolites was conducted by comparing chromatographic and spectroscopic data with the standards. The identification of the substance was also performed on the basis of the melting point [15].

2.4. Analysis Using High-Performance Liquid Chromatography (HPLC)

HPLC with UV detection was used to analyze and identify the individual constituents of the extracts. The analysis was performed using the Agilent 1200 Series (Agilent Technologies, Santa Clara, CA, USA) with a C18 column (ZORBAX Eclipse XDB-C18; 25 cm × 4.6 mm; 5 µm; (Agilent Technologies, Santa Clara, CA, USA)). Detection was carried out using a Diode Array Detector (DAD) at 280, 330, and 350 nm, and the absorption spectra of the components were recorded in the range of 200–400 nm. The dissolved samples were filtered through a 0.45 µm pore size filter. Chromatographic separation was conducted using an acetonitrile–water–phosphoric acid solvent system (90:10:0.1, *v*/*v*/*v*). The mobile phase flow rate was 1 mL/min, and 10 µL of the sample was injected. The column was maintained at a temperature of 30 °C. This procedure was previously described and employed [16,17]. Chromatograms and UV spectral data were collected at a wavelength of 254 nm.

The identification of the secondary metabolites of the acetone and methanolic extracts of the lichen *U. crustulosa* was made by comparing the retention times (t_R) and UV spectra of the metabolites with standards (λ = 200–400 nm). The standards used for HPLC identification were obtained from the following sources: gyrophoric acid (t_R = 6.70 min; λ_{max} = 214, 270, 304 nm) from *Acarospora fuscata*, methyl orsellinate (t_R = 3.54 min; λ_{max} = 218, 270, 308 nm) from *Usnea longissima*, lecanoric acid (t_R = 4.22 min; λ_{max} = 220, 270, 312 nm) from *Parmotrema tinctorum*, methyl lecanorate (t_R = 5.62 min; λ_{max} = 228, 270, 308 nm), atranorin (t_R = 20.43 min; λ_{max} = 210, 252, 321 nm) and chloroatranorin (t_R = 25.90 min; λ_{max} = 213, 252, 315, 350 nm) from lichen *Evernia prunastri*, physodic acid (t_R = 9.61 min; λ_{max} = 212, 263, 314 nm) from *Hypogimnia physodes*, crustinic acid (t_R = 4.51 min; λ_{max} = 220, 268, 308 nm) from *Umbilicaria cinereorufescens* and haematommic acid (t_R = 6.13 min; λ_{max} = 202, 236, 258, 280, 344 nm) from *Alectoria sulcata*. The standard compounds were previously isolated in our laboratory and their structures were confirmed by mass spectrometry, ^{1}H, and ^{13}C-NMR.

2.5. Measurement of the Total Phenol Content

The total polyphenol content in the extracts was assessed using the Folin–Ciocalteu reagent and the spectrophotometric method [18,19]. A mixture containing 0.5 mL of the extract (1 mg/mL), 2.5 mL of Folin–Ciocalteu reagent, and 2.5 mL of Na_2CO_3 solution (7.5%) was prepared. The quantitative estimation of polyphenols was conducted by measuring the absorbance at 760 nm. The total polyphenol content was then calculated using an equation derived from a standard gallic acid calibration curve. The parameter was determined from the standard gallic acid curve, as per Equation (1):

$$\text{Absorbance} = 0.0026 \times \text{Total phenol} + 0.0808 \ (R^2 = 0.9978) \quad (1)$$

The results are presented as milligrams of gallic acid equivalents per gram of dry extract (mg GA/g).

2.6. DPPH Method

The ability to scavenge free radicals was assessed using 1,1-diphenyl-2-picryl-hydrazyl (DPPH), following the method outlined by Dorman et al. [20] with some modifications. Methanolic solutions of DPPH radical at concentrations of 40 µg/mL were prepared in a dark room. The sample solutions were mixed with 3 mL of the DPPH radical solution, and the mixture was left for 30 min at room temperature in the dark. After incubation, the absorbance was measured at 517 nm. Ascorbic acid and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) from Acros Organics (Fair Lawn, NJ, USA) were employed as positive controls. The capacity to neutralize free radicals was calculated using Equation (2):

$$\text{Inhibition capacity of the DPPH radical (\%)} = \text{Ac}-\text{As}/\text{Ac} \times 100 \quad (2)$$

Ac represents the absorbance of the control solution (negative control), while As is the absorbance of the sample solution or standard. The IC_{50} value (µg/mL), which is defined as the concentration of extract required to reduce the DPPH radical concentration by 50%, was determined from the linear regression equation.

2.7. ABTS Method

The ability to neutralize free radicals was tested using 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radicals according to the method previously described by Tabassum et al. [21], with modification. During the preparation of the experiment, the mixture of 7 mM ABTS and 2.45 mM potassium persulfate was incubated at room temperature without the presence of light for 24 h. A mixture was prepared by combining 300 µL of extract or standard solution with 600 µL of ABTS solution. The mixture was then incubated at room temperature for 30 min, after which the absorbance was measured at 734 nm. Ascorbic acid and Trolox were employed as positive controls. The concentration of the ABTS radical was calculated using Equation (3):

$$\text{Inhibition capacity of the ABTS radical (\%)} = \text{Ac}-\text{As}/\text{Ac} \times 100 \quad (3)$$

Ac represents the absorbance of the control, which includes all reagents except for the tested extract or standard, while As is the absorbance of the sample. Using these values, a nonlinear calibration curve was constructed to determine the concentration of the tested sample that inhibits 50% of ABTS radicals (IC_{50}).

2.8. Reduction Capacity

The method previously described by Oyazu et al. was used [22]. One milliliter of each sample was mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of $K_3[Fe(CN)_6]$ (1%). Afterward, 2.5 mL of CCl_3COOH was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. A volume of 2.5 mL of the supernatant

was extracted, and then 2.5 mL of distilled water and 0.5 mL of $FeCl_3$ were added. The absorbance of the resulting solution was measured spectrophotometrically at 700 nm. Ascorbic acid and Trolox were utilized as positive controls. The rise in absorbance of the solution demonstrates the increase in reducing power.

2.9. Animal Studies–Acute Oral Toxicity and Anti-Inflammatory Activity

In vivo evaluation of the acute oral toxicity and anti-inflammatory activity of UCA and UCM was conducted at the Faculty of Medical Sciences, University of Kragujevac, Serbia. The study was conducted in accordance with the principles of Good Laboratory Practice, the European Council Directive (86/609/EEC), and the regulations of the Committee for the Welfare of Laboratory Animals of the Faculty of Medical Sciences. The experimental study included male Wistar albino rats procured from the Military Medical Academy in Belgrade, Serbia. The rats were kept in a controlled environment at a temperature of 22 ± 2 °C, with 12 h of consistent lighting every day. They were provided with commercial rat food (20% protein rat food; Veterinary Institute Subotica, Subotica, Serbia) and had unrestricted access to water.

The toxicity class was determined using the Organization for Economic Cooperation and Development (OECD) 423 procedure, which is specifically designed for testing acute oral toxicity [23]. Since there is data on the traditional use of lichen *U. crustulosa* for medicinal purposes, and in order to reduce the number of experimental animals that will be used, it was decided to start with the OECD procedure 423 at a dose of 2000 mg/kg body weight of the experimental animal. The male Wistar albino rats, 10 weeks old at the beginning of the experiment, were used for this part of the investigation.

A total of twelve animals (male Wistar albino rats) were treated with *U. crustulosa* extracts, more precisely six animals with acetone (UCA) and six animals with methanolic extract (UCM), with three animals in each of two separate cycles at a dose of 2000 mg/kg, which is recommended by the OECD 423 procedure [23]. The extracts were given orally as a solution in 1% sodium–carboxymethylcellulose (CMC) using a gavage method. Following the administration of UCA and UCM, the animals were closely monitored for the initial day and subsequently for the following 14 days, as per the protocol. Observations included alterations in the eyes, mucous membranes, skin, and fur. The animals' behavior, breathing heart activity, and any neurological issues were monitored. Furthermore, monitoring for tremors, convulsions, diarrhea, lethargy, increased salivation, prolonged sleep duration, and coma was planned. Following the experiment, the animals were euthanized to conduct a necropsy and pathohistological examination of their internal organs, focusing on the liver, kidneys, heart, brain, and testicles.

The anti-inflammatory activity was assessed using the carrageenan-induced rat paw edema model [24]. Inflammation was induced in all rats by injecting 1 mL of 0.5% carrageenan solution in saline into the left hind paw. A total of eighty male Wistar albino rats weighing between 200 and 260 g and 10 weeks old were categorized into the following groups:

- Ctrl–rats were treated with 1% CMC solution in water, per os, 60 min before inflammation induction.
- 50 UCA–rats were treated with 50 mg/kg of the *U. crustulosa* acetone extract per os dissolved in 1% CMC solution in water, per os, 60 min before inflammation induction.
- 100 UCA–rats were treated with 100 mg/kg of the *U. crustulosa* acetone extract per os dissolved in 1% CMC solution in water, per os, 60 min before inflammation induction.
- 200 UCA–rats were treated with 200 mg/kg of the *U. crustulosa* acetone extract per os dissolved in 1% CMC solution in water, per os, 60 min before inflammation induction.
- 50 UCM–rats were treated with 50 mg/kg of the *U. crustulosa* methanolic extract per os dissolved in 1% CMC solution in water, per os, 60 min before inflammation induction.
- 100 UCM–rats were treated with 100 mg/kg of the *U. crustulosa* methanolic extract per os dissolved in 1% CMC solution in water, per os, 60 min before inflammation induction.

- 200 UCM–rats were treated with 200 mg/kg of the *U. crustulosa* methanolic extract per os dissolved in 1% CMC solution in water, per os, 60 min before inflammation induction.
- Ind–rats were treated with 10 mg/kg indomethacin, per os, 60 min before inflammation induction.

Indomethacin (as positive control) and carrageenan (as inductor of inflammation) were obtained from Sigma Aldrich (St. Louis, MO, USA).

In order to assess the anti-inflammatory impact, the thickness of the left paw tissue in each rat was measured at specific time intervals: prior to inducing inflammation (moment 0) and at 1, 2, 3, and 4 h (moments 1, 2, 3, and 4) following the onset of inflammation. The thickness of the tissue in the center of the rat paw was measured using a digital vernier caliper (Aerospace, Beijing, China). The percentage of decrease in paw swelling was calculated using Equation (4).

$$\% \text{ Inhibition} = 100 \times [1 - (Yt/Yc)] \quad (4)$$

where Yt represents the mean increase in paw thickness in the group of rats that received treatment between two measurements, while Yc represents the mean increase in paw thickness in the group of rats that did not receive treatment between two measurements.

3. Results

3.1. HPLC Analysis

Chromatograms obtained after HPLC analysis of the acetone and methanol extracts of the lichen *U. crustulosa*, as well as the isolated individual fractions, are presented in Figure 1. A total of nine different compounds were identified in both extracts and individual fractions, with gyrophoric acid being the most abundant compound.

Comprehensive data regarding the retention time and absorption maxima of the identified secondary metabolites are provided in Table 1. The content of these metabolites is also included.

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

Compound	Retention Time ($t_R \pm$ SD) * (min)	Absorbance Maxima (nm)	Relative Abundance (%)	
			UCA	UCM
Methyl orsellinate	3.54 ± 0.02	218, 270, 308	2.89 ± 0.01	5.24 ± 0.03
Lecanoric acid	4.22 ± 0.04	220, 270, 312	7.41 ± 0.15	11.43 ± 0.07
Crustinic acid	4.51 ± 0.01	220, 268, 308	3.23 ± 0.27	ND
Methyl lecanorate	5.62 ± 0.01	228, 270, 308	ND	2.63 ± 0.22
Haematommic acid	6.13 ± 0.01	202, 236, 258, 280, 344	ND	0.49 ± 0.01
Gyrophoric acid	6.70 ± 0.10	214, 270, 304	59.27 ± 0.70	58.32 ± 0.45
Physodic acid	9.61 ± 0.18	212, 263, 314	1.95 ± 0.06	1.70 ± 0.01
Atranorin	20.43 ± 0.01	210, 252, 321	6.65 ± 0.40	ND
Chloroatranorin	25.90 ± 0.01	213, 252, 315 m, 350	3.34 ± 0.11	ND

* Values are the means of three determinations ± SD; m—minor absorbance maximum; ND—not detected; UCA—acetone extract of lichen *U. crustulosa*; UCM—methanol extract of lichen *U. crustulosa*.

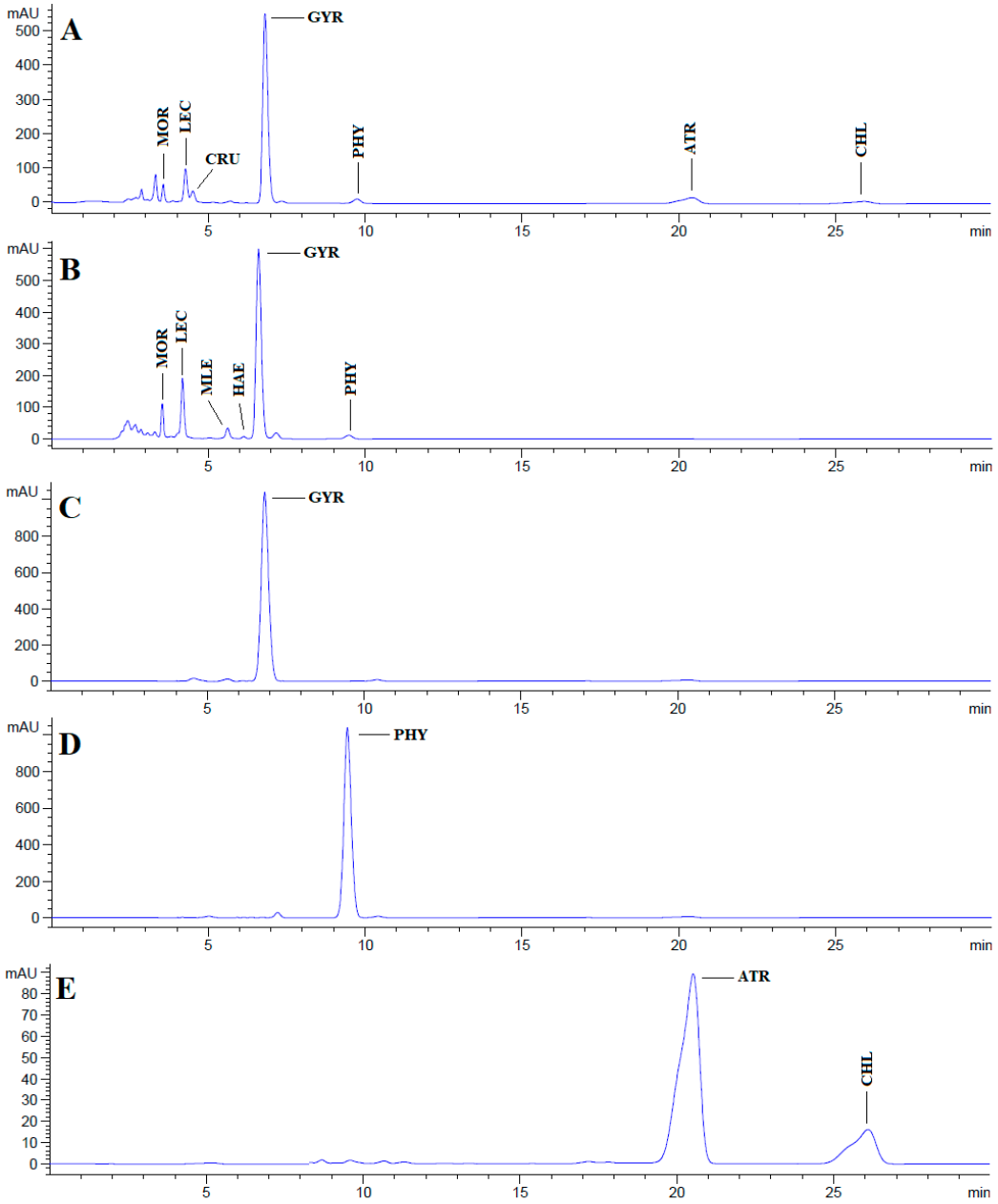
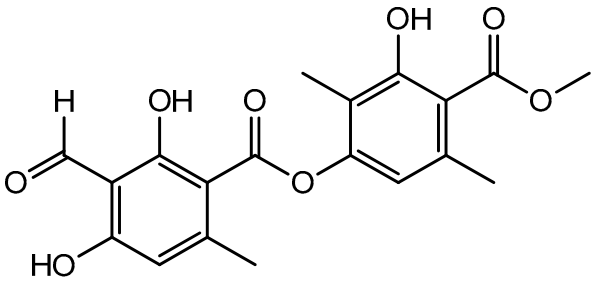
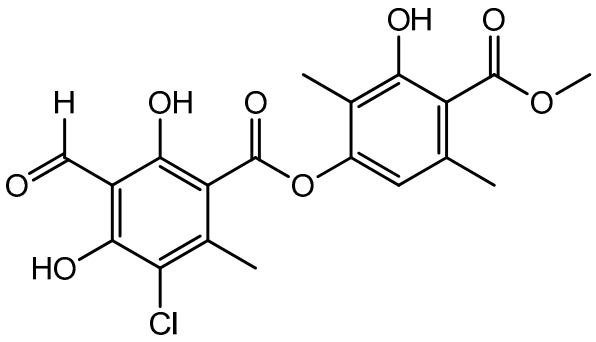


Figure 1. HPLC chromatograms of the acetone and methanol extracts of the lichen *U. crustulosa* and the isolated compounds obtained at 254 nm. (**A**) Chromatogram of *U. crustulosa* acetone extract; (**B**) chromatogram of *U. crustulosa* methanolic extract; (**C**) chromatogram of isolated compound gyrophoric acid; (**D**) chromatogram of isolated physodic acid; (**E**) chromatogram of isolated fraction (compounds atranorin/chloroatranorin); 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.

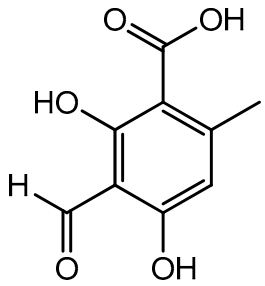
The chemical structures of the compounds identified in extracts and isolated fractions are presented in Figure 2.



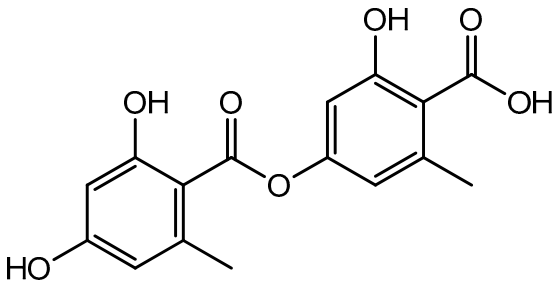
Atranorin



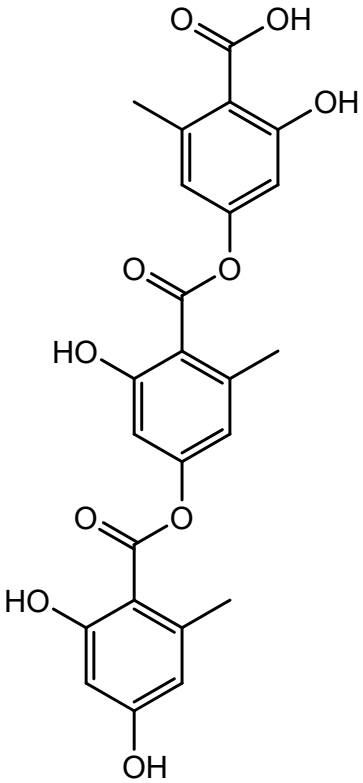
Chloroatranorin



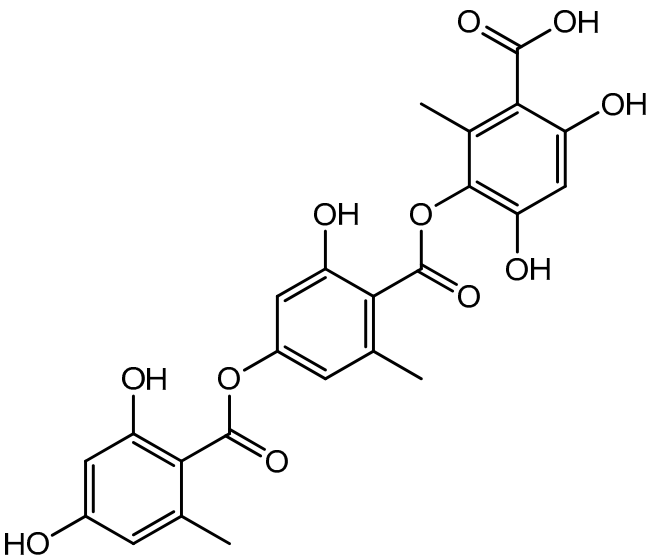
Haematommic acid



Lecanoric acid



Gyrophoric acid



Crustinic acid

Figure 2. *Cont.*

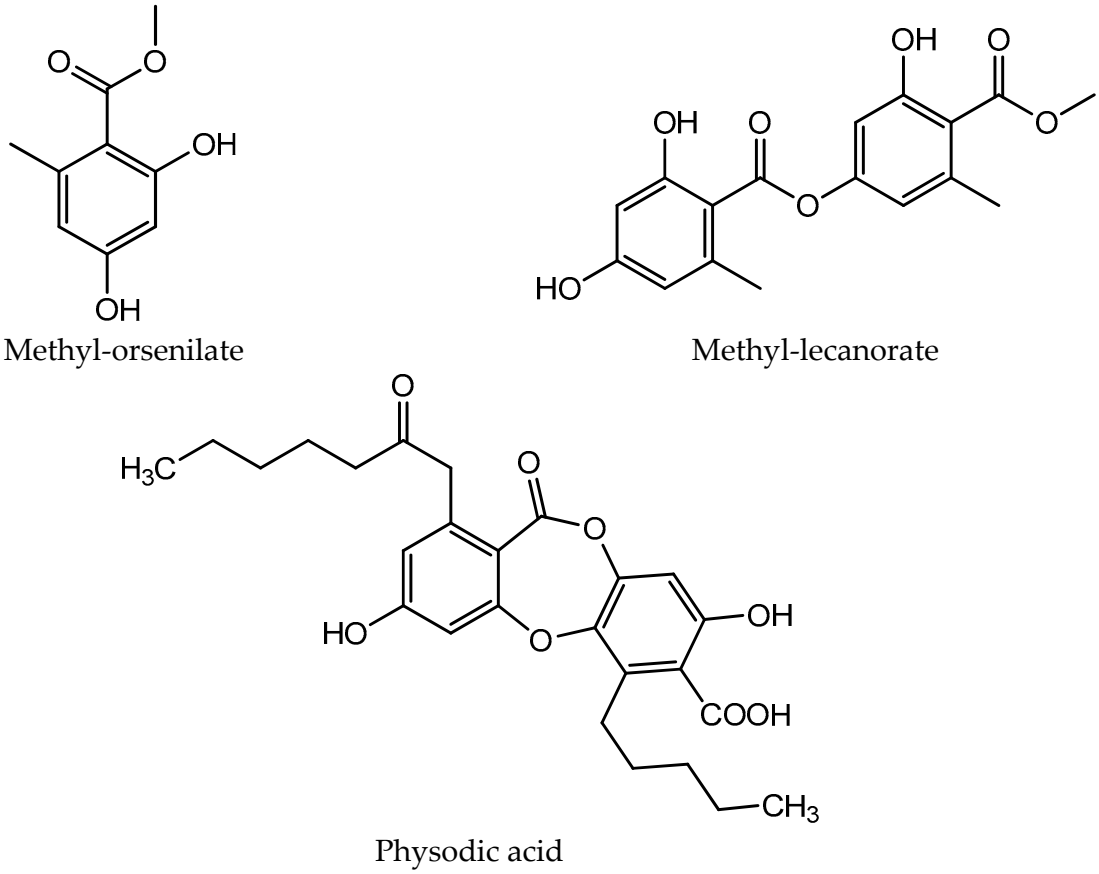


Figure 2. The chemical structures of compounds identified in extracts and isolated fractions.

3.2. Total Phenolic Content

The results of the total phenolic content (TPC) of the acetone and methanol extracts are presented in Table 2. The acetone extract showed significantly higher values for TPC compared to the other extract.

Table 2. The total polyphenols content of the extracts of the lichen *Umbilicaria crustulosa*.

Lichen Extracts	Phenolics Content (mg GA/g)
UCA	205.46 ± 0.39
UCM	156.36 ± 0.59

Values are expressed as mean ± SD of triplicate measurements; GA—gallic acid equivalents; UCA—acetone extract of lichen *U. crustulosa*; UCM—methanol extract of lichen *U. crustulosa*.

3.3. Antioxidant Activity

The antioxidant activity of two extracts of *U. crustulosa* and isolated compounds was evaluated using three methods: DPPH scavenging activity, ABTS scavenging activity, and reducing power (Tables 3 and 4).

Table 3. The antioxidant activity of the extracts of the lichen *U. crustulosa* and isolated compounds.

Lichen Extracts/Compound	IC_{50} (μg/mL)	
	DPPH Scavenging	ABTS Scavenging
UCA	390.10 ± 4.02	239.60 ± 3.45
UCM	245.69 ± 2.33	216.80 ± 3.52
GYR	>500	246.99 ± 11.88
PHY	158.97 ± 2.02	107.58 ± 1.42
ATR/CHL	150.77 ± 2.46	43.94 ± 2.27
Ascorbic acid	9.08 ± 1.96	8.28 ± 0.24
Trolox	14.26 ± 3.81	12.40 ± 0.40

Values are expressed as mean ± SD of triplicate measurements; UCA—acetone extract of lichen *U. crustulosa*; UCM—methanol extract of lichen *U. crustulosa*; GYR—gyrophoric acid; PHY—physodic acid; ATR/CHL—Fraction of acetone extract which contain atranorin and chloroatranorin.

Table 4. Reducing power of the extracts of lichen *U. crustulosa* and isolated compounds.

Lichen Extract/ Compound	Absorbance (700 nm)					
	1000 μg/mL	500 μg/mL	250 μg/mL	125 μg/mL	62.5 μg/mL	31.25 μg/mL
UCA	0.515 ± 0.003	0.333 ± 0.006	0.23 ± 0.002	0.0167 ± 0.006	0.013 ± 0.005	0.098 ± 0.002
UCM	0.701 ± 0.004	0.448 ± 0.004	0.287 ± 0.011	0.201 ± 0.007	0.015 ± 0.004	0.12 ± 0.006
GYR	0.237 ± 0.016	0.236 ± 0.018	0.193 ± 0.008	0.178 ± 0.004	0.133 ± 0.012	0.119 ± 0.003
PHY	0.786 ± 0.011	0.471 ± 0.009	0.3 ± 0.002	0.198 ± 0.002	0.152 ± 0.002	0.13 ± 0.003
ATR/CHL	0.637 ± 0.001	0.407 ± 0.010	0.198 ± 0.002	0.145 ± 0.003	0.112 ± 0.004	0.065 ± 0.004
Ascorbic acid	2.219 ± 0.012	2.147 ± 0.010	1.582 ± 0.008	1.17 ± 0.004	0.736 ± 0.002	0.281 ± 0.006
Trolox	1.96 ± 0.009	1.521 ± 0.020	0.904 ± 0.008	0.513 ± 0.001	0.312 ± 0.001	0.204 ± 0.018

Values are expressed as mean ± SD of triplicate measurements; UCA—acetone extract of lichen *U. crustulosa*; UCM—methanol extract of lichen *U. crustulosa*; GYR—gyrophoric acid; PHY—physodic acid; ATR/CHL—Fraction of acetone extract which contains atranorin and chloroatranorin.

The results of the reducing power of the tested lichen extracts and isolated compounds are shown in Table 4, where the examined extracts had slightly less activity compared to ascorbic acid and Trolox. Physodic acid shows the highest reducing power, while gyrophoric acid shows the weakest at higher concentrations, but at lower concentrations, it is in the same range as the other tested extracts and metabolites.

3.4. Animal Studies

3.4.1. Acute Oral Toxicity Study

The research discovered that giving UCA and UCM at a dosage of 2000 mg/kg did not result in any toxic symptoms or deaths in the animals, and that both extracts can be classified in Class 5 in accordance with the OECD Procedure 423 [23]. No behavioral changes were observed over 14 consecutive days of continuous observation. The histopathological results were within normal limits, and there were no instances of drug-related illness or death. Hence, the UCA and UCM were deemed non-toxic and safe for additional biological activity testing. Data from the acute oral toxicity study are presented in Tables S1–S5.

3.4.2. Anti-Inflammatory Activity

Inflammation caused by carrageenan was observed through swelling and measured by assessing the increase in paw edema. Paw edema enlargement was influenced by the time intervals following carrageenan injection (1, 2, 3, and 4 h) and the dosage of the lichen extract administered (50, 100, and 200 mg/kg), as presented in Table 5.

Table 5. Anti-inflammatory activity of the extracts of the lichen *U. crustulosa* in the carrageenan-induced rat paw edema model.

Experimental Groups	Rat Paw Thickness (mm) (% Inhibition)				
	0 h	**1 h**	**2 h**	**3 h**	**4 h**
Ctrl	5.24 ± 0.12	7.25 ± 0.18	7.43 ± 0.4	7.15 ± 0.36	7.04 ± 0.36
50 UCA	5.27 ± 0.18	6.82 ± 0.12 (22.89)	7.05 ± 0.4 (18.72)	6.57 ± 0.26 (31.94)	6.34 ± 0.27 (40.56)
100 UCA	5.30 ± 0.17	6.72 ± 0.17 (29.35)	6.50 ± 0.24 (45.21) *	6.79 ± 0.20 (21.99)	6.50 ± 0.23 (33.33)
200 UCA	5.43 ± 0.20	6.57 ± 0.13 (43.28) *	6.69 ± 0.35 (42.47) *	6.84 ± 0.40 (26.18)	6.39 ± 0.29 (46.67) *
50 UCM	5.29 ± 0.14	7.00 ± 0.15 (14.93)	6.57 ± 0.29 (41.55) *	6.86 ± 0.21 (17.8)	6.30 ± 0.22 (43.89)
100 UCM	5.44 ± 0.13	6.64 ± 0.19 (40.30) *	6.93 ± 0.38 (31.96)	6.58 ± 0.27 (40.31)	6.54 ± 0.28 (38.89)
200 UCM	5.60 ± 0.10	6.60 ± 0.10 (50.25) *	6.56 ± 0.20 (56.16) *	6.53 ± 0.32 (51.31) *	6.36 ± 0.20 (57.78) *
Ind	5.33 ± 0.23	6.38 ± 0.15 (47.76) *	6.44 ± 0.18 (49.32) *	6.23 ± 0.26 (52.88) *	6.01 ± 0.15 (62.22) *

The results are presented as the mean value ± standard deviation; * A statistically significant difference at the level of $p < 0.05$ in relation to the Ctrl; UCA—acetone extract of lichen *U. crustulosa*; UCM—methanol extract of lichen *U. crustulosa*; Ctrl–rats were treated per os with 1% water solution of carboxymethyl cellulose (CMC) 60 min before inducing inflammation; 50 UCA–rats received 50 mg/kg lichen extract UCA per os dissolved in 1% water solution of CMC 60 min before inducing inflammation; 100 UCA–rats received 100 mg/kg lichen extract UCA per os dissolved in 1% water solution of CMC 60 min before inducing inflammation; 200 UCA–rats received 200 mg/kg lichen extract UCA per os dissolved in 1% water solution of CMC 60 min before inducing inflammation; 50 UCM–rats received 50 mg/kg lichen extract UCM per os dissolved in 1% water solution of CMC 60 min before inducing inflammation; 100 UCM–rats received 100 mg/kg lichen extract UCM per os dissolved in 1% water solution of CMC 60 min before inducing inflammation; 200 UCM–rats received 200 mg/kg lichen extract UCM per os dissolved in 1% water solution of CMC 60 min before inducing inflammation; Ind–rats received 10 mg/kg indomethacin per os 60 min before inducing inflammation.

Both obtained extracts at all doses effectively decreased rat paw edema in comparison to the control group. The most significant reduction in edema was seen at two and four hours after carrageenan injection. During the second hour, the acetone extract and methanolic extract of the lichen *U. crustulosa* at the lowest dose exhibited inhibition percentages of 18.72 and 41.55, respectively. At a dose of 100 mg/kg, acetone extract showed an inhibition percentage of 45.21, while methanolic extract showed an inhibition percentage of 31.96. The most significant effect two hours after carrageenan injection occurred when methanolic was administered at the highest dose of 200 mg/kg. The paw edema inhibition percentage compared to the control was 56.16%. A decrease in paw edema reduction values was observed in the third hour. In the fourth hour, a significant effect could only be observed when the highest dose was applied, resulting in a 46.67% reduction for acetone extract and a 57.78% reduction for methanolic extract.

The data show that the examined extracts begin to show a significant effect already after the first hour and that this effect persists until the end of the experiment compared to the control group. Also, time dependence and dose dependence can be clearly observed, that is, the effects of UCA and UCM administration are most pronounced after 4 h from the moment of administration and in the highest administered dose of 200 mg/kg. The effect of UCM administration in the highest dose is almost comparable to indomethacin administration after 2, 3, and 4 h of administration, when the percentage of inhibition was greater than 50%.

4. Discussion

HPLC analysis of the acetone and methanol extracts of *U. crustulosa* lichen showed that the main components included two monoaromatic compounds, four didepsides, two tridepsides, and depsidone. The predominant phenolic compound in both extracts was tridepside gyrophoric acid (GYR). Apart from gyrophoric acid, methyl orsellinate (MOR), lecanoric acid (LEC), crustinic acid (CRU), physodic acid (PHY), atranorin (ATR), chloroatranorin (CHL), methyl lecanorate (MLE), and haematommic acid (HAE) were also identified in the chromatograms. Gyrophoric acid, the characteristic lichen substance of the *Umbilicaria* species, was detectable in 31 out of 33 investigated species. Orselinic acid (monoaromatic compound) and lecanoric acid (didepside) are biochemical precursors

of gyrophoric acid [25]. The simultaneous presence of gyrophoric and lecanoric acids suggests that lecanoric acid may be a hydrolysis product of gyrophoric acid [26]. While crustinic acid (tridepside) is a common component of the *Umbilicaria* species [25], in this research, it was only detected in the acetone extract. Methyl orsellinate (monoaromatic compound) and methyl lecanorate (didepside) are known to be an intermediate product in depside biosynthesis [26]. Physodic acid (depsidone) has been previously documented in the lichen *Hypogymnia physodes* and other lichen genera [27]. Nonetheless, this study marks the first confirmation of its presence and isolation in the lichen *U. crustulosa*. The presence or absence of depsidones is also important for classification purposes [25]. Atranorin (didepside) and chloratranorin (didepside) were identified in the acetone extract but not found in the methanol extract. In the *Umbilicaria* species, atranorin and chloratranorin are typically present in small quantities. [11,26]. Atranorin is a frequent companion of the presence of gyrophoric acid in the *Umbilicaria* species [25]. Haematommic acid (monoaromatic compound) was identified as a satellite peak in the chromatogram of the methanol extract. The ester form of this acid (ethyl haematommate) has already been found in some *Umbilicaria* species [11]. In addition, the isolation of gyrophoric acid as the most abundant component in the extract, as well as the isolation of the less abundant component in the extract of physodic acid and the fraction of extract which contains atranorin and chlorotranorin (ATR/CHL) (Figure 1), were reported, and these samples were used for further investigations.

In comparison to previous studies on *Umbilicaria* species [28], the examined extracts demonstrated significantly elevated levels of the total phenols. Various factors such as extraction method, climatic conditions, soil quality, timing of lichen collection, lichen age, and developmental stage may have contributed to these observed differences [29].

Several studies have examined the antioxidant potential of various lichen species [8,11,30,31]. Recent studies have focused on identifying the specific secondary metabolites within lichen extracts responsible for their antioxidant activity. Unlike previous research that utilized whole lichen extracts, these studies aim to pinpoint the individual metabolites contributing to the antioxidant properties [32,33]. In some earlier studies, the antioxidant activity of *U. crustulosa* extracts was investigated [12], but the antioxidant activity of its isolated metabolites has not been investigated so far. Precisely this correlation will help us to discover on what basis the antioxidant activity of the extracts originates. The assessment of the antioxidant activity showed that the tested extracts and isolated compounds were able to scavenge DPPH radicals (Table 3).

While several reports have demonstrated a positive correlation between the total phenolic content of lichens and their antioxidant activity [8,11,27,28], it is important to acknowledge that this relationship is not consistently observed in all studies [34,35]. In this study, no correlation was found between the antioxidant activity and the total phenolic content of the extracts. Although the acetone extract of *U. crustulosa* had higher values of total phenols, it showed a weaker antioxidant activity than the methanol extract in all experiments. The variation in correlation may be attributed to the differing antioxidant activities of individual phenols and potential interactions, whether antagonistic or synergistic, with other compounds within the lichens. The isolated fraction ATR/CHL and compound physodic acid showed the highest DPPH free radical scavenging activity (IC_{50} = 150.77 µg/mL; IC_{50} = 158.97 µg/mL). These metabolites showed higher antioxidant activity than the tested extracts, while the isolated gyrophoric acid showed the lowest activity (IC_{50} = <500 µg/mL). The ABTS method shows similar results. Fraction ATR/CHL and physodic acid showed the highest ABTS radical scavenging activity (IC_{50} = 43.94 µg/mL; IC_{50} = 107.58 µg/mL), while the isolated gyrophoric acid showed the lowest activity (IC_{50} = 246.99 µg/mL). As can be seen in Table 3, the DPPH and ABTS scavenging activity of the tested extracts and isolated metabolites were generally lower compared to the standard substances.

These results of the antioxidant activity are in correlation with the previous results of other authors who showed that depsides (norstictic acid and atranorine) and depsidone (fumarprotocetraric acid) show strong antioxidant activity in free radical and superoxide anion scavenging, as well as in reducing power assays. The isolated metabolites showed better antioxidant activity than the extract [7,36]. It has also been shown that depsidones exhibit slightly stronger antioxidant activity than depsides [27,37]. Depsidones in lichens are thought to be formed by the oxidative cyclization of depsides. Furthermore, some researchers have discovered that depsidones exhibit greater DPPH, superoxide anion radicals scavenging activity, and reducing power compared to depsides and dibenzofurans [27]. The increased effectiveness of depsidones may be linked to their greater incorporation into lipidic microdomains [38].

We selected the carrageenan-induced inflammation model for our research experiment because it is commonly used in the literature to evaluate the effectiveness of various agents in reducing local edema [39,40]. This study showed that using both acetone and a methanolic extract of *U. crustulosa* reduced paw edema in a dose-dependent pattern at 1, 2, 3, and 4 h after carrageenan injection. The results indicate that both extracts at doses of 50, 100, and 200 mg/kg can effectively inhibit acute inflammation, as demonstrated by a significant reduction in paw edema in rats 4 h after carrageenan injection. Also, both extracts at the highest dosage showed the highest efficiency four hours after inflammation induced by carrageenan.

The literature indicates that the acute inflammatory response consists of two phases: the initial phase and the latter phase [24]. Following carrageenan administration, the first phase begins around 1–2 h later and involves mast cells producing histamine and serotonin. The later phase is characterized by the involvement of prostaglandin and various cytokines [41]. NSAIDs demonstrate anti-inflammatory effects in the later stage of carrageenan-induced inflammation by inhibiting cyclooxygenase and suppressing prostaglandin production through COX enzyme inhibition [42].

Atranorin was previously found to inhibit the enzyme cyclooxygenase 1 in a dose-dependent manner [43]. Atranorin was also the only compound from those identified in the extracts that were tested in a carrageenan-induced paw edema reduction model, where it showed a significant effect [44]. Also, there is data on in-silico studies of the effects of atranorin and lecanoric acid on enzymes associated with the inflammatory response [45]. The anti-inflammatory potential of physodic acid has been demonstrated in in vitro studies [46] but has never been investigated in vivo in a model of carrageenan-induced paw edema.

It is clear that the extracts of lichen have the potential to have an anti-inflammatory effect; however, it is up to future research to determine the precise molecular mechanism that underlies the anti-inflammatory effect.

Given that the effect of the applied extracts can be observed already after the first hour of inducing the inflammatory process with carrageenan, but the effect remains until the very end of the experiment, it can be assumed that the components of the extract are involved in the inhibition of the inflammatory response both in the initial and in the later phase, but additional research is necessary to examine in detail the mechanism of anti-inflammatory action of both *U. crustulosa* extracts and their individual components.

5. Conclusions

This study marks the first detailed chemical analysis of the lichen *U. crustulosa* collected from Stara Planina mountain in Serbia and the identification of physodic acid from this lichen. This study's significance lies in its pioneering evaluation of pure compounds isolated from the extract of the lichen *U. crustulosa* for its antioxidant activity. Notably, certain compounds exhibited substantial antioxidant effects, marking a noteworthy discovery. This is the first time that the anti-inflammatory activity of *U crustulosa* lichen extracts has been investigated. Furthermore, the acetone and methanolic extracts demonstrated dose-dependent anti-inflammatory effects in a carrageenan-induced inflammation model. The highest dose of both extracts (200 mg/kg) resulted in the most significant reduction in paw

edema, similar to the standard drug indomethacin. Additional research is needed to clarify the fundamental mechanisms of its biological effects and to identify and describe particular active compounds for potential drug development and therapeutic uses. This research highlights the potential of lichens as a valuable source of novel biological agents, which could find applications across various industries such as food, cosmetics, and pharmaceuticals. Future investigations will be focused on isolating larger amounts of pure compounds and determining their anti-inflammatory activity as well as other biological activities.

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