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MEDICINAL PLANTS

ANTIMICROBIAL ACTIVITY OF SOME LICHEN EXTRACTS

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Cladonia rangiferina is a bushy lichen of the genus *Cladonia* and has a multi-regional distribution. This plant contains usnic acid as the main biologically active substance and has been tested against some opportunistic microorganisms. Among the studied *C. rangiferina* extracts, only glycerol extracts obtained at an extraction temperature of 40°C were biologically active and only against Gram-positive microorganisms (*S. aureus*) and fungi (*C. albicans*). The antimicrobial activity of the extracts decreased if the extraction temperature was raised to 90°C. The results showed that *C. rangiferina* lichen could be a potential rich source of effective antimicrobial agents.

Keywords: antimicrobial activity, lichen extracts, usnic acid.

Lichens and their metabolites possess various biological activities such as antiviral [1, 2], antibiotic [3 – 5], and antitumor [6]; exhibit allergenic properties; inhibit enzymes and plant growth; and play an ecological role [7]. The territory of the Khanty-Mansi Autonomous Okrug (KhMAO) – Yugra has a variable and well-developed flora of lichens. The genus *Cladonia* includes the greatest variety of lichens (31.9%) and is one of the most complex with respect to the chemistry of them [8]. Discovery and development of new pharmacologically active compounds is an important problem of the contemporary pharmaceutical industry [9]. Secondary metabolites of lichens form a large group of aromatic compounds, depsides and depsidones, dibenzofurans and polyhydroxynones, xanthones, and terpene derivatives. Their effectiveness has not been proven. They are active metabolites of cellular metabolism and play important roles in various physiological processes [10]. Usnic acid, which is one of the most active lichen compounds [11], is rather commonly used in pharmaceuticals. The present study reports an evaluation of the antimicrobial activity of extracts of *Cladonia rangiferina* growing in KhMAO – Yugra.

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EXPERIMENTAL PART

Collection and identification of lichen samples

Specimens of *C. rangiferina* were collected in September 2020 in Nizhnevartovsk District, KhMAO – Yugra. Samples were dried at room temperature for 48 h. High-performance liquid chromatography (HPLC) was used to determine the contents in the extracts of biologically active constituents and to standardize them.

Preparation of extracts

A weighed portion of each sample of plant raw material was placed into a glass beaker. Extractant was added in a 1:10 ratio. The mixture was thoroughly stirred, covered with a lid, and stored in a drying cabinet at 40°C for 24 h. Then, the extract was separated from the remaining plant raw material by filtration through filter paper. A control sample of the extractant was obtained by storing it in a glass beaker at 40°C for 24 h. The extractants were glycerol, rapeseed oil, and olive oil. The plant raw material was extracted at two temperatures (40 and 90°C). Extracts and control samples of the extractants were analogously obtained by storing the extraction mixtures and extractants at 90°C for 24 h. The remaining plant raw material was separated by centrifugation (15,000 rpm, 30 min, 4°C). The supernatant was filtered and

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TABLE 1. Samples of Studied Extracts and Extractants

No.	Sample	No.	Sample
	Temperature 40°C, 24 h		**Temperature 90°C, 24 h**
1	Lichen sample No. 1 in glycerol (1:10)	1	Lichen sample No. 1 in glycerol (1:10)
2	Lichen sample No. 1 in rapeseed oil (1:10)	2	Lichen sample No. 1 in rapeseed oil (1:10)
3	Lichen sample No. 1 in olive oil (1:10)	3	Lichen sample No. 1 in olive oil (1:10)
4	Lichen sample No. 2 in glycerol (1:10)	4	Lichen sample No. 2 in glycerol (1:10)
5	Lichen sample No. 2 in rapeseed oil (1:10)	5	Lichen sample No. 2 in rapeseed oil (1:10)
6	Lichen sample No. 2 in olive oil (1:10)	6	Lichen sample No. 2 in olive oil (1:10)
7	Glycerol, control	7	Glycerol, control
8	Rapeseed oil, control	8	Rapeseed oil, control
9	Olive oil, control	9	Olive oil, control
		10	Usnic acid, 1%

evaporated to dryness at reduced pressure and/or lyophilized. In this manner, 12 different extracts were obtained. Table 1 presents their data.

The usnic acid content in a particular sample was determined by HPLC with spectrophotometric detection. Samples were analyzed on an analytical HPLC system consisting of a

TABLE 2. Antimicrobial Activity of *Cladonia rangiferina* Extracts Against *S. aureus*

No.	Sample	Result	Interpretation
1	Usnic acid, 1% (reference drug)	12 ± 0.48	S
	Temperature 40°C, 24 h		
2	Lichen sample No. 1 in glycerol (1:10)	12 ± 0.41	S
3	Lichen sample No. 1 in rapeseed oil (1:10)	7 ± 0.15	I
4	Lichen sample No. 1 in olive oil (1:10)	7 ± 0.11	
5	Lichen sample No. 2 in glycerol (1:10)	9 ± 0.25	
6	Lichen sample No. 2 in rapeseed oil (1:10)	6 ± 0.11	
7	Lichen sample No. 2 in olive oil (1:10)	8 ± 0.20	
8	Glycerol, control	0	R
9	Rapeseed oil, control	0	
10	Olive oil, control	0	
	Temperature 90°C, 24 h		
11	Lichen sample No. 1 in glycerol (1:10)	8 ± 0.17	I
12	Lichen sample No. 1 in rapeseed oil (1:10)	0	R
13	Lichen sample No. 1 in olive oil (1:10)	0	
14	Lichen sample No. 2 in glycerol (1:10)	5 ± 0.11	I
15	Lichen sample No. 2 in rapeseed oil (1:10)	0	R
16	Lichen sample No. 2 in olive oil (1:10)	5 ± 0.09	I
17	Glycerol, control	0	R
18	Rapeseed oil, control	0	
19	Olive oil, control	0	
20	Lichen sample No. 1	0	
21	Lichen sample No. 2	0	

Milichrom A-02 liquid chromatograph (IKh ECONOVA) using a column (75 × 2 mm) packed with ProntoSIL-120-5-C18 AQ BD-2003 adsorbent, column temperature 40°C, 1.7 MPa maximum pressure, detection at 210 nm, eluent A [(4 M $LiClO_4$–0.1 M $HClO_4$):H_2O = 1:19] and eluent B (MeCN) in gradient mode with the content of eluent B varying smoothly from 5 to 100%. The chromatograph was controlled using Alfakhrom software (v. 1.0). Chromatograms of analyzed samples were processed using Alfaspektr software (v. 1.0) and the BD-2003 computer database that allowed automatic identification of usnic acid peaks in chromatograms and determination of their concentration without using standard samples. The retention volume (V_R, μL), peak area at the detection wavelength of 210 nm (S_{210}, opt. density units, μL), and spectral ratio as the ratio of the peak area at all used wavelengths to the peak area at the base wavelength ($S_{\ddot{e}}/S_{210}$) were calculated in the obtained chromatograms using a data processing program for each compound. The collection of such sets of chromatographic and spectral parameters of the compounds comprised the BD-2003 database. The contents of usnic acid in extracts No. 1 and No. 2 were (0.115 ± 0.012)% and (0.111 ± 0.011)%; the contents of extracted compounds (6.34 ± 0.63)% and (7.18 ± 0.72)%, respectively.

Bacterial strains

Standard strains from the American Type Culture Collection (ATCC) *Staphylococcus aureus* 25932, *Escherichia coli* 25922, *Pseudomonas aeruginosa* 27853, and *Candida albicans* 24433 were used in the research.

Determination of antimicrobial activity by the disk-diffusion method

The sensitivity by the disk-diffusion method was determined by placing a bacterial suspension of known density (usually equivalent to McFarland standard turbidity 0.5) onto the surface of agar in a Petri dish and then adding disks soaked with the extract being tested. Diffusion of the extracts into the agar led to the formation of microorganism growth inhibition zones around the disks if the extract exhibited antimicrobial activity. The dishes were incubated in a thermostat at 35 – 37°C overnight. Results were calculated by measuring the diameter (mm) of the zone around a disk.

TABLE 3. Antimicrobial Activity of *Cladonia rangiferina* Extracts Against *C. albicans*

No.	Sample	Result	Interpretation
1	Usnic acid, 1% (reference drug)	10 ± 0.31	S
Temperature 40°C, 24 h			
2	Lichen sample No. 1 in glycerol (1:10)	10 ± 0.35	S
3	Lichen sample No. 1 in rapeseed oil (1:10)	0	R
4	Lichen sample No. 1 in olive oil (1:10)	0	
5	Lichen sample No. 2 in glycerol (1:10)	10 ± 0.35	S
6	Lichen sample No. 2 in rapeseed oil (1:10)	0	R
7	Lichen sample No. 2 in olive oil (1:10)	0	
8	Glycerol, control	0	
9	Rapeseed oil, control	0	
10	Olive oil, control	0	
Temperature 90°C, 24 h			
11	Lichen sample No. 1 in glycerol (1:10)	8 ± 0.16	I
12	Lichen sample No. 1 in rapeseed oil (1:10)	0	R
13	Lichen sample No. 1 in olive oil (1:10)	0	
14	Lichen sample No. 2 in glycerol (1:10)	7 ± 0.14	I
15	Lichen sample No. 2 in rapeseed oil (1:10)	0	R
16	Lichen sample No. 2 in olive oil (1:10)	0	
17	Glycerol, control	0	
18	Rapeseed oil, control	0	
19	Olive oil, control	0	
20	Lichen sample No. 1	0	
21	Lichen sample No. 2	0	

The composition of the LB growth agar for determining the antimicrobial activity (g/L) was agar, 20 g; tryptone, 10.0; yeast extract, 5.0; and NaCl, 6.0. The pH was 7.0.

Samples were dissolved in distilled H_2O for the investigations. The reference drug was (+)-usnic acid.

Study of anti-lysozyme activity of microorganisms by a photometric method

Anti-lysozyme activity (ALA) of the bacteria was found using a photometric method. Biomass of the studied bacteria was inoculated into growth bullion (3 mL) with the extract being tested and cultivated in a thermostat at 37°C for 24 h. Then, the optical density of the bullion culture was measured against the growth bullion on a spectrophotometer. The supernatant was separated from bacterial cells by centrifugation at 3,000 rpm for 15 min.

The test strain for determining ALA was a one-day agar culture of *Micrococcus luteus* ATCC 15307. Outgrown bacterial cells of the test strain were killed by $CHCl_3$ for 2 h, rinsed with phosphate buffer (1.15 M, pH 6.2), filtered through a large-pore filter, and rinsed. The suspension was titrated to optical density 0.300 (0.28 – 0.32).

A solution of lysozyme (20 μg/mL) was prepared in phosphate buffered saline (PBS). The supernatant of the studied microorganism cultures (0.9 mL) was mixed with the prepared lysozyme solution (0.1 mL) and incubated at 37°C for 60 – 120 min. The mixture of supernatant and lysozyme (0.5 mL) was treated with the test-strain suspension (2.0 mL). The optical density of the obtained mixture was measured after 30 and 150 sec against PBS on the spectrophotometer. The control was a mixture of growth bullion with lysozyme in a 9:1 ratio. The anti-lysozyme activity of the studied culture was calculated from the degree of lysis of the test-culture suspension using the formula:

$$\mathrm{ALA} = \frac{V_1 C\left(\dfrac{1-\Delta D_{\mathrm{o}}}{\Delta D_k}\right)}{V_2 D_{\mathrm{m}}},$$

where ALA is the anti-lysozyme activity (μg/mL opt. density units); V_1, volume of lysozyme solution of initial concentration (mL, 0.1 mL); V_2, volume of supernatant of bullion culture of studied strain (mL, 0.9 mL); C, initial lysozyme con-

TABLE 4. Antimicrobial Activity of *Cladonia rangiferina* Extracts Against *E. coli*

No.	Sample	Result	Interpretation
1	Usnic acid, 1% (reference drug)	0	R**
Temperature 40°C, 24 h			
2	Lichen sample No. 1 in glycerol (1:10)	5 ± 0.07	I
3	Lichen sample No. 1 in rapeseed oil (1:10)	6 ± 0.10	
4	Lichen sample No. 1 in olive oil (1:10)	5 ± 0.07	
5	Lichen sample No. 2 in glycerol (1:10)	5 ± 0.09	
6	Lichen sample No. 2 in rapeseed oil (1:10)	6 ± 0.11	
7	Lichen sample No. 2 in olive oil (1:10)	7 ± 0.14	
8	Glycerol, control	5	
9	Rapeseed oil, control	5	
10	Olive oil, control	0	R
Temperature 90°C, 24 h			
11	Lichen sample No. 1 in glycerol (1:10)	5 ± 0.07	I
12	Lichen sample No. 1 in rapeseed oil (1:10)	0	R
13	Lichen sample No. 1 in olive oil (1:10)	7 ± 0.12	I
14	Lichen sample No. 2 in glycerol (1:10)	5 ± 0.07	
15	Lichen sample No. 2 in rapeseed oil (1:10)	0	R
16	Lichen sample No. 2 in olive oil (1:10)	0	
17	Glycerol, control	0	
18	Rapeseed oil, control	0	
19	Olive oil, control	0	
20	Lichen sample No. 1	0	
21	Lichen sample No. 2	0	

centration (μg/mL, 20 μg/mL); D_m, optical density of bullion culture of studied strain (opt. density units); ΔD_0, change of optical density of test-culture suspension in test between 30 and 150 sec; ΔD_k, change of optical density of test-culture suspension in control between 30 and 150 sec [12].

RESULTS AND DISCUSSION

Lichens synthesize many metabolites, i.e., lichen compounds, that include derivatives of amino acids, polyols, aliphatic acids, macrocyclic lactones, monocyclic aromatic compounds, quinones, chromones, xanthones, dibenzofurans, depsides, depsidones, depsones, terpenoids, steroids, carotenoids, and diphenyl ethers [10]. Lichen compounds form a group of natural compounds that do not occur in other organisms. About 500 individual lichen compounds are now known, of which usnic acid is most often encountered in lichen thalli and is well studied. It was first detected and isolated from lichens of the genera *Ramalina* and *Usnea* already in the middle of the 19th century. Usnic acid is an available lichen metabolite, the biological activity of which is highly varied and of pharmacopoeial interest [13].

TABLE 5. Antimicrobial Activity of *Cladonia rangiferina* Extracts Against *P. aeruginosa*

No.	Sample	Result	Interpretation
1	Usnic acid, 1% (reference drug)	0	R**
Temperature 40°C, 24 h			
2	Lichen sample No. 1 in glycerol (1:10)	5 ± 0.08	I
3	Lichen sample No. 1 in rapeseed oil (1:10)	6 ± 0.11	
4	Lichen sample No. 1 in olive oil (1:10)	6 ± 0.12	
5	Lichen sample No. 2 in glycerol (1:10)	5 ± 0.07	
6	Lichen sample No. 2 in rapeseed oil (1:10)	6 ± 0.12	
7	Lichen sample No. 2 in olive oil (1:10)	6 ± 0.12	
8	Glycerol, control	0	R
9	Rapeseed oil, control	0	
10	Olive oil, control	0	
Temperature 90°C, 24 h			
11	Lichen sample No. 1 in glycerol (1:10)	5 ± 0.07	I
12	Lichen sample No. 1 in rapeseed oil (1:10)	0	R
13	Lichen sample No. 1 in olive oil (1:10)	0	
14	Lichen sample No. 2 in glycerol (1:10)	5 ± 0.08	I
15	Lichen sample No. 2 in rapeseed oil (1:10)	0	R
16	Lichen sample No. 2 in olive oil (1:10)	0	
17	Glycerol, control	5 ± 0.07	I
18	Rapeseed oil, control	5 ± 0.09	
19	Olive oil, control	5 ± 0.09	
20	Lichen sample No. 1	0	R
21	Lichen sample No. 2	0	

TABLE 6. Anti-lysozyme Activity (μg/mL, rel. units) of *S. aureus* and *C. albicans* Affected by Extracts and 1% Usnic Acid in Meat-Peptone Broth (for Bacteria) and Sabouraud Bullion (for Fungi)

Microorganism strain from ATCC	Without extract	Lichen sample No. 1 in glycerol (1:10), DHQ 0.1%, 40°C	Lichen sample No. 2 in glycerol (1:10), 40°C	UA, 1%
S. aureus 25923	1.25 ± 0.01	1.02 ± 0.02	1.01 ± 0.02	0.95 ± 0.01
Ñ. albicans 24433	1.78 ± 0.15	1.01 ± 0.15	1.01 ± 0.15	1.55 ± 0.10

The effect of lichen extracts on the biological properties of opportunistic microorganisms was studied in the present research (Table 1). Tables 2 – 5 present the antimicrobial activities of several lichen extracts. The designations are R, no antimicrobial activity (resistance); I, intermediate antimicrobial activity; and S, strong antimicrobial activity.

Table 2 shows that oil extracts of *C. rangiferina* exhibited antimicrobial activity against *S. aureus*. The glycerol extract obtained at 40°C was highly active against *S. aureus*. The biological activities of the extracts decreased if the extraction temperature was increased.

Antifungal activity against *C. albicans* was found for glycerol extracts obtained at 40°C (Table 3).

Tables 4 and 5 show that extracts of *C. rangiferina* had intermediate and low activity against *P. aeruginosa* and *E. coli*. According to screening results of several lichen extracts for antimicrobial activity, we think that inhibition of bacteria can vary depending on the lichen extract, solvent used for the extraction, and bacteria being tested. Extracts of *C. rangiferina* were inferior to usnic acid (1%) with respect to level of antimicrobial activity against *S. aureus* strains although they had more effective antifungal activity.

Pathogens devise various methods of eluding innate and adaptive immunity factors to survive in hosts. Such factors can include the ability to inactivate lysozyme, i.e., anti-lysozyme activity (ALA) [12, 14]. ALA is a universal biological phenomenon for pathogenic and indigenous microbiota and garners enormous significance in the pathogenesis of infectious diseases [12]. The abundance and degree of ALA in pathogens determine the possible development and duration of various infections. Two glycerol extracts with antimicrobial activity that were obtained at 40°C with added DHQ (0.1%) and without it were selected for the study. The effect of the glycerol extracts on ALA was studied only against *S. aureus* and *C. albicans* because antimicrobial activity was found only against these microorganisms. Table 6 presents the results for photometric determination of ALA against *S. aureus* and *C. albicans*.

ALA in the experiment with the glycerol extracts decreased by 20% for *S. aureus* and by 43% for *C. albicans*. An assessment of the antimicrobial activity and ALA of separately isolated pure biologically active usnic acid (1%) confirmed previously obtained results for the extracts.

The absence of antimicrobial activity against *E. coli* and *P. aeruginosa* for usnic acid and the presence of moderate antimicrobial activity for samples No. 1 and No. 2 indicated that the extracts contained other antimicrobial constituents that could supplement the antimicrobial properties of usnic acid.

Thus, only the glycerol extracts of *C. rangiferina* obtained at 40°C were biologically active and only against Gram-positive microorganisms (*S. aureus*) and fungi (*C. albicans*). The antimicrobial activity of the extracts decreased if the extraction temperature was increased to 90°C.

It was previously established that lichens possessed inhibitory activity against many bacteria such as *Bacillus*, *Pseudomonas*, *E. coli*, *Streptococcus*, *Staphylococcus*, *Enterococcus*, and *Mycobacterium* [10, 15, 16]. It was shown that Me_2CO and MeOH extracts of the lichen *Usnea ghattensis* were effective against *Bacillus licheniformis*, *B. megaterium*, *B. subtilis*, and *S. aureus*. Thus, several extracts prepared from lichens possessed potential antimicrobial activity. Therefore, further research is needed to isolate effective metabolites from lichens. The antimicrobial effect of the tested extracts could possibly be explained by new studies using various solvents for the extraction and other bacteria.

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