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Original Research Article

Chemical composition and bioactive properties of the lichen, *Pleurosticta acetabulum*

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Abstract

Purpose: *To investigate the chemical composition and bioactivity of the acetone extract of Pleurosticta acetabulum lichen.*
Methods: *Phytochemical analysis of the acetone extract of the lichen (Pleurosticta acetabulum) was carried out by high-performance liquid chromatography (HPLC). The antioxidant activity of the lichen extract was evaluated by determining the radical scavenging capacity on 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals and reducing power. To determine total phenolics and flavonoids, we used spectrophotometric methods. Antimicrobial activity was estimated by determination of the minimal inhibitory concentration using broth microdilution method. Anticancer activity of the lichen extract was tested using 3-4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT).*
Results: *Salazinic, norstictic, protocetraric, evernic acid and atranorin were identified as compounds of lichen. P. acetabulum extract exhibited moderate free radical scavenging activity (half-maximum inhibitory concentration, IC_{50} of 151.7301 μg/mL). The spectrophotomeric absorbance of the extract for reducing power varied from 0.035 to 0.127, while the total phenolics and flavonoids in the extract were 35.39 μg PE/mg and 12.74 μg RE/mg, respectively. Minimum inhibitory concentration (MIC) was in the range of 1.25 to 20 mg/mL while cytotoxic activity (based on IC_{50} values) ranged from 24.09 to 45.94 μg/mL.*
Conclusion: *The results confirm that lichen extract contains secondary metabolites that possess antioxidant, antimicrobial and anticancer activities, which opens up some possibilities for the extract to be developed as food supplements and pharmaceutical raw materials.*

Keywords: *Pleurosticta acetabulum, Antioxidant activity, Antimicrobial activity, Cytotoxic activity, Lichen*

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INTRODUCTION

A great diversity of living organisms can be used for the improvement of human health, such as biologically active components extracted from the plants, mushrooms and lichens [1-3]. In searching for new therapeutic alternatives, many different lichen species were investigated, due to significant antiviral, antimicrobial, anticancer, antihyperglycemic, cardioprotective, antiparasitic,

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anti-inflammatory and antibiotic effects that they have exhibited so far [3-5].

Many lichen species exert interesting biological and pharmacological activities. However, in the literature, only a few studies have investigated the potential of *Pleurosticta acetabulum* lichen.

Thus, the aim of this study is to present the results of the phytochemical analysis of the acetone extract of *P. acetabulum* lichen and its antioxidant, antimicrobial and anticancer activities in order to search for available natural antioxidant, antibiotic and anticancer agents that can be used as possible food supplements, raw materials for the pharmaceutical industry and remedies for the treatment of various diseases.

EXPERIMENTAL

Collection and identification of lichen sample

The samples of lichen *P. acetabulum* (Neck.) Elix & Lumbsch were collected from the mountain Kopaonik, Serbia, during May 2013. Identification was done using the relevant key and monographs by Ranković[2] [6,7]. The samples of collected species of lichen have been herbarium-stored at the Department of Biology and Ecology of Kragujevac, Faculty of Science, (voucher no. 109) for future reference.

Preparation of lichen extract

The samples of Lichen *Parmelia acetabulum* (100 g) were extracted with a Soxhlet extractor using acetone. The extract was filtered and then concentrated under reduced pressure in a rotary evaporator. The dried extract was dissolved in DMSO (5 % dimethyl sulphoxide) for further experiments.

High-performance liquid chromatography (HPLC)

The dry lichen extract was dissolved in 500 µL of acetone and carried out on a 1200 Series HPLC (Agilent Technologies) instrument with C18 column (C18; 25 cm 4.6 mm, 10 m). A UV spectrophotometric detector was used with methanol-water-formic acid (70:30:0.8, v/v/v) as a solvent. The detection wavelength was 254 nm and the injection volume was 5 µL, with a flow rate of 1 ml/min. Deionized water was purified using a Milli-Q academic water purification system (Milford, MA, USA). Formic acid was used as an analytical-grade reagent. HPLC-grade methanol was purchased from Merck (Darmstadt, Germany). These lichen substances are not available commercially.

Secondary metabolites were isolated manually from the lichen species using thin-layer chromatography (TLC) and column chromatography. The purity of the isolated metabolites was checked by HPLC. The standards used were obtained from the following sources: salazinic acid (t_R = 1.56 ± 0.20 min) isolated from the lichen *Lobaria pulmonaria*, norstictic acid (t_R = 2.70 ± 0.10 min) from the lichen *Ramalina farinacea and* protocetraric acid (t_R = 3.24 ± 0.20 min) from the lichen *Toninia candida*. Evernic acid (t_R = 5.08 ± 0.10 min) and atranorin (t_R = 14.88 ± 0.10 min) were isolated from the lichen *Evernia prunastri*.

Determination of antioxidant activity

Scavenging DPPH radicals

DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) radical was used to evaluate the free radical scavenging activity of the lichen extract [8]. Two millilitres of 0.05 mg/mL methanol solution of DPPH radical and 1 mL of the lichen extract (1 mg/mL) were placed in cuvettes. The mixture was stored at room temperature for 30 min. Then, the absorbance was measured at 517 nm in a spectrophotometer (Jenway, UK). Ascorbic acid was used as a positive control. DPPH radical scavenging activity (D) was calculated using Eq 1.

$$D (\%) = \{(A_0 - A_1)/A_0\}100 \ldots\ldots\ldots\ldots\ldots (1)$$

where A_o is the absorbance of the negative control (DPPH solution) and A_1 is the absorbance of the reaction mixture or standard. All the measurements were repeated three times. The inhibitory concentration (IC_{50}) was the parameter used to compare the radical scavenging activity.

Reducing power

The reducing power of the extract was determined according to the method of Oyaizu [9]. One milliliter of extract (1 mg/mL) was mixed with 2.5 mL of phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide (2.5 mL, 1 %). The mixtures were incubated at 50 °C for 20 min. Trichloroacetic acid (10 %, 2.5 mL) was added to the mixture and centrifuged. The upper layer was mixed with distilled water (2.5 mL) and ferric chloride (0.5 mL, 0.1 %). The absorbance of the solution was measured at 700 nm in a spectrophotometer (Jenway UK). Ascorbic acid was used as a positive control. The greater the absorbance of the reaction mixture the higher the reducing power of the samples.

Determination of total phenolic compounds

Total soluble phenolic compounds in the lichen extract were determined with Folin-Ciocalteu method [10], using pyrocatechol as a standard phenolic compound. One millilitre of the lichen extract (1 mg/mL) was diluted with distilled water (46 mL), and the content was mixed in a volumetric flask after adding one millilitre of Folin-Ciocalteu reagent After 3 min, 3 mL of 2 % sodium carbonate was added and left for 2 h with intermittent shaking. The reaction mixture absorbance was measured at 760 nm in a spectrophotometer (Jenway UK). The total concentration of phenolic compounds in the extract was expressed as microgram of pyrocatechol equivalent (PE) per milligram of dried extract. The total phenolics content was determined as the pyrocatechol equivalent using an equation obtained from a standard pyrocatechol graph (y = 0.0057 x total phenols {µg PE/mg of dry extracts} - 0.1646, R^2=0.9934).

Evaluation of total flavonoid content

The total flavonoids content in the lichen extract was determined with the spectrophotometric method using aluminium trichloride based on flavonoid-aluminium complex formation [11]. Two millilitres of 2 % aluminium trichloride in methanol was mixed with the same volume of the extract solution (1 mg/mL). The mixture was incubated at room temperature for 10 min, and the absorbance was measured at 415 nm in a spectrophotometer (Jenway UK) against a reagent blank (consisting of all the reagents except the extract or rutin standard solution being substituted with methanol). The total flavonoid content was determined as microgram of rutin equivalent (RE) per milligram of dried extract. The total amount of flavonoid compounds was determined as the rutin equivalent using an equation obtained from a standard rutin graph (y= 0.0296 x total flavonoid [µgRE/mg of dry extracts] + 0.0204, R^2=0.9992).

Assessment of antimicrobial activity

Antimicrobial activity of the tested sample was evaluated against 15 microorganisms, including five strains of bacteria: *Bacillus cereus* (ATCC 11778), *B. subtilis* (ATCC 6633), *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Proteus mirabilis* (ATCC 12453) and 10 species of fungi: *Aspergillus flavus* (ATCC 9170), *A. niger* (ATCC 16888), *Candida albicans* (ATCC 10231), *Mucor mucedo* (ATCC 20094), *Trichoderma viride* (ATCC 13233), *Cladosporium cladosporioides* (ATCC 11275), *Fusarium oxysporum* (ATCC 62506), *Alternaria alternata* (ATCC 11680), *Penicillium expansum* (ATCC 20466), *P. chrysogenum* (ATCC 10106) obtained from the American Type Culture Collection (ATCC).

The bacterial isolates were isolated from overnight cultures using Müller-Hinton agar and the suspensions were prepared in sterile distilled water by adjusting the turbidity to match that of a 0.5 McFarland standard (approximately 10^8 CFU/mL). Fungal suspensions were prepared from 3-7-day-old cultures that grew on a potato dextrose agar, except for *C. albicans* which was maintained on Sabourad dextrose (SD) agar. Sterile distilled water was used to rinse the spores, the turbidity was measured spectrophotometrically at 530 nm, and then further diluted to a concentration of approximately 10^6 CFU/mL according to NCCLS recommendations [12].

In order to determine the minimum inhibitory concentration (MIC) of the active extract, the 96-well micro-titre assay using resazurin as the indicator of cell growth was employed [13]. The starting solutions of the tested extract were obtained by dissolving it in 5 % dimethyl-sulphoxide. Serial twofold dilutions of the extract were made within a concentration range from 0.04 to 40 mg/mL in sterile 96-well plates containing Mueller–Hinton broth for bacterial cultures and Sabouraud Dextrose SD broth for fungal cultures. Resazurin solution was added as an indicator to each well. The MIC was determined visually and defined as the lowest concentration of the tested extract that prevented resazurin colour change from blue to pink. Streptomycin and ketoconazole were used as positive controls, while 5 % DMSO was used as a negative control.

Determination of cytotoxic activity

Human epithelial carcinoma Hela cells, human lung carcinoma A549 cells and human colon carcinoma LS174 cells were obtained from American Type Culture Collection (Manassas, VA, USA). All cancer cell lines were cultured as monolayers in the RPMI 1640 nutrient medium with 10 % Fetal Bovine Serum (inactivated at 56 ºC), 3 mM of L-glutamine, and antibiotics, at 37 ºC in a humidified air atmosphere with 5 % CO_2.

In vitro assay for cytotoxic activity of the investigated sample was performed when the cells reached 70 – 80 % confluence. A stock solution of the extract was dissolved in the corresponding medium to the required working concentrations. Neoplastic Hela, A549 and LS174 cells (5000 cells line per well) were seeded into 96-well microtiter plates, and 24 h

later, after cell adherence, five different double-diluted concentrations of the extract were added to the wells. The final concentrations of the extract were 12.5, 25, 50, 100, and 200 μg/mL, except for the control wells where only the nutrient medium was added. The cultures were incubated for the next 72 h.

The cancer cell-survival effect was determined 72 h after adding the extract, using the MTT (3-4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide) assay [14]. To each well was added 20 μL of MTT solution (5 mg/mL PBS) and they were further incubated in humidified air containing 5 % CO_2 at 37 °C for 4 hours Subsequently, 100 μL of 10 g/L SDS was added to solubilise the MTT formazan crystals converted by mitochondrial dehydrogenases in viable cells. The absorbance proportional to the number of viable cells was measured at 570 nm using a microplate reader (Multiskan EX, Thermo Scientific, Finland). Each experiment was performed in triplicate and independently repeated at least four times. Cis-dichlorodiammineplatinum (cis-DDP) was used as a positive control.

Statistical analysis

All data are presented as the mean ± standard deviation (mean ± SD) of three parallel measurements. Statistical analyses were performed using Microsoft Excel and SPSS software (version 18) package. Student's *t*-test was used to determine statistically significant differences which were considered significant at $p < 0.05$.

RESULTS

Secondary metabolites in the lichen *P. acetabulum* were identified using HPLC. The chromatograms for standards (salazinic, norstictic, protocetraric, evernic acid and atranorin) and lichen acetone extract eluted by HPLC are represented in Figure 1 and Figure 2.

Identification of these compounds was achieved by comparison of their retention times (t_R) and UV spectra (200 - 400 nm) from HPLC-UV with the standard substances previously isolated from lichens in our laboratory. The dominant peak in the chromatogram (t_R = 2.70 ± 0.10 min) originates from depsidone compound, norstictic acid (bryopogonic acid, 1,3-Dihydro-1,4,10-trihydroxy-5,8-dimethyl-3,7-dioxo-7H-isobenzofuro(4,5-b)(1,4)benzodioxepin-11-carboxaldehy-de). The UV spectrum of norstictic acid has 3 absorption maxima (212, 239 and 320 nm). Besides norstictic acid, the tested extract of *P. acetabulum* contains salazinic acid (t_R= 1.56 ± 0.20 min), protocetraric acid (t_R= 3.24 ± 0.20 min), evernic acid (t_R= 5.08 ± 0.10 min) and atranorin (t_R= 14.88 ± 0.10 min) in different amounts.

Protocetraric, evernic acid and atranorin have very small peaks and present satellite substances in the chromatogram. The UV spectra of salazinic (212, 238 and 310 nm) and protocetraric (212, 242 and 320 nm) are very similar to those of norstictic acid. Norstictic,

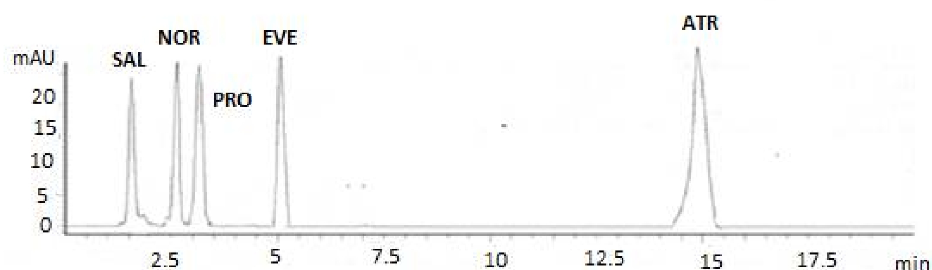

Figure 1: HPLC chromatogram for lichen standards at 254 nm Peaks: SAL= salazinic acid; NOR= norstictic acid; PRO= protocetraric acid; EVE= evernic acid; ATR= atranorin

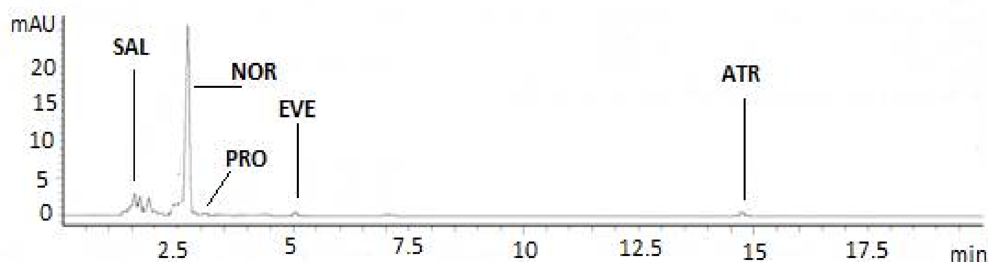

Figure 2: Chromatogram of the acetone extract of *Pleurosticta acetabulum* at 254 nm

salazinic and protocetraric acid are categorized into β-orcinoldepsidones. Absorbance maxima at 213, 270 and 305 nm are characteristic for evernic acid and at 212, 278 and 312 nm for atranorin. Salazinic and protocetraric acid belong to depsidones while atranorin and evernic acid belong to depsides. The retention times and UV absorbance maxima of the standards are shown in Table 1.

Table 1: Retention times of the lichen compounds and their absorbance maxima (nm)

Compound	Retention time (t_R±SD)* (min)	Absorbance maxima (nm) UV spectrum
Salazinic acid	1.56±0.20	212, 238, 310
Norstictic acid	2.70±0.10	212, 239, 320
Protocetraric acid	3.24±0.20	212, 242, 320
Evernic acid	5.08±0.10	213, 270, 305
Atranorin	14.88±0.10	212, 278, 312^m

Values are mean ± SD (n = 3); m = minor absorbance maximum

The structures of the detected compounds are shown in Figure 3. The antioxidant activity (scavenging DPPH radicals and reducing power) of the acetone extract is presented in Table 2.

The IC_{50} value of the lichen extract was 151.01 µg/mL for DPPH radicals. As shown in Table 2, reducing power was concentration dependent. The values of absorbance for reducing power varied from 0.035 to 0.127. In various antioxidant activities, there was a statistically significant difference between the extract and the control ($p < 0.05$).

The amounts of total phenolics and flavonoids in the extract were 35.39 µg PE/mg and 12.74 µg RE/mg, respectively.

The antimicrobial activity of the lichen extract against the test microorganisms is shown in Table 3.

The MIC for the acetone extract of *P. acetabulum* fluctuated in a range of 1.25 – 20 mg/mL for bacteria and 5 – 20 mg/mL for fungi. The extract did not show inhibitory activity against *E. coli* and *A. flavus,* which have been shown to be the most resistant bacteria, and fungi. The antimicrobial activity was compared with the streptomycin and ketoconazole which were more active than the tested lichen. In a negative control, DMSO had no inhibitory effect on the tested organisms.

The data obtained for the anticancer effect of *P. acetabulum* extract are shown in Table 4.

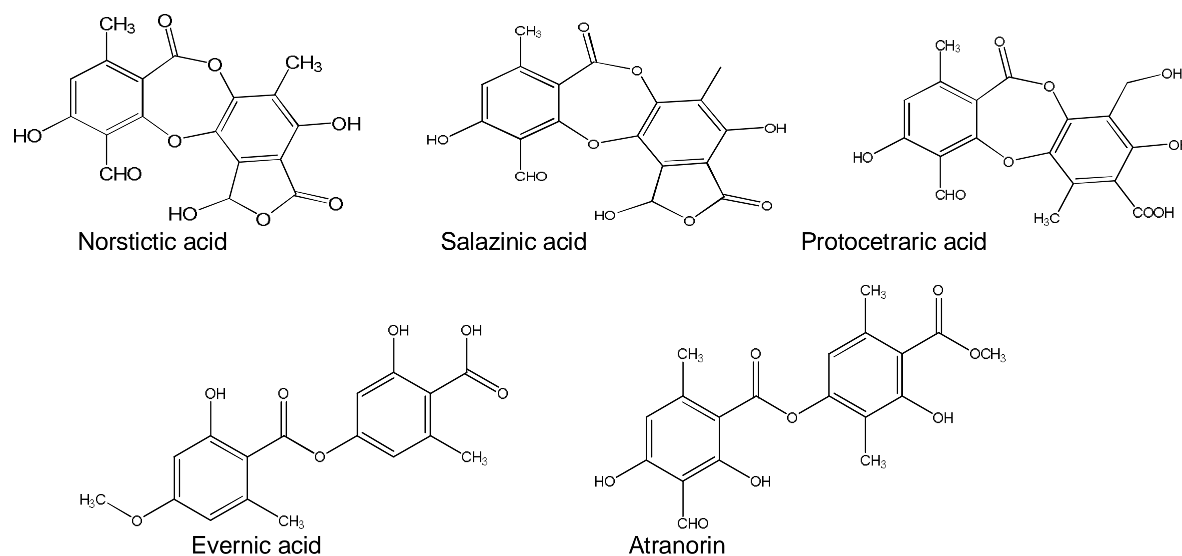

Figure 3: Chemical structures of the isolated compounds

Table 2: DPPH radical scavenging and reducing power of the acetone extracts of *Pleurosticta acetabulum*

Antioxidant test	DPPH radical scavenging IC_{50} (µg/mL)	Reducing power Absorbance (700 nm)			
		1000 µg/mL	***500 µg/mL***	***250 µg/mL***	***125 µg/mL***
Pleurostia acetabulm	151.01± 1.91	0.127±0.011	0.055±0.005	0.046±0.005	0.035±0.001
Ascorbic acid	6.42±0.18	2.113±0.032	1.654±0.021	0.0957±0.008	0.0478±0.004

Values are expressed as mean ± SD (n = 3)

Table 3: Minimum inhibitory concentration (MIC) of the acetone extracts of *Pleurosticta acetabulum*

Microorganism	*Pleurosticta acetabulum*	Streptomycin	Ketoconazole
Staphylococcus aureus	20	0.031	/
Bacillus subtilis	5	0.016	/
Bacillus cereus	1.25	0.016	/
Escherichia coli	/	0.062	/
Proteus mirabilis	10	0.062	/
Mucor mucedo	10	/	0.156
Trichoderma viride	10	/	0.078
Cladosporium cladosporioides	5	/	0.039
Fusarium oxysporum	5	/	0.078
Alternaria alternata	10	/	0.078
Aspergillus flavus	/	/	0.312
Aspergillus niger	20	/	0.078
Candida albicans	5	/	0.039
Penicillium expansum	20	/	0.156
Penicillium chrysogenum	10	/	0.078

Values given as mg/mL; Antibiotics: S – streptomycin, K – ketoconazole. Slash (/)-No activity

Table 4: Growth inhibitory activity of acetone extracts of *Pleurosticta acetabulum* on Hela, A549 and LS174 cell lines

Sample	IC_{50} (μg/mL)		
	Hela	A549	LS174
Pleurosticta acetabulum	26.95±1.54	24.09±0.36	45.94±1.28
cis-DDP	0.83±0.19	3.56±0.23	2.58±0.16

IC_{50} values are expressed as mean ± SD determined from the results of MTT assay in three independent experiments

The IC_{50} values of the lichen extract against Hela, A549 and LS174 cell lines were 26.95, 24.09 and 45.94 μg/mL, respectively.

DISCUSSION

The identification of secondary metabolites in the acetone extract of *P. acetabulum* and its antioxidant, antimicrobial and anticancer potentials were presented in this study.

By analyzing the composition of lichen *P. acetabulum* the presence of five secondary metabolites has been confirmed. Protocetraric and evernic acid were identified from the lichen *P. acetabulum* for the first time during this study research.

Norstictic acid as major lichen substance in the tested extract is a widespread secondary metabolite produced by lichen-forming fungi [15]. The identified metabolites could be used in the taxonomic classification of lichen species and as sources of commercial products.

In this study, the lichen extract showed relatively powerful levels of antioxidant activity. Some metabolites of lichens, including depsides, depsidones and dibenzofurans, contain phenolic groups considered to have an important role in antioxidative efficiency. The lichen used for the investigation contains secondary metabolites that have been shown to exhibit powerful antioxidant activity. Free radical scavenging and antioxidant activities of atranorin were evaluated using various *in vitro* assays for scavenging activity against hydroxyl radicals, hydrogen peroxide, superoxide radicals and nitric oxide.

Kosanic *et al* and Melo *et al* found that atranorin exerts differential effects towards reactive species production, enhancing hydrogen peroxide and nitric oxide production and acting as a superoxide scavenger, and then the activity towards hydroxyl radical production scavenging was observed [16,17].

Also, total reactive antioxidant potential and total antioxidant reactivity analysis indicate that atranorin acts as a general antioxidant, although it appeared to enhance peroxyl radical-induced lipoperoxidation *in vitro* [16]. Similarly, strong antioxidant activities were found for salazinic, protocetraric, evernic and norstictic acids [4,5,17].

In the literature sources that we examined, no data were found about the antimicrobial activity of *P. acetabulum* extracts. However, a relatively strong antimicrobial effect against numerous bacteria and fungi was found in the extract of the lichen *Parmelia acetabulum* where secondary metabolites were identified. Manojlović *et al* [5] reported about the antimicrobial activity of

salazinic and protocetraric acids. Also, strong antimicrobial activity was found for atranorin [18,19].

The strong antimicrobial activities of evernic and norstictic acids have previously been reported [4,17]. They observed that they both exerted antibacterial activity against Gram-positive and Gram-negative bacteria, but Gram-negative bacteria were more resilient. This resistance is likely due to the fact that Gram-negative bacteria have a wall associated with an outer complex membrane, which slows down the passage of hydrophobic compounds. Lacking outer membrane, Gram-positive bacteria are more susceptible to antibiotic agents [17].

Compared to bacteria, fungi are more resistant due to the more complex structure of the cell wall [3]. Similar to this research, the acetone extract of the lichen *P. acetabulum* and its major compound norstictic acid were previously tested for anti-proliferative activity towards HT29 cells [20]. The results showed that acetone extracts of *P. acetabulum* had the strongest anticancer activity with an IC_{50} value of 6 μg/mL after 48h treatment. Also, other constituents of *P. acetabulum* (evernic, salazinic, protocetraric acids and atranorin) have been shown as promising anticancer agents [5,17,21].

CONCLUSION

The foliose lichen, *Pleurosticta acetabulum*, is a source of versatile bioactive compounds, which provide tremendous opportunities for the production of new antioxidant, antimicrobial and anticancer agents. Lichen is also a potentially suitable source of raw material for food and pharmaceutical industries due to their activities.

However, further studies to elucidate its mechanism of actions are necessary to establish the potential biological properties of lichen extract and its compounds.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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