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Full Paper

Screening of Lichen Extracts Using Tyrosinase Inhibition and Toxicity Against *Artemia salina*

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Abstract: Nine lichen extracts were evaluated for tyrosinase inhibition and toxicity against *Artemia salina* larvae. Extract compositions were investigated by TLC and NMR analysis. The activity of constituents against tyrosinase was evaluated by bioautography, and the percent of inhibition was calculated based on the dopachrome produced during a set time interval. *Cladia aggregata*, *Cladonia dimorphoclada*, *Stereocaulon ramulosum* and *Stereocaulon microcarpum* extracts were active for tyrosinase inhibition. Barbatic, usnic, anziaic acids and an unidentified compound present in the extracts, are possibly responsible for tyrosinase inhibition. *Cladia aggregata*, *Cladonia crispatula*, *Cladonia furcata*, *Lobaria erosa*, *Punctelia canaliculata* and *S. microcarpum* proved to be less toxic to *A. salina* (LC_{50} > 500 μg/mL) than *Cladonia confusa* and *S. ramulosum* (LC_{50} 45.0 and 100.3 μg/mL, respectively), while the extract of *C. dimorphoclada* was highly toxic (LC_{50} < 10 μg/mL).

Keywords: lichens; phenolic compounds; tyrosinase; *Artemia salina*

1. INTRODUCTION

Lichens—associations between a fungus (the mycobiont) and one or more algae or cyanobacteria (the photobionts)—produce, in addition to primary metabolites (carbohydrates, proteins, lipids, and other compounds), secondary metabolites that have been extensively investigated, revealing more than 1000 compounds, mostly phenols like depsides, depsidones, furan heterocycles, xanthones, anthraquinones and chromones, besides other classes [1]. The interest in lichen extracts and compounds isolated from them lies in their biological and pharmacological properties, which comprise antibiotic, antifungal, antiviral, antioxidant, allelopathic, antitumor, and many other activities [2-7].

Phenolic substances produced by plants or lichens have shown inhibition/activation effects on the activity of enzymes such as phenolases, including tyrosinase [8-10]. This enzyme catalyzes a step in the biosynthesis of melanin, which is responsible for skin, eye, and hair pigmentation in a number of animal orders and for cuticle formation in insects [11-13]. More recent studies have been conducted on the role of tyrosinase in Parkinson's and other degenerative diseases, and as a tool for the treatment of melanoma [14-15]. Oxidization of *o*-diphenols to *o*-quinones catalyzed by phenolases can cause browning in some vegetables and fruits, with consequent losses in visual attractiveness and nutritional quality. Tyrosinase inhibitors have become important in medicine and the cosmetic industry, to prevent hyperpigmentation; in agriculture and public health, in the development of new insecticides; and for applications in the food industry [16-18]. Considering the relevance of new tyrosinase inhibitors, the present study investigated the effect of extracts obtained from the lichens *Cladia aggregata* (Sw.) Nyl., *Cladonia confusa* R. Santesson, *Cladonia crispatula* (Nyl.) Ahti, *Cladonia dimorphoclada* Robbins in Sandstede, *Cladonia furcata* (Hudson) Schaerer, *Lobaria erosa* (Eschw.)

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Trev., *Punctelia canaliculata* (Lynge) Krog, *Stereocaulon microcarpum* Müll. Arg., *Stereocaulon ramulosum* (Sw.) Räusch, all collected in Southern Brazil, on tyrosinase activity and toxicity against the microcrustacean *Artemia salina.*

2. MATERIAL AND METHODS

General procedures

NMR analysis – ^{1}H, ^{13}C, and DEPT-135 NMR spectra were obtained in DMSO-d_6 and $CDCl_3$. Chemical shifts were calibrated using the solvent signal as reference. All NMR experiments were conducted on a Bruker Advance DPX300 instrument (operating at 300.13 MHz for ^{1}H and 75.48 MHz for ^{13}C). The absorbance was measured on a Bioespectro SP220 instrument. TLC – The extracts were chromatographed on aluminum plates coated with GF_{254} silica gel (0.20 mm, Macherey-Nagel), using the following eluents: (I) toluene : dioxane : acetic acid, 180:45:5 v/v/v; (II) toluene : acetic acid, 85:15 v/v [19]. The spots were visualized under UV (254 nm) and then sprayed with methanol: sulfuric acid (10%) and heated until complete appearance of spots, followed by *p*-anisaldehyde: sulfuric acid and reheating. Migration of substances was expressed as retention fator (*R*f). Tyrosinase enzyme inhibition – Mushroom tyrosinase (*Agaricus bisporus*) and L-3,4-dihydroxyphenylalanine (L-DOPA) were purchased from Sigma-Aldrich and kept below –10 °C. Analytical-grade organic reagents were acquired from Synth, Aldrich and Tedia.

Lichens and extract preparation

All lichens were collected in Southern Brazil. The following ones from Rio Grande do Sul State: *Lobaria erosa* (Eschw) Trev. (Piratini Municipality), *Cladia aggregata*, *Cladonia confusa*, *C. crispatula*, *C. furcata*, *Stereocaulon microcarpum* and *S. ramulosum*, (Cambará do Sul Municipality), and *Punctelia canaliculata* (Rio Pardo Municipality). *Cladonia dimorphoclada* was collected in Urubici Municipality, Santa Catarina State. Voucher specimens were deposited at the Campo Grande Herbarium of the Universidade Federal de Mato Grosso do Sul (CGMS 39232, CGMS 40957, CGMS 39230, CGMS 40953, CGMS 39229, CGMS 37946, CGMS 40954, CGMS 40952, CGMS 40955).

Fragments of each lichen (240.0–960.0 mg) were cleaned, fragmented, and exhaustively extracted with acetone at room temperature. After solvent evaporation, the extracts were kept in a desiccator. Yields ranged from 2.7% to 8.2%. TLC and NMR methods were employed for analysis of the extracts. **Supplementary Material** available: NMR data (^{1}H, ^{13}C and Dept-135) of the extracts.

Tyrosinase enzyme inhibition

All aqueous solutions were prepared with deionized water on the first day of the experiment, then frozen, and used within 3 days. Phosphate buffer (Na_2HPO_4-NaH_2PO_4) at 66.7 mmol/L, pH 6.8, was used in all reactions.

Bioautography assay

In order to identify the compounds responsible for tyrosinase inhibition, TLC assays were performed. Extracts (100 µg) of each lichen were solubilized in acetone and spotted on Si-gel plates that were eluted in appropriate solvent mixtures. After development of chromatograms and total elimination of solvents, a tyrosinase solution (100 µg/mL in 0.066 M phosphate buffer, pH 6.8) was spread on the plates followed by spray of a L-DOPA solution (0.3 % m/v in distilled water) in order to identify the compounds responsible for tyrosinase inhibition. The positive results (spots with tyrosinase inhibitor appeared white against a brownish-purple background) were observed and photographed until color shifts were no longer perceived.

Determination of enzyme activity

In a glass cuvette, 1.6 mL of tyrosine solution (24 µg/mL in phosphate buffer) and 0.2 mL of substrate (0.6 mM L-DOPA in water) were mixed, and absorbance was measured at 475 nm every 30 s for 5 min, to calculate the rate of dopachrome formation (ε = 3.700 mol/L cm) [20]. The enzyme solution was used as the control.

Effect of solvent on enzyme activity

To evaluate the effect of DMSO on enzyme activity, the reaction was prepared with 1.6 mL of a solution containing 24 µg/mL of enzyme and 0.2 mL of DMSO. After 3 min of contact between enzyme

and solvent, absorbance readings were taken. The enzyme solution was used as a blank. A 0.2 mL aliquot of L-DOPA solution (0.6 mM) was subsequently added to each cuvette and the mixture was stirred. Only the enzyme solution and solvent were employed in the blank. Readings were taken in triplicate every 30 s for 5 min.

Evaluation of extract effect on enzyme activity

The extracts of *Stereocaulon microcarpum, S. ramulosum, Cladia aggregata,* and *Cladonia dimorphoclada,* which showed higher inhibition potential in the bioautography assay, were selected for quantitative evaluation of the inhibition of enzymatic activity. A 0.2 mL aliquot of extract (2 mg/mL in DMSO) was added to 1.6 mL of enzyme solution (24 µg/mL), and the absorbance read after 3 min, followed by addition of 0.2 mL of L-DOPA (0.6 mM). Absorbance was recorded every 30 s for 5 min. A solution of kojic acid in DMSO (2 mg/mL) was used as positive control. A blank experiment without the compounds was also carried out. All the experiments were performed in triplicate at 28–30 °C. Percent inhibition (%I) was calculated based on absorbance in the blank system (B) and absorbance in the system (A) after 5 min of reaction, applying the formula [(B – A)/B] × 100. Graphs were constructed using Origin 6.0 software.

Toxicity to *Artemia salina*

Approximately 100 mg of *A. salina* eggs were transferred to 1 L of saline solution (38 g/L). After hatching (approximately 48 h), the larvae were transferred to solutions at a ratio of 10 larvae per vial. The extracts (solubilized in saline solution containing 3% DMSO) were evaluated at concentrations of 500, 400, 200, 150, 100, 50, 20, and 10 µg/mL. Quinidine sulfate was used as the positive control; the saline solution was the negative control. Survivors were counted after 24 h [21]. The results were employed to determine LC_{50} (95% confidence interval) using the probit method [22]. Extracts with LC_{50} < 500 µg/mL were considered toxic.

3. RESULTS AND DISCUSSION

The chemical profiles of the lichens extracts were determined by TLC and NMR analysis. Figure 1 shows the chromatograms of the extracts.

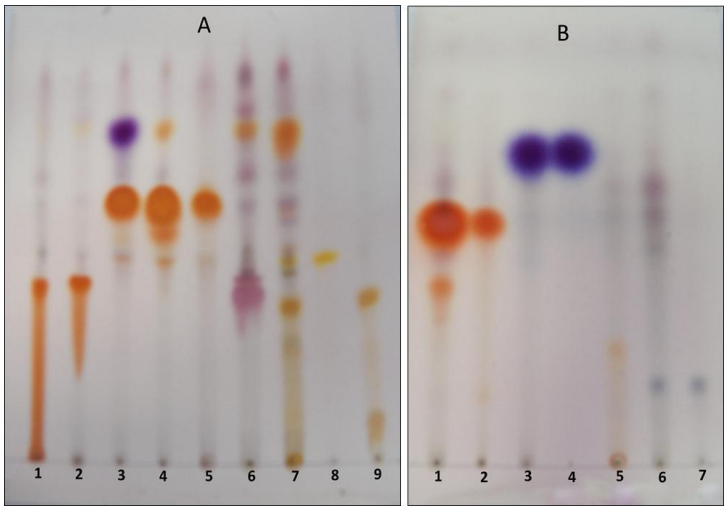

Figure 1. – Chromatograms of lichen extracts. **A**) 1 – *L. erosa;* 2 – Gyrophoric acid; 3 – *C. confusa*; 4 – *S. ramulosum*; 5 – Perlatolic acid; 6 – *P. canaliculata*; 7 – *S. microcarpum;* 8 – Norstictic acid; 9 – Stictic and constictic acids. **B**) 1 – *C. aggregata*; 2 – Barbatic acid; 3 – *C. dimorphoclada;* 4 – Usnic acid; 5 – *C. crispatula*; 6 – *C. furcata*; 7 – Fumarprotocetraric acid. Eluents: **A**) toluene : dioxane : acetic acid, 180:45:5 v/v/v; **B**) toluene : acetic acid, 85:15 v/v. Visualization with methanol : sulfuric acid (90:10 v/v) and heating, followed by *p*-anisaldehyde : sulfuric acid and reheating.

The tyrosinase inhibition effects of the lichens extracts were initially assessed by bioautography, and the extracts with promising activities were subjected to quantitative evaluation. All extracts were evaluated for toxicity against *A. salina*, widely employed for preliminary assessment of the toxicity of extracts and pure substances. Figure 2 shows the structures of the compounds identified in the extracts. *Lobaria erosa*

contains gyrophoric acid, identified by TLC and NMR spectra. The ^{1}H NMR spectrum exhibits signals at 2.35, 2.43, and 2.50 ppm (methyl groups) and six ArH signals (6.22-6.67 ppm). ^{13}C NMR and DEPT-135 spectra confirmed the presence of methyl groups at 19.7, 21.7, and 22.1 ppm, in addition to methine carbons at 100.96, 107.57, 107.64, 110.27, 114.0, and 114.6 ppm and other signals corroborating the gyrophoric acid structure. These data are in agreement with results reported by Narui *et al.* (1998) [23]. Further to gyrophoric acid, the NMR spectra showed signals indicative of alditols and waxes, as well as low-intensity signals attributed to the depside atranorin.

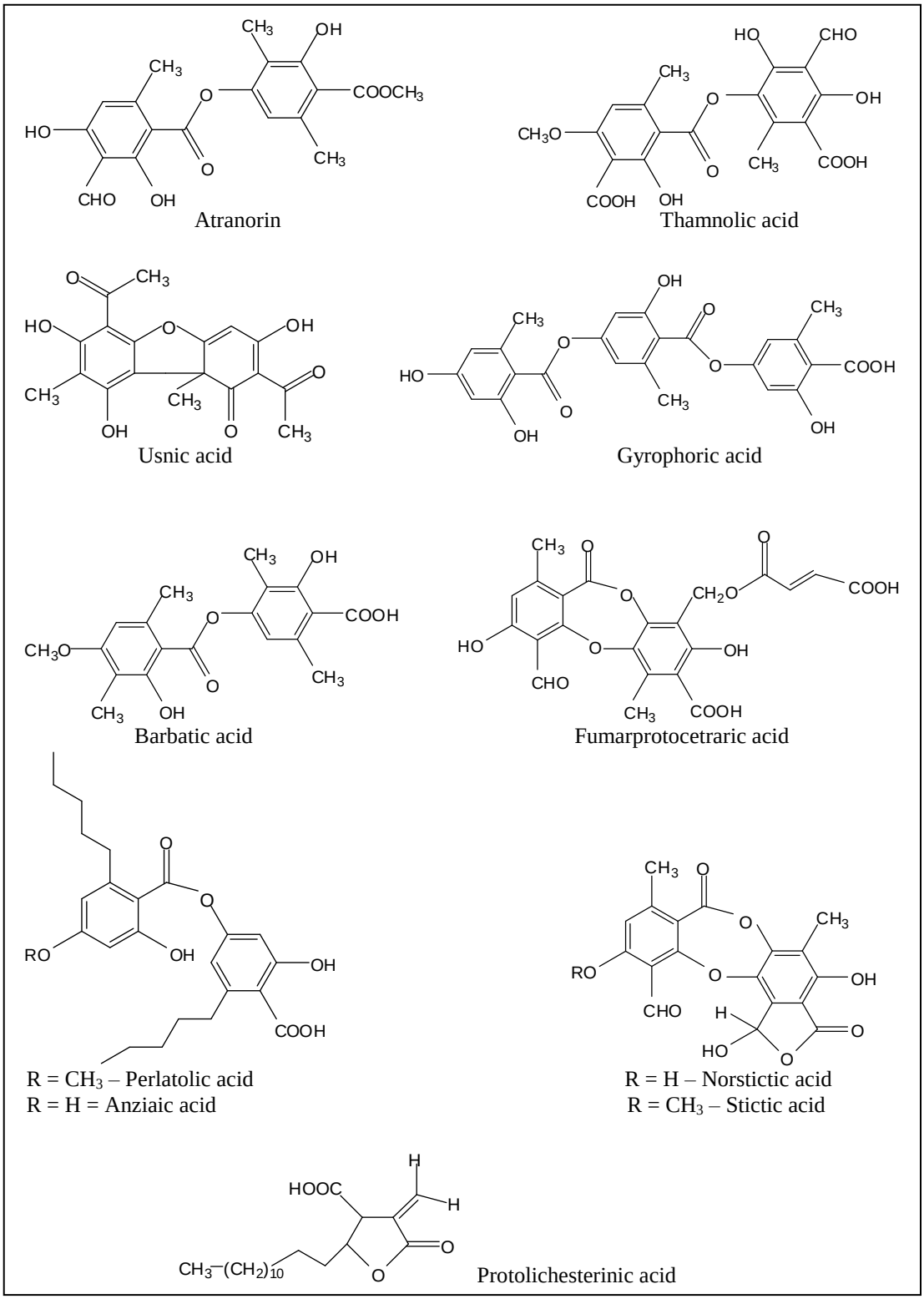

Figure 2. Structures of compounds identified in the lichen extracts.

Cladonia confusa contains perlatolic acid and usnic acid, while *Stererocaulon ramulosum* contains perlatolic acid, atranorin and a compound (Rf_I = 0.50) that migrates slightly less than perlatolic acid. Perlatolic acid was identified by comparison between its chromatographic behavior and the isolated perlatolic acid obtained in our laboratory. According to Sipman [24], *S. ramulosum* also contains a compound that migrates slightly less than perlatolic acid and probably corresponds to anziaic acid, which differs from perlatolic acid only by demethylation at C-4 (Figure 2). *Punctelia canaliculata* produces atranorin, protolichesterinic acid, and an aliphatic acid possibly similar to caperatic acid [25]. ^{1}H NMR spectrum of the extract showed an intense signal at 1.22 ppm and others at 0.84 and 1.63 (dublet), in addition to a large signal at 4.39 ppm, suggesting an aliphatic chain structure. Two singlets, at 6.2 and 5.9 ppm, indicated the presence olefinic hydrogens. These signals could be assigned to (+) and (−) isomers of the protolichesterinic acid. The spectrum contains no signals attributable to other aliphatic compounds, unless these occur in trace amounts. Stictic acid is the main component of the *S. microcarpum* extract, in addition to norstictic acid, atranorin, and other unidentified compounds. Its ^{1}H NMR spectrum exhibited signals corresponding to two aromatic methyl groups (2.17 and 2.49 ppm), one methoxy group (3.90 ppm), one aromatic hydrogen (7.09 ppm), and an aldehyde hydrogen (10.20 ppm). Two signals, at 8.20 and 6.64 ppm, correspond to a hydroxyl and a lactol hydrogen, respectively, indicating a butyrolactone unit. The ^{13}C and DEPT-135 NMR spectra exhibit signals at 9.59, 21.57, 56.8, and 186.79 ppm that correspond to two methyl, one methoxyl, and one aldehyde group, in addition to other signals indicative of the stictic acid structure [26]. The spectra showed lower-intensity signals indicative of other substances in the *S. microcarpum* extract. *Cladia aggregata* contains barbatic acid (figures 1 and S9–S11). Usnic acid is the principal compound in *Cladonia dimorphoclada (*figures 1 and S16–S17). Fumarprotocetraric acid is present in *C. furcata* (figures 1 and S6–S8).

Thamnolic acid is present in *Cladonia crispatula*, whose ^{13}C and DEPT-135 NMR spectra showed signals corresponding to two $ArCH_3$ groups (14.4 and 21.8 ppm), one $ArOCH_3$ (56.7 ppm), one ArH (106.0 ppm), and one ArCHO group (194.4 ppm). The ^{13}C spectrum exhibits signals at 170.3 and 171.7 ppm corresponding to two carboxyl groups, in addition to other signals that suggest thamnolic acid as the principal compound in the *C. crispatula* extract [26].

The preliminary bioautography assay for tyrosinase using 100 μg of each extract indicated that the anziaic, usnic and barbatic acids and an unidentified compound (Rf_I = 0.24), were the active compounds present in *S. ramulosum*, *Cladonia dimorphoclada*, *Cladia aggregata*, and *S. microcarpum* extracts (Figure 3), respectively.

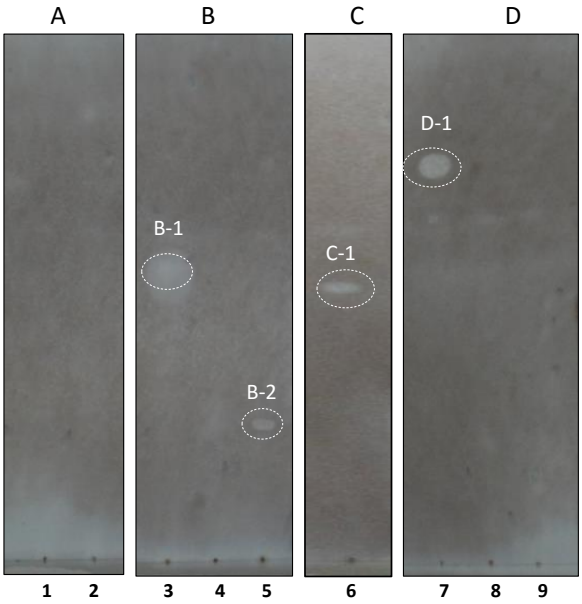

Figure 3. Chromatograms of lichen extracts developed with tyrosinase solution. 1 – *L. erosa*; 2 – *C. confusa;* 3 – *S. ramulosum*; 4 – *P. canaliculata*; 5 – *S. microcarpum*; 6 – *Cladia aggregata*; 7 – *Cladonia dimorphoclada*; 8 – *C. crispatula*; 9 – *C. furcata*. Chromatograms A and B were eluted with toluene : dioxane : acetic acid 180:45:5 v/v/v; C and D with toluene : acetic acid 85:15 v/v. Compounds that inhibited tyrosinase activity: B-1, anziaic acid; B-2, not identified; C-1, barbatic acid; D-1, usnic acid.

Once it was possible to assess which extract were active and the substances responsible for activity, quantitative assessment of enzyme inhibition were performed. The relationship between enzyme activity (9.10^{-3} mmol/min/mg) and inhibition was expressed by the concentration of dopachrome present in the reaction medium as a function of time. Figure 4 shows the effect of enzyme inhibition by the extracts (at a concentration of 200 μg/mL) and the concentration of dopachrome formed (mmol/L). This assay showed that the extract of *S. microcarpum* caused stronger inhibition of the enzyme (32.4%), possibly by action of the unidentified compound (Rf_I = 0.24). The extracts of *S. ramulosum* and *Cladia aggregata* had similar inhibitory effects (23.8% and

21.5%, respectively) and the extract of *Cladonia dimorphoclada* was less active in inhibiting tyrosinase (16.1%) (Table 1).

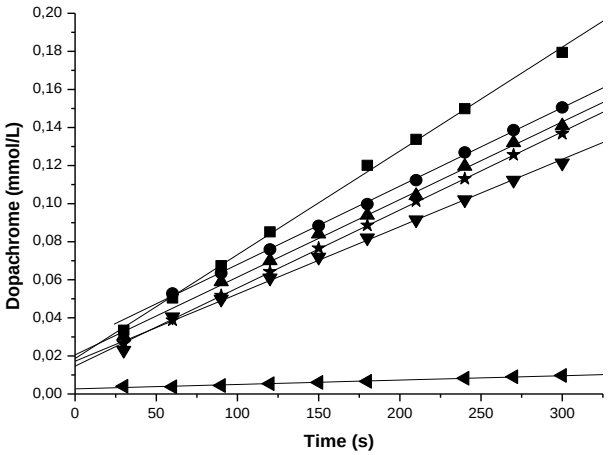

Figure 4. Concentrations (mM) of dopachrome formed under the effect of lichen extracts on tyrosinase, as a function of time. ■ Control (corresponding to tyrosinase activity without test sample); ● *Cladonia dimorphoclada*; ▲ *Cladia aggregata*; ▼ *Stereocaulon microcarpum*; ★ *Stereocaulon ramulosum*; ◄ kojic acid (positive control).

Table 1. Composition and activity of lichen extracts against *A. salina* and in tyrosinase inhibition assay.

Lichens	Compounds identified and *Rf* values	*Artemia salina* (LC_{50}, 95% CI)	Tyrosinase activity
Lobaria erosa	Gyrophoric acid (Rf_I = 0.39) and atranorin (trace) (Rf_I =0.74)	>500 µg/mL	
Cladonia confusa	Perlatotic acid (Rf_I = 0.56) and usnic acid (Rf_I = 0.72)	45.04 µg/mL (21.6-53.0)	
Stereocaulon ramulosum	Perlatolic acid (Rf_I = 0.56), atranorin (Rf_I = 0.74) and anziaic acid (Rf_I = 0.50)	100.32 µg/mL (61.22-164.40)	23.8%
Punctelia canaliculata	Protolichesterinic acids (Rf_I 0.36 and 0.40), atranorin (Rf_I = 0.74), and unidentified compounds	>500 µg/mL	
Stereocaulon microcarpum Müll.	Stictic acid (Rf_I = 0.33), norstictic acid (Rf_I = 0.44), atranorin (Rf_I = 0.74), and an unidentified compound (Rf_I = 0.24).	>500 µg/mL	32.4%
Cladia aggregata	Barbatic acid (Rf_{II} = 0.52)	>500 µg/mL	21.5%
Cladonia dimorphoclada	Usnic acid (Rf_{II} = 0.70)	<10 µg/mL	16.1%
Cladonia crispatula	Thamnolic acid (Rf_{II} = 0.25)	>500 µg/mL	
Cladonia furcata	Fumarprotocetraric acid (Rf_{II} = 0.19) and atranorin (trace) (Rf_{II} = 0.69)	>500 µg/mL	
Quinine sulfate	—	46.59 µg/mL (23.43-92.64)	NT
Kojic acid	—	NT	94.6%

LC_{50}: concentration that causes 50% death; CI: confidence interval; Rf_I and Rf_{II} : retention fator of the substances present in extracts chromatographed on TLC in the eluents (I) and (II), respectively. NT: not tested

The toxicity of the extracts on *A. salina* was determined. In this assay, *Cladonia crispatula*, *Cladia aggregata*, *L. erosa*, *P. canaliculata*, *Cladonia furcata*, and *S. microcarpum* extracts exhibited low toxicity against microcrustacean larvae, with LC_{50} > 500 μg/mL, whereas the extracts of *Cladonia confusa* and *S. ramulosum* showed LC_{50} values of 45.0 and 100.3 μg/mL, respectively. The extract of *C. dimorphoclada* proved highly toxic, with LC_{50} < 10 μg/mL (Table 1).

These are promising results, given the inhibitory activity of *S. microcarpum* and *C. aggregata* extracts on tyrosinase and their low toxicity against *A. salina*. Although some of the extracts failed to inhibit tyrosinase, the compounds present in the species investigated are known for other important properties. Gyrophoric acid is a potent protein tyrosine phosphatase 1B (PTP1B) inhibitor, with an IC_{50} value of 3.6 ± 0.04 μM. Selective inhibition of this enzyme is a target for the treatment of type-2 diabetes and obesity [27]. This acid is also a potent antiproliferative agent against growth of human keratinocytes (HaCaT cells), with an IC_{50} value of 1.7 μM, and a topoisomerase I inhibitor (25 μM) [28-29]. Pandey *et al*. [30], reported the anti-*Malassezia* action of the ethanolic extract of *C. aggregata* against three fungal species that cause pityriasis versicolor and seborrheic dermatitis. Atranorin and, more effectively, usnic acid inhibit cell proliferation and induce cell death, as revealed by evaluating these compounds against nine human cancer cell lines [31]. The cytotoxicity mechanisms of these compounds have been investigated [32]. Protolichesterinic acid has been reported as active against several tumor cell lines [33-35].

4. CONCLUSION

In conclusion, our results showed that, out of the nine extracts tested, four contained compounds that inhibit tyrosinase (*S. ramulosum*, *Cladonia dimorphoclada*, *Cladia aggregata* and *S. microcarpum*). The activity of these extracts can be attributed to anziaic, usnic, barbatic, and stictic acids, the principal compounds in these lichen species. *Cladia aggregata* and *S. microcarpum* were not toxic to *A. salina* (LC_{50} > 500 μg/mL), while *S. ramulosum* and *Cladonia dimorphoclada* inhibited the enzyme (23.8% and 16.1%, respectively) and were toxic to *A. salina* (100.32 and <10 μg/mL, respectively). The study was novel in revealing the inhibitory effects of the selected lichens extracts against tyrosinase. Further investigations of the effects of the isolated compounds are expected to identify the mechanisms and types of the inhibition processes.

5. ACKNOWLEDMENTS

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