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Expectorant and antioxidant activities of purified fumarprotocetraric acid from *Cladonia verticillaris* lichen in mice

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ABSTRACT

The lichen *Cladonia verticillaris* produces bioactive secondary metabolites, such as fumarprotocetraric (FUM) and protocetraric acids. Species of the genus *Cladonia* demonstrate anti-tumor, anti-inflammatory and antipyretic activities and have been used in folk medicine to treat respiratory diseases (throat irritation, cough, asthma and tuberculosis). The aim of the present study was to evaluate the expectorant and mucolytic activities of fumarprotocetraric acid in albino Swiss mice. FUM was extracted and purified from an acetone extract of *C. verticillaris*. The phenol red quantification method was used on the bronchoalveolar lavage fluid following the administration of FUM (25, 50 or 100 mg/kg orally or intraduodenally and 12.5, 25 or 50 mg/kg, intraperitoneally) for the evaluation of expectorant activity. Control groups received either saline solution (7.5 mL/kg) or ambroxol (1 mg/kg) through the same administration routes. Antioxidant activity was evaluated using the thiobarbituric acid reactive species assay in mouse lung tissue treated with the FUM at 25, 50 or 100 mg/kg orally, followed by a lipopolysaccharide solution at 1 mg/kg intrapleurally. The same protocol was used for the control groups using either saline solution (7.5 mL/kg, orally) or N-acetylcysteine (20 mg/kg, orally). Results: Orally administered FUM at doses of 25 and 50 mg/kg promoted significantly greater dose-dependent phenol red activity in the bronchoalveolar lavage and expectorant activity in comparison to the controls ($p < 0.05$). Lipid peroxidation (malondialdehyde equivalent) was reduced by 50% in the lung tissue. Conclusion: The results confirm the expectorant and antioxidant properties of fumarprotocetraric acid produced by the lichen *C. verticillaris*.

1. Introduction

Respiratory diseases are major causes of illness and death in adults and children [1,2]. While expectorants and similar drugs are widely prescribed for the treatment of lung diseases, the efficacy of such drugs is questioned [3–5]. Lichens are organisms consisting of a combination of fungi and algae or cyanobacteria in a symbiotic relationship [6]. Lichens produce substances resulting from their metabolism known as lichen acids, which are responsible for the biological activities of the organism [7]. The chemical nature of these substances is almost entirely phenolic [8–10]. Depsidones produced by the lichen *Cladonia verticillaris* [11] give rise to fumarprotocetraric (FUM) and protocetraric (PRO) acids, which have been found to have a photo protective effect [12] and inhibit both integrase in HIV-1 [13] and 5-lipoxygenase in leukocytes [14].

C. verticillaris is a lichen commonly found in northeastern Brazil with a similar composition to that of *Cetraria islandica* [6–8]. *C. islandica*, which is found in flat regions near the banks of lakes in Europe [15], is a species that has been used in the form of

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expectorants and cough syrups to treat sore throat, cough and bronchitis [3,16]. *C. verticillaris* belongs to the division Ascomycota, order Lecanorales and family Cladoniaceae [15,17]. Both species (*C. verticillaris* and *C. islandica*) have large concentrations of fumarprotocetraric acid, which have demonstrated biological actions, such as antitumor [18], anti-inflammatory [19], antibacterial [20] and insecticidal [21] activities.

The aim of the present study was to evaluate the antioxidant and expectorant activities of fumarprotocetraric acid produced by *C. verticillaris*.

2. Materials and methods

2.1. Collection and storage of lichen

Samples of *C. verticillaris* (200 g) were collected from sandy flat regions with gentle slopes near ponds in the city of Alhandra Paraíba, state of Paraíba, northeastern Brazil (Latitude: −7.43933, Longitude: −34.9136, 7°26′22″ South, 34°54′49″ West), as described elsewhere [22]. Specimens were placed in paper bags and dried at room temperature (28 °C). The lichen was identified by the chemical and morphological characters of the talus at the Natural Product Chemistry Laboratory of the Federal University of Pernambuco (Brazil) and a voucher specimen was deposited in the Geraldo Mariz Herbarium of the same university (n°. 361638).

2.2. Acquisition of organic extracts

Organic extracts were obtained from 60 g of fresh talus from *C. verticillaris* at room temperature (28 °C), as described elsewhere [22]. The stem was ground and subjected to successive extractions with 250 mL of diethyl ether under mechanical stirring for 1 h and cooled to 4 °C for 24 h, followed by filtration. The residue was submitted to extraction with acetone in a similar manner as that described for diethyl ether. The extracts were evaporated in a water bath at 40 °C until dry and then placed in a desiccator until achieving a constant weight of 3.6 g.

2.3. Extraction, identification and purification of fumarprotocetraric acid

Fumarprotocetraric acid was isolated from the acetone extract and purified through successive crystallizations, as described elsewhere [7]. Samples were identified by thin layer chromatography (TLC) [23] and purity was determined using high performance liquid chromatography (HPLC), based on Legaz et al. [22]. The organic extracts were also analyzed by HPLC using an Hitachi liquid chromatographer (655A-11, Tokyo, Japan) coupled to an UV detector (CG437-B) set at 254 nm, with a C-18 reverse phase column (MicroPack MCH-18, Berlin, Germany) measuring 300 × 4 mm. The mobile phase consisted of methanol, de-ionized water and acetic acid (Merck KGaA, Darmstadt, Germany) at proportions of 80:19.5:0.5 v/v, with a flow rate of 1 mL min^{-1}, injection volume of 10 μL and 0.04 attenuation at room temperature (28 °C), based on the method described by Legaz [22]. Dry residues were dissolved in methanol at 0.01 g dm^{-3} and injected into the column. Quantization of FUM and PRO was achieved by injecting pure standards (FUM and PRO) for the construction of calibration functions.

2.4. Animals and study design

Male Albino mice, Swiss Webster (25–40 g) were obtained from the Keizo Asami Immunopathology Laboratory (Pernambuco, Brazil) and kept in cages under standard environmental conditions (22 °C, 12/12 h light/dark cycle) with free access to chow (Labina, Purina, Brazil) and water. Animals in the experimental groups ($n = 6$ animals/group) were treated with FUM (dissolved in 0.9% saline solution) administered orally (25, 50 or 100 mg/kg), intraduodenally (25, 50 or 100 mg/kg) or intraperitoneally (12.5, 25 or 50 mg/kg). To access the duodenum the animals were anesthetized with an intraperitoneal injection of a combination of ketamine and xylazine (75 and 15 mg/kg, respectively). Subsequently, a midline laparotomy was made to expose the stomach and duodenum. Using a 0.5 mL syringe with a 28 gauge, the FUM was administered intraduodenally (25, 50 or 100 mg/kg). Same procedure was adopted for the three control groups. a) Ambroxol solution, known for its expectorant activity (1 mg/kg administered orally), b) 0.9% saline (7.5 mL/kg administered orally) or c) N-acetylcysteine (NAC), known for their antioxidant activity (20 mg/kg administered orally). At the end of the procedure the incision was sutured using cotton yarn with a simple isolated point. Three control groups were also determined: treatment with a) ambroxol, known for its expectorant activity (1 mg/kg, administered orally), b) 0.9% saline solution (7.5 mL/kg, administered orally) or c) N-acetylcysteine (NAC), known for its antioxidant activity (20 mg/kg, administered orally). The experiments were approved by the Ethics Committee for Animal Experimentation (number 23076.023133/2011-37) of the Federal University of Pernambuco, Recife, Brazil.

2.5. Pharmacological tests

2.5.1. Determination of expectorant activity of FUM

Expectorant activity was determined by measuring phenol red, as described elsewhere [16]. Thirty minutes after treatment, the animals received an intraperitoneal injection of phenol red (10 mg/mL) at a dose of 200 mg/kg. Thirty minutes after administration, the animals were anesthetized; the anterior-superior portion of the neck was shaved and exposure of the trachea was performed. Tracheobronchial lavage was then performed using 2 mL of 0.9% saline solution with the recovery of 1.0 mL. The lavage fluid was centrifuged at 1600 rpm (638 G) for 10 min. The supernatant (1 mL) was removed and 0.5 mL of sodium hydroxide (NaOH 0.01N) were added. The concentration of phenol red was measured spectrophotometrically at a wavelength of 535 nm and the results were expressed as mg/mL. A standard curve (0.055–10 mg/mL) of phenol red was used to normalize the data.

2.5.2. Lipid peroxidation assay

The formation of thiobarbituric acid reactive species (TBARS) during an acid-heating reaction was used as an index of lipid peroxidation, as described elsewhere [24]. Animals were treated orally with FUM (25, 50 or 100 mg/kg) and compared to the positive control (treated NAC, 20 mg/kg) and negative control (treated with 0.9% saline solution, 7.5 mL/kg). Thirty minutes after treatment, an intrapleural injection of 1 mg/kg of lipopolysaccharide (LPS) from *Escherichia coli* 055; B5 was performed. Sixty minutes after oxidative stress induction by LPS, mice were euthanized with an overdose of anesthetic (ketamine 10%). Briefly, organs were homogenized in 50 mM phosphate buffer using a Potter-Elvehjem homogenizer. An aliquot (200 μL) was mixed with trichloroacetic acid 15% (400 μL) and centrifuged for 10 min (4000 × g). The supernatant was mixed with an equal volume of thiobarbituric acid 0.67%. This system was heated in a boiling water bath for 15 min and TBARS were determined by absorbance at 535 nm. Protein content was assessed using the Lowry assay [25] and the results were expressed as nmol of malondialdehyde (MDA) equivalent/mg of protein [26].

2.6. Statistical analysis

The results were expressed as mean and standard error. The Kolmogorov–Smirnov test was used to determine the distribution