Antinociceptive Activity of Crude Extracts and Atranorin Obtained from the Lichen Cladina dendroides (des Abb.) Ahti

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SUMMARY. The aim of this study was to isolate and characterize atranorin, one of the major constituents presents in Cladina dendroides (des Abb.) Ahti., and analyze its antinociceptive effect. The antinociceptive activity was verified in acetatic acid-induced (1%, 10 ml/Kg; i.p.) writhing test with mice. Both crude extracts (50 mg/Kg; i.p.) as well as atranorin (25 mg/Kg; i.p.) were found to have a significant activity against acetatic acid-induced nociception. The ether extract was the most active, and with the highest content of atranorin (72.4%), and low amount of fumarprotocetraric acid (27.5%). The acetone extract that contained 55.5% atranorin and 44.5% fumarprotocetraric acid also showed a remarkable antinociceptive action. The chloroform extract that presented similar content of atranorin as of ether extract, but no fumarprotocetraric acid in its composition, exerted lowest antinociceptive activity indicating that the presence of this acid in the extract seem be important to antinociceptive activity. All the extracts and atranorin exhibited low toxicity potential, and an antinociceptive effect more pronounced than aspirin (50 mg/Kg; i.p.). Taken together, the data suggest that the antinociceptive activity of crude extracts are primarily due to atranorin. A facilitator role for fumarprotocetaric acid should not be ruled out.

RESUMEN. “Actividad antinociceptiva de extractos crudos y de atranorina obtenidos a partir del liquen Cladina dendroides (des Abb.) Ahti” . El objeto de este estudio fue el aislamiento y la caracterización de atranorina, uno de los principales constituyentes presentes en Cladina dendroides (des Abb.) y analizar su efecto antinociceptivo. La actividad antinociceptiva fue verificada por medio del ensayo en ratones de las contorsiones inducidas por ácido acetático (1%, 10 ml/Kg; i.p.). Tanto los extractos crudos (50 mg/Kg; i.p.) como la atranorina (25 mg/Kg; i.p.) demostraron tener una actividad significativa contra la nocicepción inducida por ácido acetático. El extracto etéreo fue el más activo y con el mayor contenido de atranorina (72,4%) y menor cantidad de ácido fumarprotocetrarico (27,5%). El extracto acetónico contuvo 55,5% de atranorina y 44,5% de ácido fumarprotocetrarico y también mostró una destacada acción antinociceptiva. El extracto cloroformico, que presentó similar contenido de atranorina que el extracto etéreo, pero no el ácido fumarprotocetrarico en su composición, exhibió la menor actividad antinociceptiva, indicando que la presencia de este ácido en el extracto parece ser importante para que se manifieste la actividad antinociceptiva. Todos los extractos y la atranorina exhibieron bajo potencial toxicológico y un efecto antinociceptivo más pronunciado que la aspirina (50 mg/Kg; i.p.). Los datos sugieren que la actividad antinociceptiva de los extractos crudos es debida principalmente a atranorina, aunque un rol facilitador del ácido fumarprotocetrarico no debería ser descartado.

INTRODUCTION

Lichens and their secondary metabolites have attracted the interest of many researchers. Some species have been shown to contain substances with remarkable biologic activity, as: antimicrobial, mainly against Gram positive bacteria 1-3; antineoplastic acting on solid and ascitic tumors, or in culture cells 4; antiviral, that include inhibition of HIV virus replication 5,6; hypotensive and anti-inflammatory 7, and spasmylic effects 8.

Lichen substances are phenolic compounds biosynthesized through acetate polymalonate pathway. Polysaccharides are also referred to lichens. Some of them are exclusive to this taxon 9-11, and are also potent antitumor agents 12. In Brazilian northeastern area, the lichen species varies according to the environment, since the

KEY WORDS: Antinociceptive activity, Lichen substances, Atranorin, Cladina dendroides.

PALABRAS CLAVE: Actividad antinociceptiva, Atranorina, Cladina dendroides, Sustancias liquénicas.

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coastal zone (humid), until the interior of the region, where the climate is semi arid (caatinga). In these habitats several species are mentioned to be bioactive. In this study the antinociceptive property of crude extracts and atranorin obtained from Cladina dendroides (des Abb.) Ahti is described.

**MATERIALS AND METHODS**

**Lichen material**

Cladina dendroides (des Abb.) Ahti that grows on sandy soils of Savannah like vegetation ("tabuleiro/cerrado") of Alhandra County, Paraíba-Brazil, was used throughout this work. Lichen identification suggests the use of morphological and chemical characteristics (chemo-taxonmy) for ratifying the species nomination, for inclusion of the data in specific keys for the studied species. The lichen samples were identified by morphological and chemical characteristics by one of the authors (E.C.P.). The chemical identification was performed through thallus color reaction by use of KOH (20%) that evidences a deep yellow coloration in presence of atranorin. This assay was followed by microcrystal test using GE (glycerin/ acetic acid, 1:1, v/v) added to acetonic extract of the thallus. The reaction provided crystal formation in needle shape, visualized under light microscope.

The material was kept in paper bags, at room temperature (28 ± 3º C ), until pharmacological and chemical assays.

**Preparation of extracts**

Extracts were prepared by macerating lichen samples with diethyl ether. After filtration, the lichen was successively extracted with chloroform and acetone. The organic extracts were evaporated at room temperature, and kept in a desiccator for future use.

**Chemical assays**

**TLC tests**

The organic extracts were spotted in silica gel F254+366 plates (20 x 20 cm), and developed in solvent systems A (toluene/dioxan/acet acid, 180:45:5, v/v) and B (hexane/diethyl ether/formic acid, 130:80:20, v/v), according Culberson’s method. The spots were visualized under UV long and short wavelengths, and latter sprayed with H2SO4 (10%), heated at 100 ºC for 1h, for color reaction. The bands were identified by RF values and compared with the used standards (atranorin, protocetraric and fumarprotocetraric acids).

**HPLC assays**

The extracts were dissolved in methanol at 1 mg/mL and injected in a Gilson Liquid Chromatograph, coupled to an UV detector at 254 nm. The chromatography conditions were: reverse phase column C18 (250 x 4,6 mm -10 µm), flow of 2 mL/min, attenuation 6, pression 84 atm, mobile phase: methanol/water/acet acid (80:19,5:0,5, v/v), isocratically, according method of Legaz & Vicente. The lichen substances were identified, and their amounts were determined by retention time and peak area, respectively. The obtained values were compared with the used standards (atranorin, protocetraric and fumarprotocetraric acids).

**Isolation and purification of atranorin**

The major compound of C. dendroides, atranorin, was isolated by soxhlet extraction with diethyl ether, during 48 h at 40 ºC. The extract was evaporated and resuspended in chloroform and heated at 60 ºC for 4 h. The concentrated material was precipitated with cold ethanol during 24 h at 4 ºC. The isolated material was crystallized several times until purity.

**Pharmacological assays**

The DL50 assays were realized in two stages: the preliminary and definitive assays. In the preliminary test it was determined the highest applied dose that did not kill any animal (D1), and the lowest one capable of kill all of them (D2). Five groups of mice (Mus musculus Swiss; 25 -30 g) with four animals each were used. Doses up 100 to 800 mg/Kg for crude extracts, and 100 to 500 mg/Kg for atranorin, dissolved in vehicle (saline 0,9% and Tween 80), were administrated intraperitoneally. The control groups received vehicle (5mL/Kg). The definitive assays were carried out with doses between D1 and D2, in four groups with 5 mice in each. The different used doses were calculated in a geometric progression in a rate 1,5. All the animals were observed for 48 h after crude extracts and atranorin administration, for detecting sings of toxicity. The DL50 was calculated as described by Berlion, using the following formulae: $DL_{50} = Df - \Sigma (a \times b)/n$, where (a) is a difference between two consecutive doses; (b) is an average of death per group, between two used doses; (n) the number of tested animals in each group, and (Df) is the lowest applied dose capable of killing all animals.
Analgesic assays
The analgesic activity was evaluated in writhing test induced by acetic acid, as described by Koster 19. Writhing were induced in mice (20-30 g; n = 7/group) by 1% acetic acid (10 ml/Kg, i.p.) 30 min after administration of the organic extracts (50 mg/Kg; i.p.) or atranorin (25 mg/Kg; i.p.) dissolved in vehicle (saline 0,9% and Tween 80). The control groups received vehicle (5 ml/Kg; i.p.), or Aspirin (50 mg/Kg; i.p.), dissolved in vehicle. Previous assays with crude extract alone (50 mg/Kg; i.p.) were carried out. Writhing scores were noted three minutes after acetic acid injection, over a period of 10 min.

Statistical analysis
Data were expressed as the means (± SE) and analyzed using student’s t test. Differences were accepted as significant P < 0,05.

RESULTS AND DISCUSSION
The lichen Cladina dendroides (des Abb.) Ahti was also described as Cladonia sandestedei f. dendroides (des Abb.) Ahti, and Cladonia dendroides (des Abb.) Ahti 14. Its chemical composition shows the atranorin as the main compound, with low content of fumarprotocetraric and protocetraric acids, and the substance Cph-2, also referred as confumarprotocetraric acid 14. In addition to these phenolics, Vicente et al. 20 reported the presence of orselinic acid and methyl β-orcinol carboxilate, and an unknown compound to this species, whose samples were collected in Paraíba (Brazil). Legaz et al. 21 detected the usnic acid in C. dendroides from the same area, being this species considered as non-producer of this substance. On the other hand, Pereira 22 reported a seasonal influence on metabolites production by Cladoniaceae species, considering the dry season (summer) the most productive one.

The samples analyzed in this work have been obtained from the same area, and the TLC assays showed the presence of atranorin and the fumarprotocetraric acid in both ether and acetone extracts. Only in the later extract the protocetraric acid was present, probably due to its high polarity, and low amounts of this compound was found in the lichen thallus (Fig. 1a, b). In the same chromatogram was possible to observe four additional spots. According to the literature, these spots may be the other substances mentioned by Vicente et al. 20. However, Pereira 23 report that immobilized cells of C. dendroides produce besides atranorin, derivatives of the fumarprotocetraric acid, as hypoprotocetraric acid and its aldehyde. This fact is common in species that produce fumarprotocetraric acid, since in the metabolic pathway of this substance the mentioned compounds are synthesized in a posterior step of atranorin production 24. The HPLC chromatograms (Fig. 2) confirms the TLC data registering the occurrence of atranorin (Fig. 3) in all extracts, RT 10.6 min to 11.1 min, and fumarprotocetraric acid, RT 2.1 min to 2.4 min, but with different contents due to the solvent capacity of extracting.
The remarkable analgesic activity of ether extract (94%) coincides with the high content of atranorin (72.4%) in its composition. On the other hand, this extract also has 27.5% of fumarprotocetraric acid. The chloroformic extract has similar content of atranorin as the ether extract, but has no fumarprotocetraric acid in its composition and its analgesic activity was the lowest one (57%). However the acetone extract present the lowest amount of atranorin (55.5%) and the activity. At the same dose the chloroformic and acetone extracts were less active. No writhing was registered when the crude extracts or atranorin alone were injected intraperitoneally to mice.

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<table>
<thead>
<tr>
<th>Standards and Extracts</th>
<th>Substances</th>
<th>Retention Time (min)</th>
<th>Area</th>
<th>Concentration (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standards</td>
<td>FUM</td>
<td>2.133</td>
<td>570</td>
<td>100.00</td>
</tr>
<tr>
<td></td>
<td>ATR</td>
<td>10.883</td>
<td>550</td>
<td>100.00</td>
</tr>
<tr>
<td>Ether</td>
<td>FUM</td>
<td>2.483</td>
<td>235.84</td>
<td>27.5</td>
</tr>
<tr>
<td></td>
<td>ATR</td>
<td>10.666</td>
<td>582.26</td>
<td>72.2</td>
</tr>
<tr>
<td>Chloroform</td>
<td>FUM</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>ATR</td>
<td>10.800</td>
<td>401.25</td>
<td>72.2</td>
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<tr>
<td>Acetone</td>
<td>FUM</td>
<td>2.216</td>
<td>196.16</td>
<td>44.5</td>
</tr>
<tr>
<td></td>
<td>ATR</td>
<td>11.116</td>
<td>231.06</td>
<td>55.5</td>
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Table 1. Liquid chromatography data of organic extracts from Cladina dendroides (des Abb.) Ahti. FUM: fumarprotocetraric acid standard. ATR: atranorin

<table>
<thead>
<tr>
<th>Test Substances</th>
<th>Dose (mg/Kg; i.p.)</th>
<th>N° of writhing movements (mean ± SE)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
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<tr>
<td>Control (Vehicle)</td>
<td>10 ml</td>
<td>29 ± 1.0*</td>
<td>-</td>
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<tr>
<td>AAS</td>
<td>50</td>
<td>9.2 ± 0.8*</td>
<td>68</td>
</tr>
<tr>
<td>Ether extract</td>
<td>50</td>
<td>1.8 ± 0.4*</td>
<td>94</td>
</tr>
<tr>
<td>Chloroform extract</td>
<td>50</td>
<td>12.2 ± 1.6*</td>
<td>57</td>
</tr>
<tr>
<td>Acetone extract</td>
<td>50</td>
<td>3.8 ± 0.8*</td>
<td>87</td>
</tr>
<tr>
<td>Atranorin</td>
<td>25</td>
<td>3.8 ± 1.3*</td>
<td>87</td>
</tr>
</tbody>
</table>

Table 2. Effect of organic extracts (50 mg/Kg; i.p.) and atranorin (25 mg/Kg; i.p.) obtained from Cladina dendroides (des Abb.) Ahti, on the writhing response induced by 1% acetic acid (10 ml/Kg; i.p.) in mice. n = seven animals for group, * p<0.05.
highest of fumarprotocetraric acid (44.5%). Its activity was similar to that of purified atranorin (87%), and identical to ether extract. This substance showed similar activity to the acetone extract. For clarifying the possibility of a facilitator role of fumarprotocetraric acid upon the antinociceptive effect of atranorin, it should be useful to test the effects of each mentioned substances, alone and in combination. Another interesting aspect observed was that crude extracts and atranorin exhibited higher effect than aspirin, the standard drug.

The data on DL50 of crude extracts and atranorin from C. dendroides are interesting. The ether extract showed no toxicity up to a dose of 800 mg/Kg, as well as atranorin, tested up to 500 mg/Kg, that is almost 20 times the effective doses in the antinociceptive assays (25 mg/Kg).

The animals treated with the highest doses of chloroformic and acetone extracts showed, in the first 15 min, clinical signs as piloerection, stereotypy and tachycardia, after by 30 min all of these were subsided, but the animals became drowsy, indicating a probable depressor effect on central nervous system. At doses higher to 800 mg/Kg the depressive signs were more intense, leading to animal mortality (Table 3).

<table>
<thead>
<tr>
<th>Extracts</th>
<th>Dose (mg/Kg; i.p)</th>
<th>(a)</th>
<th>Deaths/group after 48h</th>
<th>(b)</th>
<th>LD50 = Df - Σ(a × b)/n</th>
</tr>
</thead>
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<tr>
<td>Chloroform</td>
<td>375</td>
<td>0/5</td>
<td></td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>562.5</td>
<td>1/5</td>
<td></td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>843.4</td>
<td>4/5</td>
<td></td>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1265.60</td>
<td>5/5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetone</td>
<td>300</td>
<td>0/5</td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>450</td>
<td>2/5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>675</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1012.50</td>
<td>5/5</td>
<td></td>
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</table>

Table. Determination of DL50 of chloroformic and acetonic extracts from Cladina dendroides (des Abb.) Ahti. (a) Diff. between two consecutive doses; (b) average of death/group between 2 doses; (n) the number of tested animals in each group, and (Df) is the lowest applied dose capable of killing all animals. * Standard atranorin was tested up to 100 to 500 mg/kg; i.p., and no toxicity was detected.

In conclusion, the data in this study indicate that crude extracts and atranorin obtained from C. dendroides were able to inhibit the abdominal constriction response (writhing movements) induced by i.p. injection of acetic acid. The mechanism whereby the compounds produce this effect is unclear. Our data do not allow reaching a final conclusion whether the reduced writhing movements result of the sedation or change of motor function observed at toxicity study, or to peripheral mechanism. Further studies, however, are needed to elicit the mechanism(s) involved in the prevention of nociception sensitization, and the part played by atranorin and other substances present in this lichen.

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