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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 acetic 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 acetic 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 fumarprotocetraric 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 acético (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 acético. El extracto etéreo fue el más activo y con el mayor contenido de atranorina (72,4%) y menor cantidad de ácido fumarprotocetrárico (27,5%). El extracto acetónico contuvo 55,5% de atranorina y 44,5% de ácido fumarprotocetrárico y también mostró una destacada acción antinociceptiva. El extracto clorofórmico, que presentó similar contenido de atranorina que el extracto etéreo, pero no ácido fumarprotocetrárico en su composición, exhibió la menor actividad antinociceptiva, indicando que la presenecia 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 fumarprotocetrárico 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]; antineoplasic 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 spasmolytic 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[12, 13]. 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 (chemotaxonomy) for ratifying the species nomination, for inclusion of the data in specific keys for the studied species. The lichen samples were identified by morphological [14] and chemical [14,15] 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) [15], 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 $F_{254+366}$ plates (20 x 20 cm), and developed in solvent systems A (toluene/dioxan/acetic acid, 180:45:5, v/v) and B (hexane/diethyl ether/formic acid, 130:80:20, v/v), according Culberson's method [16]. The spots were visualized under UV long and short wavelengths, and latter sprayed with H_2SO_4 (10%), heated at 100 ºC for 1h, for color reaction. The bands were identified by Rf values and compared with the used standards (atranorin, fumaprotocetraric and protocetraric 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 C_{18} (250 x 4,6 mm -10 µm), flow of 2 mL/min, attenuation 6, pression 84 atm, mobile phase: methanol/water/acetic acid (80:19,5:0,5, v/v), isocratically, according method of Legaz & Vicente [17]. 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 DL_{5O} 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 (D_1), and the lowest one capable of kill all of them (D_2). 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 D_1 and D_2, in four groups with 5 mices 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 DL_{50} was calculated as described by Berlion [18], 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.

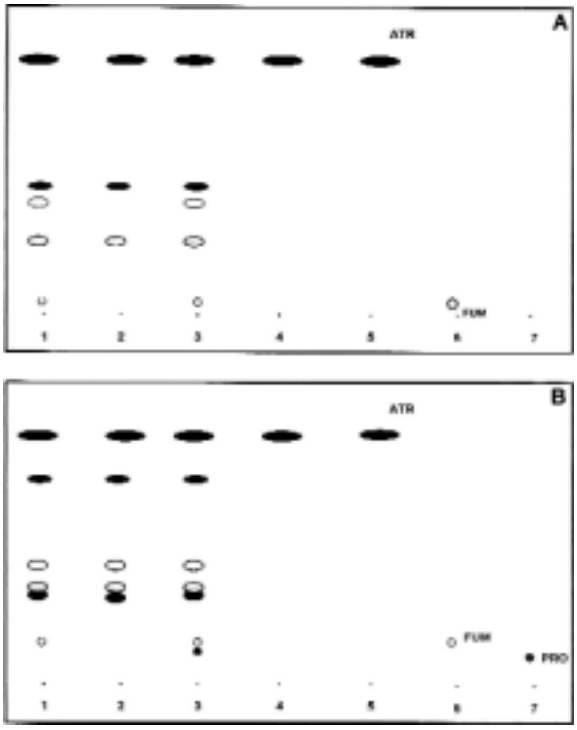

Figure 1. Thin layer chromatograms of organic extracts and purified atranorin obtained from *Cladina dendroides* (des Abb.) Ahti. (a) Substances developed in A solvent system; (b) substances developed in B solvent system. (1) Ethereous extract, (2) Chloroformic extract, (3) Acetonic extract, (4) Purified atranorin, (5) Atranorin standard, (6) Fumarprotocetraric acid standard and (7) Protocetraric acid standard.

It is apparent from the Table 1 that atranorin is present in highest content in ether and chloroform extracts (72.4% and 72.2%, respectively), but the acetone extract shown 55.5% of this compound and besides it contained 44.5% of fumarprotocetraric acid that is absent in the chloroform one. In the ether extract it is possible to

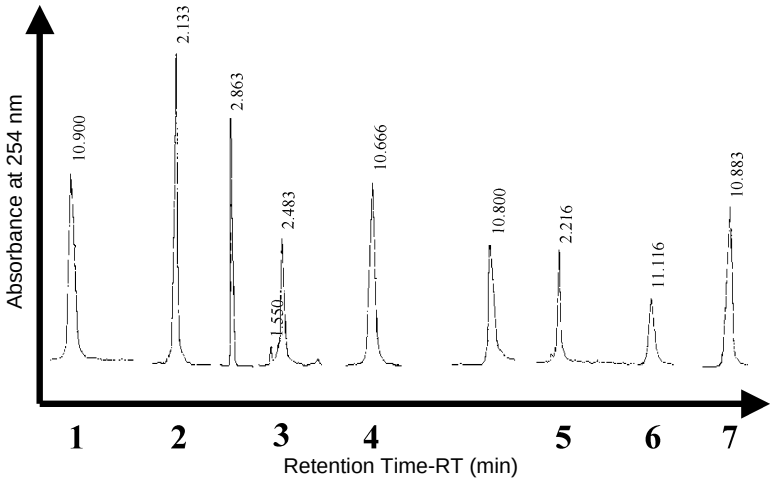

Figure 2. High performance liquid chromatograms of organic extracts and purified atranorin obtained from *Cladina dendroides* (des Abb.) Ahti.
(1) Atranorin standard
(2) Fumarprotocetraric acid standard
(3) Protocetraric acid standard
(4) Ethereous extract
(5) Chloroformic extract
(6) Acetonic extract and
(7) Purified atranorin.

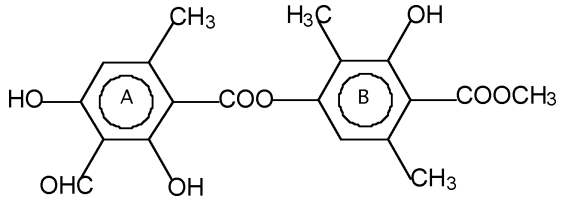

Figure 3. Structural formulae of atranorin, a lichen substance.

observe only 27.5% of this substance. The Table 2 presents the data related to antinociceptive activity of organic extracts and atranorin against acetic acid-induced nociception in mice. The ether extract exhibited a marked antinociceptive 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.

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

Standards and Extracts	Substances	Retention Time (min)	Area	Concentration (%)
Standards	FUM	2.133	570.0	100.00
	ATR	10.883	550.0	100.00
Ether	FUM	2.483	235.84	27.5
	ATR	10.666	582.26	72.4
Chloroform	FUM	-	-	-
	ATR	10.800	401.25	72.2
Acetone	FUM	2.216	196.16	44.5
	ATR	11.116	231.06	55.5

Table 1. Liquid chromatography data of organic extracts from *Cladina dendroides* (des Abb.) Ahti. FUM: fumarprotocetraric acid standard. ATR: atranorin

Test Substances	Dose (mg/Kg; i.p.)	N° of writhing movements (mean^n ± SE)	Inhibition (%)
Control (Vehicle)	10 ml	29 ± 1,0*	-
AAS	50	9,2 ± 0,8*	68
Ether extract	50	1,8 ± 0,4*	94
Chloroform extract	50	12,2 ± 1,6*	57
Acetone extract	50	3,8 ± 0,8*	87
Atranorin	25	3,8 ± 1,3*	87

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 DL_{50} 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).

Extracts	Dose (mg/Kg; i.p)	(a)	Deaths/group after 48h	(b)	LD_{50}= Df - Σ (a x b)/n
	375		0/5		
		187.5		0.5	
	562.5		1/5		
Chloroform		280.9		2.5	LD_{50} = 1265.6 - 581.38 **LD_{50} = 684.22 mg/Kg**
	843.4		4/5		
		422.2		4.5	
	1265.60		5/5		
	300		0/5		
		150		1	
Acetone	450		2/5		LD_{50} = 1012.5 - 468.7 **LD_{50} = 543.8 mg/Kg**
		225		3	
	675		4/5		
		337.5		4.5	
	1012.50		5/5		

Table. Determination of DL_{50} of chloroformic and acetonic extracts from *Cladina dendroides* (des Abb.) Ahti. (**a**) Dif. 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 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 on the prevention of nociception sensitization, and the part played by atranorin and other substances present in this lichen.

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