Purification, Physicochemical Properties, Thermal Analysis and Antinociceptive Effect of Atranorin Extracted from *Cladina kalbii*

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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; antineoplasic acting on solid and ascetic tumors, or in culture cells; antiviral; hypotensive; and spasmolytic effects. The aim of this study was to isolate and characterize atranorin, one of the major constituents which presents in *Cladina kalbii* (des Abb.) Ahti., and analyze its antinociceptive effect. The antinociceptive activity was verified in acetic acid-induced writhing test and formalin test with mice. In this work it was observed that atranorin was effective in significant reducing ($p<0.05$) abdominal writhing at doses of 200 and 400 mg/kg (*p.o.*) by 52.6 and 61.3%, respectively, when compared to control group (vehicle). The formalin test showed in 200 and 400 mg/kg (*p.o.*) that atranorin injection was able to inhibit the inflammatory processes (second phase) dose dependently.

Key words *Cladina kalbii*; antinociceptive effect; thermal analysis; atranorin

As lichens are symbiotic associations between a fungus (mycobiont) and an alga (photobiont), a number of lichens were screened for antibacterial activity in the 1940s and 1950s following the discovery of penicillin from a fungus.[1,2] Chemotaxonomic studies have shown that the most unique lichen metabolites belong to the chemical classes of depsides, depsidones and dibenzofurans. Slow growth, and often harsh living conditions, make production of protective metabolites a necessity to lichens, and many secondary constituents are believed to serve as antigrowth, antimicrobial or antiherbivore agents.[3]

In Brazilian northeastern area, the lichen species varies according to the environment, since the 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 physicochemical characterization, thermal analysis and antinociceptive property of atranorin (Fig. 1) obtained from *Cladina kalbii* (des Abb.) Ahti. is described.

MATERIALS AND METHODS

Lichen Material *Cladina kalbii* was collected in March, 2006, in the Itabaiana county, Sergipe State, northeastern Brazil (10°44′S, 37°23′W). *C. kalbii* was identified by M. P. Marcelli (Botanical Institute of São Paulo-SP, Brazil). SP 393235.

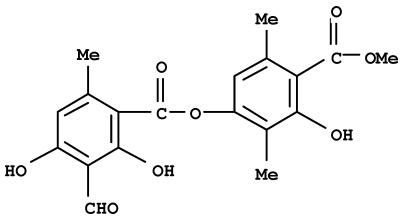

Fig. 1. Molecular Structure of the Atranorin

Extraction and Isolation of Atranorin Atranorin ($C_{19}H_{18}O_8$) was isolated from the crude extract of the lichen *C. kalbii*. The air-dried parts (100 g) of *C. kalbii* were extracted with 150 ml of chloroform using a Soxhlet apparatus to isolate atranorin. The crude extract was filtered and stored at 4 °C for 24 h to precipitate atranorin. The atranorin precipitates were collected and subjected to silica gel (70—230 mesh) column chromatography (CC) eluting with chloroform : hexanes (80 : 20) as the solvent system. At the end of this process, 840 mg of atranorin was obtained with a 0.84% (w/w) yield.

Physical–Chemical Characterization of Atranorin C, H, N (carbon, hydrogen and nitrogen) contents were determined by elemental analysis using a Perkin-Elmer analyzer (Model 2400).

IR (infrared) of atranorin was obtained at room temperature in the range 4000—400 cm^{-1} in KBr pellets using a Nicolet spectrophotometer, model Magna 550.

DSC (differential scanning calorimetry) analyses were obtained in a DSC-50 cell (Shimadzu) using aluminium crucibles with about 2 mg of samples, under dynamic nitrogen atmosphere (50 $ml \cdot min^{-1}$) and heating rate of 10 $°C \cdot min^{-1}$ in the temperature range from 25 to 600 °C. The DSC cell was calibrated with indium (mp 156.6 °C; $\Delta H_{fus.} = 28.54\ J \cdot g^{-1}$) and zinc (mp 419.6 °C). TG/DTG (thermogravimetry/derivative thermogravimetry) analyses were obtained with a thermobalance model TGA 50 (Shimadzu) in the temperature range 25—900 °C, using platinum crucibles with *ca.* 3 mg of samples, under dynamic nitrogen atmosphere (50 $ml \cdot min^{-1}$) and heating rate of 10 $°C \cdot min^{-1}$.

XRD (X-ray diffraction) patterns were obtained on a Siemens, model D5000, with tube of Cu$K\alpha$, in the range of 3 to 65° (2θ) and 1 s of pass time, using the powder X-ray diffraction method.

The NMR (nuclear magnetic resonance, as ^{13}C-, ^{1}H-NMR) spectra of atranorin was taken on a Bruker DRX500 (^{1}H: 500 MHz; ^{13}C: 125 MHz) spectrometer.

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Animals Male Swiss mice (25—30 g), with 2—3 months of age, were used throughout this study. The animals were randomly housed in appropriate cages at 22±1 °C on a 12 h light/dark cycle (lights on 06:00—18:00) with free access to food (Purina) and water. They were used in groups of 10 animals each. Experimental protocols and procedures were approved by the Federal University of Sergipe Animal Care and Use Committee (CEPA/UFS No. 55/07).

Analgesic Activity Acetic Acid-Induced Abdominal Writhing: This was carried out according to the method described previously.[4] The atranorin (100, 200, 400 mg/kg, *p.o.*) or distilled water with one drop of tween 80 0.2% (vehicle) were administered orally (*p.o.*) to mice before intraperitoneal (i.p.) injection of acetic acid (0.85% v/v in normal saline, 10 ml/kg). Acetylsalicylic acid (200 mg/kg, *p.o.*) was used as the reference drug. The number of writhes was counted for 15 min.

Formalin Test: The method used was similar to that described previously.[5,6] Twenty microlitres of 1% formalin was injected subcutaneously into the right hind paw of mice. The time (in seconds) spent in licking and biting responses of the injected paw was taken as an indicator of pain response. Responses were measured for 5 min after formalin injection (first phase) and 15—30 min after formalin injection (second phase). Atranorin (100, 200, 400 mg/kg, *p.o.*) and acetylsalicylic acid (200 mg/kg, *p.o.*) were administered 30 min before formalin injection. Control animals received the same volume of distilled water orally.

Statistical Analysis The parameters data were evaluated by one-way ANOVA (analysis of variance) followed by Dunnett's *t*-test. Differences were considered to be statistically significant when $p<0.05$. The percent of inhibition by an antinociceptive agent was determined for each experimental group using the following formula[7] and were evaluated by Fisher's Exact Test.

$$\text{inhibition } \% = 100 \cdot (\text{control} - \text{experiment})/\text{control}$$

RESULTS

Physical–Chemical Characterization of Atranorin Analytical data of C, H, N percentages (found/calculated) for atranorin were determined by elementar analysis are C: 60.94/60.96%, H: 4.71/4.85% and N: 0.00/0.01. These data are consistent with the general formula ($C_{19}H_{18}O_8$).

The IR spectrum of the atranorin showed the bands in the region IR (KBr) ν_{max} (cm^{-1}): 3450, 1770, 1730, 1650, 1580, 1260, 1145. Powder XRD patterns of atranorin is shown in Fig. 2. As can be observed the atranorin has well-defined Bragg reflections confirming its crystallinity.

^{1}H- (500 MHz, $CDCl_3$) and ^{13}C- (100 MHz, $CDCl_3$) NMR: Table 1. Its ^{1}H-NMR spectrum showed the presence of singlet signals at δ_H 2.09, 2.54, 2.68 and 3.98 corresponding to three methyl groups bonded to sp^2 carbons and one methoxyl group, respectively. This spectrum also revealed six deshielded singlet signals corresponding to two aromatic hydrogens (δ_H 6.39, 6.51), a formyl hydrogen (δ_H 10.35) and three quelated hydroxyl groups (δ_H 11.93, 12.49, 12.54). The ^{13}C-NMR spectra (Table 1) showed twelve quaternary sp^2 carbon signals, three CH (δ_C 112.86, 116.02, 193.86) and four CH_3 (δ_C 9.37, 24.00, 25.60, 52.36) consistent with the data obtained by ^{1}H-NMR analysis. The chemical shifts of quaternary sp^2 carbons [δ_C 102.83, 108.54, 110.25, 116.78, 139.88, 151.98, 152.46, 162.88, 167.48, 169.09, 169.71 (C=O), 172.21 (C=O)] were used to propose two aromatic rings sustaining five substituents and one hydrogen atom [δ_H 6.51 (s), 6.39 (s)] each.

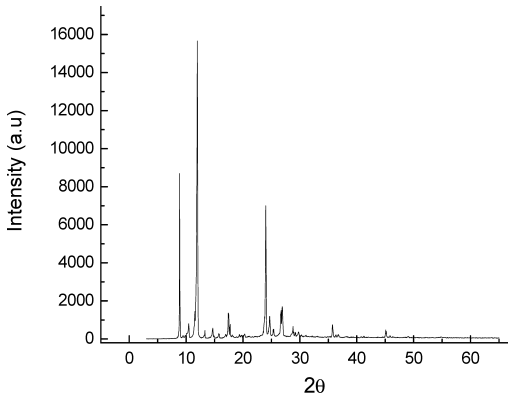

Fig. 2. X-Ray Diffraction of Atranorin

Table 1. ^{1}H- (500 MHz) and ^{13}C- (100 MHz) NMR Spectral Data of Atranorin, in $CDCl_3$

C/H	δ_C (CH_X)	δ_H (n_H, m)
1	102.83 (C)	—
2	169.09 (C)	—
3	108.54 (C)	—
4	167.48 (C)	—
5	112.86 (CH)	6.39 (1, s)
6	152.46 (C)	—
7	169.71 (C)	—
8	193.86 (CH)	10.35 (1, s)
9	25.60 (CH_3)	2.68 (3, s)
1′	151.98 (C)	—
2′	116.78 (C)	—
3′	162.88 (C)	—
4′	110.25 (C)	—
5′	139.88 (C)	—
6′	116.02 (CH)	6.51 (1, s)
7′	172.21 (C)	—
8′	9.37 (CH_3)	2.09 (3, s)
9′	24.00 (CH_3)	2.54 (3, s)
H_3C–O	52.36 (CH_3)	3.98 (3, s)
OH-2	—	12.49 (1, s)
OH-4	—	12.54 (1, s)
OH-3′	—	11.93 (1, s)

Thermal Behavior of Atranorin DSC curve for pure atranorin showed a sharp endothermic transition at 193.4 °C (on-set temperature) corresponding to the melting point of the compound (Fig. 3). An observed second event corresponds to the decomposition of the material, by means of an endothermic reaction at 248 °C. The TG/DTG curves indicate that the thermal decomposition process of UA occurs in two stages in the following temperature ranges (weight loss): 193.4—280 °C (Δm=63.4%) and 280—900 °C (Δm= 36.5%) (Fig. 3). In the first stage partial thermal decomposition of atranorin occurs with elemental carbon formation due to sample carbonization. Between 350 and 900 °C the elemental carbon is released slowly.

Acetic Acid-Induced Abdominal Writhing As shown in Table 2, atranorin was effective in significantly reducing

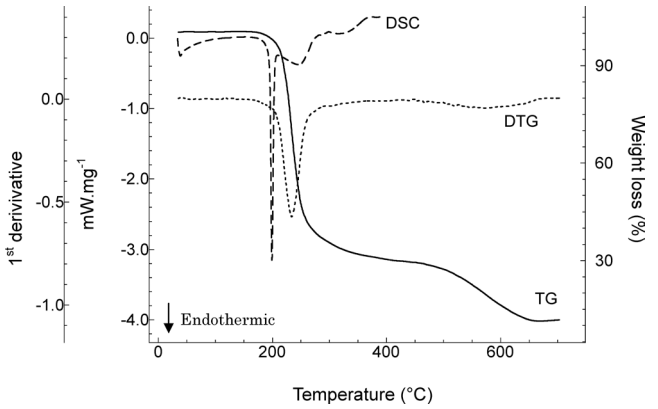

Fig. 3. DSC and TG/DTG Curves of Atranorin Recorded in Dynamic Nitrogen Atmosphere (50 ml·min[1]), and Heating Rate 10 °C·min^{-1}

Table 2. Effect of Atranorin or Acetylsalicylic Acid on Acetic Acid-Induced Writhing in Mice

Group	Dose (mg/kg)	No. of writhings[a] (per 15 min)	% Inhibition
Vehicle	—	19.4±3.9	—
Atranorin	100	16.8±3.7	13.4
Atranorin	200	9.2±2.9*	52.6***
Atranorin	400	7.5±4.3*	61.3#
Acetylsalicylic acid	200	1.8±0.9**	90.7#

$n=10$. *a*) Values represent mean±S.E.M. * $p<0.05$ (one-way ANOVA and Dunnett's test), significantly different from control. ** $p<0.01$ (one-way ANOVA and Dunnett's test), significantly different from control. *** $p<0.05$ (Fisher's test), significantly different from control. # $p<0.01$ (Fisher's test), significantly different from control.

Table 3. Effect of Atranorin or Acetylsalicylic Acid on Formalin-Induced Pain in Mice

Group	Dose (mg/kg)	0—5 min[a]	% Inhibition	15—30 min[a]	% Inhibition
Vehicle	—	47.6±17.8	—	70.8±15.3	—
Atranorin	100	41.5±17.3	12.8	42.1±21.7	40.5***
Atranorin	200	44.2±15.9	7.1	35.2±14.5*	50.3#
Atranorin	400	42.3±21.2	11.1	24.9±16.9**	64.8#
Acetylsalicylic acid	200	39.3±14.7	17.4***	21.3±12.8**	69.9#

$n=10$. [a] Values represent mean±S.E.M. * $p<0.05$ (one-way ANOVA and Dunnett's test), significantly different from control. ** $p<0.01$ (one-way ANOVA and Dunnett's test), significantly different from control. *** $p<0.05$ (Fisher's test), significantly different from control. # $p<0.01$ (Fisher's test), significantly different from control.

($p<0.05$, Dunnett's test) abdominal writhing at doses of 200 and 400 mg/kg (*p.o.*) by 52.6% and 61.3%, respectively, when compared to control group (vehicle).

Formalin Test The oral administration of the atranorin, all doses, was infective in alters first phase of formalin test. However, atranorin in doses 200 and 400 mg/kg, (*p.o.*) exhibited greater effects on the second phase of the nociceptive response when compared to control group (vehicle). Therefore, significantly decreased of pain stimuli ($p<0.01$) by 50.3% and 64.8% on second phase, respectively. Acetylsalicylic acid (200 mg/kg, *p.o.*) significantly reduced the licking time in second phase (Table 3).

The analgesic effect of the atranorin was evaluated using the acetic acid-induced writhing and the formalin (first and second phases) tests. Both tests are proven to be effective in evaluating the antinociceptive activity.[8)]

Atranorin (200, 400 mg/kg, *p.o.*) inhibited acetic acid-induced writhing in mice hence it can be suggested that the analgesic effect of the extract is also peripherally mediated.[9)] This method is not only simple and reliable but also affords rapid evaluation of peripheral type of analgesic action. However, in acetic acid-induced abdominal writhing, pain is elicited by the injection of an irritant such as acetic acid into the peritoneal cavity which produces episodes of characteristic stretching (writhing) movements and inhibition of number of episodes by analgesics is easily quantifiable.[10)] Furthermore, these results support the hypothesis of atranorin participation in the inhibition of prostaglandin synthesis since the nociceptive mechanism of abdominal writhing induced by acetic acid involves the process or release of arachidonic acid metabolites *via* COX (cyclooxygenase), and prostaglandin biosynthesis.[11)]

The formalin test is believed to represent a significant model of clinical pain.[8)] The first phase of the formalin test is probably due to direct chemical stimulation of nociceptors.[5,8)] Experimental data indicate that formalin also evokes activity in C fibres in the first phase.[12)] On the other hand, peripheral inflammatory processes are involved in the second phase.[8)] We showed that 200 and 400 mg/kg (*p.o.*) of atranorin injection was able to inhibit the inflammatory processes (second phase) dose dependently. Since the atranorin's effect was on inflammatory pain, it meant its possible site and mechanism of action was inhibition of inflammatory mediators notably prostaglandins synthesis as well as receptor blockade.[13)]

Based on the results of this study we came to the conclusion that atranorin has consistently shown to act peripherally on inflammatory mediators especially prostaglandins. The blockade of phase 2 of formalin test was typical of substances, which antagonize cyclooxygenase—an enzyme which produces prostaglandins responsible for the genesis of fever and inflammation.[14)]

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