

Antinociceptive Activity of Atranorin in Mice Orofacial Nociception Tests

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Physicochemical characterization and antinociceptive and anti-inflammatory activities of atranorin (AT) extracted from *Cladina kalbii* Ahti in formalin- and capsaicin-induced orofacial pain and anti-inflammatory tests in rodents were studied. Physicochemical characterization showed that AT has the general formula C₁₉H₁₈O₈. Male Swiss mice were pretreated with AT (100, 200, and 400 mg/kg, i.p.), morphine (3 mg/kg, i.p.), or vehicle (0.9% saline with two drops of 0.2% Tween 80) before formalin (20 µl, 2%) or capsaicin (20 µl, 2.5 µg) were injected into the right vibrissa. Our results showed that i.p. treatment with AT displayed marked inhibitory effects in different orofacial pain tests in mice. AT (400 mg/kg, i.p.) was effective in reducing the nociceptive face-rubbing behavioural response in both phases of the formalin test, which was also naloxone-sensitive. Additionally, AT produced a significant antinociceptive effect at all doses in the capsaicin test. Such results were unlikely to be provoked by motor abnormality, since AT-treated mice exhibited no performance alteration on the rota rod apparatus. AT exhibited significant anti-inflammatory activity in the acute model of inflammation (leukocyte migration to the peritoneal cavity), carrageenan- and arachidonic acid-induced hind paw edema in rats. Additionally, AT exhibited a dose-dependent antioxidant activity *in vitro*, as assessed by total radical-trapping antioxidant parameter and total antioxidant reactivity assays. All these findings suggest that AT might represent an important tool for the management of orofacial pain and/or inflammatory disorders.

Key words: Antioxidant, Atranorin, Nociception, Orofacial Pain

Introduction

The orofacial region is one of the most densely innervated areas of the body, and the trigeminal nerve plays an important role in this process. Orofacial pain focuses some of the most common acute pains, *i.e.*, those accompanying the pathologic states of the teeth and related structures. It is also the site of frequent chronic post-herpetic neuralgia, migraine, and referred pains (Pelissier *et al.*, 2002; Raboisson and Dallel, 2004).

A wide variety of drugs are indicated to treat orofacial pain, which include nonsteroidal anti-inflammatory drugs (NSAIDs), muscle relaxants, opioids, and antidepressants. However, side-effects and toxicity are connected with long-term administration (Dionne, 2001). In recent years, there has been a constant search for alternative drugs that are more efficient and safe in reducing inflammatory and neuropathic pain, aiming to halt the transition from acute to chronic pain (Holanda Pinto *et al.*, 2008). In this regard,

natural products have been demonstrated to be an important source of new drugs with relevant biological activity (Almeida *et al.*, 2001; Quintans-Júnior *et al.*, 2008).

Lichens have been used for medicinal purposes throughout the ages (Vartia, 1973; Ingólfssdóttir *et al.*, 1998). In the Brazilian northeastern area, there is a variation of occurrence of lichen species according to the shift from the coastal zone (humid) to the inner part of the country, where the climate is semiarid (an ecosystem known as “caatinga”). In these habitats several lichen species were mentioned to be bioactive (Maia *et al.*, 2002). Atranorin (AT), an important lichen metabolite, has been used for medicinal, perfumery, cosmetic as well as ecological applications (Hiserodt *et al.*, 2000; Ingólfssdóttir, 2002). A recent study suggested a possible antinociceptive effect of AT in unspecific tests (Melo *et al.*, 2008).

Therefore, the aim of the present study was to perform a physicochemical and redox characterization, and to investigate the antinociceptive and anti-inflammatory activities of AT extracted from *Cladina kalbii* Ahti in formalin and capsaicin-induced orofacial nociception and in inflammatory experimental protocols.

Materials and Methods

Lichen material and extraction and isolation of atranorin

Cladina kalbii was collected in March 2006 in the Itabaiana Country, 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). AT was isolated from *C. kalbii* as previously described (Melo *et al.*, 2008).

Physicochemical characterization of atranorin

Carbon, hydrogen and nitrogen (C, H, N) contents were determined by elemental analysis using a Perkin-Elmer analyzer (Model 2400).

Differential scanning calorimetry (DSC) analyses were obtained in a DSC-50 cell (Shimadzu) using aluminium crucibles with about 2 mg of samples, under dynamic nitrogen atmosphere (50 ml min⁻¹) and heating rate of 10 °C min⁻¹ in the temperature range from 25 to 600 °C. The DSC cell was calibrated with indium (m.p. 156.6 °C; ΔH_{fus} = 28.54 J g⁻¹) and zinc (m.p. 419.6 °C).

The nuclear magnetic resonance (NMR) spectra of AT were taken on a Bruker DRX500 (¹H, 500 MHz; ¹³C, 125 MHz) spectrometer.

The infrared (IR) absorption spectrum of AT was obtained at room temperature in the range of 4000–400 cm⁻¹ in KBr pellets using a Nicolet spectrophotometer (model Magna 550).

Animals assays

Male Swiss mice (28–33 g) were used for all experiments. Animals were housed under conditions of controlled temperature [(25 ± 1) °C] and lighting (light on: 6 am to 6 pm) and had free access to food and water. All procedures described in the present study were approved by the Animal Research Ethics Committee of the Federal University of Sergipe (CEPA/UFS # 55/07) and were in compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication 85–23, revised 1996; <http://www.nap.edu/readingroom/books/labrats/index.html>).

Formalin test

Orofacial nociception was induced in mice by subcutaneous (s.c.) injection of 20 µl of 2% formalin into the right upper lip (perinasal area), using a 27-gauge needle (Luccarini *et al.*, 2006). This volume and content of formalin was selected from our pilot studies that showed a pain-related biphasic behavioural response (face-rubbing) of greater intensity at periods of 0–5 min (first phase) and 15–40 min (second phase), following its injection into the perinasal area. Nociception was quantified at these time periods by measuring the time period that the animals spent face-rubbing the injected area with its forepaws or hindpaws. To assess the effects of test drugs, groups of different mice (*n* = 8) were pretreated systemically with vehicle (0.9% saline with two drops of 0.2% Tween 80, the solvent for AT) or AT [100, 200, and 400 mg/kg, intraperitoneally (i.p.)], 0.5 h before the local injection of formalin. Morphine (MOR, 3 mg/kg, i.p.), administered 0.5 h before the algogen, was included as a positive control. In separate groups, possible involvement of the opioid mechanism in the antinociception was assessed produced by AT (400 mg/kg, i.p.) or MOR with naloxone (NAL, 1.5 mg/kg, i.p.), an opioid antagonist, injected simultaneously. Trying to have

a rational management of animals, NAL was used only in this experiment.

Capsaicin test

Orofacial pain was induced by capsaicin as described by Pellisier *et al.* (2002) and Holanda Pinto *et al.* (2008), with alterations. Mice ($n = 8$) were injected with capsaicin (20 μ l, 2.5 μ g) subcutaneously into the right upper lip (perinasal area), using a 27-gauge needle. Capsaicin was dissolved in dimethyl sulfoxide and distilled water (2:8). Vehicle of capsaicin was evaluated and not demonstrated to induce nociceptive behaviour (data not shown). In pilot studies, mice manifested pain-related face-rubbing nociceptive behaviour immediately following the injection of capsaicin with a higher intensity after a 10–20 min period. Therefore, nociception quantification was performed after this time period by measuring the time period the animals spent face-rubbing the injected area with the forepaws or hindpaws. AT was administered in doses of 100, 200, and 400 mg/kg (i.p.). The reference drug MOR (3 mg/kg, i.p.) was diluted in vehicle (0.9% saline with two drops of 0.2% Tween 80) and administered intraperitoneally to different groups of mice 0.5 h before the local injection of capsaicin.

Rota rod test

To evaluate the possible nonspecific muscle-relaxant or sedative effects of AT, mice were submitted to the rota rod apparatus (Vaz *et al.*, 1996). Rota rod (AVS®, São Paulo, Brazil) consisted of a bar with a diameter of 4 cm, subdivided into six compartments. The bar rotated at a constant speed of seven revolutions per minute. The animals were selected 24 h previously by eliminating those mice which did not remain on the bar for two consecutive periods of 180 s. Animals were treated with diazepam (DZP, 1.5 mg/kg, i.p.), AT (100, 200, and 400 mg/kg, i.p.), or vehicle and were placed on a rotating rod 0.5 h after injection. The latency to falling was measured up to 180 s. The results are expressed as the average time(s) that the animals remained on the rota rod in each group.

Leukocyte migration to the peritoneal cavity

The leukocyte migration was induced by injection of carrageenan (500 μ g/cavity, i.p., 500 μ l) into

the peritoneal cavity of mice 1 h after administration of AT (100, 200, and 400 mg/kg, i.p.), vehicle (0.9% saline with two drops of 0.2% Tween 80, the solvent for AT), or dexamethasone (DEXA, 2 mg/kg, s.c., $n = 6$) using a modified technique previously described by Bastos *et al.* (2007). Mice were euthanized by cervical dislocation 4 h after carrageenan injection, shortly after phosphate buffered saline (PBS)-containing EDTA (1 mM, i.p., 10 ml) was injected. Immediately a brief massage was done for further fluid collection, which was centrifuged (3000 rpm, 5 min) at room temperature. The supernatant was disposed and 1 ml of PBS was introduced to the precipitate. An aliquot of 10 μ l from this suspension was dissolved in 200 μ l of Turk solution, and the total number of cells was counted in a Neubauer chamber, under an optic microscope. The results were expressed as the number of leukocytes/ml. The percentage of leukocyte inhibition was calculated according to the formula: leukocyte inhibition = $(1 - T/C) \cdot 100$, where T represents the treated groups leukocyte counts, and C represents the control group leukocyte counts.

Carrageenan-induced hind paw edema in rats

The acute hind paw edema was produced by injecting 0.1 ml of carrageenan (prepared as 1% suspension in distilled water) locally into the plantar aponeurosis of the right hind paw of rats (Winter *et al.*, 1962). AT (100, 200, and 400 mg/kg, i.p.) was administered to different groups ($n = 6$), while the other groups served as negative and positive controls and received vehicle (control) and standard drug, DEXA (2 mg/kg, i.p.), respectively. AT and DEXA were administered 1 h prior to the injection of carrageenan. The rat pedal volume up to the ankle joint was measured using a plethysmometer (LE 7500, PanLab, Spain) 0 (just before) and 4 h after the injection of carrageenan. Increase in the paw edema volume was considered as the difference between 0 and 4 h.

Arachidonic acid-induced rat paw edema

Rat paw edema was induced in animal groups ($n = 6$) by subplantar injection of 0.1 ml 0.5% arachidonic acid dissolved in carbonate buffer, pH 8.5, into the right hind paw. Norhydroguaiaretic acid (NDGA, 100 mg/kg) as reference and AT (400 mg/kg) were administered intraperitoneally 60 min before arachidonic acid injection. The ede-

ma volume was measured by a plethysmometer immediately after arachidonic acid injection and at 30-min intervals thereafter for a period of 2 h (Arrigoni-Blank *et al.*, 2004).

Total radical-trapping antioxidant parameter (TRAP) and total antioxidant reactivity (TAR)

An adapted method of the TRAP assay was used to determine the capacity of AT to trap the flow of water-soluble peroxy radical produced at constant rate, through thermal decomposition of 2,2'-azobis(2-amidopropane) dihydrochloride (AAPH) (Silva *et al.*, 2007). Briefly, the reaction mixture containing 4 ml of the free radical source (10 mM AAPH) in glycine buffer (0.1 M), pH 8.6, 10 ml of the test samples at different concentrations, and 10 ml luminol (4 mM) as external probe to monitor radical production was incubated at 25 °C. Trolox (water-soluble vitamin E analogue) was used as reference peroxy radical scavenger molecule. The chemiluminescence produced was directly proportional to the radical generation and measured in the out-of-coincidence mode (Wallac 1409 DSA Liquid Scintillation Counter, Wallac Oy, Turku, Finland) as counts per minute (CPM). The TRAP of AT was evaluated for instantaneous inhibition of chemiluminescence as area under curve (AUC). The TAR was calculated as the ratio of light intensity in the absence of

samples (I_0) to light intensity right after CT addition (I) and expressed as percentage of inhibition. AUC and radical basal production were acquired by software (GraphPad Software Inc., San Diego, CA, USA) analysis of data.

Statistical analysis

All data was presented as mean $\pm$ standard error of the mean (S.E.M), and the differences between control and treated groups were evaluated by one-way analysis of variance (ANOVA) followed by Dunnett's *t* or Fisher's tests. In all cases differences were considered significant if $P < 0.05$.

The percentile of inhibition by an antinociceptive agent was determined for the acetic acid-induced writhing and formalin tests using the following formula (Reanmongkol *et al.*, 1994): inhibition (%) = $100 \cdot (\text{control} - \text{experiment}) / \text{control}$, where control is the score of pain of the vehicle and experiment of the study groups at first or second phase.

Results and Discussion

Physicochemical characterization of atranorin

Analytical data of C, H, N percentages (found/calculated) for AT determined by elemental analysis are: C, 60.94/60.96; H, 4.71/4.85; and N,

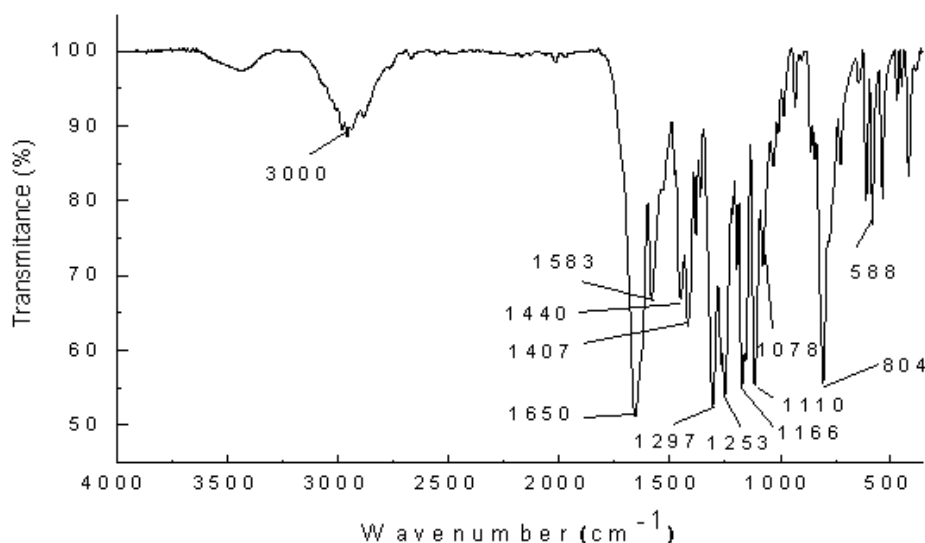
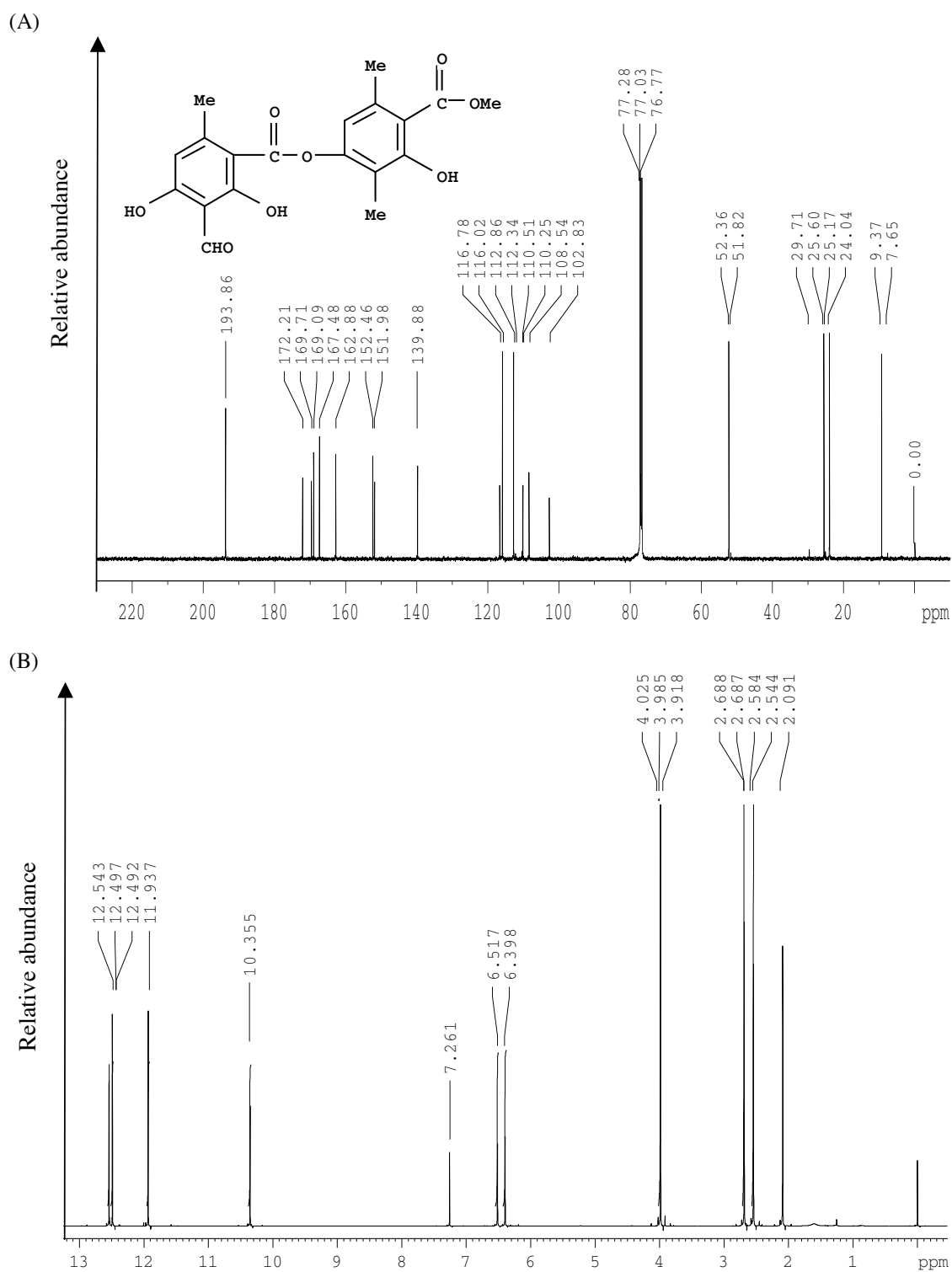


Fig. 1. Infrared spectrum of atranorin [3-formyl-2,4-dihydroxy-6-methylbenzoic acid 3-hydroxy-4-(methoxycarbonyl)-2,5-dimethylphenyl ester], in KBr.

Fig. 2. (A) ^{13}C NMR spectrum (100 MHz) and (B) ^1H NMR spectrum (500 MHz) of atranorin in CDCl_3 .

0.00/0.01. These data are consistent with the general formula $C_{19}H_{18}O_8$.

The DSC curve for pure AT showed a sharp endothermic transition at 193.4 °C (onset temperature) corresponding to the melting point of the compound and a purity of 99.64%. An observed second event corresponds to the decomposition of the material, by means of an endothermic reaction at 248 °C.

The infrared spectrum of AT is shown in Fig. 1. It shows stretching bands in the 3000 cm^{-1} wave number region assigned to $\nu(CH)$ aromatic, a $\nu(C=O)$ methyl ester band at 1650 cm^{-1} , a $\nu(C=C)$ aromatic band at 1583 cm^{-1} , bands at 1440 and 1407 cm^{-1} assigned to $\delta(CH_2)$ and $\delta(CH_3)$, a band at 1297 cm^{-1} assigned to ring stretches, a band at 1253 cm^{-1} assigned to $\nu(C-O)$ methyl ester, a band at 1166 cm^{-1} assigned to $\nu(C-O)$ carboxylic acid, $\nu(C-O)$ chain ester and $\nu(C-O)$ methyl ester bands at 1110 cm^{-1} and 1078 cm^{-1} , respectively; the bands at 804 and 588 cm^{-1} correspond to $\nu(C-O)$ chain carboxylic acid and hydrogen bonds. These data are similar with the related ones by Edwards *et al.* (2003).

The 1H (500 MHz, $CDCl_3$) and ^{13}C (100 MHz, $CDCl_3$) NMR spectra of AT are shown in Fig. 2. The 1H NMR spectrum (Fig. 2B) shows the presence of singlet signals at δ_H 2.09, 2.54, 2.68 and 3.98 ppm corresponding to three methyl groups bound to sp^2 carbon atoms and one methoxy group, respectively. This spectrum also reveals six deshielded singlet signals corresponding to two aromatic hydrogen atoms (δ_H 6.39 and 6.51 ppm), a formyl hydrogen atom (δ_H 10.35 ppm) and three

chelated hydroxy groups (δ_H 11.93, 12.49 and 12.54 ppm). The ^{13}C NMR spectrum (Fig. 2A) shows twelve quaternary sp^1 carbon signals, three CH (δ_C 112.86, 116.02 and 193.86 ppm) and four CH_3 (δ_C 9.37, 24.04, 25.60 and 52.36 ppm) signals consistent with the data obtained by 1H NMR analysis. The chemical shifts of quaternary sp^2 carbon atoms [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), and 172.21 ppm (C=O)] were used to propose two aromatic rings sustaining five substituents and one hydrogen atom [δ_H 6.51 (s) and 6.39 (s)] each. These data match those by Huneck (1996) and Ingólfssdóttir *et al.* (1998).

Biological tests

The orofacial region focuses some of the most common acute or chronic pains such as post-herpetic and trigeminal neuralgia. However, the precise mechanisms of these pains and their treatments are still poorly understood (Moalem and Tracey, 2006). Throughout history man has used many different forms of therapy for pain relief, and natural products are highlighted due to their wide popular use (Almeida *et al.*, 2001). Additionally, natural products are an important source of biological molecules with therapeutic activity (Almeida *et al.*, 2001; Quintans-Júnior *et al.*, 2008). Thus, the antinociceptive effect of AT on formalin- and capsaicin-induced orofacial pain in mice was evaluated. Our results showed that acute administration of AT (400 mg/kg, i.p.) in formalin-induced orofacial pain results in a pronounced an-

Table I. Effect of AT and MOR on formalin-induced orofacial pain-related face-rubbing response in mice.

Treatment	Dose [mg/kg]	Face rubbing			
		0–5 min		15–40 min	
		Score of pain ^a [s]	Inhibition (%)	Score of pain ^a [s]	Inhibition (%)
Vehicle	–	81.5 ± 10.1	–	104.5 ± 10.4	–
AT	100	64.1 ± 6.7	21.3	58.5 ± 4.4 ^c	44.0 ^d
AT	200	57.8 ± 11.4	29.1 ^d	32.7 ± 7.7 ^c	68.7 ^c
AT	400	49.5 ± 9.1 ^b	39.3 ^d	38.8 ± 9.1 ^c	62.9 ^c
AT + NAL	400 + 1.5	69.5 ± 9.8	14.7	112.3 ± 35.1	–7.5
MOR	3	8.2 ± 2.7 ^c	89.9 ^f	12.3 ± 4.0 ^c	88.2 ^f
MOR + NAL	1.5	72.5 ± 5.7	11.0	89.7 ± 11.3	14.2

^a Values represent means ± S.E.M; $n = 8$.

^b $P < 0.05$ (one-way ANOVA and Dunnett's test), significantly different from control.

^c $P < 0.001$ (one-way ANOVA and Dunnett's test), significantly different from control.

^d $P < 0.05$ (Fisher's test), significantly different from control.

^e $P < 0.01$ (Fisher's test), significantly different from control.

^f $P < 0.001$ (Fisher's test), significantly different from control.

Table II. Effect of AT and MOR on capsaicin-induced orofacial pain-related face-rubbing response in mice.

Treatment	Dose [mg/kg]	Face rubbing [s] ^a	Inhibition (%)
Vehicle	–	31.3 ± 2.6	–
AT	100	6.2 ± 3.6 ^c	80.2 ^c
AT	200	5.2 ± 1.2 ^c	83.4 ^c
AT	400	12.9 ± 3.3 ^b	58.8 ^d
MOR	3	3.3 ± 1.4 ^c	89.5 ^e

^a Values represent means ± S.E.M; *n* = 8.

^b *P* < 0.05 (one-way ANOVA and Dunnett's test), significantly different from control.

^c *P* < 0.001 (one-way ANOVA and Dunnett's test), significantly different from control.

^d *P* < 0.01 (Fisher's test), significantly different from control.

^e *P* < 0.001 (Fisher's test), significantly different from control.

tinociception (*P* < 0.05) as evidenced by decreased face-rubbing nociceptive behaviour in the first and second phases of the formalin test. All doses of AT significantly inhibited (*P* < 0.001) the face-rubbing behaviour at the second phase, mimicking the peripheral analgesic activity (Table I).

These results suggest that AT (400 mg/kg, i.p.) has a central analgesic effect. To confirm such effect, we tested the blocking effect of naloxone, a specific antagonist of morphinomimetic receptors (Belvisi *et al.*, 1998; Munday *et al.*, 2000) in both phases of the formalin test. The effect of AT was indeed inhibited by naloxone (Table I).

Systemic and topical NSAIDs are a mainstay of over-the-counter analgesic preparations, and cyclooxygenase (COX) enzymes (also called prostaglandin H synthase) inhibition lies in their mechanism of action (Smith *et al.*, 2000). Two human isoforms of COX have been described, COX-1 and COX-2. COX-1 is constitutively expressed in a variety of cell types and is involved in normal cellular homeostasis. COX-2 is also constitutively expressed in some tissues but is often induced during the inflammatory process (Xie *et al.*, 1991). Opioids and NSAIDs are the main agents used to relieve acute and chronic pain, such as orofacial pain (Miranda *et al.*, 2009). Melo *et al.* (2008) suggested a possible antinociceptive effect of AT to act peripherally on the inflammatory mediators, especially prostaglandins, and Bugni *et al.* (2009) have showed that part of the antinociceptive effect obtained with AT may be due to the inhibition of the COX enzymes. Additionally it was demonstrated that AT effectively inhibits the biosynthesis of leukotriene B₄ in bovine polymorphonuclear leukocytes, which could also lead to an anti-inflammatory effect (Kumar and Muller, 1999).

In another orofacial pain test, acute treatment with AT, in all doses, significantly reduced (*P* < 0.001) the face-rubbing behaviour induced by capsaicin. These treatments had a similar effect like the positive control-pretreatment with morphine (3 mg/kg, i.p.) (Table II). AT inhibited the neurogenic inflammatory pain induced by capsaicin injection into the upper lip. The inhibition by AT (100, 200, and 400 mg/kg, i.p.) in capsaicin- and in the second phase of formalin-induced facial grooming may have resulted from inhibition of Substance P release or due to a direct blocking action on its receptor neurokinin-1 (NK-1) (Holanda Pinto *et al.*, 2008).

At the rota rod test, AT-treated mice showed no significant motor performance alterations with the doses of 100, 200, or 400 mg/kg (Fig. 3). As expected, the central nervous system (CNS)-

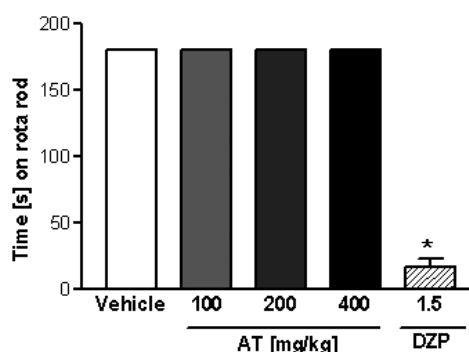


Fig. 3. Time on the rota rod observed in mice after i.p. treatment with vehicle (control), atranorin (AT, 100, 200, and 400 mg/kg), or diazepam (DZP, 1.5 mg/kg). The motor response was recorded for the following 180 s after drug treatment. Each value represents the mean ± S.E.M. of 8 animals. * *P* < 0.001 compared to control (ANOVA followed by Dunnett's test).

depressant diazepam (1.5 mg/kg, i.p.), a standard drug, reduced the time period of treated animals on the rota rod after 30 min [(17.0 ± 5.8) s]. Previous studies suggested that the CNS depression and the nonspecific muscle relaxation effect can reduce the response of motor coordination and might invalidate the results of formalin and capsaicin tests. Our results showed that AT-treated mice had no performance alteration in the rota rod test with the doses used.

Cell recruitment during inflammation depends on the orchestrated release of local mediators that are responsible for local vascular and tissue changes as well as for the recruitment of host defense cells (Luster *et al.*, 2005). The inflammation induced by carrageenan involves cell migration, plasma exudation and production of mediators, such as nitric oxide, prostaglandin E₂, interleukin (IL)-1 β , IL-6, and tumour necrosis factor (TNF)- α (Salvemini *et al.*, 1996; Loram *et al.*, 2007). These mediators are able to recruit leukocytes, such as

neutrophils, in several experimental models. Fig. 4 shows the inhibitory effect of AT on carrageenan-induced leukocyte migration to the peritoneal cavity 4 h after stimulation in all doses (31.9, 35.9, and 42.5% at doses of 100, 200, and 400 mg/kg, respectively, $P < 0.05$). The results obtained with the control group support the effect of AT since the vehicle presented no activity, and the control drug dexamethasone (2 mg/kg, s.c.) inhibited leukocyte migration (92.2%, $P < 0.001$) as compared with the control group. Since AT inhibited leukocyte migration induced by injection of carrageenan it is suggested that these activities may be inhibition of the synthesis of many inflammatory mediators whose involvement in the cell migration is well established.

As shown in Table III, AT (100, 200, and 400 mg/kg, i.p.) significantly ($P < 0.05$) reduced the mean paw edema volume 3 h after carrageenan injection. AT (200 and 400 mg/kg, i.p.) exhibited anti-inflammatory activity with the percent inhibition

Table III. Effect of AT and DEXA on carrageenan-induced hind paw edema in rats.

Treatment	Dose [mg/kg]	Mean $\pm$ S.E.M. [ml] ^a				Edema inhibition (%)
		1 h	2 h	3 h	4 h	
Vehicle	–	0.36 $\pm$ 0.04	0.49 $\pm$ 0.03	0.57 $\pm$ 0.04	0.45 $\pm$ 0.02	
AT	100	0.29 $\pm$ 0.03 ^b	0.32 $\pm$ 0.03 ^b	0.39 $\pm$ 0.04 ^b	0.36 $\pm$ 0.07	30.6 ^d
AT	200	0.25 $\pm$ 0.02 ^c	0.23 $\pm$ 0.04 ^c	0.32 $\pm$ 0.06 ^c	0.27 $\pm$ 0.02 ^c	44.9 ^e
AT	400	0.20 $\pm$ 0.02 ^c	0.20 $\pm$ 0.03 ^c	0.25 $\pm$ 0.02 ^c	0.30 $\pm$ 0.02 ^c	51.0 ^e
DEXA	2	0.10 $\pm$ 0.01 ^c	0.15 $\pm$ 0.02 ^c	0.19 $\pm$ 0.02 ^c	0.23 $\pm$ 0.04 ^c	65.3 ^e

^a Values represent means $\pm$ S.E.M.; $n = 6$.

^b $P < 0.05$ (one-way ANOVA and Dunnett's test), significantly different from control.

^c $P < 0.001$ (one-way ANOVA and Dunnett's test), significantly different from control.

^d $P < 0.05$ (Fisher's test), significantly different from control.

^e $P < 0.001$ (Fisher's test), significantly different from control.

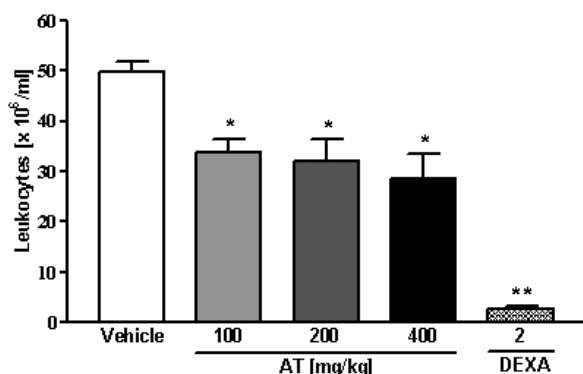


Fig. 4. Effect of atranorin on leukocyte migration into the peritoneal cavity induced by carrageenan in mice. Groups of rats were pretreated with vehicle (control group, 10 ml/kg, i.p.), dexamethasone (DEXA, 2 mg/kg, s.c.), or atranorin (AT, 100, 200, and 400 mg/kg, i.p.) 30 min before carrageenan (500 μ g/cavity, 250 μ l, i.p.)-induced peritonitis. Cell counts were performed 4 h after injection of carrageenan. Values represent the means $\pm$ S.E.M. * $P < 0.05$ and ** $P < 0.001$ when compared to control group. ANOVA followed by Dunnett's test ($n = 6$, per group).

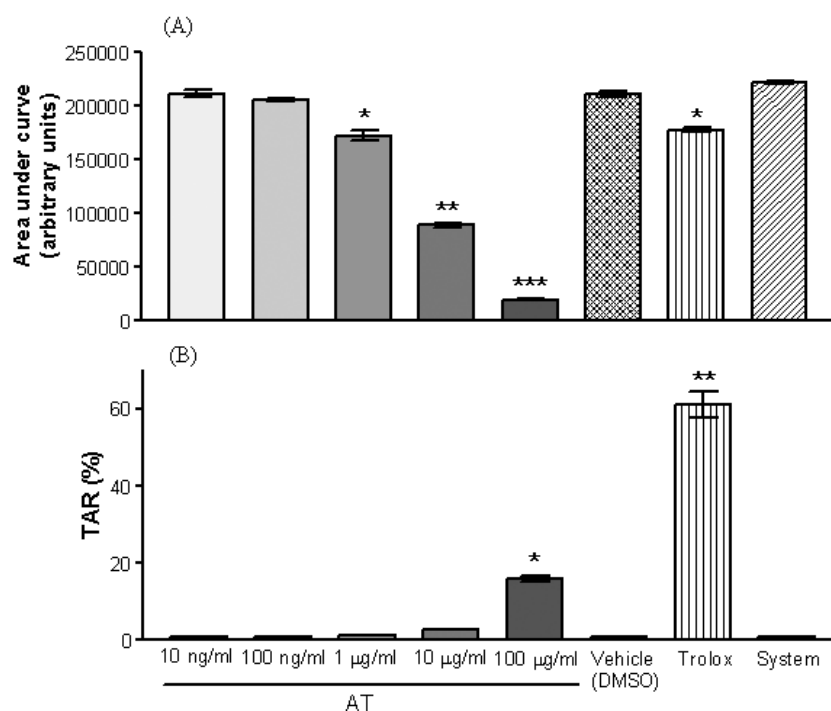


Fig. 5. *In vitro* antioxidant activity of atranorin (AT). (A) Total radical-trapping antioxidant parameter (TRAP) of atranorin at different concentrations. Bars represent area under curve (arbitrary units) generated by AAPH, a free radical source, in the presence of different concentrations of atranorin, as described in "Materials and Methods". System is the area generated during reading the luminescence by AAPH alone. Trolox is the standard antioxidant. (B) The total antioxidant reactivity (TAR) was calculated as the ratio of light intensity in the absence of samples and the light intensity right after atranorin addition and is expressed as percent of inhibition (I_0/I) using the same raw data. Values represent means $\pm$ S.E.M., $n = 5$, experiments in triplicate, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, one-way ANOVA with Duncan's *post hoc* analysis.

Table IV. Effect of AT on arachidonic acid-induced hind paw edema in rats.

Time [min]	Arachidonic acid-induced hind paw edema volume [ml] ^a		
	Vehicle	NDGA	400 mg/kg AT
30	0.31 $\pm$ 0.02	0.10 $\pm$ 0.02 ^d	0.27 $\pm$ 0.12
60	0.50 $\pm$ 0.02	0.12 $\pm$ 0.01 ^d	0.31 $\pm$ 0.03 ^c
90	0.51 $\pm$ 0.03	0.16 $\pm$ 0.02 ^d	0.33 $\pm$ 0.02 ^c
120	0.53 $\pm$ 0.03	0.25 $\pm$ 0.02 ^d	0.39 $\pm$ 0.05 ^c
% Inhibition ^b	–	65.2 ^f	28.3 ^e

^a Values represent means $\pm$ S.E.M; $n = 6$.

^b Percent inhibition of total edema response.

^c $P < 0.05$ (one-way ANOVA and Dunnett's test), significantly different from control.

^d $P < 0.001$ (one-way ANOVA and Dunnett's test), significantly different from control.

^e $P < 0.05$ (Fisher's test), significantly different from control.

^f $P < 0.001$ (Fisher's test), significantly different from control.

of paw edema of 44.9 and 51.0, respectively, as compared with the control group. However, the standard drug dexamethasone (2 mg/kg, i.p.) showed highly significant ($P < 0.001$) anti-inflammatory activity with the percent inhibition of 65.3. Additionally, AT (400 mg/kg, i.p.) reduced edema formation when administered intraperitoneally, similarly with norhydroguaiaretic acid (reference drug) (Table IV).

Various mediators are released by carrageenan in the rat paw. Thus, while the initial phase may be due to the release of histamine and serotonin, kinins may play a role in the middle phase (Di Rosa and Sorrentino, 1968) and prostaglandins could be the most important mediators in the final 3–5 h of post-carrageenan response (Vinegar *et al.*, 1969; Arrigoni-Blank *et al.*, 2004). Table III shows that AT stronger inhibited paw edema,

suggesting that the extract has a possible selective inhibitory effect on the release or actions of prostaglandins mediators. However, the paw edema induced by arachidonic acid is a widely used method for distinguishing between 5-lipoxygenase and cyclooxygenase inhibitors (Griswold *et al.*, 1987). On the basis of the present results, we may propose that AT has anti-inflammatory action interfering with prostaglandin synthesis.

In vitro antioxidant evaluation

The ability of AT to act as an antioxidant/free radical scavenger was evaluated using the TRAP/TAR assays, which are widely adopted to establish the nonenzymatic antioxidant potential of isolated substances or mixtures (Fig. 5). AT showed a dose-dependent antioxidant activity in the TRAP assay. TAR analysis demonstrated that AT exerts a significant free radical scavenger effect at the dose of 100 $\mu\text{g/ml}$. These results indicate that AT is able to act as a general antioxidant in a free radical-generating system suggesting an ability of AT to prevent against oxidative damage induced by metabolic stress or xenobiotics. These results could also suggest that biological processes medi-

ated by redox-active molecules, such as inflammation and cell division activation, could be modulated as a consequence of the interaction between AT and free radicals in biological systems.

Taken together, the results presented herein strongly suggest that AT extracted from *Cladonia kalbii* Ahti modulates neurogenic and inflammatory pain in the test models of orofacial pain induced by capsaicin and formalin through a naloxone-sensitive mechanism and redox activity. The precise mechanisms through which AT exerts its action are currently under investigation, but possibly they could be related with the arachidonic acid cascade and/or modulation of pro-inflammatory molecules production.

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