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Pharmacology, Biochemistry and Behavior 83 (2006) 90 – 99
PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR
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Antinociceptive properties of the ethanolic extract and of the triterpene 3β,6β,16β-trihidroxilup-20(29)-ene obtained from the flowers of *Combretum leprosum* in mice

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Received 10 May 2005; received in revised form 28 November 2005; accepted 9 December 2005
Available online 3 February 2006

Abstract

The present study examined the antinociceptive effects of the ethanolic extract (EE) and of the triterpene 3β,6β,16β-trihidroxilup-20(29)-ene obtained from the flowers of *Combretum leprosum* in chemical and thermal behavioural models of pain in mice. The EE (10–1000 mg/kg) given orally (p.o.), 1 h prior to testing, produced dose-dependent inhibition of acetic acid-induced visceral pain, with mean ID_{50} value of 131.9 mg/kg. In the formalin test, the EE (10–300 mg/kg, p.o.) also caused significant inhibition of both the early (neurogenic pain) and the late (inflammatory pain) phases of formalin-induced licking, however, it was more potent and efficacious in relation to the late phase of the formalin test, with mean ID_{50} values for the neurogenic and the inflammatory phases of ~300 and 88.8 mg/kg, respectively. The EE (10–1000 mg/kg, p.o.) also caused significant and dose-dependent inhibition of capsaicin- and glutamate-induced pain, with mean ID_{50} values of 160.5 and 38.3 mg/kg, respectively. Furthermore, the triterpene 3β,6β,16β-trihidroxilup-20(29)-ene (1–30 mg/kg), given p.o., 1 h prior to testing, also produced dose-related inhibition of glutamate-induced pain, with a mean ID_{50} value of 5.6 mg/kg. When assessed in a thermal model of pain, the EE (10–300 mg/kg, p.o.) and fentanyl (100 μg/kg, s.c.) caused a significant and marked increase in the latency response on the hot-plate test (50 °C). The antinociception caused by EE (100 mg/kg, p.o.) in the glutamate test was significantly attenuated by intraperitoneal (i.p.) treatment of mice with naloxone (opioid receptor antagonist, 1 mg/kg), pindolol (a 5-$HT_{1A/1B}$ receptor/β adrenoceptor antagonist, 1 mg/kg), WAY100635 (a 5-HT_{1A} receptor antagonist, 0.7 mg/kg) or ketanserin (a 5-HT_{2A} receptor antagonist, 0.3 mg/kg). In contrast, EE (100 mg/kg, p.o.) antinociception was affected neither by L-arginine (precursor of nitric oxide, 600 mg/kg) nor by ondansetron (a 5-HT_3 receptor antagonist, 0.5 mg/kg) i.p. treatment. It was not associated with non-specific effects such as muscle relaxation or sedation. Together, these results indicate that EE produces dose-related antinociception in several models of chemical and thermal pain through mechanisms that involve an interaction with opioid and serotonergic (i.e., through 5-$HT_{1A/1B}$ and 5-HT_{2A} receptors) systems.

Keywords: *Combretum leprosum*; Triterpene; Antinociception; Opioid; Nitric oxide system; 5-HT receptors; Mice

1. Introduction

The plants of the family Combretaceae comprise 20 genera with approximately 600 species, the largest of which are *Combretum*, with about 370 species, and *Terminalia*, with about 200 species (McGaw et al., 2001; Katerere et al., 2003). Species from the genus *Combretum* and to a lesser extent *Terminalia* are most widely used for medicinal purposes (McGaw et al., 2001). These genera are widespread in parts of Africa where they are often the most abundant species. It has been demonstrated that some of the extracts or active principles obtained from *Combretum* species have a broad spectrum of biological activities, including antibacterial, antiprotozoal, anticancer, cytotoxic, analgesic, anti-inflammatory, hepatoprotective and antiviral activities (Nabha et al.,

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doi:10.1016/j.pbb.2005.12.010

2000; McGaw et al., 2001; Griggs et al., 2001; Asres et al., 2001; Adnyana et al., 2001; Fyrquist et al., 2002; Ancolio et al., 2002; Ali et al., 2002; Lira et al., 2002; Olajide et al., 2003; Cirla and Mann, 2003; Nam, 2003; Young and Chaplin, 2004; Martini et al., 2004; Benssong et al., 2005).

Combretum leprosum Mart., a member of the family Combretaceae, is a plant from the north of Brazil, known by the popular name of "mufumbo" or "mofumbo" or "cipoaba" (Lira et al., 2002). *C. leprosum* is the major species of tree reported in the natural habitat of Ceará State (Facundo et al., 1993). Infusions prepared with the aerial (stems, leaves and flowers) part and roots of *C. leprosum* are used in folk medicine for the treatment of haemorrhages and as a sedative (Lira et al., 2002). In spite of the aerial (leaves and stems) parts of *C. leprosum* being used popularly, until the present moment no data exists about the possible biological activity of the flowers of this plant. However, preliminary studies conducted by Lira et al. (2002) have demonstrated that the ethanolic extract (EE) of the roots from *C. leprosum* has antinociceptive activity in two models of pain (tail immersion and formalin tests) in rats and mice.

Phytochemical studies carried out with some species belonging to the genus *Combretum* have demonstrated the occurrence of many classes of constituents, including triterpenes, flavonoids, lignans, non-protein amino acids, among others (Facundo et al., 1993; Masika and Afolayan, 2002; Katerere et al., 2003; Chowdhury and Islam, 2004).

Taking into account the biological activities of *C. leprosum*, it is surprising that no pharmacological study has been carried out on the possible antinociceptive effects of the EE from the flowers of *C. leprosum* up to now. Here, we have therefore examined the possible antinociceptive action of the EE from the flowers of *C. leprosum* in chemical and thermal models of nociception in mice. Attempts have been made to further investigate some of the possible mechanisms that underlie the antinociceptive action of *C. leprosum* extract. In addition, we also analysed the possible antinociceptive effect of the triterpene 3β,6β,16β-trihidroxilup-20(29)-ene isolated from this plant.

2. Materials and methods

2.1. Animals

Experiments were conducted using male Swiss mice (25–35 g), housed at 22±2 °C under a 12-h light/12-h dark cycle (lights on at 06:00 h) and with access to food and water ad libitum. Animals were acclimatised to the laboratory for at least 1 h before testing and were used only once throughout the experiments. The experiments were performed after approval of the protocol by the Institutional Ethics Committee and were carried out in accordance with the current guidelines for the care of laboratory animals and the ethical guidelines for investigations of experimental pain in conscious animals (Zimmermann, 1983). The numbers of animals and intensities of noxious stimuli used were the minimum necessary to demonstrate the consistent effects of the drug treatments.

2.2. Preparation of ethanolic extract, isolation and chemical identification of the active compound

Botanical material was collected in May 2001 at Viçosa, Ceará State, Brazil, and was classified by Dr. Afrânio Fernandes (Universidade Federal do Ceará, Fortaleza) as *C. leprosum* Mart. A voucher specimen of this plant was deposited in the Herbarium Prisco Bezerra of the Biology Department, Universidade Federal do Ceará, Brazil, under number 12446.

The dried flowers (2.7 kg) were powdered and extracted with ethanol (5 l), being stirred and macerated at room temperature (24±3 °C) for approximately 24 h, with this procedure being repeated three times. The solvent was fully evaporated under reduced pressure, and the extract (yield 58.3 g) was concentrated and stored in a freezer at −20 °C until use.

Part of the extract (32.0 g) was chromatographed on a silica gel column, eluted successively with hexane, $CHCl_3$, EtOAc and MeOH, respectively. The fraction eluted with $CHCl_3$ was subjected to column chromatography over silica gel and then eluted with hexane–EtOAc, with increasing polarity. A total of 3 fractions (30 mL each) eluted with hexane–EtOAc (30:70) were combined on the basis of TLC analysis and after removal of the solvent, the precipitate was observed, which, through recrystallization from Et_2O, was identified as being the triterpene 3β,6β,16β-trihidroxilup-20(29)-ene (Fig. 1), previously identified in leaves of this plant (Facundo et al., 1993).

2.3. Drugs

The following substances were used: acetic acid, formalin and morphine hydrochloride (Merck, Darmstadt, Germany); N^{ω}-nitro-L-arginine, L-arginine hydrochloride, capsaicin, L-glutamic acid hydrochloride, naloxone hydrochloride, pindolol, WAY100635 (Sigma Chemical Co., St. Louis, USA); ketanserin tartarate (Tocris Cookson Inc., Ellisville, USA); ondansetron hydrochloride (Cristália, São Paulo, Brazil). The drugs were dissolved in saline, with the exception of EE, triterpene and capsaicin that were dissolved in Tween 80/DMSO plus saline, Tween 80 plus saline and absolute ethanol. The final concentration of Tween 80, DMSO and ethanol did not exceed 5% and did not cause any "per se" effect.

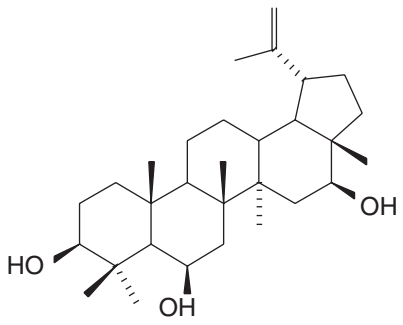

Fig. 1. Molecular structure of triterpene 3β,6β,16β-trihidroxilup-20(29)-ene.

2.4. Assessment of antinociceptive effect of EE from *C. leprosum* and triterpene

2.4.1. Formalin-induced nociception

The procedure used was essentially the same as that described previously (Santos and Calixto, 1997; Santos et al., 1999). Animals received 20 μl of a 2.5% formalin solution (0.92% formaldehyde) made up in saline, injected intraplantar (i.pl.) in the ventral surface of the right hindpaw. Animals were observed from 0 to 5 min (neurogenic phase) and 15 to 30 min (inflammatory phase) and the time spent licking the injected paw was recorded with a chronometer and considered as indicative of nociception. Animals received EE of *C. leprosum* (10–300 mg/kg, p.o.) 1 h beforehand on the basis of a previous time–response curve. Control animals received vehicle (10 ml/kg, p.o.).

2.4.2. Abdominal constriction response caused by intraperitoneal injection of acetic acid

The abdominal constrictions were induced according to procedures described previously (Santos et al., 1999) and resulted in contraction of the abdominal muscle together with a stretching of the hind limbs in response to an i.p. injection of acetic acid (0.8%) at the time of the test. Mice were pre-treated with EE of *C. leprosum* by p.o. (10–1000 mg/kg) route, 1 h before injection of the irritant. Control animals received a similar volume of vehicle (10 ml/kg). After the challenge, the mice were individually placed into glass cylinders of 20 cm diameter, and the abdominal constrictions were counted cumulatively over a period of 20 min. Antinociceptive activity was expressed as the reduction in the number of abdominal constrictions, i.e., the difference between control animals (mice pre-treated with vehicle) and animals pre-treated with EE.

2.4.3. Capsaicin-induced nociception

In an attempt to provide more direct evidence concerning its possible antinociceptive effect on neurogenic nociception, EE of *C. leprosum* was investigated in capsaicin-induced licking in the mouse paw. The procedure used was similar to that described previously (Santos and Calixto, 1997). After an adaptation period (20 min), 20 μl of capsaicin (1.6 μg/paw prepared in saline) was i.pl. injected in the ventral surface of the right hindpaw. Animals were observed individually for 5 min following capsaicin injection. The amount of time they spent licking the injected paw was recorded with a chronometer and was considered as indicative of nociception. Animals were treated with EE of *C. leprosum* (10–1000 mg/kg, p.o.) 1 h before capsaicin injection. Control animals received vehicle by p.o. (10 ml/kg) route.

2.4.4. Glutamate-induced nociception

In an attempt to provide more direct evidence concerning the interaction of EE with the glutamatergic system, we separately investigated whether or not EE was able to antagonise glutamate-induced licking of the mouse paw. The procedure used was similar to that described previously (Beirith et al., 2002). A volume of 20 μl of glutamate (10 μmol/paw prepared in saline) was i.pl. injected in the ventral surface of the right hindpaw. Animals were observed individually for 15 min following glutamate injection. The amount of time they spent licking the injected paw was recorded with a chronometer and was considered as indicative of nociception. Animals were treated with EE (10–300 mg/kg, p.o.) or triterpene (1–30 mg/kg, p.o.) 1 h before glutamate injection. Control animals received a similar volume of vehicle by p.o. (10 ml/kg) route.

2.4.5. Hot-plate test

The hot-plate test was used to measure the response latencies according to the method described by Santos et al. (1999), with minor modifications. In these experiments, the hot-plate (Ugo Basile, model-DS 37) was maintained at 50±1 or 56±1 °C. Animals were placed into a glass cylinder of 24-cm diameter on the heated surface, and the time between placement and shaking or licking of the paws, or jumping, was recorded as the index of response latency. An automatic 30-s cut-off was used to prevent tissue damage. Each animal was tested before administration of drugs in order to obtain the baseline. Animals were treated with EE (10–300 mg/kg, p.o.), fentanyl (100 μg/kg, s.c. — used as positive control) or with

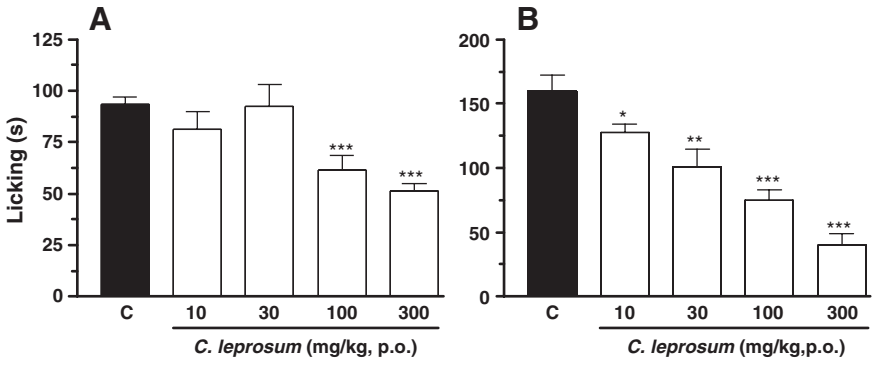

Fig. 2. Effect of EE of *C. leprosum* administered orally against formalin-induced licking (first phase, panel A, and second phase, panel B) in mice. Each column represents the mean of 8–12 animals and the error bars indicate the S.E.M. Control values (C) indicate the animals injected with vehicle and the asterisks denote the significance levels when compared with control groups (one-way ANOVA followed by Newman–Keuls test) $*P<0.05$; $**P<0.01$ and $***P<0.001$.

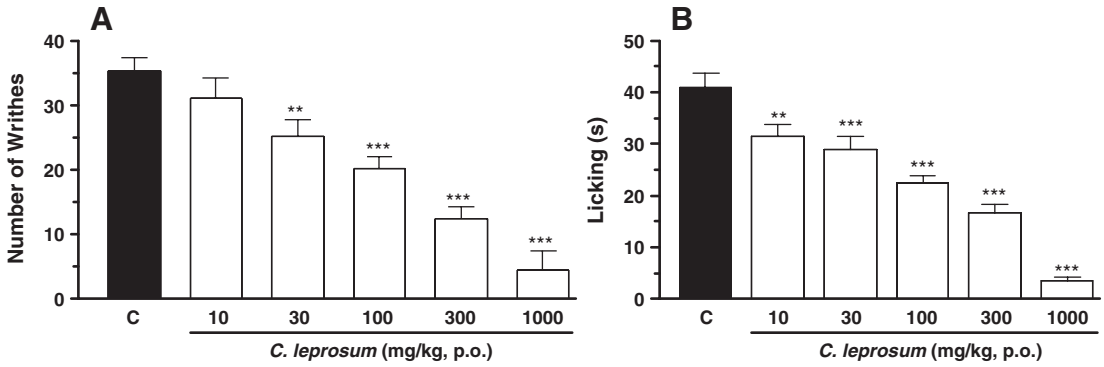

Fig. 3. Effect of the EE of *C. leprosum* administered orally against either acetic acid-induced writhing movements (A) or capsaicin-induced licking (B) in mice. Each column represents the mean of 8–12 animals and the error bars indicate the S.E.M. Control values (C) indicate the animals injected with vehicle and the asterisks denote the significance levels when compared with control groups (one-way ANOVA followed by Newman–Keuls test) $^{**}P<0.01$; $^{***}P<0.001$.

vehicle (10 ml/kg, i.p.), 1, 0.5 and 1 h before testing, respectively. The maximal percentage of the effect (MPE) of drugs-induced antinociception was calculated as follows: $\%MPE = (postdrug - predrug)/(30 - predrug) \times 100$.

2.5. Measurement of motor performance and locomotor activity

In order to evaluate the possible non-specific muscle-relaxant or sedative effects of EE of *C. leprosum*, mice were submitted to the rota-rod task (Santos et al., 1999) and open-field test (Rodrigues et al., 2002). The rota-rod apparatus (Ugo Basile, Model 7600) consisted of a bar with a diameter of 2.5 cm, subdivided into four compartments by disks 25 cm in diameter. The bar rotated at a constant speed of 22 revolutions per min. The animals were selected 24 h previously by eliminating those mice which did not remain on the bar for three consecutive periods of 60 s. Animals were treated with EE of *C. leprosum* (10–300 mg/kg, p.o.), diazepam (2 mg/kg, i.p.) or with vehicle (10 ml/kg, i.p.) 1, 0.5 and 1 h beforehand. Results are expressed as the time (s) for which animals remained on the rota-rod. Cut-off time used was 180 s.

The ambulatory behaviour was assessed in an open-field test as described previously (Rodrigues et al., 2002). The apparatus consisted of a wooden box measuring $40 \times 60 \times 50$ cm. The floor of the arena was divided into 12 equal squares, and the number of squares crossed with all paws crossing was counted in a 6-min session. Mice were treated with EE of *C. leprosum* (10–1000 mg/kg, p.o.) or vehicle (10 ml/kg, p.o.) 1 h beforehand.

2.6. Analysis of possible mechanism of action of EE

To address some of the mechanisms by which EE of *C. leprosum* causes antinociception in the glutamate-induced nociception, animals were treated with different drugs. The doses of the drugs used were selected on the basis of literature (Santos et al., 1999, 2005) data and also based on previous results from our laboratory.

2.6.1. Involvement of opioid system

To assess the possible participation of the opioid system in the antinociceptive effect of EE of *C. leprosum*, mice were pre-treated with naloxone (1 mg/kg, i.p., a non-selective opioid receptor antagonist), and after 20 min the animals received an injection of EE (100 mg/kg, p.o.), morphine (5 mg/kg, s.c.) or vehicle (10 ml/kg, p.o.). The nociceptive responses to glutamate were recorded 60, 30 and 1 h after the administration of EE, morphine, or vehicle, respectively. Another group of animals was pre-treated with vehicle and after 20 min, received

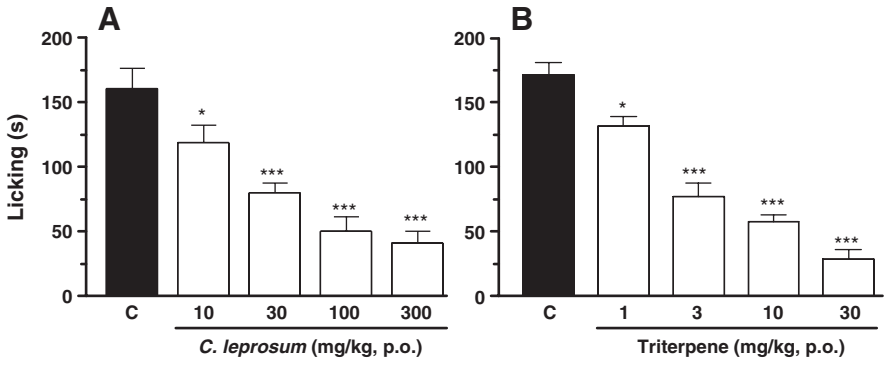

Fig. 4. Effect of the EE (A) and triterpene (B) obtained from *C. leprosum* administered orally against glutamate-induced licking in mice. Each column represents the mean of 8–12 animals and the error bars indicate the S.E.M. Control values (C) indicate the animals injected with vehicle and the asterisks denote the significance levels when compared with control groups (one-way ANOVA followed by Newman–Keuls test) $^{*}P<0.05$; $^{***}P<0.001$.

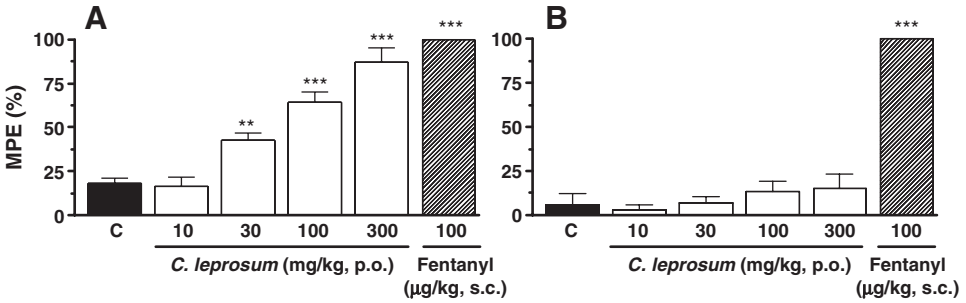

Fig. 5. Effect of EE of *C. leprosum* and fentanyl in the hot-plate test at 50 °C (panel A) and 56 °C (panel B) in mice. Each column represents the mean of 8–12 animals and the error bars indicate the S.E.M. Control values (C) indicate the animals injected with vehicle and the asterisks denote the significance levels when compared with control groups (one-way ANOVA followed by Newman–Keuls test) $^{**}P<0.01$ and $^{***}P<0.001$.

EE, morphine or vehicle, 1, 0.5 and 1 h before glutamate injection, respectively.

2.6.2. Involvement of L-arginine-nitric oxide pathway

To investigate the role played by the L-arginine-nitric oxide pathway in the antinociception caused by EE of *C. leprosum*, mice were pre-treated with L-arginine (600 mg/kg, i.p., a nitric oxide precursor) and after 20 min, they received EE (100 mg/kg, p.o.), N^{ω}-nitro-L-arginine (L-NOARG, 75 mg/kg, i.p., a nitric oxide inhibitor) or vehicle (10 ml/kg, p.o.). The nociceptive responses to glutamate were recorded 1, 0.5 and 1 h after the administration of EE, L-NOARG, or vehicle, respectively. Another group of animals was pre-treated with vehicle (10 ml/kg, i.p.) and after 20 min received EE, L-NOARG or vehicle, 1, 0.5 and 1 h before glutamate injection, respectively.

2.6.3. Involvement of serotonergic system

In order to investigate the participation of the serotonergic system in the antinociceptive action of EE of *C. leprosum*, mice were pre-treated with pindolol (1 mg/kg, i.p., a 5-$HT_{1A/1B}$ receptor/β adrenoceptor antagonist), WAY100635 (0.7 mg/kg, i.p., a selective 5-HT_{1A} receptor antagonist), ketanserin (0.3 mg/kg, i.p., a selective 5-HT_{2A} receptor antagonist), ondansetron (0.5 mg/kg, i.p., a 5-HT_3 receptor antagonist) or vehicle (10 ml/kg, i.p.) and after 20 min they received EE (100 mg/kg, p.o.) or saline injection, one hour before glutamate i.pl. injection (Santos et al., 2005). Another group of animals was pre-treated with vehicle and after 20 min they received EE or vehicle, 1 h before the glutamate injection, respectively.

2.7. Statistical analysis

The results are presented as mean+S.E.M., except the ID_{50} values (i.e., the dose of EE or triterpene reducing the nociceptive response by 50% relative to the control value), which are reported as geometric means accompanied by their respective 95% confidence limits. The ID_{50} value was determined by linear regression from individual experiments using linear regression GraphPad software (GraphPad software, San Diego, CA, USA). The statistical significance of differences between groups was detected by ANOVA followed by Newman–Keuls' test. *P*-values less than 0.05 ($P<0.05$) were considered as indicative of significance.

3. Results

3.1. Formalin-induced nociception

The results depicted in Fig. 2 (A and B) show that EE of *C. leprosum* caused significant inhibition of both neurogenic (0 to 5 min) and inflammatory (15 to 30 min) phases of formalin-induced licking. However, its antinociceptive effects were significantly ($P<0.05$) more pronounced against the second phase of this model of pain. The calculated mean ID_{50} values for these effects were: ~300 and 88.8 (59.9–131.5) mg/kg and the inhibitions observed were 45±4% and 75±6% at a dose of 300 mg/kg, respectively, for the first and second phases.

The antinociceptive effect of EE was long-lasting and significant when given by p.o. route (results not shown). EE produced marked antinociception as early as 1 h after p.o. administration, an action that remained significant up to 6 h after administration. Thus, the time point (1 h for p.o. route) of maximum effect of EE was chosen for all further studies with independent groups of animals.

3.2. Abdominal constriction response caused by intraperitoneal injection of acetic acid

The results depicted in Fig. 3A show that EE of *C. leprosum*, given by p.o. route 1 h earlier, produced dose-related inhibition of the acetic acid-induced abdominal constrictions in mice, with mean ID_{50} value (and its respective 95% confidence limits) of 131.9 (91.8–189.6) mg/kg and inhibition of 88±6% at a dose of 1000 mg/kg.

Table 1
Effect of EE of *C. leprosum* and diazepam on the motor performance (rota-rod test) and locomotor activity (open-field test) in mice

	Rota-rod (s)	Open field (number of crossing)
Control	179.0±0.8	128.8±2.5
Diazepam (2 mg/kg, i.p.)	8.1±0.5	–
EE (10 mg/kg, p.o.)	179.2±0.8	–
EE (30 mg/kg, p.o.)	178.3±1.7	–
EE (100 mg/kg, p.o.)	175.3±4.7	130.2±3.5
EE (300 mg/kg, p.o.)	177.3±1.7	99.8±19.9
EE (1000 mg/kg, p.o.)	–	132.0±1.0

Data are expressed as mean±S.E.M of 6 animals.

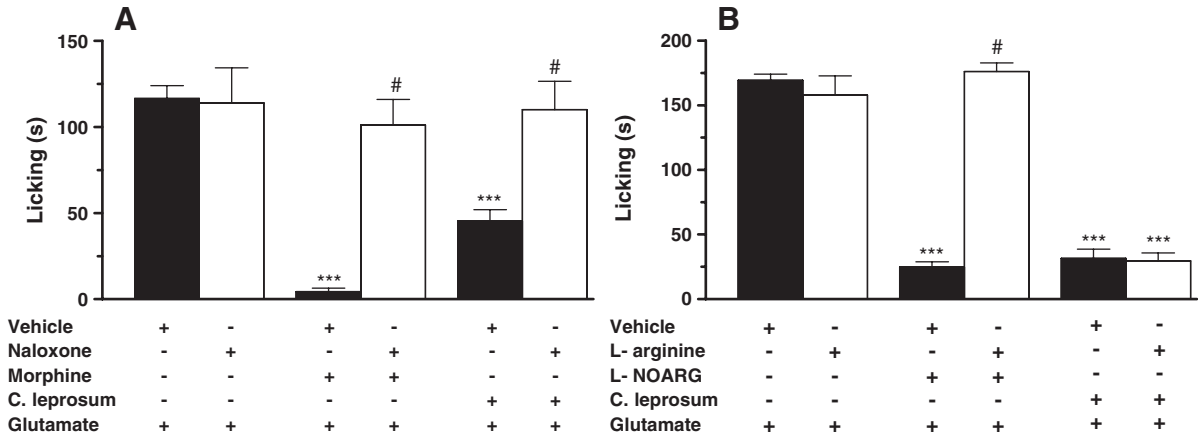

Fig. 6. Effect of pre-treatment of animals with naloxone (1 mg/kg, panel A) or L-arginine (600 mg/kg, i.p., panel B) on the antinociceptive profiles of EE of *C. leprosum* (100 mg/kg, p.o.), morphine (5 mg/kg) and L-NOARG (75 mg/kg, i.p.) against the glutamate-induced licking in mice. Each column represents the mean of 8–12 animals and the error bars indicate the S.E.M. The symbols report the significance level $^{***}P<0.01$ compared with control group (animals injected with the vehicle alone) and $^{\#}P<0.001$ compared with EE, morphine and L-NOARG treatment.

3.3. Capsaicin-induced nociception

The p.o. administration of EE of *C. leprosum* also produced dose-dependent attenuation of capsaicin-induced neurogenic pain (Fig. 3B). The mean ID_{50} value from these results was 160.5 (105.0–245.2) mg/kg with inhibition of 91±2% at a dose of 1000 mg/kg.

3.4. Glutamate-induced nociception

The results presented in Fig. 4A show that EE of *C. leprosum*, given by p.o. route, caused a significant inhibition of the glutamate-induced nociception, with a mean ID_{50} value of 38.3 (29.9–70.2) mg/kg and the peak of inhibition observed was 74±6%. Interestingly, when triterpene, isolated from *C. leprosum*, was administered p.o. to mice, it produced dose-related inhibition of glutamate-induced pain, with a mean ID_{50} value of 5.6 (4.5–6.9) mg/kg and the peak of inhibition observed was 79±6% (Fig. 4B). In addition, the triterpene had a 6.8-fold greater potency than EE when analysed in the glutamate test.

3.5. Hot-plate test

The results in Fig. 5 (A and B) show that EE of *C. leprosum*, given by p.o. route, caused a significant and dose-related increase in the pain latency in the hot-plate test at 50 °C. In contrast, EE at the same doses did not produce any significant increase in the pain latency in the hot-plate test at 56 °C. In addition, fentanyl (given s.c.) caused a significant and marked increase in the pain latency in the hot-plate test against both temperatures studied (Fig. 5 A and B).

3.6. Evaluation of motor performance and locomotor activity

The EE of *C. leprosum* given by p.o. route did not affect significantly the motor behaviour of animals when tested for motor performance on the rota-rod task and locomotor activity

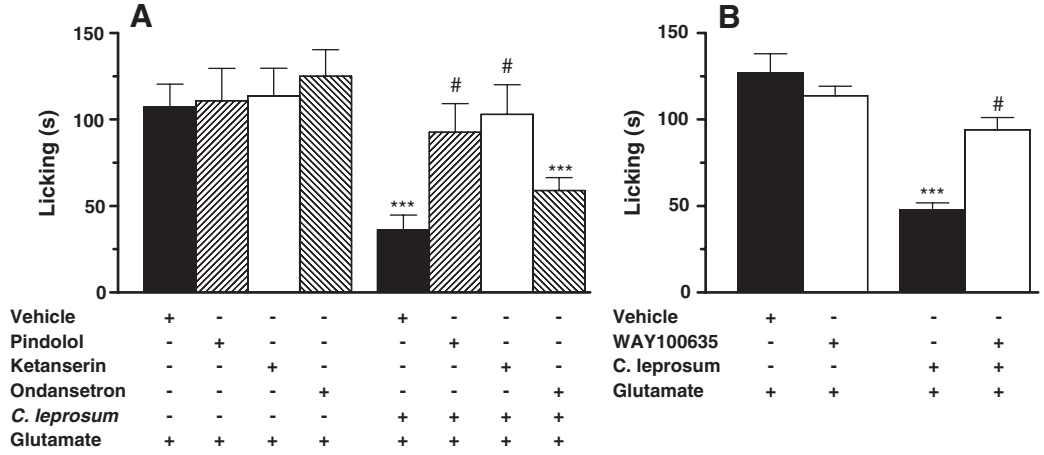

Fig. 7. Effect of pre-treatment of animals with pindolol (1 mg/kg, i.p., panel A), WAY100635 (0.7 mg/kg, i.p., panel B) or ketanserin (0.3 mg/kg, i.p., panel A) or ondansetron (0.5 mg/kg, i.p., panel A) on the antinociceptive profile of EE of *C. leprosum* (100 mg/kg, p.o.) against the glutamate-induced licking in mice. Each column represents the mean of 8–12 animals and the error bars indicate the S.E.M. The symbols report the significance level $^{***}P<0.01$ compared with control group (animals injected with the vehicle alone) and $^{\#}P<0.001$ compared with EE treatment.

in the open-field test, when compared with animals that received vehicle (control group) (Table 1). However, diazepam (2 mg/kg, i.p.) significantly ($P<0.01$) reduced the motor coordination of animals on the rota-rod test (Table 1).

3.7. Analysis of possible mechanism of action of EE

The results presented in Fig. 6A show that the pre-treatment of mice with naloxone (1 mg/kg, i.p., a non-selective opioid receptor antagonist), given 20 min beforehand, completely reversed the antinociception caused by EE of *C. leprosum* (100 mg/kg, p.o.) and morphine (5 mg/kg, s.c.) when analysed against glutamate-induced pain (Fig. 6A).

The systemic pre-treatment of mice with the nitric oxide precursor L-arginine (600 mg/kg, i.p., a nitric oxide precursor), given 20 min earlier, completely reversed the antinociception caused by L-NOARG (100 mg/kg, i.p., a nitric oxide inhibitor) when analysed against glutamate-induced nociception (Fig. 6B). Under the same conditions, L-arginine did not significantly modify the antinociception caused by EE in the glutamate test (Fig. 6B).

The results depicted in Fig. 7 (A and B) show that the previous treatment of mice with pindolol (1 mg/kg, i.p., a 5-$HT_{1A/1B}$ receptor/β-adrenoceptor antagonist), WAY100635 (0.7 mg/kg, i.p., a selective 5-HT_{1A} receptor antagonist) or ketanserin (0.3 mg/kg, i.p., a selective 5-HT_{2A} receptor antagonist), 20 min before, but not ondansetron (0.5 mg/kg, i.p.), significantly reversed the antinociception caused by EE (100 mg/kg, p.o.) against glutamate-induced pain.

4. Discussion

The present study demonstrates that systemic (p.o.) administration of EE obtained from the flowers of *C. leprosum* elicits a dose-dependent inhibition of the acetic acid-induced visceral nociceptive response in mice. The additional findings of the present work are that, (i) p.o. administration of EE also causes significant inhibition against both neurogenic and inflammatory pain responses to the i.pl. injection of formalin, and against the neurogenic pain caused by activation of vanilloid receptors by the agonist capsaicin in the mouse paw; (ii) the algesic response caused by i.pl. injection of glutamate was also greatly inhibited by EE; (iii) the treatment of mice with triterpene, isolated from *C. leprosum*, also caused a marked and dose-related inhibition of glutamate-induced pain; (iv) the antinociceptive action of EE in the glutamate test was significantly reversed by i.p. treatment of animals with naloxone, WAY100635, pindolol and ketanserin, but not by L-arginine and ondansetron; (v) EE was effective in increasing the response latency of animals in a thermal nociceptive model (hot-plate test at 50 °C); and (vi) the dose of EE that caused significant antinociception did not produce any statistically significant motor dysfunction.

A considerable number of studies have suggested that extracts or active principles obtained from *Combretum* species have a broad spectrum of biological activities, including antibacterial, antiprotozoal, anticancer, cytotoxic, analgesic, anti-inflammatory, hepatoprotective and antiviral activities (Nabha et al., 2000; McGaw et al., 2001; Griggs et al., 2001; Asres et al., 2001; Adnyana et al., 2001; Fyrquist et al., 2002; Ancolio et al., 2002; Ali et al., 2002; Lira et al., 2002; Olajide et al., 2003; Cirla and Mann, 2003; Nam, 2003; Young and Chaplin, 2004; Martini et al., 2004; Benssong et al., 2005). Regarding analgesic effects, preliminary studies have shown that extracts from the roots of *C. leprosum* are capable of reducing the nociceptive response in two experimental pain models, the formalin and tail immersion tests in rats (Lira et al., 2002). However, in spite of the fact that the extract from the roots of *C. leprosum* has been shown to possess an antinociceptive effect, the putative antinociceptive activities of the ethanolic extract obtained from the flowers of *C. leprosum* as well as its mechanisms of action have not yet been demonstrated.

In the present study, we attempted to characterise some of the mechanisms through which EE exerts its antinociceptive action in chemical and thermal models of nociception in mice. The results reported here indicate that oral administration of EE produced marked and dose-related antinociception when assessed in acetic acid-induced visceral nociception, at doses that did not produce any statistically significant motor dysfunction. To our knowledge this is the first report of this kind in the literature. The acetic acid-induced writhing reaction in mice, described as a typical model of inflammatory pain, has long been used as a screening tool for the assessment of analgesic or anti-inflammatory properties of new agents (Vinegar et al., 1979; Tjølsen and Hole, 1997). At the cellular level, protons depolarize sensory neurones by directly activating a non-selective cationic channel localized on cutaneous, visceral and other types of nocisponsive peripheral afferent C-fibres (Reeh and Kress, 2001; Julius and Basbaum, 2001). This method shows good sensitivity, as it allows for the effects of weak analgesics, but shows poor specificity because the abdominal writhing response may be suppressed by muscle relaxants and other drugs, leaving scope for the misinterpretation of results (Le Bars et al., 2001). This can be avoided by complementing the test with other models of nociception and a performance motor test. For this reason, EE of *C. leprosum* was examined for its action in the rota-rod and open-field tests. In both tests, we observed that there was no statistically significant interference in the patterns of performance or motor activity at a dose that produced marked suppression of the writhing response.

Also of interest are the results showing that EE of *C. leprosum* caused significant and dose-related antinociception when administered by p.o. route against both neurogenic (early phase) and inflammatory (late phase) pain responses caused by formalin injection in mice. These observations are in agreement with and extend previous findings (Lira et al., 2002) that demonstrated that the EE of the roots of *C. leprosum* has antinociceptive activity in the formalin test in rats and mice. The neurogenic pain is caused by direct activation of nociceptive nerve terminals, while the inflammatory pain is mediated by a combination of peripheral input and spinal cord sensitization (Tjølsen et al., 1992). It has been shown that i.pl.

injection of formalin in rodents produces a significant increase in spinal levels of different mediators, such as excitatory amino acids, PGE_2, nitric oxide, tachykinin, kinins, among other peptides (Tjølsen et al., 1992; Malmberg and Yaksh, 1995, Santos and Calixto, 1997; Santos et al., 1998). Another interesting finding of the present study is the demonstration, for the first time, that EE of *C. leprosum*, given orally, has a dose-dependent antinociceptive effect on the capsaicin-induced neurogenic paw licking response. It has been shown that capsaicin (8-methyl-N-vanillyl-6-nonenamide), the pungent algesic substance obtained from hot red chilli peppers, is a valuable pharmacological tool for studying a subset of mammalian primary sensory C-fibres and Aδ afferent neurones including polymodal nociceptors and warm thermoceptors (for review see Holzer, 1991; Jancso, 1992). In addition, it has been proposed that the capsaicin-induced nociception is brought about by the activation of the capsaicin receptor, also known as the vanilloid receptor (TRPV), termed TRPV1, a ligand-gated nonselective cation channel present in primary sensory neurons (Szallasi and Blumberg, 1993; Caterina et al., 1997; Tominaga et al., 1998). Studies have shown that capsaicin evokes the release of neuropeptides, excitatory amino acids (glutamate and aspartate), nitric oxide and pro-inflammatory mediators in the periphery, and transmits nociceptive information to the spinal cord (Szallasi and Blumberg, 1993; Santos and Calixto, 1997; Sakurada et al., 1996, 2003).

Our results also show that p.o. administration of EE of *C. leprosum* produced a significant and dose-dependent inhibition of the nociceptive response caused by i.pl. injection of glutamate into the mouse hindpaw. Furthermore, the EE of *C. leprosum* was 2.3- to 4.2-fold more potent in inhibiting the nociception caused by the glutamate when compared with the nociception caused by acetic acid, formalin and capsaicin in mice. Beirith et al. (2002) found that the nociceptive response induced by glutamate appears to involve peripheral, spinal and supraspinal sites of action and is largely mediated by both NMDA and non-NMDA receptors as well as by the release of nitric oxide or by some nitric oxide-related substance. Therefore, the suppression of the capsaicin-, formalin- and glutamate-induced licking response and of the acetic acid-induced abdominal writhing response, caused by treatment with EE, are complementary indications that the antinociceptive action of this plant could be associated with its ability to inhibit NO production or through interaction with the glutamatergic system.

Another interesting result of the current study was the fact that p.o. administration of EE of *C. leprosum* produced a significant antinociception in a thermal model of nociception: the hot-plate test. We observed a significant and dose-dependent increase in the reaction time of animals in the hot-plate test at 50 °C but not at 56 °C. Under very similar conditions, fentanyl caused a marked increase in the reaction time of animals in the hot-plate test at both temperatures studied. The hot-plate test, at a constant temperature, produces two kinds of behavioural response: paw licking and jumping. Both of these are considered to be supraspinally integrated responses (Chapman et al., 1985). The reason for such a difference in the action of EE in the hot-plate test still remains unclear. However, it has been suggested that ~45% of small- to medium-diameter neurones in the dorsal horn (C and type II Aδ nociceptors, respectively) exhibit heat-evoked membrane currents with a moderate threshold of ~45 °C, whereas another 5–10% of cells (I Aδ nociceptors) respond with a high threshold of ~52 °C and are insensitive to capsaicin (for review see Julius and Basbaum, 2001). Thus, the EE may depress the response in the dorsal horn produced by C and type II Aδ nociceptors more easily than those produced by I Aδ nociceptors. However, additional studies are necessary to address this hypothesis.

Further experiments were undertaken to elucidate the mechanism by which EE of *C. leprosum* exerted its antinociceptive activity. The present results lead to the conclusion that the opioid system is likely to be involved. This is drawn from the fact that pre-treatment of animals with naloxone, a non-selective opioid receptor antagonist, at a dose that produced no significant effect on glutamate-induced pain, completely inhibited the antinociceptive effect caused by both morphine and EE. Furthermore, the results of the present study provide consistent evidence to suggest that the L-arginine-nitric oxide pathway is unlikely to be involved in the antinociceptive action of EE. This conclusion derives from the fact that pre-treatment of animals with the substrate for NOS, L-arginine, at a dose that produced no significant effect on glutamate-induced pain, significantly reversed the antinociception caused by L-NOARG (a known nitric oxide inhibitor), but did not effect the antinociceptive action of EE.

It is well known that serotonin (5-HT) pathways within the CNS arise from a series of nuclei situated in the midline of the brain stem, the raphe nuclei, which represent the richest source of neuronal 5-HT synthesised in the mammalian brain (Fields et al., 1991; Millan, 2002). In addition, several studies have shown that the bulbospinal serotonin system may suppress incoming noxious input to the spinal cord and inhibit pain transmission (Basbaum and Fields, 1984; Alhaider et al., 1991; Millan, 1995). Descending serotonergic pathways modulate the activity of projection neurons directly, as well as via interneurones (Alhaider et al., 1991). The multiple 5-HT receptor types within the spinal cord appear to fulfil different roles in the control of nociception, reflecting their contrasting patterns of coupling to intracellular transduction mechanisms (Millan, 1995; Bardin et al., 2000). The activities of 5-HT receptors are complex and sometimes even contrasting, and can depend on: (1) the receptor subtype being activated, (2) the relative contributions of pre- versus postsynaptic actions of receptors, and (3) the nociceptive paradigm in terms of quality and intensity of stimulus (Sawynok and Reid, 1996; Millan, 2002), and (4) the dose-related effect, which can be pro- or antinociceptive, of agonists and antagonists of serotonergic receptor subtypes (Hylden and Wilcox, 1983). Several pieces of evidence point to 5-HT_{1A}, 5-HT_2 and 5-HT_3 receptors modulating nociceptive transmission, as activation of these receptors in the spinal cord produces antinociception in the formalin test and other models (Bardin et al., 2000; Sasaki et al., 2001; Millan, 2002). Furthermore, the results of the present

study show that EE produces antinociception that appears to be mediated by an interaction with 5-HT_1 and 5-HT_2 receptors. This assertion is supported by the demonstration that 1) selective antagonists of 5-HT_{1A} and 5-HT_{2A} receptors, namely WAY100635 and ketanserin, respectively, consistently reversed the antinociception caused by systemic administration of EE when analysed against the glutamate-induced pain. In marked contrast, 5-HT_3 receptors appear not to account for the antinociceptive action of EE. This notion comes from the data showing that targeting the 5-HT_3 receptors sensitive to ondansetron with the doses and scheme of treatment at which this substance effectively antagonizes responses mediated by activation of the receptor by agonists (Takeshita and Yamagushi, 1995) largely failed to prevent the antinociception caused by EE in the glutamate test.

Finally, the chemical studies carried out on this ethanolic extract allowed us to isolate and identify the triterpene 3β,6β,16β-trihidroxilup-20(29)-ene in *C. leprosum*, which seem to be responsible, at least in part, for the antinociceptive properties reported for the EE of *C. leprosum*. In addition, at the ID_{50} level, this triterpene had a 6.8-fold greater potency than the EE when analysed in the glutamate test.

In summary, the present results provide convincing evidence that EE exerts a rapid onset, relatively long-lasting and pronounced systemic antinociception in chemical (acetic acid-, formalin-, capsaicin-, and glutamate-induced pain) and thermal (hot-plate at 50 °C) models of nociception in the mouse at a dose that does not interfere with the motor performance. In addition, the antinociceptive effect of EE involves an interaction with opioid and serotonergic (through 5-HT_{1A} and 5-HT_{2A} receptors) systems, but not with the L-arginine nitric oxide pathway. Furthermore, the triterpene isolated and identified as 3β,6β,16β-trihidroxilup-20(29)-ene contributes to the explanation of the antinociceptive properties reported for the EE. Pharmacological and chemical studies are continuing in order to characterise the precise mechanism(s) responsible for the antinociceptive action, and also to identify other active compounds present in *C. leprosum*. Finally, the antinociceptive action demonstrated in the present study supports, at least in part, the ethnomedical uses of this plant.

Acknowledgements

This work was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), the Programa de Apoio aos Núcleos de Excelência (PRONEX) and Fundação de Apoio a Pesquisa Científica e Tecnológica do Estado de Santa Catarina (FAPESC), Brazil.

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