Anti-inflammatory and antinociceptive activities of an acid fraction of the seeds of *Carpotroche brasiliensis* (Raddi) (Flacourtiaceae)

**Abstract**

*Carpotroche brasiliensis* is a native Brazilian tree belonging to the Oncobeae tribe of Flacourtiaceae. The oil extracted from its seeds contains as major constituents the same cyclopentenyl fatty acids hydnocarpic (40.5%), chaulmoogric (14.0%) and gorlic (16.1%) acids found in the better known chaulmoogra oil prepared from the seeds of various species of *Hydnocarpus* (Flacourtiaceae). These acids are known to be related to the pharmacological activities of these plants and to their use as anti-leprotic agents. Although *C. brasiliensis* oil has been used in the treatment of leprosy, a disease that elicits inflammatory responses, the anti-inflammatory and analgesic activities of the oil and its constituents have never been characterized. We describe the anti-inflammatory and antinociceptive activities of *C. brasiliensis* seed oil in acute and chronic models of inflammation and in peripheral and central nociception. The mixture of acids from *C. brasiliensis* administered orally by gavage showed dose-dependent (10-500 mg/kg) anti-inflammatory activity in carrageenan-induced rat paw edema, inhibiting both the edema by 30-40% and the associated hyperalgesia. The acid fraction (200 mg/kg) also showed significant antinociceptive activity in acetic acid-induced constrictions (57% inhibition) and formalin-induced pain (55% inhibition of the second phase) in Swiss mice. No effects were observed in the hot-plate (100 mg/kg; N = 10), rota-road (200 mg/kg; N = 9) or adjuvant-induced arthritis (50 mg/kg daily for 7 days; N = 5) tests, the latter a chronic model of inflammation. The acid fraction of the seeds of *C. brasiliensis* which contains cyclopentenyl fatty acids is now shown to have significant oral anti-inflammatory and peripheral antinociceptive effects.
Introduction

*Carpotroche brasiliensis* (Raddi) Endl. (syn. *Mayna brasiliensis* Raddi) is a native Brazilian tree belonging to the Oncoboe tribe of Flacourtiaceae, which occurs in the high altitude forests of the states of Rio de Janeiro, Minas Gerais, Espírito Santo, Bahia, São Paulo, and Piauí. It is commonly known as sapucainha and has several other local popular names such as canudeiro, babado fruit, comona fruit, cotia fruit, leprosy fruit, monkey fruit, mata-piolho, papo de anjo, pau de anjo, pau de cachimbo, pau de cotia, pau de lepra, and ruchuchu (1). The oil extracted from the seeds, whose major fatty acids are hydnocarpic, chaulmoogric and gorlic acids (2,3), has parasiticidal and anti-leprotic properties (Figure 1) (1,2,4,5).

This class of cyclopentenyl fatty acids seems to be restricted to the seed oils of two tribes of the Flacourtiaceae family, Oncobae and Pangieae (3). *C. brasiliensis* oil, like the better known chaulmoogra oil prepared from the seeds of various Flacourtiaceae, was used in medicine for the treatment of leprosy until 1940 when diaminodiphenyl sulfone began to be used with better results (4).

Leprosy is a disease characterized by a spectrum of clinical and immunological manifestations, including inflammation and pain. One of the major modifications in the inflammatory reactions observed in leprosy seems to be the increase in tumor necrosis factor-α (TNF-α) synthesis. Drugs that inhibit TNF-α production, such as thalidomide, have demonstrated a beneficial effect in the therapy of the inflammatory reactions of leprosy and other immune inflammatory diseases like rheumatoid arthritis (6). It was also demonstrated that prostaglandin E2 (PGE2) plays a regulatory role in the immune response to *Mycobacterium leprae* (7).

Although *C. brasiliensis* oil has been used in the treatment of leprosy, the anti-inflammatory and analgesic activities of the oil and its constituents have never been characterized.

We report here the results of a study of the anti-inflammatory, anti-arthritic and analgesic activities of the cyclopentenyl fatty acid mixture from *C. brasiliensis*.

Material and Methods

Plant material and preparation of the acid fraction of *C. brasiliensis*

Fruits of *C. brasiliensis* were collected in July 2001 at Serra do Caparaó, MG, Brazil. A voucher specimen was deposited in the herbarium of the Museu Nacional of the Federal University of Rio de Janeiro (R-203170).

The acid fraction of *C. brasiliensis* was obtained by maceration and extraction of the dried seeds (30.3 g) with 260 ml isopropanol/dichloromethane (2:1). After filtration, concentration and extraction with 100 ml chloroform/methanol (1:2), the organic fraction was concentrated under vacuum. The resulting oil was then saponified by heating under reflux with 100 ml 10% (w/v) ethanolic potassium hydroxide for 3 h. After ethanol evaporation and addition of 150 ml water, the aqueous phase was acidified with 100 ml 10% (v/v) aqueous hydrochloric acid. The free acids were extracted into diethyl ether to provide the acid fraction in 29% (w/w) yield after concentration and drying over sodium sulfate (8). The chemical composition of the acidic fraction was established by high-resolution gas chromatography (HRGC; Agilent 6890, Avondale, PA, USA), HRGC-mass spectrometry (HRGC-
MS; Agilent 6890 gas chromatograph coupled to an Agilent 6973 mass-selective detector; after methylation with a 30% solution of diazomethane in diethyl ether) and by 1H- and 13C-NMR analyses of the mixture (Bruker DRX-300, Rheinstetten, Germany) (9,10). HRGC (FID at 280ºC) and HRGC-MS (ionization energy at 70 eV, ion source at 200ºC) were carried out on a DB-5 (J&W, Folsom, CA, USA) capillary column (12 m x 0.25 µm x 0.22 mm). Helium was used as carrier gas (1.3 ml/min) and the injection port in the splitless mode at 270ºC (0.5 min). Oven temperature was programmed from 120ºC (3ºC/min) to 280ºC (15 min). The compounds observed are reported in terms of relative areas.

**Animals**

Wistar rats (120-200 g) and Swiss albino mice (18-25 g) of both sexes were obtained from the LASSBio breeding unit (Faculty of Pharmacy, UFRJ, Brazil). The animals were maintained under standardized conditions, with only water *ad libitum* for 12 h before the experiment. Animal experiments were performed according to the “Principles of Laboratory Animal Care and Use in Research” (Colégio Brasileiro de Experimentação Animal - COBEA/Instituto Brasileiro Carlos Chagas Filho - IBCCF², Brazil), based on international guidelines for the care and use of laboratory animals and ethical guidelines for the investigation of experimental pain in conscious animals (11).

**Reagents**

Acetic acid, indomethacin, Tween 80, ethanol and all other chemicals were purchased from Merck (Darmstadt, Germany). Arabic gum was from Sigma (St. Louis, MO, USA), carrageenan was from Cialgas (Taboão da Serra, SP, Brazil) and morphine sulfate was from Cristália (Itapira, SP, Brazil). A solution of 2.5% (v/v) formalin was prepared with formaldehyde (Merck) in saline (0.9% NaCl).

**Carrageenan-induced rat paw edema and hyperalgesia**

Anti-inflammatory activity was determined *in vivo* using the carrageenan-induced rat paw edema test (12). The acid fraction from *Carpotroche* seeds was administered orally at doses of 10, 50, 100, 200, and 500 mg/kg (0.1 ml/20 g) as a suspension in EtOH/Tween 80/H₂O (2:2:20, v/v/v; vehicle) 1 h before carrageenan. Control animals received an equal volume of vehicle. Animals were then injected subplantarily with either 0.1 ml of 1% carrageenan solution in saline (0.1 mg/paw) or sterile saline (0.9% NaCl) into one of the hind paws. Paw volumes were measured every hour up to 4 h after subplantar injection using a glass plethysmometer coupled to a peristaltic pump. The edema was considered to be the difference in volume between the carrageenan- and saline-treated paws. Anti-inflammatory activity was reported as percent inhibition of the edema compared with the vehicle control group. Ulcerogenic effects in rats were investigated (13). Briefly, animals were euthanized and the stomachs excised along the greater curvature for visualization of gastric lesions with a stereomicroscope.

Carrageenan-evoked hyperalgesia was quantified in separate groups of animals as a measure of the nociceptive response to a thermal stimulus using the hot-plate test with the temperature adjusted to 51 ± 1ºC (14). The withdrawal latency of each hind paw was determined and hyperalgesia defined as a decrease in the Δ latency (s) calculated as the difference between carrageenan and saline paw latency times. The withdrawal latency of the saline-injected paws remained stable (11.2 ± 0.9 s), as did the withdrawal latency of non-injected paws (data not shown). The *C. brasiliensis* seed acid fraction was administered orally by gavage at a dose of 200 mg/kg. For both assays a positive control group received indomethacin (10 mg/kg, po).
Acetic acid-induced abdominal constrictions in mice

The antinociceptive activity was determined in vivo in mice using the abdominal constriction test induced by 0.6% acetic acid (0.1 ml/10 g, ip) (12). The C. brasiliensis seed acid fraction was administered orally (100-200 mg/kg; 0.1 ml/20 g) as a suspension in EtOH/Tween 80/H₂O (2:2:20, v/v/v; vehicle) 1 h before the ip injection of acetic acid. Ten minutes after ip injection of acetic acid the number of constrictions per animal was recorded for 20 min. Control animals received an equal volume of vehicle and indomethacin (10 mg/kg, po) was used as a positive control. Antinociceptive activity is reported as percent inhibition of constrictions compared with the vehicle control group.

Formalin-induced pain in mice

The formalin induced pain test was carried out as described by Hunskaar and Hole (15). Animals were injected subplantarly with 20 µl 2.5% formalin into one hind paw. The acid mixture (200 mg/kg), indomethacin (10 mg/kg) or vehicle was administered po 60 min before formalin injection. The time the mice spent licking or biting the injected paw or leg was recorded. Two distinct periods of intensive licking activity were identified and scored separately unless otherwise stated. The first period (earlier or neurogenic phase) was recorded 0-5 min after formalin injection and the second period (later or inflammatory phase) was recorded 15-30 min after injection.

Hot-plate test

Central analgesic activity was investigated using the hot-plate test (16). In these experiments, the hot-plate apparatus (Model-DS 37, Ugo Basile, Varese, Italy) was maintained at 56 ± 1°C. Mice were placed on the heated surface at 0, 30, 60, 90, and 120 min after oral administration of vehicle, morphine (30 mg/kg, used as positive control) or C. brasiliensis seed acid fraction (100 mg/kg, by gavage) and the time between placement and the first sign of paw licking or jumping was recorded as latency. The basal latencies were 6-10 s. A cut-off time of 30 s was established to prevent injury to the paws.

Motor performance

In order to evaluate nonspecific muscle relaxant or sedative effects of the C. brasiliensis seed acid fraction, mice were tested on the rota-rod (17). The rota-rod apparatus (AVS, São Paulo, SP, Brazil) consisted of a horizontal bar 2.5 cm in diameter divided into 5 compartments by disks measuring 25 cm in diameter. The bar rotated at a constant speed of 10 rotations per minute. The animals were trained for three days before the assay and the mice that did not remain on the rod for 120 s (cut-off time) were eliminated from the experiment. On the day of the experiment, mice were placed on the rod for 120 s before C. brasiliensis administration (200 mg/kg, po; control). One hour later, the time(s) during which animals remained on the rotating bar was recorded.

Acute toxicity

The acute toxicity of the C. brasiliensis seed acid extract was investigated using a single oral administration of the acid mixture in mice. In this assay, increasing doses of the test substance were orally administered to groups of 5 animals per dose (100-500 mg/kg). The animals were observed for 14 days and at the end of this period the number of survivors was counted and body and spleen weight was recorded. The acute toxicologic effect was estimated by a previously described method (18).

Adjuvant-induced arthritis

Anti-arthritic activity was determined in
vivo using the adjuvant arthritis model in rats (19). The arthritic syndrome was induced by a subplantar injection of 0.05 ml of a suspension of heat-killed \textit{M. tuberculosis} in liquid paraffin (0.5%, w/v) into the right hind paw. Fourteen days after inoculation, the rats were selected and divided into two groups (N = 5). One group received the acid mixture orally (50 mg/kg) and the other received the vehicle once a day for seven days. The hind paw thickness was measured using a caliper rule before subplantar injection, at the onset of arthritis (day 14) and every other day until day 22. Animal weights were recorded daily and the development of secondary lesions was also investigated.

**Statistical analysis**

Data are reported as means ± SEM for “N” animals per group and were analyzed statistically by the Student \( t \)-test and one-way ANOVA, with the level of significance set at \( P < 0.05 \). When appropriate, the ID\textsubscript{50} value (i.e., the dose that reduces the response by 50%) was determined by non-linear regression using GraphPad Prism software.

**Results**

**Acid fraction composition**

After HRGC and HRGC-MS analysis of the methylated acid fraction obtained from the seeds of \textit{C. brasiliensis}, the major compounds identified were methyl hydnocarpate (40.5%), methyl gorlate (16.1%), methyl chaulmoograte (14.0%), methyl hexadecanoate (6.0%), and methyl octadecanoate (3.0%). Other methyl fatty esters were present as minor constituents.

**Acute toxicity of \textit{C. brasiliensis}**

In the acute toxicity trial no deaths were observed for an oral dose of up to 300 mg/kg but the mice showed some lethargy. No significant changes in body or spleen weight were observed at this dose level (data not shown). However, a 30% death rate was observed at an oral dose of 500 mg/kg. The results for rats submitted to this dose in the edema experiment were similar. Both mice and rats were observed for one week.

**Anti-inflammatory activity of \textit{C. brasiliensis}**

The results for the determination of anti-inflammatory activity of the extract on the rat paw edema and on hyperalgesia are illustrated in Figure 2. The acid mixture assayed at oral doses of 10, 50, 100, 200, and 500 mg/kg showed a dose- and time-dependent effect: 10 mg/kg had no anti-inflammatory...
effect, 50 and 100 mg/kg inhibited the edema from the 2nd hour after carrageenan injection to the same extent, reaching a maximum inhibition by the 4th hour (31.6 and 29.2%, respectively, for 50 and 100 mg/kg). With increasing doses (200 and 500 mg/kg) a significant inhibition of edema was observed from the 1st hour and during the subsequent 3 h, with maximum inhibition by the 2nd hour (41.3 and 44.1%, respectively). The ID$_{50}$ values for each time of edema formation were 192.4 mg/kg (1 h), 80.2 mg/kg (2 h), 19.8 mg/kg (3 h), and 12.2 mg/kg (4 h).

*C. brasiliensis* seed acid fraction at a dose of 200 mg/kg also significantly increased the $\Delta$ withdrawal latency, inhibiting the inflammatory hyperalgesia response. Indomethacin (10 mg/kg) inhibited both the edema and hyperalgesia by 70%, showing an ID$_{50}$ of 0.96 mg/kg at the 3rd hour under our experimental conditions (data not shown). No gastric irritation was observed even at the dose of 500 mg/kg.

In the adjuvant arthritis model, the major parameter considered was the decrease of swelling of the paw injected 14 days earlier with a suspension of *M. tuberculosis*. The seed acid mixture administered daily for a week was not able to reduce the edema at the dose employed (50 mg/kg, po). However, no loss of body weight or secondary lesions on the tail were observed, which are other parameters considered in this model (data not shown).

### Analgesic activity of *C. brasiliensis*

Table 1 illustrates the highly significant antinociceptive activity of the acid mixture at doses of 100 and 200 mg/kg (po), which inhibited the acetic acid-induced constrictions dose-dependently by 27.9 and 56.9%, respectively. Indomethacin at 10 mg/kg (po) showed 44.5% inhibition.

The acid mixture (200 mg/kg, po) as well as indomethacin (10 mg/kg, po) also inhibited significantly (55.3 and 52.5%, respectively) the inflammatory second phase but not (0%) the neurogenic first phase of the formalin-induced algesic response (Figure 3).

At a dose of 100 mg/kg (po) the acid mixture did not affect the latency time response in the hot-plate test, a classic model of thermal pain, recorded 60 min after administration. The control latency time was 8.5 ± 0.9 vs 9.6 ± 0.7 s for the seed acid fraction.

### Rota-rod test

*C. brasiliensis* (200 mg/kg), given orally 60 min prior to placing the mice on the bar, did not significantly affect the motor coordination of the animals. The control response in the rota-rod test was 120 vs 118.4 ± 2.2 s in the presence of *C. brasiliensis* (N = 9).
Discussion

*C. brasiliensis* seed oil, like the better known chaummoogra oil, contains the cyclopentenyl fatty acids hydnocarpic and chaummoogric acids as major constituents. Although chaummoogra oil used to be widely employed in the treatment of leprosy, this activity has only been experimentally demonstrated as activity against mycobacterial species and by its ability to inhibit the multiplication of *M. leprae* in mice (4,5). Since leprosy is a disease characterized by a spectrum of clinical and immunological manifestations including inflammation and pain in which PGE2 plays a regulatory role (7), it was important to investigate the anti-inflammatory and antinociceptive properties of *C. brasiliensis* oil.

Development of the edema and hyperalgesia induced by carrageenan corresponds to events occurring in the acute phase of inflammation, mediated by histamine, bradykinin and prostaglandins (20-22). The increases in edema and hyperalgesia were both prevented by pretreatment with *C. brasiliensis* seed acid extract. From the ID50 values observed during edema formation it can be seen that the acid mixture of *C. brasiliensis* is more potent at a later stage of the edema (Figure 2), suggesting that *C. brasiliensis* seed extract is more selective in the inhibition of the mediators involved at this later stage, such as prostaglandins. However, larger doses seem to result in a nonspecific effect, since inhibition was observed after the 1st hour of edema (Figure 2).

The constrictions induced by acetic acid in mice result from an acute inflammatory reaction related to the increase in the peritoneal fluid levels of PGE2 and PGF2α (20-22). The fact that the seed acid fraction was able to inhibit constrictions showed that this fraction has a peripheral antinociceptive effect, interfering with the acute phase of the inflammatory process.

Earlier reports have shown that indomethacin, a cyclooxygenase inhibitor, also attenuates the pain response in the second phase but not in the first phase of the formalin test in mice. Generally, anti-inflammatory drugs are completely inactive in preventing the first phase of the formalin test (15,23). The behavior of *C. brasiliensis* seed extract was similar to that of indomethacin in the formalin test, reinforcing its antinociceptive profile.

The hot-plate test is commonly used to assess narcotic analgesics or other centrally acting drugs (23), and the present results showed that *C. brasiliensis* seed extract has no central action, suggesting that its antinociceptive effects are related to the anti-inflammatory action observed. Also, antinociception is not related to nonspecific central effects, since no detectable effect was observed in the rota-rod test.

Taken together, the results obtained in the four models employed in the present study to assess pain and in the rota-rod test point to a peripheral action of the *C. brasiliensis* seed acid mixture.

The present results show for the first time that the mixture of acids obtained from the seeds of *C. brasiliensis*, when orally administered, has significant anti-inflammatory and antinociceptive effects and that these may contribute to the use of *C. brasiliensis* seed oil as an anti-leprotic agent, since leprosy is an unstable disease characterized by immunologically mediated inflammatory reactive states such as the reversal reaction (type 1 reaction) and erythema nodosum leprosum (type 2 reaction), occurring mainly in borderline lepromatous leprosy and lepromatous leprosy, respectively. Common to both types of leprotic reaction are pain and swelling at sites of infection (24) and these reactions are characterized by increased inflammatory activity in nerves and skin lesions that are regulated by the synthesis of cytokines and TNF-α (6,25-27).

Another feature of leprosy is the acute arthritis occurring in association with erythema nodosum leprosum and arthritis in leprosy, respectively. Common to both types of leprotic reaction are pain and swelling at sites of infection (24) and these reactions are characterized by increased inflammatory activity in nerves and skin lesions that are regulated by the synthesis of cytokines and TNF-α (6,25-27).
thea nodosum leprosum reactions (24). Although *C. brasilien sis* seed extract at the doses used here did not interfere with paw swelling in arthritic rats, we cannot rule out an action on chronic inflammatory diseases, since the oil had anti-inflammatory and antinociceptive effects in acute models of inflammation and pain. The effect of higher doses of the *C. brasilien sis* seed acid fraction on arthritic animals is currently under investigation.

In preliminary toxicologic studies, deaths were observed only at the level of 500 mg/kg, demonstrating a certain degree of safety. In addition, the acid mixture from the seeds of *C. brasilien sis* did not cause lesions in the gastric mucosa of the animals.

The present results show that the mixture of acids extracted from *C. brasilien sis*, which contains cyclopentenyl fatty acids, possesses important anti-inflammatory and peripheral antinociceptive effects that may contribute to its use and effectiveness as an anti-leprosy agent. Since inhibition of both TNF-α and PGE2 promotes anti-inflammatory and analgesic effects (28), the ability of *C. brasilien sis* to interfere with these mediators deserves further investigation.

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**References**


