Programa de Mestrado em Ciências Farmacêuticas (PMCF) e Núcleo de Investigações Químico Farmacêuticas (NIQFAR)/CCS[1], Universidade do Vale do Itajaí (UNIVALI), Itajaí, SC, Brazil; Departamento de Química[2], Universidade Federal de Santa Catarina (UFSC), Florianópolis, SC, Brazil; Centro Chimica Recettori (CNR)[3], Rome, Italy

Pharmacological and phytochemical investigations of different parts of *Calophyllum brasiliense* (Clusiaceae)

D. E. B. Isaias[1], R. Niero[2], V. F. Noldin[1], F. de Campos-Buzzi[1], R. A. Yunes[2], F. Delle-Monache[3], V. Cechinel-Filho[1]

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Prof. Dr. Valdir Cechinel Filho, NIQFAR/CCS/UNIVALI, Rua Uruguai 458, Cx. P. 360, 88302-202 Itajaí, SC, Brasil
cechinel@univali.br

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Continuing our search for antinociceptive agents from natural sources, this study analyzed the antinociceptive effects of some fractions obtained from different parts (roots, flowers and fruits) of *Calophyllum brasiliense*, a Brazilian medicinal plant used to treat several diseases, including inflammation and pain. For this purpose, the writhing and formalin induced-pain models in mice were used. We also analyzed the chemical composition of these different parts and tested two pure compounds isolated from chloroform fraction (roots) identified as friedelin (**1**) and 1,5-dihydroxyxanthone (**3**), by direct comparison with authentic samples. The results showed that some fractions and both compounds exhibited considerable antinociception properties, particularly against the writhing test, and that these are more potent than acetyl salicylic acid and acetaminophen, two reference drugs used here for comparison.

1. Introduction

The genus *Calophyllum* (Clusiaceae), consists of a large group of tropical trees and has been proven to be a source of bioactive compounds. Biological studies have demonstrated that these plants produce several active principles including coumarins, xanthones, steroids and triterpenes (Dharmaratne et al. 1998; Morel et al. 2002; Oger et al. 2003; Shen et al. 2003). *Calophyllum brasiliense*, known as "guanandi", is a medicinal and ornamental plant which commonly grows in Brazil and which is used in folk medicine for the treatment of ulcers, inflammation and pain (Oliveira et al. 1994). Previous studies confirmed that this species produces compounds that possess antisecretory and cytoprotective properties (Sartori et al. 1999). We have demonstrated that extract, fractions and some phenolic compounds obtained from the leaves of this plant display pronounced antinociceptive action in mice (Da Silva et al. 2001). More recently, the isolation and identification of several xanthones and three new 4-substituted coumarins from this plant has been reported, which showed inhibition of tumor-promoting activity by means of a short-term *in vitro* assay for TPA-induced EBV-EA activation of the Raji cells (Ito et al. 2002, 2003).
In this study, we extended our previous investigation on this plant, evaluating the chemical composition and analgesic effects of *C. brasiliense*, particularly of the chloroform extract and pure compounds obtained from roots, using the writhing and formalin models in mice.

2. Investigations, results and discussion

In recent years, the therapeutic potential and determination of the active principles of several plants have been reported by our laboratories, particularly against pain (Calixto et al. 2000; Cechinel Filho 2000; De Souza et al. 2003).
The previous study carried out with *C. brasiliense*, showing its promising antinociceptive activity (Da Silva et al. 2001), encouraged us to continue the pharmacological and chemical investigations in order to identify other active principles of this plant. Thus, we initially prepared methanolic extracts and two fractions, denoted non polar (soluble in chloroform) and polar (non soluble in chloroform) from different parts of the plant (roots, flowers and fruits) in order to determine where the possible active compounds are located. The

Table 1: Antinociceptive action of extracts, fractions or compounds obtained from different parts of *C. brasiliense* and two reference drugs against acetic acid-induced abdominal constrictions in mice (3–10 mg/kg, i.p.)

Treatment	DI_{50} (μmol/Kg)	Maximal inhibition (%)*
Ext. MeOH (flowers)	ND	70.0 ± 5.5**
Ext. MeOH (fruits)	ND	65.0 ± 2.5**
Ext. MeOH (roots)	ND	80.0 ± 3.5**
Polar fraction (roots)	ND	81.0 ± 8.0**
Non polar fraction (roots)	ND	98.5 ± 5.5**
Friedelin (**1**)	12.0 (10.0–14.5)	84.4 ± 3.6**
1,5-Dihydroxyxanthone (**3**)	30.0 (27.0–32.0)	94.0± 2.2**
[a]ACE	125.0 (140.0–240.0)	38.0 ± 1.0*
[a]ASA	133.0 (73.0–247.0)	35.0 ± 2.0*

ND = Not Determined; *At 10 mg/kg; ACE = Acetaminophen; ASA = acetyl salicylic acid. *$P < 0.05$; **$P < 0.01$. Each group representing the mean ± S.E.M of six to eight experiments.
[a] From Bresciani et al. (2003).

pharmacological results indicated in Table 1 clearly show that the roots exhibited the most pronounced antinociceptive activity in the writhing test (10 mg/kg, i.p.), causing inhibition of 80.0 ± 3.5%, whereas the reference drugs acetaminophen and acetyl salicylic acid caused inhibition of 38.0 ± 1% and 35.0 ± 2% respectively, in the same model and dose. However, the flowers and fruits also exhibited notable antinociceptive activity, but were not studied in detail because of their low yield. For this reason, we evaluated the phytochemical profile of all parts by TLC, using the following phenolic compounds previously isolated (Da Silva et al. 2001) from the leaves as standard: protocatechuic acid, (±)epicatechin, gallic acid, quercetin, hyperoside and amentoflavone. The results showed that gallic acid and protocatechuic acid are present in the fruits in highest concentration and hyperoside in small quantities whereas quercetin, amentoflavone and (±)epicatechin were not detected. With respect to the flowers, only protocatechuic acid and (±)epicatechin were detected. However, the roots showed a different chemical composition when compared with the other parts, and phenolic compounds were not detected. However, other classes of compounds, such as terpenes and xanthones were found. Since the chloroform fraction practically abolished the abdominal constrictions induced by acetic acid at 10 mg/kg, i.p. (Table 1), it was selected for confirmation in another model of pain (formalin-induced pain) and phytochemical studies.

Table 2 shows that both methanolic extract and chloroform fraction from the roots inhibited only the second phase (inflammatory phase) on the formalin test, with inhibition of 87.0 ± 3.5 and 76.0 ± 8.0%, respectively. The antiinflammatory and analgesic drugs, acetyl salicylic acid and indomethacin, exhibited similar pharmacological profiles, but were less active than *C. brasiliense*. Fractionation of the chloroform fraction led to the isolation of two known triterpenes, friedelin (**1**) and betulinic acid (**2**), and one xanthone, identified as 1,5-dihydroxyxanthone (**3**). All the compounds were identified based on spectroscopic evidence (^{1}H and ^{13}C, NMR, IR) and direct comparison with authentic samples. The data are in accordance with those reported in the literature.

Friedelin (**1**) and 1,5-dihydroxyxanthone (**3**), when given intraperitoneally, caused graded dose-dependent inhibition of abdominal constrictions (Figs. 1 and 2), with DI_{50} values of 12.0 (10.0–14.5) and 30.0 (27.0–32.0) µmol/kg, respectively. Compound **1** was about 9 to 13-fold more potent than the reference drugs, whereas compound **3** was about 4 to 5-fold more active than these reference compounds. Betulinic acid (**2**) was not included in this study because we have previously reported its antinociceptive action, when isolated from *Ipomoea pes-caprae* (Krogh et al. 1999).

Table 2: Antinociceptive action of methanolic extract, chloroform fraction, acetyl salicylic acid and indomethacin against formalin-induced pain in mice at 10 mg/kg, i.p.

Treatment	Inhibition (%) First phase[1]	Second phase[2]
MeOH extract (roots)	14.0 ± 3.5	87.0 ± 3.5**
$CHCl_3$ fraction (roots)	inactive	76.0 ± 8.0**
[3]ASA	inactive	39.0 ± 4.0*
[3]IND	inactive	33.0 ± 5.0*

Each group representing the mean ± S.E.M of six to eight experiments. P values < 0.05 were considered as indicative of significance
[1] 0–5 min licking (s); [2] 15–30 min licking (s)
[3]From Bresciani et al. (2003)
ASA = Acetyl salicylic acid; IND = Indomethacin

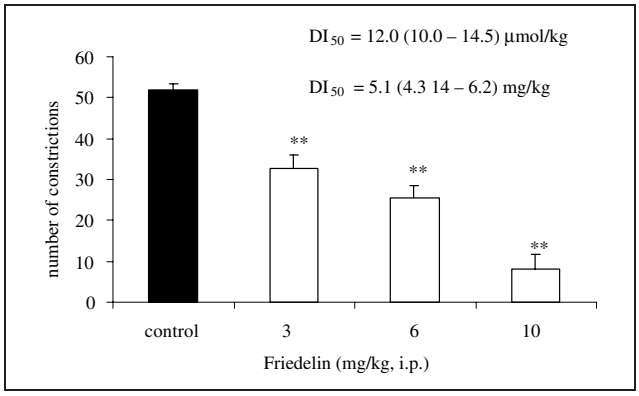


Fig. 1: Effect of friedelin (**1**) against acetic acid-induced abdominal constrictions in mice. Each column represents mean ± s.e.m. of six to eight experimental values. *p < 0.05; **p < 0.01

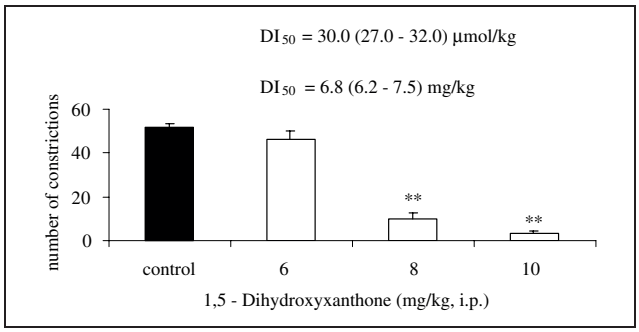
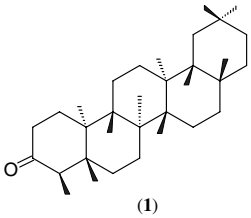


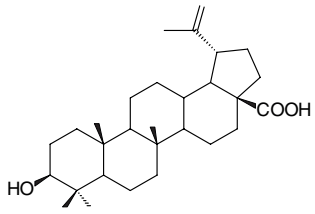
Fig. 2: Effect of 1,5-dihydroxyxanthone (**3**) against acetic acid-induced abdominal constrictions in mice. Each column represents mean ± s.e.m. of six to eight experimental values. *p < 0.05; **p < 0.01

Although compounds **1** and **3** are known to be biologically active (Rocha et al. 1994; Augustin et al. 2002; Shimizu et al. 1994; Singh et al. 2001), this is the first report, to the best of our knowledge, to demonstrate their antinociceptive potential.

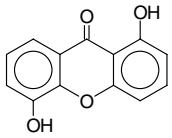
Finally, our results extended previous investigations on *C. brasiliense*, showing the presence of triterpenes and



(**1**)



(**2**)



(**3**)

xanthones with potent antinociceptive activity. These findings encourage further studies on the polar fraction, for which experimental procedures are in progress in our laboratories and will be published elsewhere. In addition, compounds **1** and **3** could be used as models for obtaining new and potent antinociceptive derivatives.

3. Experimental

3.1. Plant material

Roots, flowers and fruits of *C. brasiliense* were collected from the Gardens of the Federal University of Santa Catarina (Florianópolis – SC – Brazil) in April, September and December 2001, respectively. The material was classified by Dr. Ademir Reis (Department of Botany, UFSC). A voucher was deposited at the Barbosa Rodrigues Herbarium (Itajaí) under number VC Filho 007.

3.2. Extraction and isolation

Air-dried material from different parts of the plant (roots, flowers and fruits, 330 g of each) were powdered and macerated separately with methanol at room temperature for seven days. After removal of the solvent under reduced pressure, the extracts were dissolved in chloroform. The soluble parts were denominated as non polar and the non soluble parts as polar fractions. They were preliminarily analyzed by TLC and specific reagents, according to the methodology previously described (Ugaz 1994; Cechinel Filho and Yunes 1998).

Considering that the non polar fraction (chloroform soluble) from the roots showed the most interesting analgesic action, 2 g of this fraction was submitted to a chromatographic column on silica gel, eluted with $CHCl_3$: MeOH gradient and monitored by TLC. Similar fractions, which showed a positive reaction with $FeCl_3$ or anisaldehyde sulphuric reagents, were combined and rechromatographed as in the previous case. From the roots, three compounds were isolated and identified as friedelin **1** (16 mg), betulinic acid **2** (9 mg) and 1.5-dihydroxyxanthone **3** (25 mg). These were directly compared with authentic samples (Co-TLC) and their spectral data (IR, NMR ^1H and ^{13}C) compared with those published in the literature. The purity of all the isolated substances was examined by TLC using Merck silica pre-coated aluminum plates of 200 μm in thickness with several solvent systems of different polarities. Spots were visualized by short-wave UV light, sulphuric acid and $FeCl_3$ reagents.

3.3. Pharmacological assays in mice

3.3.1. Abdominal constriction response caused by intraperitoneal injection of diluted acetic acid

Abdominal constriction was induced by intraperitoneal injection of acetic acid (0.6%), according to the procedures described previously (Collier et al. 1968; Bresciani et al. 2003) with minor modifications. Male Swiss mice (25–30 g) were pre-treated with fractions or compounds (3–10 mg/kg), intraperitoneally (i.p.), 30 min before the acetic acid injection (six to eight animals in each group). The control animals received a similar volume of 0.9% NaCl (10 ml/kg, i.p.). All experiments were carried out at 23 ± 2 °C. After the challenge, pairs of mice were placed in separate glass funnels and the number of contractions of the abdominal muscles, together with stretching, were counted cumulatively over a period of 20 min. Antinociceptive activity was expressed as the reduction in the number of abdominal contractions between control animals and mice pretreated with the test materials.

3.3.2. Formalin-induced pain

The procedure used was essentially similar to that described previously (Mendes et al. 2000; Bresciani et al. 2003). Animals of the same strain were anaesthetized with ether, except when used to analyze the first phase, and 20 μl of 2.5% (0.92% formaldehyde), made up of PBS (phosphate-buffered saline containing: NaCl 137 mM; KCl 2.7 mM and phosphate buffer 10 mM), was injected intraplantarly on the left hind paw. The animals were pre-treated with fractions by i.p. (10 mg/kg), 30 min before formalin injection. The control animals received a similar volume of a vehicle i.p. (10 ml/kg), intraplantarly (20 μl/paw). After application of the intraplantar irritant, the animals were immediately placed in glass cylinders (20 cm in diameter). The time spent by animals licking or biting the injected paw was timed with a chronometer and was considered indicative of pain.

3.3.3. Statistical analysis

The results are presented as mean ± S.E.M., and statistical significance between the groups was analyzed by means of the t test of variance followed by Dunnett's multiple comparison test. P values of less than 0.05 were considered significant. When appropriate, the ID_{50} values (the dose of the compound that reduced formalin or acid-induced pain by 50% relative to control) were estimated by graphical interpolation from individual experiments.

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