Phytochemical and Pharmacological Investigations of *Virola oleifera* Leaves

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A methanolic extract and two fractions (*n*-hexane and ethyl acetate) from *Virola oleifera* leaves and some compounds (one lignan and two flavonoids) were investigated to verify the analgesic activity by using the writhing test in mice. The crude methanolic extract showed a moderate analgesic effect (about 40% of inhibition in this test at 10 mg/kg), whereas *n*-hexane and ethyl acetate fractions caused inhibition of 51.3 ± 5.9% and 50.5 ± 6.3%, respectively. Oleiferin-C (**1**), a lignan isolated from the *n*-hexane fraction, showed an interesting analgesic potential in this model when compared to two standard drugs, paracetamol (4-acetamidophenol) and aspirin (acetylsalicylic acid). The ID_{50} calculated for this compound was 17.25 μmol/kg, with confidence interval between 13.7 and 21.3 μmol/kg, being about 8 times more potent than the standard drugs. The mixture of two glycoside-flavonoids, identified as astilbin (**2**) and quercitrin (**3**), also exhibited good analgesic activity, causing 63% of reduction of abdominal constriction in mice. These results suggest beneficial effect of this plant to treat dolorous processes.

Introduction

Myristicaceae represents one of the greatest families, with 18 genus and 300 species (Joly, 1998), including the genus *Virola*, which comprises approximately 35 species and are typically found in tropical forests. In Brazil, these plants are distributed mainly in the Amazonian forests. *Virola oleifera* is one of the few species of this genus existing in the Atlantic forest, southeastern region of Brazil (Sartorelli *et al.*, 1998).

V. oleifera, known in Brazil as "bicuíba", "ücuúba-branca" or "bocuva", has been widely used in folk medicine as a anti-inflammatory, antirheumatic, anti-asthmatic, and to stimulate memory and cerebral intelligence (Rodrigues, 1980). Its leaves have been previously studied and some known compounds such as galbacin, eupomatenoid-8, (+)-aristolignin (Fernandes *et al.*, 1993), galbulin, verrucosin (Sartorelli *et al.*, 1998), otobafenol in the arils, and otobain, methylaustrobailignan-6 and arylalkanones in kernels (Sartorelli and Kato, 1996) were reported. Other ten new lignans were previously isolated, named oleiferin-A, B, C, D, E (Fernandes *et al.*, 1993), F, G, H, 4-hydroxy-5,3',4'-trimethoxy-2,7'-cyclolignan (Sartorelli *et al.*, 1998) and an arylalkanone, denoted oleiferinone (Azevedo *et al.*, 1997).

Some of these lignans were tested to verify their antifungal activities against *Cladosporium sphaerospermun* and *C. cladosporoides.* They showed moderate activities, but they were not efficient when compared to traditional fungicides (Sartorelli *et al.*, 1998).

Material and Methods

Plant material

Leaves from *V. oleifera* (Schott) A.C. Smith were collected in Ilhota, State of Santa Catarina, Brazil, during spring 1999 (September) and a

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voucher specimen (V.C. Filho – **020**) was deposited at the Barbosa Rodrigues Herbarium, Itajaí (Santa Catarina). The plant was identified by Dr. Ademir Reis from the Department of Botany, Santa Catarina Federal University (UFSC, Florianópolis).

Isolation and identification of active compounds

Dried leaves of *V. oleifera* were ground (500g) and macerated with methanol at room temperature during 10 days. The solvent was concentrated (reduced pressure) to 100 ml and then successively partitioned with *n*-hexane and ethyl acetate, respectively. Each solvent was evaporated under reduced pressure to dryness, yielding 27.7g (*n*-hexane) and 16.6g (ethyl acetate), which were submitted to pharmacological tests.

Part of the *n*-hexane fraction (1.5 g) was chromatographed on silica-gel column using *n*-hexane-ethyl acetate gradient, yielding 100 fractions of 10 ml each. The fractions were monitored by TLC and combined according to their similarities. Fractions 49–53 were combined, yielding 28 mg of a yellow oil, pure by TLC. The spectral data (IR, ^{1}H and ^{13}C-NMR) allowed us to conclude that this compound is oleiferin-C (**1**). The spectroscopic data are in good accordance with those of literature (Fernandes *et al.*, 1993).

3.0 g of ethyl acetate fraction was chromatographed on a silica-gel column using a $CHCl_3$: MeOH gradient as eluent, yielding 74 fractions of 10 ml each. Fractions 45–52 were combined (468 mg) because they were very similar on TLC, showing two spots, and were chromatographed following the same procedure described above, yielding 109 fractions of 10 ml each. Fractions 33–36 (19.8 mg) presented only one spot by TLC, obtained by a positive test like $FeCl_3$ reagent. The spectral data (IR, ^{1}H and ^{13}C-NMR) allowed us to conclude that fractions 33–36 consist of a mixture of two flavonoids (about 2:1 w/w), identified as quercitrin (**2**) and astilbin (**3**). HPLC analysis using authentic samples confirmed these compounds.

Pharmacological analysis: writhing test

Male Swiss mice (25–30 g) were kept in a temperature controlled environment (23±2 °C) under a 12-h light-dark cycle. Food and water were freely available. The abdominal constrictions resulting from intraperitoneal injection of acetic acid (0,6%), consisting of a constriction of the abdominal muscle together with a stretching of hind limbs, was carried out according to procedures previously described (Collier *et al.*, 1968; Santos *et al.*, 1995). Animals were pretreated with extracts, fractions or compounds through intraperitoneal administration 10 mg/kg, 30 min before the acetic acid injection. The methanolic extract and n-hexane and ethyl acetate fractions were prepared at 1 mg/ml (concentration of dry residue). These solutions were injected in mice according to its weight, corresponding in volumes at 0.25 to 0.30 ml for each animal, resulting in a final concentration of 10 mg/kg. Oleiferin-C was tested in the doses of 3, 6 and 10 mg/kg (or 8.8, 17.5 and 29.2 μmol/Kg). Control animal received a similar volume of 0.9% NaCl (10 ml/kg, i.p.). After challenge, each mice was placed in a separated glass funnel and the number of abdominal contractions of abdominal muscles together with stretching was cumulatively counted over a period of 20 min. Analgesic activity was expressed as the reduction of the number of abdominal contractions between control animals (saline pre-treated) and mice pre-treated with extract, fractions or compounds.

Statistical analysis

The results are presented as mean ± s.e.m., except the mean ID_{50} values (i.e. the dose of extracts or compounds that reduced responses by 50% in relation to control value) which are reported as geometric means accompanied by their respective 95% confidence limits. These values were determined by linear regression from individual experiments with linear regression GraphPad software (GraphPad Software, San Diego, CA) according to Mendes *et al* (2000). The statistical significance between the groups was analyzed by means of analysis of variance followed by Dunnet's multiple comparison test. P values < 0.01 or $P< 0.05$ were considered as indicative of significance.

Results and Discussion

In order to investigate the analgesic activities of *Virola oleifera* leaves the methanolic extract and subsequent *n*-hexane and ethyl acetate fractions were prepared and tested in the writhing test in

mice at 10 mg/kg. Table I shows the percentage of inhibition of abdominal constriction at 10 mg/kg, i.p.. The results evidenced a good analgesic activity of these extracts, mainly when they are compared to two reference drugs, aspirin (acetylsalicylic acid) and paracetamol (4-acetamidophenol). *n*-Hexane and ethyl acetate fractions, at 10 mg/kg, caused 51.3 ± 5.9% and 50.5 ± 6.3% of inhibition in the number of abdominal contractions of the abdominal muscle in mice, respectively. These drugs were more active than standard ones, which caused inhibitions of 35% (aspirin / acetylsalicylic acid) and 38% (paracetamol / 4-acetamidophenol). Both extracts showed similar efficacy, suggesting that non polar (*n*-hexane) and polar (ethyl acetate) compounds of a distinct structure may be acting as antinociceptive in this plant.

Table I. Analgesic effect of methanolic extract, n-hexane and ethyl acetate fractions and compounds isolated from *Virola oleifera*, on acetic acid-induced abdominal constrictions in mice (10 mg/kg i.p.).

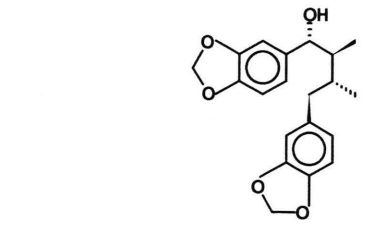
Treatment	Inhibition (%)
Methanolic extract	39.8 ± 3.9*
n-Hexane fraction	51.3 ± 5.9**
Ethyl acetate fraction	50.5 ± 6.3**
Quercitrin + astilbin	62.9 ± 5.2**
Oleiferin-C	75.9 ± 4.7**
Aspirin	35.0 ± 2.0*
Paracetamol	38.0 ± 1.0*

Each group represents the mean of six to eight animals. Significantly different from controls; P < 0.01 (**) and P < 0.05 (*).
Standard drugs were included for comparison purposes.

Preliminary phytochemical analysis by TLC using specific reagents (Marini-Bettólo *et al.*, 1981; Ugáz, 1994) showed that this plant includes phenolic compounds, particularly flavonoids, and has no alkaloids. To confirm lack of alkaloids in this plant, we prepared a specific fraction following the classic methodology for alkaloids. TLC analysis (Dragendorff reagent) proved the absence of these compounds in *V. oleifera*. According to several investigators (Cassady *et al.*, 1970; Lai *et al.*, 1973; Braz-Filho *et al.*, 1977; Schultes, 1979; Romoff and Yoshida,1997) about five or six species of the genus *Virola* are known to be used to prepare hallucinogenic agents, like tryptamines, especially 5-methoxy-*N*,*N*-dimethyltryptamine as the main psychoactive principle.

Part of the *n*-hexane fraction was chromatographed on a silica gel column, eluted with increasing amount of ethyl acetate in n-hexane, yielding only one pure compound. The spectral data (IR, ^{1}H-NMR and ^{13}C-NMR) of this compound, in comparison with literature (Fernandes *et al.*, 1993), allowed us to identify it as (7*R**,8*S**,8'*R**)-3,4:3',4'-bis(methylenedioxy)-lignan7-ol, previously named as oleiferin-C (**1**).

The pharmacological results indicated that this compound (**1**) caused a significant (P < 0.01) and dose-dependent analgesic effect against acetic acid-induced writhing response in mice (Fig.1). A dose of **1** at 29.2 μmol/kg (10 mg/kg) injected intra-



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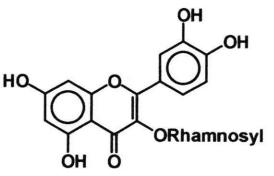

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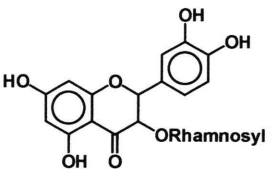

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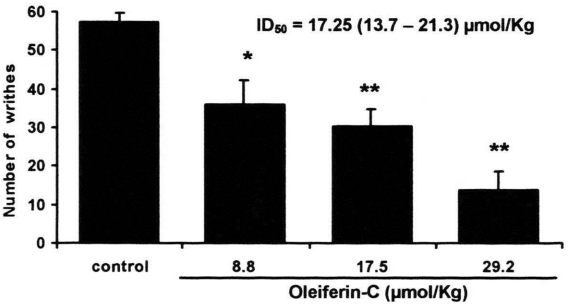

Fig. 1. Analgesic effects of oleiferin-C (**1**) given intraperitoneally, against acetic acid-induced abdominal constrictions in mice. Each group represents the mean of 6 to 8 experiments and the vertical bars indicate the s.e.m. Significantly differs from controls; P < 0.01 (**) and P < 0.05 (*).

peritoneally, it inhibited 76 ± 4.7% of the abdominal constrictions. In order to compare its potency with standard drugs, the mean ID_{50} was calculated by graphic interpolation resulting in 17.2 μmol/kg, with confidence interval between 13.7 and 21.3 μmol/kg (Fig. 1). Aspirin and paracetamol showed values of ID_{50} (μmol/kg) of 125 (73–243) and 133 (77–209), respectively. Thus, oleiferin-C was about 7.5 fold more active than these drugs. Such results show the high efficiency of this compound as analgesic in mice, and suggest the application of other analgesic models to confirm its analgesic activities. Furthermore, it might be used as a model to synthesize other more potent derivatives.

The ethyl acetate fraction was chromatographed in silica gel furnishing two glycoside-flavonoids identified as quercitrin (quercetin-3-*O*-rhamnoside) (**2**) and astilbin (taxifolin-3-*O*-rhamnoside) (**3**) based on spectral data. HPLC analysis confirmed the ratio of about 2:1 (quercitrin: astilbin).

This mixture caused reduction in abdominal muscle constriction of about 63.0 ± 5.2% (Table I). Previous studies carried out in our laboratories have shown that astilbin is practically inactive as analgesic in mice (Cechinel Filho *et al.*, 2000). For this reason, the analgesic effects demonstrated by the mixture of flavonoids may be due to the presence of quercitrin itself or to an synergistic effect between astilbin and quercitrin, but such hypothesis requires further investigations.

Although other flavonoids are known in this genus, some of them with analgesic effect (Carvalho *et al.*, 1999), compounds **2** and **3** are reported here for the first time in this plant.

In summary, our results indicate a promising analgesic effect in mice for the crude extract and fractions from *V. oleifera*, which seems be related to the presence of oleiferin-C and flavonoids.

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