In vivo Analgesic and Anti-Inflammatory Activities of Ursolic Acid and Oleanoic Acid from *Miconia albicans* (Melastomataceae)

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The aim of this work was to use *in vivo* models to evaluate the analgesic and anti-inflammatory activities of ursolic acid (UA) and oleanoic acid (OA), the major compounds isolated as an isomeric mixture from the crude methylene chloride extract of *Miconia albicans* aerial parts, in an attempt to clarify if these compounds are responsible for the analgesic properties displayed by this plant. Ursolic acid inhibited abdominal constriction in a dose-dependent manner, and the result obtained at a content of 40 mg kg^{-1} was similar to that produced by administration of acetylsalicylic acid at a content of 100 mg kg^{-1}. Both acids reduced the number of paw licks in the second phase of the formalin test, and both of them displayed a significant anti-inflammatory effect at a content of 40 mg kg^{-1}. It is noteworthy that the administration of the isolated mixture, containing 65% ursolic acid/35% oleanolic acid, did not display significant analgesic and anti-inflammatory activities. On the basis of the obtained results, considering that the mixture of UA and OA was poorly active, it is suggested that other compounds, rather than UA and OA, should be responsible for the evaluated activities in the crude extract, since the crude extract samples displayed good activities.

Key words: Miconia, Ursolic Acid, Oleanoic Acid

Introduction

Ursolic acid (UA) and its isomer, oleanoic acid (OA) (Fig. 1), belong to the triterpenoid class of compounds. They are widely distributed in the plant kingdom and have been frequently isolated as mutually isomeric mixtures. In 1995, Liu published a review article summarizing pharmacological studies on these triterpenoids, and their potential for the area of oncology has been pointed out because of their cytotoxicity, anti-mutagenic, antiviral and anti-invasive activities, as well as their ability to decrease radiation damage to the hematopoietic system caused by radiotherapy (Hsu *et al*., 1997; Kim *et al*., 1998; Li *et al*., 2002; Novotný *et al*., 2001).

Baricevic *et al*. (2001) reported that UA is the compound involved in the anti-inflammatory activity displayed by the crude chloroform extract of *Salvia officinalis* leaves. Studies on ten triterpenes using different *in vivo* models of inflammation allowed to infer that the presence of a carboxylic group in these compounds increases the anti-inflammatory activity if compared with non-acid triterpenes (Recio *et al*., 1995). It was reported that UA displays a significant COX-2 inhibitory effect, while OA was found to be less active than UA (Ringbom *et al*., 1998). Ismaili *et al*. (2001) reported that UA and OA are the main compounds responsible for the anti-inflammatory activity of *Thymus willdenowii* leaves extract. Other experimental *in vivo* studies showed the antinociceptive and anti-inflammatory effects of UA (Tapondjou *et al*., 2003).

Miconia, a genus bearing approx. 1000 species (Martins *et al*., 1996), belongs to the Melastomataceae family (Renner, 1993; Judd and Skean Jr., 1991). Previous studies on *Miconia* species have shown the presence of triterpenes (main constituents) as well as coumarins and benzoquinones (Lowry, 1968; Macari *et al*., 1990; Chan *et al*., 1992; Gunatilaka *et al*., 2001; Cunha *et al*., 2003).

Previous studies undertaken in our laboratory have described the analgesic effects of the crude

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hexane, methylene chloride, and hydroalcoholic extracts obtained from the aerial parts of *M. albicans*, using both writhing test in mice and hot plate test in rats (Vasconcelos *et al*., 2003). Oral administration of the methylene chloride extract of this plant led to significant antinociceptive activity in the writhing test. However, none of the crude extracts tested in that work produced a significant effect in the hot plate test.

The aim of this work was to use *in vivo* models to evaluate the analgesic and anti-inflammatory activities of UA and OA, the main constituents of the methylene chloride extract of *M. albicans,* in an attempt to clarify if these compounds are responsible for the analgesic properties displayed by this extract.

Materials and Methods

Plant material

M. albicans (SW.) Triana (Melastomataceae) was collected along the Franca-Claraval highway side, São Paulo, Brazil. It was identified by Dr. Angela Borges Martins at Biology Institute, UNICAMP, Campinas, Brazil, where a voucher specimen was deposited (UEC 25448).

General procedures

Vacuum-liquid chromatography (VLC) was carried out in a glass column with an internal diameter of 5 cm, using silica gel 60 (0.063–0.200 mm, Merck). Column chromatography (CC) was performed in a 450×25 mm glass column, using silica gel 60 (0.063–0.200 mm, Merck). Semi-preparative HPLC separation and analysis were carried out on a Shimadzu SCL-10 liquid chromatography system, equipped with a UV-diode array detector (UV detection: 225 nm) and a Shimadzu chromatographic column (silica 5 μm, 20×250 mm). NMR spectra were recorded on a Bruker ARX 400 spectrometer.

Extraction and isolation of UA and OA

The plant aerial parts were air-dried at 40 °C and ground. The powdered material (1.0 kg) was exhaustively extracted by maceration at room temperature, using *n*-hexane, methylene chloride and ethanol in this sequence, to afford the crude extracts (11.0 g, 9.2 g and 32.0 g, respectively).

The methylene chloride extract (7.0 g) was chromatographed over silica gel by a VLC system, using *n*-hexane/ethyl acetate and ethyl acetate/ethanol mixtures in increasing proportions, furnishing five fractions (F1–F5) of 2000 mL each, which were concentrated under reduced pressure (F1, *n*-hexane; F2, methylene chloride; F3, methylene chloride/ethyl acetate; F4, ethyl acetate; F5, ethanol). F3 (0.77 g) and F4 (0.45 g) were combined, once they exhibited similar chromatographic profiles on TLC. The combined fraction was purified over silica gel, furnishing a mixture containing 65% of UA and 35% of OA (350 mg). The individual compounds of the mixture were isolated by HPLC, using a silica column Shimadzu (5 μm, 20×250 mm) and an isocratic mobile phase consisting of *n*-hexane/isopropyl alcohol (49:1) at a 9 mL/min flow rate (Cunha *et al*., 2003). Ursolic and oleanoic acids were obtained as white amorphous solids: t_R: 5.7 min, $[\alpha]_D^{26}$ +63.4° (*c* 0.18, MeOH) and t_R: 5.0 min, $[\alpha]_D^{26}$ +68.2° (*c* 0.18, MeOH). The chemical structures of both compounds were established by ^{1}H and ^{13}C NMR analyses by comparing with data of authentic samples (Kim *et al*., 2000). Purity of the isolated compounds was considered to be above 95% by using both HPLC analysis and ^{13}C NMR spectroscopy.

Drugs and chemicals

Acetic acid (Merck, Darmstadt, Germany), indomethacin (Merck Sharp Dohm, St. Louis, MO, USA), morphine chlorohydrate (Sigma, St. Louis, MO, USA), acetylsalicylic acid (Sigma) and kappa carrageenan type III (Sigma) were used in the experiments. All the other chemicals employed in this work were of analytical grade and were purchased locally.

Animals

Male Swiss albino mice (20–25 g) were used for both the writhing and formalin tests. Male Wistar rats (160–170 g) were used for the paw oedema assay. The animals were housed in standard cages, in groups of five, at room temperature [(25 ± 3) °C] with both food and water *ad libitum*. They were transferred to the laboratory and maintained only with water *ad libitum* 12 h before the experiments. The experiments were authorized by the Ethical Committee for Animal Care of the University of Franca (Process number 089/04) in accordance with the Federal Government enforcement on animal care.

Abdominal constriction test

This test was carried out by using the method described by Koster *et al.* (1959). The writhes were induced by intraperitoneal injection of 0.6% acetic acid (v/v) (80 mg kg^{-1}) to a group of 5 mice. The number of muscular contractions was counted for 20 min, following the acetic acid injection. The treatments were undertaken orally using the following doses: 20 and 40 mg kg^{-1} for the mixture 65% UA/35% OA and 10, 20 and 40 mg kg^{-1} for pure compounds (UA and OA). Acetylsalicylic acid (100 mg kg^{-1}) was used as the reference drug for positive control.

Formalin test

The method used in this work was similar to that previously described (Shibata *et al.*, 1989; Okpo *et al.*, 2001). A 1% formalin solution (20 μL) was injected subcutaneously into the right hind paw of the rat. The time, in seconds, spent on the licking and biting responses of the injected paw was taken as an indicator of pain response. Responses were measured during the first 5 min after formalin injection (first phase) and between 15–30 min (second phase). The pure compounds UA and OA (10, 20 and 40 mg kg^{-1}, p.o.) and morphine (10 mg kg^{-1}, sc) were administered 30 min before the formalin injection. Negative control groups received orally the same volume of a 10% Tween saline solution, which had been used to dissolve the tested compounds.

Carrageenan-induced paw oedema in rats

The method used was described by Winter and Risley (1962). Carrageenan (0.1 mL, 100 μg) was injected into the right paw, while a saline solution (0.1 mL) was injected into the left paw. After the inflammatory stimulus, foot volume was measured by plethysmography (Plethysmometer Model 7140, Ugo Basile, Comerio-Varese, Italy) at one hour intervals, for 5 h. The activity was acknowledged for the third hour only, when the maximum oedema occurred. Results were obtained by measuring the difference in volume between the right and the left paws in comparison with both the negative (treated with 10% Tween saline solution) and the positive (treated with 10 mg kg^{-1} indomethacin) control groups. The treatments were undertaken using a dose of 40 mg kg^{-1} for UA, OA and the mixture of 65% UA/35% OA. This dose was established on the basis of the results obtained for the writhing test.

Statistical analysis

Data were statistically analyzed by one-way ANOVA followed by a multiple comparison test.

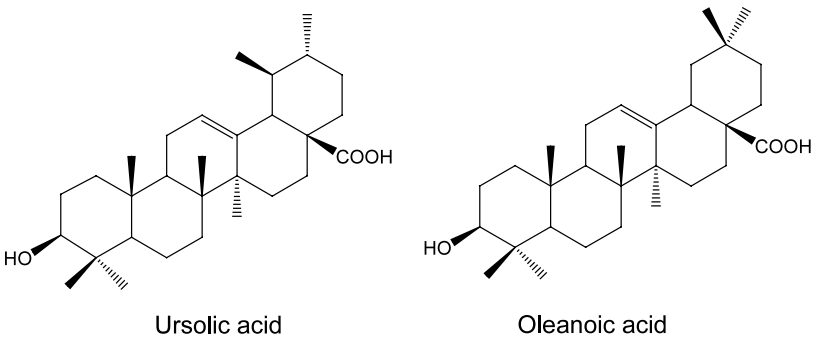
Results

Abdominal constriction test

The oral administration of both UA and OA (Fig. 1) showed a dose-dependent inhibition of acetic acid-induced abdominal writhes in mice. The inhibition due to UA (40 mg kg^{-1}) was similar to that produced by administration of acetylsalicylic acid (100 mg kg^{-1}) (Fig. 2). Interestingly, administration of a mixture of UA + OA (20 and 40 mg kg^{-1}) did not inhibit the writhes.

Paw oedema test

In the case of the carrageenan-induced paw oedema test in rats, the oral administration of UA



Ursolic acid Oleanoic acid

Fig. 1. Chemical structures of ursolic acid and oleanoic acid.

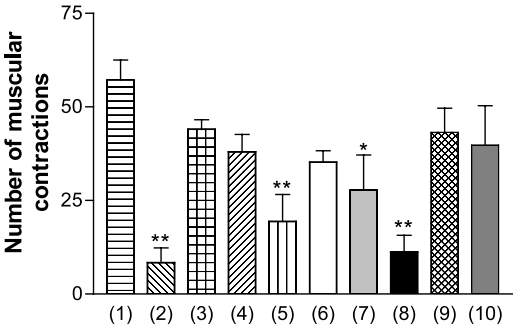


Fig. 2. Analgesic effect of ursolic acid (UA), oleanoic acid (OA) and a mixture of both evaluated by the abdominal contortion test, after intraperitoneal injection of 0.6% solution of acetic acid. (1) (–) Control (10% Tween saline solution); (2) (+) control (acetylsalicylic acid 100 mg kg^{-1}); (3) OA (10 mg kg^{-1}); (4) OA (20 mg kg^{-1}); (5) OA (40 mg kg^{-1}); (6) UA (10 mg kg^{-1}); (7) UA (20 mg kg^{-1}); (8) UA (40 mg kg^{-1}); (9) UA + OA (20 mg kg^{-1}); (10) UA + OA (40 mg kg^{-1}). Each group represents the mean ± SEM. *$P < 0.05$ and $P < 0.01$ were considered significant in comparison with negative control (analysis of variance and critical differences of the means were evaluated by Dunnett's test).

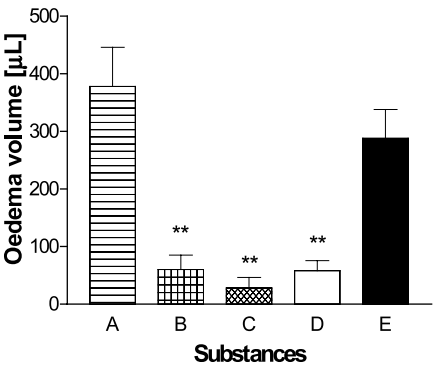


Fig. 3. Effects of ursolic acid (UA), oleanoic acid (OA) and the mixture of UA + OA (40 mg kg^{-1}) on rat paw oedema induced by carrageenan. (A) (–) control (10% Tween saline solution); (B) (+) control (indomethacin 10 mg kg^{-1}); (C) OA (40 mg kg^{-1}); (D) UA (40 mg kg^{-1}); (E) UA + OA (40 mg kg^{-1}). The results are expressed as mean ± SEM. **$P < 0.01$ (analysis of variance followed by Dunnett's multiple comparison test).

and OA (40 mg kg^{-1}) led to a significant anti-oedematous effect (Fig. 3). On the other hand, administration of the mixture of UA + OA (40 mg kg^{-1}) produced mild activity only, if compared with the effect obtained by using the individual compounds.

Formalin test

Administration of UA and OA alone did not produce a significant effect on the first phase of the formalin test. However, for all the tested doses, these compounds reduced the number of paw licks in the second phase of the formalin test (Table I). The first and second phases correspond to neurogenic and inflammatory pains, respectively.

Discussion

Our results indicate that UA and OA produced a significant anti-inflammatory effect on carrageenan-induced paw oedema. This effect is more pronounced than the one obtained by indomethacin, a well-known prostaglandin inhibitor compound. UA and OA also inhibit acetic acid-induced abdominal constriction in mice, but the results of this test alone do not allow us to conclude whether the origin of the analgesic activity lies on the central or on the peripheral actions of the tested samples. Therefore, the formalin test was undertaken to allow the investigation of the analgesic mechanism action of these compounds. We observed that OA and UA inhibited the 2nd phase of formalin-induced pain, suggesting that the analgesic effect of these compounds could be due to a peripheral mediated mechanism. Considering that these compounds lead to significant inhibition of the inflammatory pain, the possible site and mechanism of action could be due not only to the inhibition of inflammatory mediators, such as the syntheses of prostaglandins, but also to the blockage of their receptor sites. In this regard, Diaz *et al.* (2000) used *in vitro* assays and found that these compounds inhibit the enzymes of the arachidonate cascade, especially prostaglandin E2, for which the IC_{50} values were 23.51 μM and 60.91 μM for oleanolic and ursolic acids, respectively. It should be pointed out that the administration of the mixture 65% UA/35% OA did not significantly inhibit the abdominal constrictions in mice or the paw oedema in rats, when compared with both negative and positive control groups. This is

Table I. Effect of ursolic acid and oleanoic acid on formalin-induced pain.

Group	Dose [mg kg^{-1}]	0–5 min	Inhibition (%)	15–30 min	Inhibition (%)
Oleanoic acid	10	45.67 ± 8.47	24.50	1.12 ± 0.97***	96.79
	20	37.56 ± 1.36	37.91	0***	100.00
	40	31.44 ± 6.09**	48.02	0***	100.00
Ursolic acid	10	36.20 ± 4.43	40.16	5.17 ± 3.38***	85.17
	20	33.14 ± 2.88**	45.21	0***	100.00
	40	27.10 ± 6.44***	52.20	0***	100.00
Morphine	10	19.50 ± 4.24***	67.80	5.83 ± 4.16***	83.28
Control	–	60.49 ± 6.27	–	34.87 ± 6.14	–

Results expressed as mean ± SEM.
** $P < 0.01$; *** $P < 0.001$ (Bonferroni's multiple comparison test).

a very intriguing result, since on one hand the individual compounds UA and OA displayed significant analgesic and anti-inflammatory activities, and on the other hand the mixture of the compounds was poorly active. Therefore, other experiments should be undertaken aiming to investigate whether this low activity would be due either to the lower gastro-intestinal absorption of the mixture or to the interaction with the bind receptors.

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