

## Article

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# Anti-inflammatory and opioid-like activities in methanol extract of *Mikania lindleyana*, sucuriju

Frederico A. Vanderlinde,<sup>\*1</sup> Fabio F. Rocha,<sup>1</sup> David C. Malvar,<sup>1</sup>  
Raquel T. Ferreira,<sup>1</sup> Elson A. Costa,<sup>2</sup> Iziara F. Florentino,<sup>2</sup>  
Giselle M. S. P. Guilhon,<sup>3</sup> Thereza C. M. de Lima<sup>4</sup>

<sup>1</sup>Departamento de Ciências Fisiológicas, Instituto de Biologia, Universidade Federal Rural do Rio de Janeiro, Seropédica, Brazil,

<sup>2</sup>Departamento de Ciências Fisiológicas, Instituto de Ciências Biológicas, Universidade Federal de Goiás, Goiânia, Brazil,

<sup>3</sup>Departamento de Química, Centro de Ciências Exatas e Naturais, Universidade Federal do Pará, Brazil,

<sup>4</sup>Departamento de Farmacologia, Universidade Federal de Santa Catarina, Brazil.

**Abstract:** *Mikania lindleyana* DC., Asteraceae (sucuriju), grows in the Amazon region, where is frequently used to treat pain, inflammatory diseases and scarring. This study was carried out to investigate phytochemical profile accompanied by *in vivo* antinociceptive and anti-inflammatory screening of *n*-hexane (HE), dichloromethane (DME) and methanol (ME) extracts obtained from the aerial parts of the plant. The oral administration of ME (0.1, 0.3, 1 g/kg) caused a dose-related reduction (16.2, 42.1 e 70.2%) of acetic acid-induced abdominal writhing while HE and DME (1 g/kg, *p.o.*) were ineffective. In the hot plate test, ME (300 mg/kg, *p.o.*) increased the latency of heat stimulus between 30 and 120 min and inhibited the first (45%) and second (60%) phases of nociception in the formalin test. The antinociception induced by ME or positive control fentanyl (150 µg/kg, *s.c.*) in hot plate and formalin tests was prevented by naloxone (3 mg/kg, *s.c.*). When submitted to the carrageenan-induced peritonitis test, ME (0.5, 1.0, 2.0 g/kg, *p.o.*) impaired leukocyte migration into the peritoneal cavity by 46.8, 59.4 and 64.8% respectively, while positive control dexamethasone (2 mg/kg, *s.c.*), inhibited leukocyte migration by 71.1%. These results indicate that the antinociception obtained after oral administration of methanol extract of *M. lindleyana* involves anti-inflammatory mechanisms accompanied with opioid-like activity which could explain the use of the specie for pain and inflammatory diseases.

## Introduction

*Mikania lindleyana* DC., Asteraceae, is a creeper plant found in the Amazon region, known as sucuriju. The infusion of its leaves is empirically used as an anti-inflammatory, analgesic or cicatrizing and in the treatments of hepatitis, chronic gastric ulcers, varicose veins, acne and various types of cutaneous eruptions. Also is used as a diuretic and anti-hypertensive medication through orally administration of the tea leaves. The dry leaves of sucuriju are sold in some of Belém do Pará markets for medicinal purposes (Berg, 1993; Martins et al., 2005). The genus *Mikania* Willd belongs to the subtribe Mikaniinae and to the tribe Eupatorieae, from the family Asteraceae, and includes among 430 species across tropical regions.

Nowadays, several anti-inflammatory agents are used to treat different types of pain associated or not

to the inflammatory process. These agents are efficient in most cases, but the collateral effects are common, especially when they are used in chronic treatments. The most common collateral effect is gastrointestinal disturb (Peura & Goldkind, 2005). Considering the research of medicinal plants can propitiate the discovery of new molecules with innovative mechanisms or less adverse side effects (McCurdy & Scully, 2005), the ethnopharmacological profile of *Mikania lindleyana* and the absence of phytochemical and pharmacological studies with the aerial parts of this plant, the aim of this work was to assess the antinociceptive and anti-inflammatory activities and chemical studies of this specie. Here we report our first results of phytochemical and pharmacological investigations undertaken mainly with methanol extracts from the aerial parts of *M. lindleyana*.

## Material and Methods

### Extraction

*Mikania lindleyana* DC., Asteraceae, aerial parts were collected in Benevides-PA (Brazil) and samples were identified by Dr. M. E. van den Berg, botanist from Emilio Goeldi Paraense Museum (MPEG). The voucher specimens were deposited at the João Murça Pires Herbarium of MPEG under MG-01607412 number.

Botanical material were dried and submitted to extraction with hexane at room temperature, followed by dichloromethane and methanol extraction. The solutions obtained were concentrated dry under reduced pressure in a rotary evaporator, below 40 °C, to obtain the *n*-hexane (HE), dichloromethane (DME) and methanol (ME) extracts.

### Phytochemical characterization

In the phytochemical study of *M. lindleyana* extracts, the substances were isolated by chromatographic methods and identified by usual spectroscopic methods (NMR, IR and MS) being detected in the hexane and dichloromethane extracts: stigmasterol (0.27% of the extracts), stigmasta-4,24-dien-3-one (0.07%), 24-methylene-cycloartan-3-ol (0.60%), a mixture of 24-methylene-cycloartan-3-ol and cycloart-24-en-3-ol (0.51%), a mixture of 24-methylene-cycloartan-3-one and cycloart-24-en-3-one (0.29%), trans-phytol (0.29%) and also long chain esters of stigmasterol (0.57%) and fatty acids (0.25%). A mixture of the inorganic salts KNO<sub>3</sub> and KCl (0.50%) was obtained from the methanol extracts (Guilhon et al., 1998a,b,c, 1999).

When partitioned with water and chloroform, methanol extract (1 g) formed the aqueous (A-F) and chloroform (CH-F) fractions. Analyses by comparative thin layer chromatography (CCDC) on plates of silica gel (Merck F<sub>254</sub>) were eluted with butanol/acetic acid/water (BAW, 4:1:5) and chloroform-methanol 9:1 (CHCl<sub>3</sub>:MeOH) and visualized in UV chromatograms at 254 nm and 366 nm. The reagent NP/PEG (BAW) revealed the presence of flavonoids in CH-F (R<sub>f</sub> 0.70 and 0.57) and A-F (R<sub>f</sub> 0.20) for rutin and quercetin R<sub>f</sub> 0.55/R<sub>f</sub> 77 (Merck) and triterpenes (Lieberman-Burchard) AF (R<sub>f</sub> 0.21 and 0.27). The Drangendorff reagent did not detect alkaloids (Moreira et al., 2008).

### Animals

Adult male Swiss mice (25-35 g) were housed in plastic cages, with food and tap water available *ad libitum* in the colony room. Mice were acclimatized to the laboratory for at least 60 min prior to the test procedure and were fasted for 12-18 h before the gavage. All

experiments were carried out in accordance with current guidelines for the care of laboratory animals and the ethical guidelines on the use of animals in pain research (Zimmermann, 1986). The experimental protocols were approved by the local Animal Care and Use Committee (006/2006/CEPEB/UFRRJ). The minimum number of animals and duration of observation required to obtain consistent data were employed.

### Drugs

Formalin, acetic acid, acetone (Merck AG, Darmstadt, Germany), indomethacin, carrageenan, naloxone hydrochloride (Sigma Chemical Co., St. Louis, MO, USA), fentanyl (Janssen Pharmaceutical), dexamethasone (Prodome, Brazil). The *n*-hexane (HE), dichloromethane (DME) and methanol (ME) extracts were prepared and provided by Dr. Giselle M. S. P. Guilhon (Federal University of Pará, Brazil). Test solutions of extracts and drugs were diluted in water (*p.o.*) or saline (*s.c.* or *i.p.*) in such a concentration as to allow the administration of constant volumes of 10 mL/kg for each dose employed. The oral pre-treatment (*p.o.*) was always done 1 h and subcutaneous (*s.c.*) 30 min before tests.

### Acetic acid-induced writhing test

In an initial screening of antinociceptive activity, group of six mice were orally treated with vehicle (water), *n*-hexane (HE), dichloromethane (DME), methanol (ME) extracts (1.0 g/kg) or positive control indomethacin (10 mg/kg), 60 min before acetic acid injection (1.2%, 0.1 mL/10 g) and the number of writhes was counted for the following 30 min (Koster et al., 1959). The absence of activity with the HE and DME directed the study to obtain in this method the dose effects of ME (0.1, 0.3 and 1.0 g/kg).

### Hot-plate test

The latency (s) of heat stimulus (55.0±0.5 °C) was measured every 30 min, starting 30 min before and up to 150 min after pretreatment of mice (n=8) with vehicle (water), ME (300 mg/kg, *p.o.*), fentanyl (positive control - 100 µg/kg, *s.c.*), ME+naloxone (3 mg/kg, *s.c.*) or fentanyl+naloxone. The animals whose basal flick responses were bigger than 9 s were discharged and a cut-off time of 30 s was maintained through the experiment, to prevent tissue damage. (D'Amour & Smith, 1941).

### Formalin-induced nociception

Groups of 6-8 mice were orally treated with water, ME (300 mg/kg), indomethacin (10 mg/kg) 60 min prior or fentanyl (100 µg/kg, *s.c.*) 30 min prior to

injection of the formalin solution (3%, 20  $\mu$ L/paw, *s.c.*) into the plantar surface of the hind paw. To investigate the mechanism of antinociception, naloxone (300 mg/kg, *s.c.*) treatment preceded (15 min) the administration of ME or fentanyl. The time that animals spent licking the injected paw was measured with a chronometer and was considered as an index of pain (licking time) in seconds. From the formalin injection, the initial nociceptive response peaked at about 5 min (first phase) and was followed by a second peak (second phase) that occurred at 15-30 min post injection (Hunnskaar et al., 1986; Hunnskaar & Hole, 1987).

#### Peritonitis induced by carrageenan

Mice (n=10) were orally treated with vehicle (water), ME (0.5, 1.0 and 2.0 g/kg) 60 min prior to an injection of carrageenan (1% in saline; 250  $\mu$ L/mouse) into the peritoneal cavity. The positive control group was pre-treated (30 min) with dexamethasone (2 mg/kg, *s.c.*). The number of leukocytes that had migrated to the peritoneum was counted in a Neubauer chamber and results were expressed as cells $\times 10^6$ /mL and % of inhibition of leukocyte migration compared to control groups (Ferrández & Alcaraz, 1991).

#### Statistical analysis of data

Data were statistically analyzed by one-way ANOVA followed by the *Dunnet* multicomparison test. In the hot-plate test, the two-way ANOVA followed by the *Bonferroni's* test analysis was used. The values are reported as mean $\pm$ standard error of the mean (SEM). P values less than 0.05 ( $p < 0.05$ ) were considered to be significant (Sokal & Rohlf, 1981).

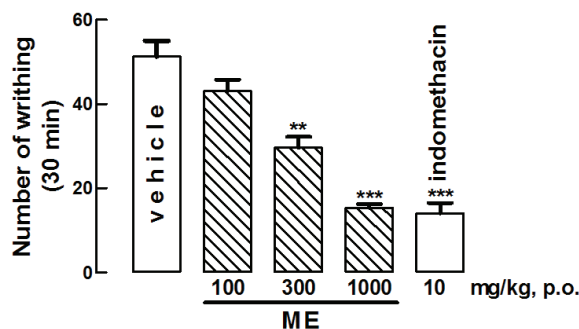
## Results

#### Acetic acid-induced writhing test

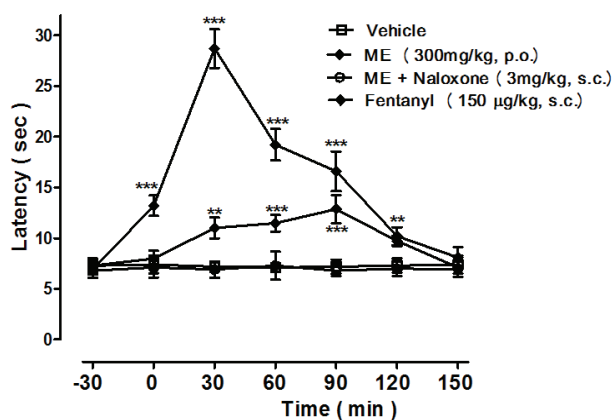
The initial screening of antinociceptive activity demonstrated that the oral treatments with hexane (HE) or dichloromethane (DME) extracts (1.0 g/kg) did not alter the number of acetic acid-induced abdominal writhing (48.3 $\pm$ 3.9 and 54.1 $\pm$ 4.0, respectively) compared to the control group (51.3 $\pm$ 3.8 writhes) while as shown in Figure 1, methanol extract (ME - 0.1, 0.3 and 1 g/kg) produced a dose-related inhibition of acetic acid-induced abdominal writhing (16.2 $\pm$ 1.1, 42.1 $\pm$ 3.8 and 70.2 $\pm$ 4.8 %, respectively; IC<sub>50</sub>=431.5 mg/kg). The group treated with indomethacin showed a decrease in accumulated abdominal writhes (14 $\pm$ 2.5 writhes).

#### Hot-plate test

In the hot-plate test Figure 2 shows that the basal latency of control group was 7.1 $\pm$ 0.6 s and orally administration of ME (300 mg/kg) increased the pain latency by 51.7 $\pm$ 4.8, 62.4 $\pm$ 4.3, 79.5 $\pm$ 8.6 and 33.8 $\pm$ 1.7% after 30, 60, 90 and 120 min respectively. The positive control fentanyl (150  $\mu$ g/kg, *s.c.*) produced a significant increased of reactivity time to heat stimulus in the intervals between zero and 120 min with a maximum latency of 3.9-fold (30 min). Pre-treatment (15 min) with naloxone (3 mg/kg, *s.c.*) inhibited antinociceptive effect of ME (figure 2) and fentanyl (results no show).



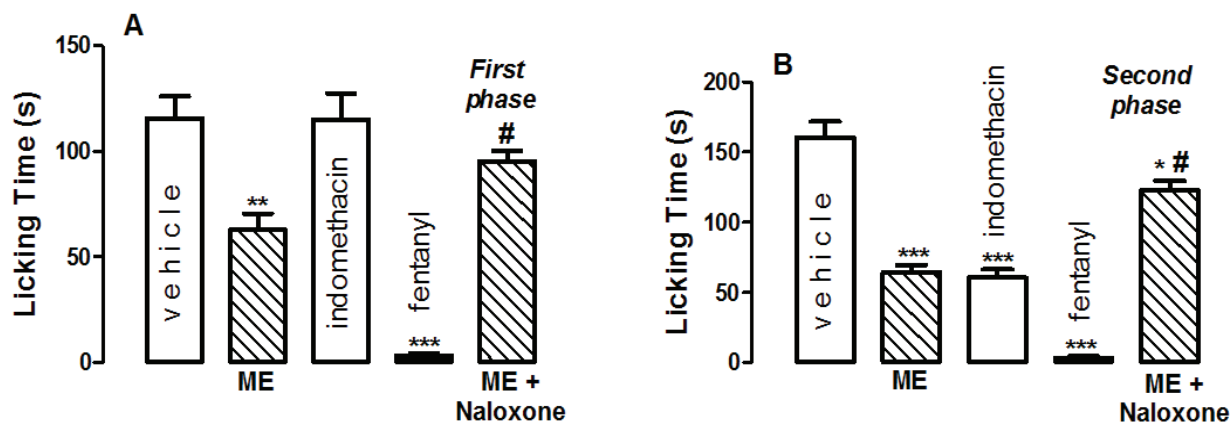
**Figure 1.** Effect of methanol extract (ME) obtained from the aerial parts of *Mikania lindleyana* or indomethacin on the acetic acid-induced writhing in mice. The vertical bars and lines express means $\pm$ SEM of the number of writhing registered for 30 min after the acetic acid injection. Significantly different from vehicle group: \*\* $p < 0.01$ ; \*\*\* $p < 0.001$  (n=6).



**Figure 2.** Latency of response to thermal stimulus (hot plate at 55 °C) in group of mice (n=8) before and after treatment with water (Vehicle), methanol extract (ME) obtained from the aerial parts of *Mikania lindleyana*, fentanyl or ME+naloxone. The symbols and vertical lines express means $\pm$ SEM of the latency for the nociceptive behavior. \*\* $p < 0.01$ , \*\*\* $p < 0.001$  when compared with the vehicle group. Symbols represent the different between the ME and fentanyl.

#### Formalin-induced nociception

In the formalin-induced nociception test, the



**Figure 3.** Effect of methanol extract of *Mikania lindleyana* (ME-300 mg/kg, *p.o.*) on the licking time (s) in the first phase (0-5 min/A) and second phase (15-30 min/B) of the formalin test in mice (n=6-8). Fentanyl (100 µg/kg, *s.c.*) and indomethacin (10 mg/kg, *p.o.*) were used as positive controls of the first and second phases respectively. The administration of naloxone (3 mg/kg, *p.o.*) preceded ME treatment. The vertical bars and lines express means±SEM of licking time. \**p*<0.05, \*\**p*<0.01; \*\*\**p*<0.001 significantly different from vehicle group (A–B) and #*p*<0.01 when compared with the ME group (B).

licking time of animals previously treated with vehicle presented 115.8±10.3 s in the first phase and 160.5±11.4 s in the second phase. ME (300 mg/kg, *p.o.*) treatment produced reduction in pain reaction to 63.0±7.4 s (Figure 3A) and 64.1±5.5 s (Figure 3B) in the first and second phases respectively. The licking times of animals treated with fentanyl (100 µg/kg, *s.c.*) were reduced in both phases (around 97%), while indomethacin treatment (10 mg/kg, *p.o.*), a positive control of second phase, inhibited 62.3% this phase without influencing the first phase of nociception. The naloxone pre-treatment (3.0 mg/kg, *s.c.*) reversed totally the antinociceptive effects of ME in the first phase (95.3±5.1 s) and partially in the second phase (122.6±7.2 s) of test (Figures 3A and 3B). The fentanyl analgesic effect also were blocked by naloxone (results no show).

**Table 1.** Effect of ME of *M. lindleyana* on carrageenan-induced peritonitis in mice.

Treatment	Dose (g/kg)	Number of leukocytes (x 10 <sup>6</sup> /mL)	Inhibition (%)
Vehicle ( <i>p.o.</i> )	-	12.8±1.7	-
ME ( <i>p.o.</i> )	0.5	6.8±1.0**	46.8
ME ( <i>p.o.</i> )	1.0	5.2±0.8***	59.4
ME ( <i>p.o.</i> )	2.0	4.5±0.9***	64.8
Dexamethasone ( <i>s.c.</i> )	0.002	3.7±0.3***	71.1

Values represent the means±SEM of ten mice. Significantly different from vehicle group. *p*<0.01, *p*<0.001.

#### Peritonitis induced by carrageenan

Using carrageenan as a stimulus, it was possible to produce an acute inflammatory response after 4 h

in the peritoneal cavity of mice, with a large number of leukocytes in the exudates. With the aim of evaluating a possible inhibitory effect of ME on the cell migration into the peritoneal cavity, the carrageenan-induced peritonitis test was used. As can be seen in Table 1 in comparison with vehicle group (12.8±1.7×10<sup>6</sup> leukocytes/mL), ME (0.5, 1.0 and 2.0 g/kg, *p.o.*) exhibited a dose-related reduction of leukocyte migration by 46.8, 59.4 and 64.8%, respectively. The positive control dexamethasone (2 mg/kg, *s.c.*) also was effective, inhibiting leukocyte migration by 71.1%.

#### Discussion

In the antinociceptive screening using acetic acid-induced abdominal writhing model and high doses of hexane (HE), dichloromethane (DME) or methanol (ME) extracts obtained from the aerial parts of the *Mikania lindleyana* DC., Asteraceae, only the ME was effective. The writhing induced by acetic acid in mice result from chemical-induced a peripheral acute inflammatory reaction (Zakaria et al., 2006a). The treatment with ME promoted a dose-related antinociceptive effect in this model, normally used for screening of synthetic and natural compounds to determinate the presence of central and/or peripheral antinociceptive activity, because it is sensitive to nonsteroidal anti-inflammatory drugs (NSAID) and opioids (Zakaria et al., 2006b; Fischer et al., 2008). Moreover, the nociception induced in writhing method has been associated with some prostanoids, like as PGE<sub>2</sub>, PGF<sub>2α</sub> and PGI<sub>2</sub> (Deraedt et al., 1980) as well as lipoxigenase products (Ballou et al., 2000; Parveen et al., 2007), neurokinin A (Julia & Buéno, 1997) and CGRP (Friese et al., 1997), for example. The consequence of the involvement of different mediators in the nociception,

which means that based only on this model, is not possible to suggest any mechanism to the antinociceptive effect of ME.

Therefore the effectiveness of ME was tested in more two models of analgesia: hot-plate and formalin-induced nociception tests. The hot plate test is a good model for studying central activity, being selectivity for drugs as opioid-derived analgesics. This assay is thought to involve the supraspinal reflex (Oliveira et al., 2008), produce an acute non-inflammatory nociception and moderate analgesic appear to have less or nothing effect in this kind of test (Zakaria et al., 2006a,b; Fischer et al., 2008). In this test, the ME affected mice latency time from the thermal stimulus, showing that ME antinociception may have the participation of central action. In fact, the reversal of this effect after prior administration of naloxone, a competitive antagonist of opioid receptors, revealed opioid-like mechanisms in the ME antinociception. This conclusion was reinforced by the results that ME reduced the licking time in the first phase (neurogenic pain) which is highly sensitive to opioid agents (Hunskar & Hole, 1987) and second phase (inflammatory pain) of formalin test. Additionally, naloxone also reversed antinociception produced of ME in both phases of the test. It has been demonstrated that intraplantar injection of formalin in rodents produces significant increases in spinal levels of different mediators, such as excitatory aminoacids, PGE<sub>2</sub>, nitric oxide and tachykinin, kinins, among other peptides (Fischer et al., 2008). The test produced a distinct biphasic nociceptive response generally regarded as the early (first) and late (second) phases. The early phase is result of direct stimulation of nociceptors by formalin and is an acute reaction observed immediately after the administration of the agent (0-5 min, tonic pain) (Zakaria et al., 2007). This phase is produced from stimulation of myelinated and unmyelinated fibers, mainly C fibers, and the release of substance P, histamine, CGRP and can be suppressed by opioid analgesic drugs like morphine. (Gonçalves et al., 2008; Oliveira et al., 2008). Histamine appears to be the principal mediator in this phase (Wang et al., 2008). The second phase (15-30 min, localized inflammatory pain) is due to the inflammatory processes, with mediators release locally, such as prostaglandins, serotonin, histamine and bradykinin, as well as activation of the neurons located in the dorsal horns, which promote facilitation of synaptic transmission at the spinal cords (Oliveira et al., 2008; Zakaria et al., 2007). This latter phase was reported to be sensitive to the action of the majority of NSAID, including acetyl salicylic acid, indomethacin or naproxen (Gonçalves et al., 2008) consequently the inhibition of the second phase suggest anti-inflammatory mechanism, although analgesics without anti-inflammatory properties such fentanyl, may exhibit this effect. The fact that naloxone reverse totally the first and partially the second

phases suggest that ME antinociception is produced by anti-inflammatory and opioid-like mechanisms. The antinociception of ME is not related to nonspecific central effects, since no detectable effect was observed after oral administration of ME high dose in the rota-rod test (results not showed).

Considering the popular use of specie to treat inflammatory diseases and antinociception obtained in the second phase of formalin test accompanied by partial reversal with naloxone pre-treatment, the anti-inflammatory hypothesis was tested in leukocyte migration model. The carrageenan-induced peritonitis was used to investigate possible alterations in the leukocyte migration. ME reduced in a dose-related manner the total leukocyte migration to the peritoneum induced by carrageenan with a highest inhibition value by 64.8%. This process is dependent of the synthesis and release of chemo-attractants such as lipoxigenase products and chemokines being constituted by three phase: leukocyte rolling, attachment and diapedesis. Drugs can reduce the leukocyte migration by inhibit the synthesis and release of chemo-attractants or by block of some leukocyte migration phase (Muller, 2002). Several studies have been reported that leukocytes migration would not be directly related to cyclooxygenase products, but the process could be inhibited by some, indicating that many mechanisms may be implicated in its control (Higgs et al., 1980; Mikami & Miyasaka, 1983; Brooks & Day, 1991). A recent study showed that PGF<sub>2α</sub> was still able to induce leukocyte migration, suggesting that the inhibitory property of NSAID appears to be primarily related to the reduced production of the PGF<sub>2α</sub> (De Menezes et al., 2005). Since several mechanisms may be involved in the leukocytes migration more studies must be done to elucidate the mechanism of the anti-inflammatory effect of ME.

These results showing that the oral administration of methanol extract of *Mikania lindleyana* produced a potent antinociceptive activity in the acetic acid-induced abdominal writhing, inhibitory effect on the reactivity of heat stimulus in the hot-plate test and the first phase (neurogenic pain) and second phase (inflammatory pain) of the formalin test with effects reversed by naloxone, are indicative that ME antinociception involves opioid-like mechanisms. The fact of ME also reduced the leukocyte migration, a characteristic activity present of anti-inflammatory drugs, allow to state that antinociception of ME is also associated with anti-inflammatory mechanisms.

The anti-inflammatory and antinociceptive effects of stigmasterol isolated from different vegetal species have been demonstrated in pharmacological models (Santos et al., 1995; Garcia et al., 1999) as flavonoids have long been recognized to possess a wide variety of biological activities such as antioxidant

and anti-inflammatory (Middleton et al., 2000), besides the blockade of phospholipase A<sub>2</sub>, inhibit the cyclooxygenase and/or the lipoxygenase pathways of arachidonate metabolism (Lindahl & Tagesson, 1997; Middleton et al., 2000; Kwak et al., 2003). Although the phytochemical study of hexane and dichloromethane extracts has detected stigmaterol compound (Guilhon et al., 1998a,b,c, 1999), high doses of these extractions were ineffective in acetic acid-induced writhing model, however the antinociceptive and anti-inflammatory activities of ME can be attributed to flavonoids compounds, such quercetin and rutin, present in this extraction (Moreira et al., 2008), without excluding the possibility of stigmaterol, and other chemical compounds present in the aerial parts of plant also participate in the analgesic and anti-inflammatory effectiveness of specie. These results could explain the use of the plant for pain and inflammatory diseases and a bioassay-guided fractioning of the extracts is now in progress to identify a greater number bioactive substances in aerial parts of *M. lindleyana* as well as the mechanisms of action involved in the effects described before.

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**\*Correspondence**

Frederico A. Vanderlinde  
Laboratório de Farmacologia, Sala 31, Pavilhão de Química,  
Universidade Federal Rural do Rio de Janeiro  
BR 465, km 07, 23890-000, Seropédica, RJ, Brazil  
vanderlinde@ufrjr.br  
Tel: +55 21 26823222