

# Phytochemical screening, antinociceptive and anti-inflammatory effects of the essential oil of *Myrcia pubiflora* in mice

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Article

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**Abstract:** This report aimed to investigate the chemical composition and possible antinociceptive and anti-inflammatory effects of the essential oil from fresh leaves of *Myrcia pubiflora* DC., Myrtaceae (EOMP), through different experimental tests. The essential oil of *M. pubiflora* (EOMP) was obtained by hydrodistillation, analyzed by GC-MS, and tested at doses of 25, 50, and 100 mg/kg (*i.p.*) in three different tests of nociception (acetic acid-induced writhing test, formalin test, and hot plate test) and one test of inflammation (leukocyte migration to the peritoneal cavity) in order to evaluate the motor activity in mice treated with EOMP. The major component of EOMP was caryophyllene oxide (22.16%). This oil significantly reduced the number of writhes in an acetic acid test and the time spent licking the paw at the second phase of the formalin test. Furthermore, EOMP inhibited the carrageenan-induced leukocyte migration to the peritoneal cavity. However, administration of EOMP did not alter reaction time in the hot plate test, and did not affect the motor coordination test. These results indicate antinociceptive and anti-inflammatory properties of EOMP probably mediated via inhibition of inflammatory mediator synthesis or other peripheral pathway.

## Keywords:

antinociceptive activity,  
anti-inflammatory activity  
caryophyllene oxide  
*Myrcia pubiflora*

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## Introduction

Pain, though an important physiological process for the individual that prevents the occurrence of diseases and/or changes that may endanger survival, has been since antiquity one of mankind's major scourges. This has encouraged scientists in laboratories and industries to search for substances capable of relieving pain. The discovery of new drugs of anesthetic action and/or analgesic drugs with fewer side effects than those available on the market could bring fortune to the holders of such a discovery (Souza et al., 2003; Quintans-Júnior et al., 2010). However, medicinal plants have been an important source of new drugs with biological activity (Quintans-Júnior et al., 2008; Melo et al., 2010; Guimarães et al., 2010).

The Myrtaceae family consists of around 129

genera and 4620 species (Mabberley, 1997), many of which have been reported to have antinociceptive and anti-inflammatory action in rodents, as *Eugenia candolleana* (Guimarães et al., 2009), *E. caryophyllata* (Daniel et al., 2009), and *Campomanesia adamantium* (Vendruscolo et al., 2005).

Some species of the *Myrcia* genera are used in folk medicine, especially *M. multiflora* (Lam.) DC., because of the hypoglycemic action of their infusion or decoction. Pharmacological studies have demonstrated activity from the extract of the leaves of *M. fallax* (Rich.) DC. against cancer cells, and antidiabetic activity (Limberger et al., 2004).

The specie *Myrcia pubiflora* DC. is unpublished and its study is based on the pharmacological activity of the Myrtaceae species. Caryophyllene oxide, a major constituent of the essential oil of species of the Myrtaceae

family, belongs to the family of terpenes that, according to the literature, have already presented antinociceptive and anti-inflammatory activity (Vendruscolo, 2005; Vallilo et al., 2006; Guimarães et al., 2010).

Taking into account the biological activities of the Myrtaceae, it is surprising that no pharmacological study has been carried out on the chemical composition and possible antinociceptive and anti-inflammatory effects of the essential oil of *M. pubiflora* DC. (EOMP) until now. Here, we have therefore examined the possible antinociceptive and anti-inflammatory actions of EOMP in experimental protocols on mice.

## Material and Methods

### *Plant material and essential oil extraction*

Leaves of the *Myrcia pubiflora* DC., Myrtaceae, were collected in Santo Amaro das Brotas-SE, Brazil (satellite positioning: S 10.47.2040/W 36.58.2508). The specie was identified by Dr. Adauto de Souza Ribeiro and Dr. Marcos E. Sobral and the voucher specimen was deposited in the Herbarium of the Department of Botanic of Federal University of Minas (BHCB n° 642, Adauto Ribeiro).

### *Isolation of essential oil*

The EOMP was obtained by hydrodistillation for 3 h using a glass Clevenger apparatus, physically separated from the water, dried with anhydrous sodium sulphate and filtered. The oil mass was determined by an analytical balance with precision of 1 mg. Samples of the oil were transferred to amber glass bottles of and stored in a freezer at -20° C, until analysis. Extractions were performed in triplicate.

### *Gas Chromatography-Mass Spectrometry*

Oil samples were analyzed using a Shimadzu QP5050A (Shimadzu Corporation, Kyoto, Japan) system comprising a AOC-20i autosample and gas chromatograph interfaced with a mass spectrometer (GC/MS) employing the following conditions: J&W Scientific DB-5MS (Folsom, CA, USA) fused silica capillary column (30 cm x 0.25 mm i.d, composed of 5% phenylmethylpolysiloxane), operating in electron impact mode at 70 eV; helium (99.999%) was used as the carrier gas at a constant flow of 1.2 mL min<sup>-1</sup> and an injection volume of 0.5 µL was employed (split ratio of 1:83) injector temperature 250 °C; ion-source temperature 280 °C. The oven temperature was programmed from 50 °C (isothermal for 2 min), with an increase of 4 °C/min., to 200 °C, then 10 °C/min to 300 °C, ending with a 10 min isothermal at 300 °C.

Mass spectra were taken at 70 eV; a scan interval of 0.5 s and fragments from 40 to 550 Da.

### *Gas Chromatography - Flame ionization Detector (GC-FID)*

Quantitative analysis of the chemical constituents was performed by flame ionization gas chromatography (FID), using a Shimadzu GC-17A (Shimadzu Corporation, Kyoto, Japan) apparatus, under the following operational conditions: ZB-5MS (5%-phenyl -arylene-95%-dimethylpolysiloxane) fused silica capillary column (30 m x 0.25 mm i.d. x 0.25 µm film thickness) from Phenomenex (Torrance, CA, USA), under the same GC-MS conditions. Quantification of each constituent was estimated by area normalization (%). Compound concentrations were calculated from the GC peak areas and arranged in order of GC elution.

### *Drugs*

Dexamethasone, acetic acid, formalin, diazepam and polyoxyethylene-sorbitan monolated (Tween 80) was purchased from Sigma (USA). Morphine was purchased from União Química (Brazil).

### *Animals*

Male Swiss mice (20-30 g), 2-3 months of age, were used throughout this study. The animals were randomly housed in appropriate cages at 25±2 °C on a 12 h light/dark cycle (lights on 6:00-18:00 h) with free access to food (Purina®) and water. They were used in groups of eight animals each. All experiments were carried out between 9am and 4pm in a quiet room. Experimental protocols and procedures were approved by the Animal Care and Use Committee at the Universidade Federal de Sergipe (CEPA/UFS N° 49/09).

### *Acetic acid-induced writhing test*

This test was done using the method described by Koster et al. (1959) and Broadbear et al. (1994). Muscular contractions were induced by intraperitoneal injection (*i.p.*) of a 0.85% solution of acetic acid (0.1 mL/10 g) to a group of eight mice. After a latency period of 5 min, the number of muscular contractions was counted for 15 min and the data represents the average of the total number of writhes observed. EOMP was administered in doses of 25, 50, and 100 mg/kg (*i.p.*). The reference drug, morphine (MOR) (3 mg/kg) was solubilized in saline+Tween-80 0.2% (vehicle) and was administered intraperitoneally to different groups of the mice 0.5 h before the acetic acid injection.

### Formalin test

The observation chamber was a glass box of 30 cm diameter on an acrylic transparent plate floor. Beneath the floor, a mirror was mounted at a 90 °C angle to allow clear observation of the paws of the animals. The animals were treated with the vehicle (saline+Tween-80 0.2%), EOMP (25, 50, and 100 mg/kg, *i.p.*) or the reference drug (MOR, 3 mg/kg, *i.p.*) 0.5 h before the formalin injection. Each mouse was placed in the chamber more than 5 min before treatment in order to allow acclimatization to the new environment. The formalin test was carried out as described by Hunskaar & Hole (1987). Twenty microliters of a 1% formalin solution (0.92% formaldehyde) in a phosphate-buffer were injected into the dorsal surface of the left hind paw. Each animal was then returned to the chamber and the amount of time that the animal spent licking the injected paw was considered to be indicative of pain. Two distinct phases of intensive licking activity were identified: an early acute phase and a late or tonic phase (0-5 and 15-30 min after formalin injection, respectively).

### Hot plate test

The hot plate test described by Jacob et al. (1974) and by Jacob & Ramabadran (1978) was used. The animals were placed on an aluminum plate that was adapted to a water bath at 55±0.5 °C. The reaction time was noted by observing either the licking of the hind paws or the rotation movements at basal, 0.5, 1.0, 1.5, and 2.0 h after *i.p.* administration of 25, 50, and 100 mg/kg of EOMP or the vehicle (saline+Tween-80 0.2%) to different groups of 8 mice. Morphine, 3 mg/kg (*i.p.*), was used as the reference drug.

### Evaluation of the motor activity

To investigate if the treatments could influence the motor activity of the animals and consequently impair the assessment of the nociceptive behavior in the experimental tests, the motor activity of the animals was evaluated in a Rota rod apparatus, according to Dunham & Miya (1957) with some modifications. Initially, the mice able to remain on the Rota rod apparatus (AVS®, Brazil) longer than 180 s (7 rpm) were selected 24 h before the test. Then the selected animals were divided into five groups (n=8) and treated *i.p.* with vehicle (control), EOMP (25, 50 and 100 mg/kg, *i.p.*), and diazepam (DZP, 1.5 mg/kg). Each animal was tested on the Rota rod and the time (s) they remained on the bar for up to 180 s was recorded 0.5, 1 and 2 h after administration.

### Leukocyte migration to the peritoneal cavity

The leukocyte migration was induced by injection of carrageenan (1%, *i.p.*, 0.25 mL) into the peritoneal cavity of mice 0.5 h after administration of EOMP (25, 50, and 100 mg/kg, *i.p.*), dexamethasone (2 mg/kg, *i.p.*) or vehicle (saline+Tween-80 0.2%) by modification of the technique previously described by Matos et al. (2003). The animals were anesthetized with sodium pentobarbital (50 mg/kg, *i.p.*) and were euthanized by cervical dislocation 4 h after carrageenan injection. Shortly after, saline containing EDTA (1 mM, *i.p.*, 3 mL) was injected. Immediately a brief massage was done for further fluid collection, which was centrifuged (5000 x g, 5 min) at room temperature. The supernatant was disposed and the precipitate was resuspended in saline. An aliquot of 10 µL from this suspension was dissolved in 200 µL of Turk solution and the total cells were counted in a Neubauer chamber, under optic microscopy. The results were expressed as the number of leukocytes/mL. The percentage of the leukocyte inhibition =  $(1 - T/C) \times 100$ , where *T* represents the treated groups leukocyte counts and *C* represents the control group leukocyte counts.

### Statistical analysis

The obtained data was evaluated by one-way analysis of variance (ANOVA) followed by Tukey's test. In all cases differences were considered significant if  $p < 0.05$ . The percent of inhibition by an antinociceptive agent was determined for the acetic acid-induced writhing and formalin tests using the following formula (Reanmongkol et al., 1994):  $\text{Inhibition\%} = 100 \cdot (\text{control} - \text{experiment}) / \text{control}$ .

## Results

### Analysis of essential oil

The essential oil of the fresh leaves of *M. pubiflora* was yellow in color and had an average yield of 1.1% (v/w). GC-MS and GC-FID analysis of the essential oil resulted in the identification of 22 compounds, by the comparison of retention indices and mass spectra from the literature (Adams, 2007), constituting 72.7% of the total oil (Table 1). The major component was caryophyllene oxide (22.2%), with other components present in appreciable content, such as: mustakone (11.3%), 1,8-cineole (5.4%), and tricyclene (5.3%).

### Acetic acid-induced writhing

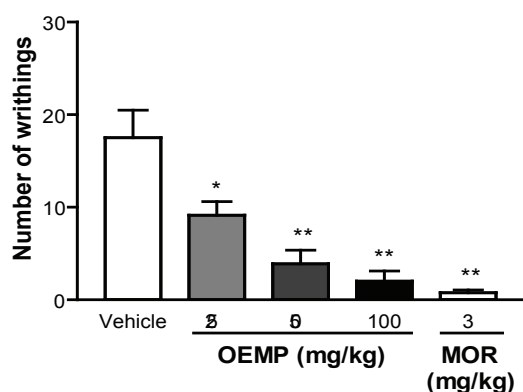
Figure 1 shows that EOMP significantly

reduced the number of writhings induced by the *i.p.* administration of acetic acid solution at doses of 25 ( $p<0.05$ ), 50, and 100 mg/kg ( $p<0.001$ ). As can be seen in Figure 1, doses of 50 and 100 mg/kg produced a similar effect to morphine (3 mg/kg).

**Table 1.** Chemical constituents of essential oil from leaves of *Myrcia pubiflora* analyzed by GC-MS.

Compound	IR exp.	IR lit.*	Percentage
Tricyclene	921	926	5.27
<i>p</i> -Cymene	1021	1024	1.95
Limonene	1026	1029	2.15
1,8-Cineole	1029	1031	5.35
$\alpha$ -Camfolenale	1124	1126	0.43
<i>Trans</i> -pinocarveole	1138	1139	4.71
Pinocarpone	1159	1164	1.61
<i>p</i> -Mentha-1,5-dien-8-ol	1169	1170	2.84
Terpinen-4-ol	1178	1177	0.49
<i>p</i> -Cymene-8-ol	1185	1182	0.86
Mirtenol	1193	1194	4.31
Verbenone	1205	1205	2.16
<i>Trans</i> -carveol	1216	1216	0.71
Carvone	1241	1243	1.45
Cyclosativene	1366	1371	0.49
$\alpha$ -copaene	1372	1374	1.72
$\delta$ -Amorfeno	1514	1512	0.89
$\alpha$ -Calacoreno	1538	1545	1.07
Caryophyllene oxide	1580	1583	22.16
Ledol	1601	1602	0.70
Mustakone	1672	1676	11.34

RI: Retention Indices; \*Adams (2007).



**Figure 1.** Effects of EOMP on the acetic acid-induced writhing test. Vehicle (control), EOMP (25, 50 and 100 mg/kg) or morphine (MOR) (3 mg/kg) were administered *i.p.* 0.5 h before acetic acid injection. Each column represents mean±SEM (n=8). \* $p<0.05$ , \*\* $p<0.001$  versus control (ANOVA followed by Tukey's test).

### Formalin test

The results of this test are shown in Table 2. Intraperitoneal administration of EOMP at doses of 25 ( $p<0.05$ ), 50, and 100mg/kg ( $p<0.001$ ) significantly reduced nociception in the second phase of the formalin test.

### Hot plate test

Table 3 shows the results of the hot plate test. All doses of EOMP were ineffective at inhibiting the time of reaction to the thermal stimulus, compared to control (vehicle). The reaction time parameter was only significantly increased ( $p<0.001$ ) where morphine was administered (3 mg/kg, *i.p.*).

### Evaluation of motor activity

In the Rota rod test, EOMP treated mice did not show any significant motor performance alterations with doses of 25, 50, or 100 mg/kg (Figure 2). As might be expected, the CNS depressant diazepam (1.5 mg/kg, *i.p.*), standard drug, reduced the time of treated animals on the Rota rod after 30 min ( $7.9\pm3.0$  s) and 60 min ( $34.6\pm21.1$  s), compared with the control group.

### Leukocyte migration to the peritoneal cavity

Figure 3 shows the inhibitory effect of EOMP on carrageenan-induced response (54.4, 56, and 70% at 25, 50, and 100 mg/kg, respectively,  $p<0.001$ ). The results obtained with the control group support the effect of EOMP since the vehicle presented no activity, and the control drug dexamethasone inhibited (72.6%,  $p<0.001$ ) the carrageenan-induced leukocyte migration to the peritoneal cavity (Figure 3).

## Discussion

The present study demonstrates the chemical constituents and antinociceptive and anti-inflammatory effects of the essential oil from leaves of *Myrcia pubiflora* DC., Myrtaceae (EOMP). EOMP was tested in three different tests of nociception (acetic acid-induced writhing test, formalin-induced paw licking test, and hot plate test), and one test of inflammation (leukocyte migration to the peritoneal cavity) in rodents, beyond the assessment of motor coordination, using the Rota rod test in mice treated with EOMP.

More than 20% of the essential oil components of *M. pubiflora* were due to a oxygenated terpenoid, caryophyllene oxide (22.2%). Another three major constituents of the leaf oil were mustakone (11.3%), 1,8-cineole (5.4%), and tricyclene (5.3%). Studies

**Table 2.** Effect of EOMP or MOR on formalin-induced pain.

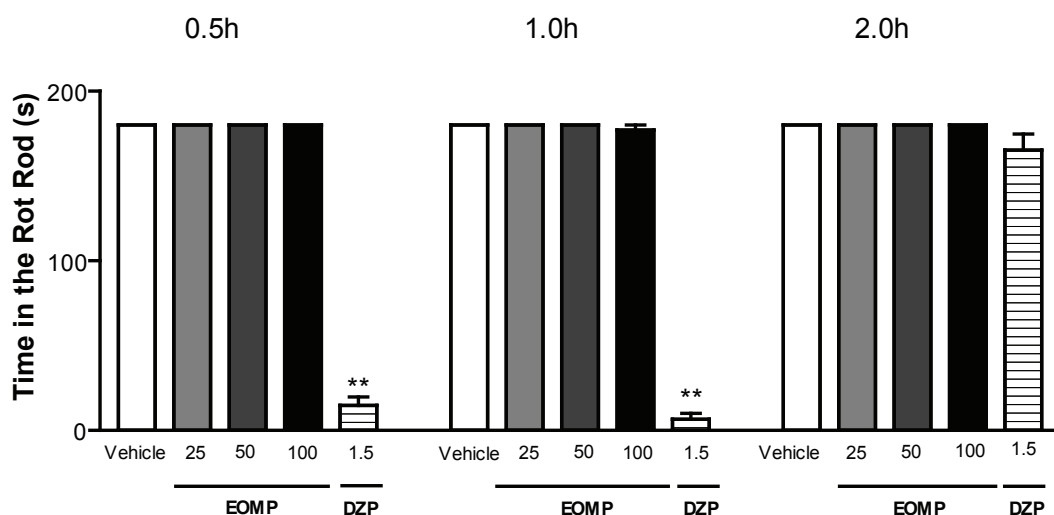
Treatment	Dose (mg/kg)	Number of licks (s)			
		0-5 min		15-30 min	
		Score of pain <sup>a</sup>	% inhibition	Score of pain <sup>a</sup>	% inhibition
Vehicle	-	39.6±4.1	-	69.9±18.5	-
EOMP	25	46.1±5.0	-16.40	27.5±12.4 <sup>b</sup>	60.48
EOMP	50	47.5±4.0	-19.88	9.8±7.9 <sup>c</sup>	85.91
EOMP	100	40.0±3.2	-0.95	1.0±0.7 <sup>c</sup>	98.56
Morphine	3	0.9±0.6 <sup>c</sup>	97.72	1.8±1.8 <sup>c</sup>	97.91

n=8; <sup>a</sup>Values represent mean±SEM; <sup>b</sup>p<0.05 and <sup>c</sup>p<0.001 (one-way ANOVA and Tukey's test), significantly different from control.

**Table 3.** Antinociceptive effect of EOMP or MOR on the hot plate test in mice.

Treatment	Dose (mg/kg)	Reaction time (s) <sup>a</sup>				
		Basal	0.5 h	1 h	1.5 h	2 h
Vehicle	-	7.3±0.9	7.3±0.9	7.1±1.7	7.5±1.2	9.1±1.8
EOMP	25	7.0±1.1	7.0±1.1	8.1±1.0	9.3±1.3	7.1±2.7
EOMP	50	9.0±1.3	9.0±1.3	8.5±1.7	9.1±1.7	9.6±1.8
EOMP	100	9.0±0.9	9.0±0.9	9.6±1.3	6.5±1.6	8.0±1.2
Morphine	3	7.1±2.8	26.5±4.5 <sup>b</sup>	29.3±3.5 <sup>b</sup>	28.7±4.1 <sup>b</sup>	29.1±5.9 <sup>b</sup>

n=8; <sup>a</sup>Values represent mean±SEM; <sup>b</sup>p<0.001 (one-way ANOVA and Tukey's test), significantly different from control.



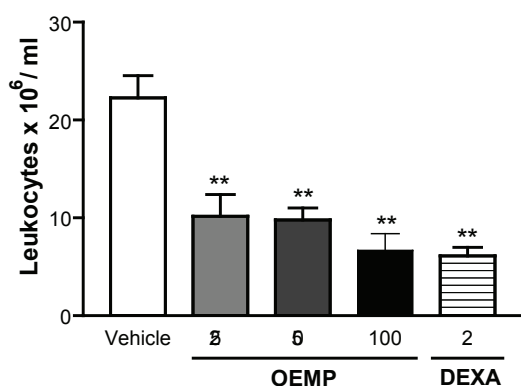
**Figure 2.** Time (s) on the Rota rod observed in mice after *i.p.* treatment with Vehicle (Control), EOMP (25, 50, and 100 mg/kg), or Diazepam (DZP, 1.5 mg/kg). The motor response was recorded for the following 180 s after drug treatment. Statistical differences vs control group were calculated using ANOVA, followed by Tukey's test (n=8). \*\*p<0.001.

performed with caryophyllene oxide exhibited antinociceptive activity by mechanisms that may involve both central and peripheral pathways (Chavan et al., 2010). Similarly, 1,8-cineole, isolated from the essential oil obtained from *Eucalyptus camaldulensis* leaves, showed the central antinociceptive properties of these monoterpenes on hot plate and tail-flick tests (Liapi et al., 2007). Another study suggested that *R*-(+)-limonene presented antinociceptive activity and that, probably, this action can be related to peripheral analgesia, but not with the stimulation of opioid

receptors (Amaral et al., 2007).

Acetic acid-induced writhing is a standard, simple, and sensitive test to evaluate central and peripheral analgesic activities (Hayes et al., 1987; Hunskaar & Hole, 1987). Additionally, although this test is a nonspecific test (e.g. anticholinergic, antihistaminic, and other agents show activity in this test), it is widely used for analgesic screening and involves local peritoneal receptors (cholinergic and histaminic receptors) (Alexandre-Moreira et al., 1999). The inhibitory effect of EOMP in the writhing test is





**Figure 3.** Effect of EOMP on leukocyte migration into the peritoneal cavity induced by carrageenan. Groups of mice were pre-treated with vehicle, dexamethasone (Dexa, 2 mg/kg) or EOMP at doses of 25, 50, and 100 mg/kg (*i.p.*) 0.5 h before carrageenan (1%, 0.25 mL, *i.p.*) induced peritonitis. Cell counts were performed at the time 4 h after the injection of carrageenan. Each value represents the mean±SEM. Asterisks denote statistical significance, \*\* $p < 0.001$  related to control group. ANOVA followed by Tukey's test ( $n=8$ ).

shown in Figure 1. These results suggest that EOMP possibly acts to inhibit endogenous substance release, responsible for stimulating nervous termination of pain, or blocking the transmission of action potentials produced by nociceptive stimulus (Khanna & Bhatia, 2003; Trongsakul et al., 2003).

The formalin test produces nociceptive response in two distinct phases involving different mechanisms. The first phase (neurogenic nociception) results from the direct chemical stimulation of myelinated and unmyelinated nociceptive afferent fibers, mainly C fibers, which can be suppressed by opioid analgesic drugs like morphine (Amaral et al., 2007). The second phase (inflammatory nociception) results from the release of inflammatory mediators in the peripheral tissues and of functional changes in the neurons of the spinal dorsal horn that, in the long term, promote facilitation of synaptic transmission at the spinal level (Campos et al., 2002; França et al., 2001). This latter phase was reported to be sensitive to the action of the majority of nonsteroidal anti-inflammatory drugs (NSAID), including acetylsalicylic acid (ASA), indomethacin, and naproxen (Dai et al., 2002; Tjolsen et al., 1992). In this test, EOMP significantly inhibited the licking response of mice ( $p < 0.001$ ) only in the second phase, indicating peripheral antinociceptive action.

Moreover, the hot plate test was used to check for a possible central antinociceptive effect of EOMP, as this is considered a specific test for the study of supraspinal and spinal response to pain. EOMP did not interfere with nociception in this test (Table 3).

The inflammation induced by carrageenan involves cell migration, plasma exsudation, and production of mediators, such as nitric oxide, prostaglandin E<sub>2</sub>, interleukin (IL)-1 $\beta$ , IL-6, and tumor necrosis factor (TNF)- $\alpha$  (Salvemini et al., 1996; Loram et al., 2007). These mediators are able to recruit leukocytes, such as neutrophils, in several experimental tests. Figure 3 shows that EOMP inhibited leukocyte migration induced by carrageenan, and a putative mechanism associated with this activity may be inhibition of the synthesis of many inflammatory mediators involved in the cell migration.

Previous studies suggested that the CNS depression and the nonspecific muscle relaxation effect can reduce the response of motor coordination and might invalidate the behavior tests' results (De Sousa et al., 2006). Our results revealed that all mice treated with EOMP, at these doses, did not have any performance alteration in the Rota rod test.

It can be concluded from the present study that the essential oil of *M. pubiflora* demonstrates antinociceptive and anti-inflammatory properties, which are probably through inhibition of inflammatory mediator release or other peripheral pathway. Further studies currently in progress will enable us to understand the precise action mechanisms.

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