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Antinociceptive effects of *Maytenus imbricata* Mart. ex. Reissek (Celastraceae) root extract and its tingenone constituent

Clarice de Carvalho Veloso[1], Vanessa Gregório Rodrigues[2], Adolfo de Oliveira Azevedo[1], Cristina da Costa Oliveira[1], Lindisley Ferreira Gomides[1], Lucienir Pains Duarte[2], Igor Dimitri Duarte[1], Andre Klein[1] and Andrea de Castro Perez[1]*

[1]Department of Pharmacology, Institute of Biological Sciences, Federal University of Minas Gerais, Belo Horizonte, Minas Gerais, Brazil.
[2]Department of Chemistry, Institute of Exact Sciences, Federal University of Minas Gerais, Belo Horizonte, Minas Gerais, Brazil.

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Plants belonging to the genus *Maytenus* (Celastraceae) are routinely used in folk medicine for the treatment of pain and inflammatory diseases. The aim of this study was to assess the *in vivo* anti-inflammatory and anti-nociceptive effects of the root extracts and tingenone, a natural triterpene, from *Maytenus imbricata* Mart. ex. Reissek. Oral pre-treatment with methanol extract (ME), ethyl acetate extract (EAE) (both 100 to 1000 mg/kg) and tingenone (5.3, 15.9 and 53 mg/kg) significantly reduced the licking time in the second phase of the formalin test. Hexane/ethyl ether (1:1) extract (HEE) reduced the licking time in both phases of the formalin test and inhibited carrageenan-induced edema formation in mice. These results show that the three extracts and tingenone had significant anti-nociceptive effects in the second phase of the chemical behavioral model of nociception. Therefore, *M. imbricata* root extracts and tingenone, a natural quinone-methide triterpene, constitute an attractive alternative to relieve pain.

Key words: Painful disorders, antinociception, *Maytenus imbricate*, triterpenes, tingenone.

INTRODUCTION

The identification of molecular components responsible for pain has led to major advances in understanding pain and developing new pharmacological tools for its treatment (Woolf, 2004). Medicinal plants constitute an alternative therapeutic approach for the treatment of painful inflammatory disorders, because they are potential sources of phytopharmaceuticals, such as flavonoids, phytosterols, triterpenoids and other constituents. It has been demonstrated that these components inhibit the molecular targets of pro-inflammatory mediators (Iwalewa et al., 2007).

The Celastraceae family comprises approximately 98 genera and 1210 species throughout the tropical and subtropical regions of the world (Simmons et al., 2008) and has been widely used in folk medicine for the treatment of inflammatory diseases, such as stomach complaints, fever, rheumatoid arthritis and cancer (Spivey et al., 2002). *Maytenus* is a genus of this family, and its

*Corresponding author. E-mail: acp@icb.ufmg.br. Tel: +0055 31 34092721.

species are used in traditional medicine for the treatment of gastric disorders, inflammatory diseases and pain, among other disorders (Baggio et al., 2009; Sosa et al., 2007; Niero et al., 2011). The hexane and ethyl acetate extracts of *Maytenus ilicifolia* inhibited formaldehyde-induced nociception and paw edema in mice and carrageenan-induced paw edema in rats (Jorge et al., 2004), while the chloroform extract of *Maytenus senegalensis* Lam. Excell reduced edema induced by croton oil in mice (Sosa et al., 2007), and the hydroalcoholic extract of *Maytenus robusta* had gastroprotective activity in rats (de Andrade et al., 2007).

Previous phytochemical studies on the roots of *Maytenus imbricata* resulted in the isolation and characterization of pentacyclic triterpenes, including tingenone (Rodrigues et al., 2012). There is a growing interest in natural triterpenoids, because they have a wide spectrum of biological activities, such as bactericidal, fungicidal, antiviral, cytotoxic, analgesic, anticancer, spermicidal, cardiovascular and antiallergic activities (Patočka, 2003). Tingenone has been shown to exhibit insecticidal effects (Avilla et al., 2000) and antitumoral activity (Gomes et al., 2011).

Considering the popular use of the species from genus *Maytenus* for the treatment of painful inflammatory diseases, the aim of this study was to evaluate the pharmacological potential of *M. imbricata* root extracts and that of tingenone isolated from one of these extracts in animal models of nociception and inflammation.

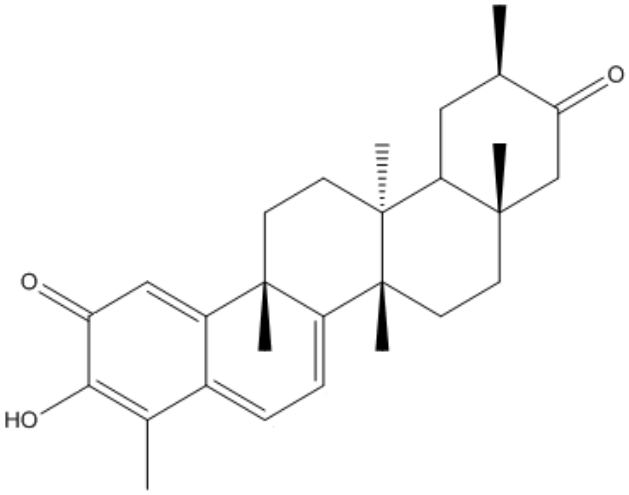

Figure 1. Tingenone structure, a natural pentacyclic triterpene, isolated from roots of *Maytenus imbricata* Mart. ex. Reissek.

MATERIALS AND METHODS

Plant

The roots of *M. imbricata* (Celastraceae) were carefully collected to prevent damage to the specimens. The collection area was Ouro Preto municipality, Minas Gerais state, Brazil. The plant material was identified by the botanists Rita M. de Carvalho Okano, Botanic Department of the Federal University of Viçosa, and M. Cristina Teixeira Braga Messias, Botanic Department of the Federal University of Ouro Preto. A voucher specimen (number 27780) was deposited in the collection of the Herbarium of the Botanic Department of the Federal University of Viçosa, Brazil.

Obtaining the extracts and tingenone

The roots of *M. imbricata* were dried at room temperature and powdered in a mill. The powder (1.5 kg) was submitted to extractions in a Soxhlet apparatus with three different organic solvents: hexane/ethyl ether (1:1), ethyl acetate and methanol (2 L of each solvent in this order). The filtrates were removed in a rotator evaporator. The following quantities were obtained for each filtrate: 16.1 g for the hexane/ethyl ether (1:1) extract (HEE), 21.2 g for the ethyl acetate extract (EAE) and 176.7 g for the methanol extract (ME). From the HEE, 1.5 g of tingenone (Figure 1) was isolated and characterized, as previously reported by Rodrigues et al. (2012). In brief, the HEE (3.0 g) was submitted to silica gel (300.8 g) cc and eluted with hexane-EtOAc. Three hundred and fifty fractions of 100 ml each were obtained and similar profiles observed in the chromatoplates were grouped together. Fractions 66 to 85 produced an orange solid (314.0 mg) with a yield of 15.7% and an mp of 145.0 to 147.9°C, which was identified as tingenone.

Phytochemical screening of the extracts

The following chemical constituents were screened in the extracts: steroids, triterpenes, saponins, glycosylated triterpenes, tannins, phenols, resins, alkaloids and flavonoids. The screening was performed on the extracts using chemical reagents according to the methodology suggested by Wagner and Bladt (2001).

Animals

Male Swiss and BALB/c mice (20 to 30 g) from the Bioterism Center of Federal University of Minas Gerais (CEBIO/UFMG) were used in the experiments. The mice were housed in standard cages and kept at a constant temperature of 23°C with a 12-h light-dark cycle and free access to food and tap water, except for the night before the experiments, when they were submitted to an overnight fast. All testing procedures were in accordance with the ethical guidelines of the International Association for the Study of Pain (IASP) (Zimmermann, 1983) and approved by the Ethics Committee in Animal Experimentation at the Federal University of Minas Gerais (protocol 115/2012).

Dose calculation

The inhibition percentage for the first and second phases of the formalin test was calculated and analyzed using the Graph Pad Prism 3.0 software to plot the log curve for the maximum effect percentage versus the extract dose. ID_{50} values (the doses producing 50% inhibition) were calculated by the graphic interpolation of this dose-effect curve. The reference dose (RD) of tingenone (5.3 mg/kg) was calculated by multiplying the ID_{50} obtained from the

Table 1. Type of secondary metabolites identified in organic extracts of *Maytenus imbricata* roots.

Class of organic compound	HEE	EAE	ME
Steroids/Triterpenes	+	+	+
Tannins	-	-	-
Phenols	-	+	+
Alkaloids	-	-	-
Resins	-	-	-
Saponin/Glycosylated triterpenes	-	-	-
Flavonoids	-	+	+

Positive: +(detected); Negative: -(no detected)

second phase of HEE (56 mg/kg) by the percentage yield of tingenone (9.4%). The values of 3 × RD and 10 × RD were calculated (15.9 and 53 mg/kg, respectively).

Formalin-induced nociception

This test was based on the method by Dubuisson and Dennis (1977) and adapted for mice by Hunskaar et al. (1985). Formalin solution, 2% in sterile saline (0.9% NaCl), was injected at a volume of 30 µl/paw into the right hind paw plantar surface (i.pl. injection) of Swiss mice. The time (s) spent licking the affected paw was rated during two time intervals after the injection: 0 to 5 min (first phase or neurogenic pain) and 15 to 30 min (second phase or inflammatory pain). The ME and EAE, HEE and tingenone were solubilized in sterile saline, dimethylsulphoxide (DMSO) (4% in sterile saline) and DMSO (1% in sterile saline), respectively. The three extracts were administered at 10, 30, 100, 300 and 1000 mg/kg doses and tingenone at 5.3, 15.9 and 53 mg/kg doses per gavage (p.o.) 60 min prior to formalin injection. The animals (n = 4 to 6 per group) in the negative control groups received sterile saline, DMSO (4% in sterile saline) or DMSO (1% in sterile saline) at a volume of 10 ml/kg, p.o. The positive control groups received morphine (5 mg/kg, i.p.) solubilized in sterile saline, administered 30 min prior to formalin injection, or indomethacin (10 mg/kg, p.o.) solubilized in tween 20:ethanol:sterile saline (1:4:45) 60 min prior to formalin injection. To analyze whether the antinociceptive effects of the HEE occur by the opioid pathway, the animals were pre-treated with naltrexone (5 mg/kg, i.p.), 30 min before the morphine (5 mg/kg, i.p.) or the HEE (198 mg/kg, p.o.) treatment.

Carrageenan-induced mouse paw edema

Paw edema was measured with a plethysmometer (Ugo Basile, mod 7140) based on the method of Levy (1969). The basal volume of the right hind paw was determined before the administration of any drug. The Swiss mice were divided into the experimental groups (n = 4 to 6 per group). The vehicle (DMSO 1%, DMSO 4% or sterile saline), ME, EAE, HEE, tingenone or indomethacin (10 mg/kg) were orally administered 1 h before the i.pl. injection of carrageenan (300 µg and 30 µl). The doses for the extracts corresponded to the ID_{50} and 3 × ID_{50} values from the second phase of the formalin test, and the doses for tingenone were 5.3, 15.9 and 53 mg/kg. The paw volume was measured 1, 2, 4 and 6 h after the injection of the inflammatory stimulus. The results are presented as the paw volume (µl) variation in relation to the basal values.

Leukocyte migration into the pleural cavity induced by carrageenan

BALB/c mice were divided into experimental groups (n = 5 to 6 per group). Vehicle (DMSO 1%, 10 ml/kg) or tingenone (5.3, 15.9 and 53 mg/kg) was orally administered 1 h before the intrapleural injection of carrageenan (200 µg, 100 µl) or PBS (100 µl). The positive control group received dexamethasone (0.5 mg/kg, i.p.) 30 min before the inflammatory stimulus. The animals were then sacrificed in a CO_2 chamber 4 h after the injection. The cells in the cavity were harvested after an intrapleural injection of 2 ml phosphate buffered saline (PBS), and total cell counts were performed in a modified Neubauer chamber using Turk's stain. Differential cell counts were performed on cytospin preparations stained with May-Grunwald-Giemsa using standard morphologic criteria to identify cell types. The results were presented as the number of cells per cavity.

Statistical analysis

The results obtained were analyzed using Graph Pad Prism 3.0 and expressed as means ± standard error of mean (SEM). Statistically significant differences among the groups were calculated by the application of an analysis of variance (ANOVA) followed by Bonferroni's test, with the level of significance set at $P < 0.05$.

RESULTS

Phytochemical screening of the extracts

The secondary metabolites were identified using the methodology suggested by Wagner and Bladt (2001) and are shown in Table 1. The analgesic activity and the ID_{50} values are shown in Figure 2. In the second phase of the formalin test, the EAE ($P = 0.0004$), ME and HEE (both $P < 0.0001$) induced a significant antinociceptive effect at doses of 100 to 1000 mg/kg p.o compared to the control group. For the ME, EAE and HEE, the ID_{50} were 57, 14 and 56 mg/kg, respectively, and the 3 × ID_{50} values were 171, 42 and 168 mg/kg, respectively. In the first phase of the formalin test, the HEE induced a significant antinociceptive effect at doses of 300 to 1000 mg/kg p.o. compared to the control group ($P < 0.0001$; Figure 2A),

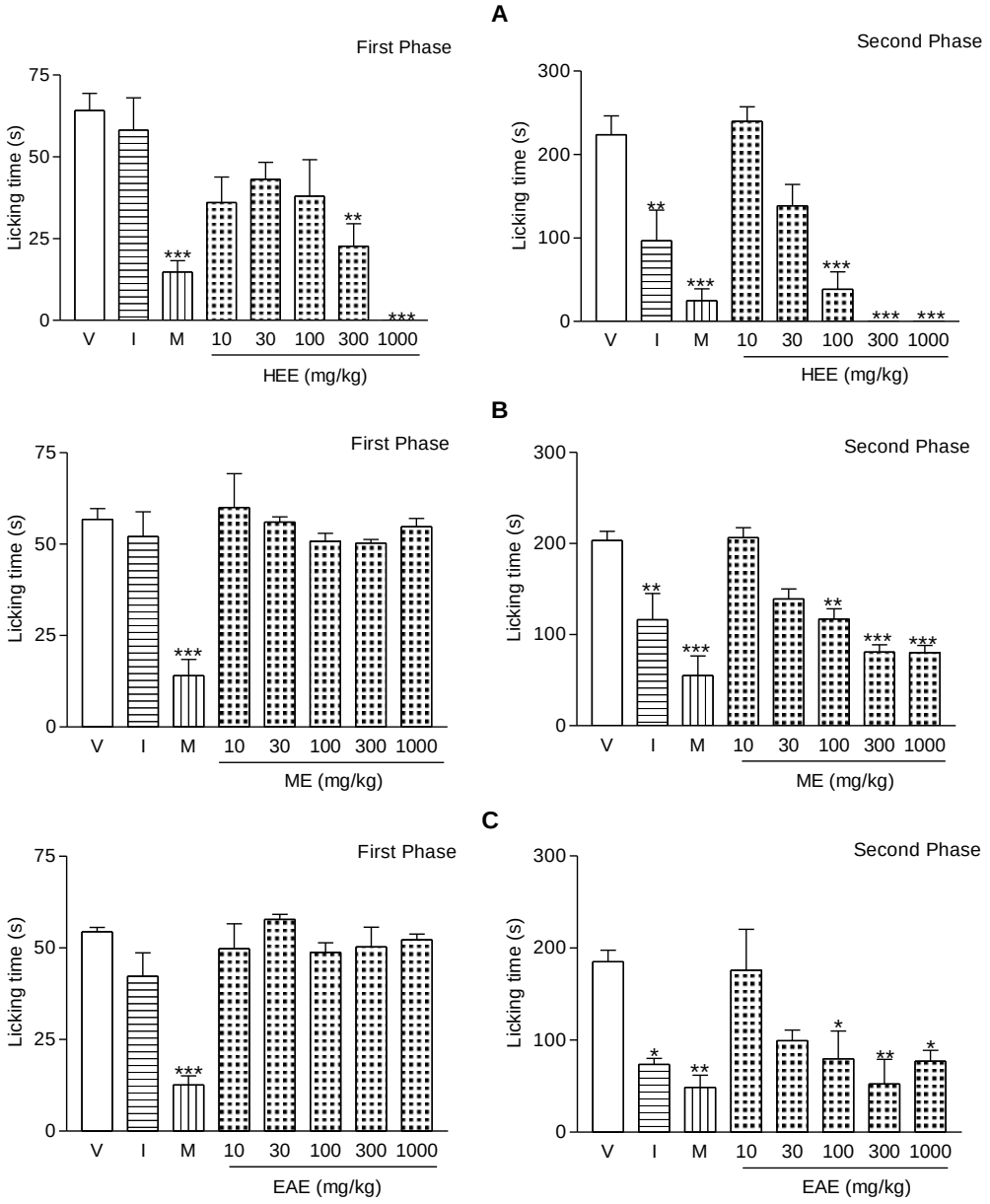

Figure 2. Effects of hexane/ethyl ether (1:1) extract (HEE) (A), methanolic extract (ME) (B) and ethyl acetate extract (EAE) (C) from *Maytenus imbricata* roots, indomethacin (I) and morphine (M) on the licking time induced by formalin in mice. The total time spent licking the hind paw was measured in the first and second phases after intraplantar injection of formalin. Each column represents the mean with SEM for 4 to 6 mice per group. The symbols denote the significance levels (one-way ANOVA followed by Bonferroni's test): *P<0.05; **P<0.01; ***P<0.001 when compared with the vehicle (V) group.

and ID_{50} and 3 × ID_{50} had values of 66 and 198 mg/kg, respectively. The ME and EAE presented no effect in this phase (Figure 2B and C). Naltrexone completely reversed the morphine antinociceptive effect but failed to reverse the antinociceptive effect of the HEE at a dose of 198 mg/kg in both phases (Figure 3). The reference drug indomethacin suppressed only the second phase of the formalin test, while morphine inhibited both phases of the test.

Tingenone significantly inhibited the second phase of the formalin test ($P < 0.0001$) at doses of 5.3, 15.9 and 53 mg/kg compared to the control group and presented

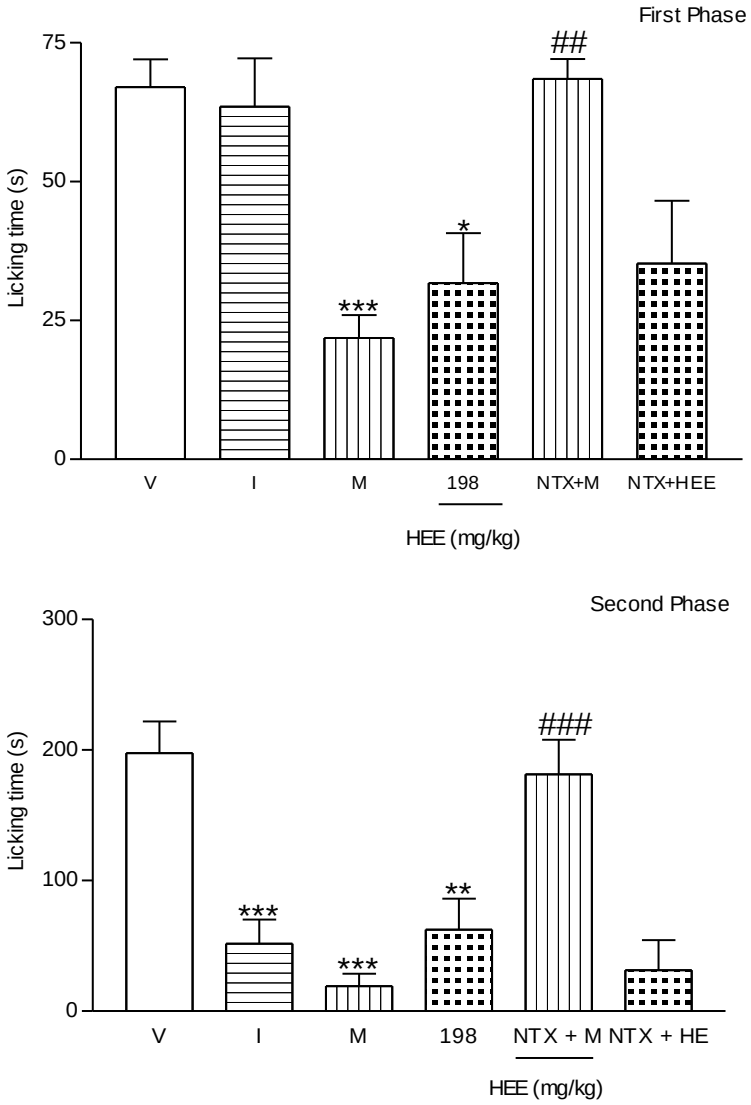

Figure 3. Effects of naltrexone in the antinociception induced by the hexane/ethyl ether (1:1) extract (HEE). Animals were pre-treated with naltrexone + morphine (NTX + M) or naltrexone + HEE (NTX + HEE). The total time spent licking the hind paw was measured in the first and second phases after intraplantar injection of formalin. Each column represents the mean with SEM for 4 to 6 mice per group.
The symbols denote the significance levels (one-way ANOVA followed by Bonferroni's test): *P<0.05; **P<0.01; ***P<0.001 when compared with the vehicle (V) group; $^{\#}$P<0.05; $^{\#\#}$P<0.01 $^{\#\#\#}$P<0.001 when compared with the morphine (M) group.

no antinociceptive effect in the first phase (Figure 4).

Carrageenan-induced mouse paw edema

The swelling in the paw started 1 h after the carrageenan injection and increased progressively for 4 h. The inhibition of edema was 72.22% 4 h post carrageenan ($P < 0.001$) for the dose corresponding to 3 × ID_{50} (second phase of formalin test) of the HEE (Figure 5A). The doses corresponding to 3 × ID_{50} of ME and EAE suppressed the edematogenic response from the fourth hour onwards (late phase), with 43.64% and 40.68% inhibition, respectively. However, these values were not significantly different (Figure 5B). Although tingenone (53 mg/kg) presented a weak inhibition of 25.69% compared to the control group, it was not statistically significant (Figure 5C). Indomethacin (10 mg/kg) inhibited edema formation

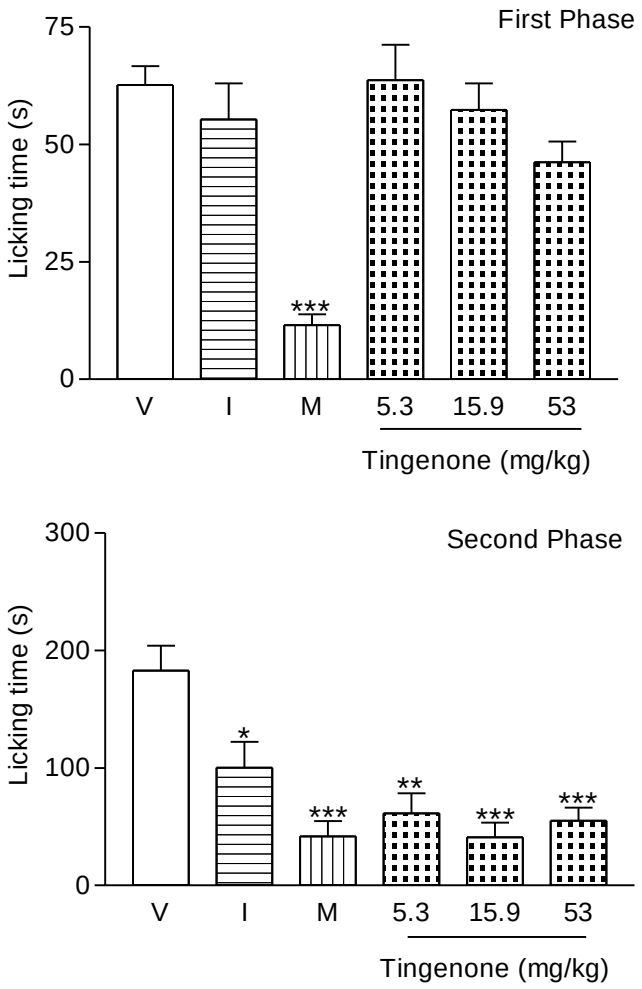

Figure 4. Effects of tingenone, from *Maytenus imbricata* roots, indomethacin (I) and morphine (M) on the licking time induced by formalin in mice. The total time spent licking the hind paw was measured in the first and second phases after intraplantar injection of formalin. Each column represents the mean with SEM for 4 to 6 mice per group. The symbols denote the significance levels (one-way ANOVA followed by Bonferroni's test): *P<0.05; **P<0.01; ***P<0.001 when compared with the vehicle (V) group.

by approximately 60%.

Neutrophil recruitment induced by carrageenan in the pleural cavity

Carrageenan significantly increased neutrophil recruitment compared with the group treated with PBS ($P < 0.001$), while dexamethasone reduced the number of neutrophils compared with the group treated with carrageenan ($P < 0.01$). Although the tingenone pretreatment (5.3, 15.9 and 53 mg/kg) presented a weak tendency to reduce the number of neutrophils in the pleural cavity, the mean values exhibited no significant difference (Figure 6).

DISCUSSION

Many compounds used globally as drugs are obtained from plants (Fabricant and Farnsworth, 2001), including phenolic compounds, particularly flavonoids, terpenes, steroids and alkaloids (Niero et al., 2011). However, this study is the first to evaluate the antinociceptive and anti-inflammatory effects of *M. imbricata* in pain and inflammation models. The main outcomes of the study were as follows: (1) oral administration of the three extracts and tingenone produced antinociceptive effects in the second phase of the formalin test; (2) only the HEE inhibited both phases of the formalin test; (3) the HEE had significant anti-inflammatory action in the paw edema test induced by carrageenan; (4) the three extracts and tingenone produced no motor performance alteration in the rota-rod test (data not shown).

The formalin-induced paw licking test is commonly employed as a model of pain, characterized by the presence of a distinct biphasic nociceptive response. The early phase corresponds to the direct activation of primary afferent sensory neurons (C-fiber), whereas the late phase has been proposed to reflect the combined effects of an inflammatory reaction in the peripheral tissue and central sensitization in the dorsal horn (Tjolsen et al., 1992; McNamara et al., 2007). The first phase is sensitive to centrally acting analgesics, such as morphine, and substances that act on the kininergic pathway (Hunskaar and Hole, 1987; Correa and Calixto, 1993). The second phase is inhibited by non-steroid anti-inflammatory drugs (NSAIDs), such as indomethacin, steroids and peripherally acting opioids (Hunskaar and Hole, 1987; Oluyomi et al., 1992). Shibata et al. (1989) reported that substance P and bradykinin participate in the early phase response, while the late phase is caused by local inflammation with the release of inflammatory and hyperalgesic mediators, such as histamine, serotonin, prostaglandin and bradykinin.

It was demonstrated that the ME and EAE administered by gavage induced significant antinociception only in the second phase. Considering the antinociceptive property of the three extracts on the late phase of the formalin test, reducing inflammatory pain, similar to indomethacin (positive control), it is likely that their antinociceptive activity is due to anti-inflammatory action. This action may occur by inhibiting prostaglandin synthesis. The HEE had an antinociceptive effect on both phases of the formalin test, reducing the non-inflammatory and inflammatory pain. To evaluate the involvement of the opioid pathway modulating the early and late phases of the formalin test (Oluyomi et al., 1992), the mice were pre-treated with

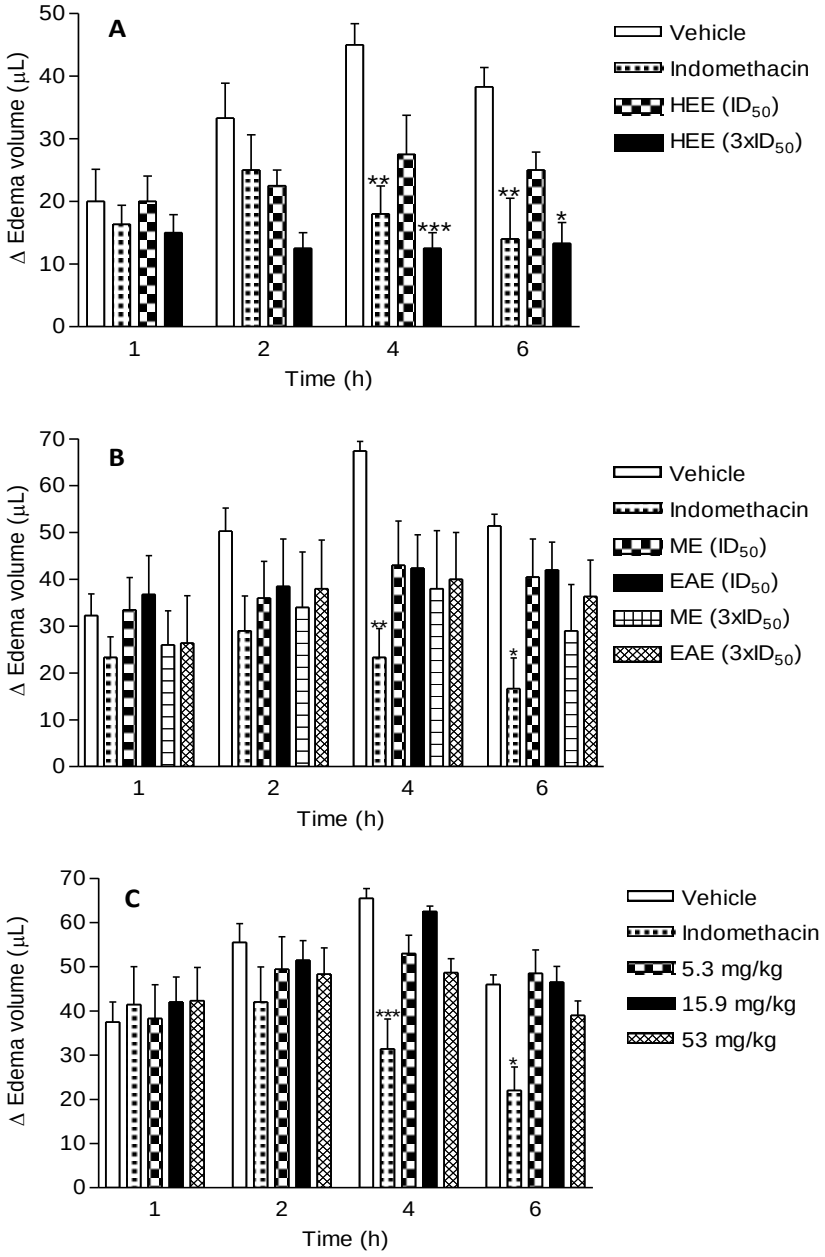

Figure 5. Effects of the hexane/ethyl ether (1:1) extract (HEE) (A), methanolic extract (ME) and ethyl acetate extract (EAE) (B) from *Maytenus imbricata* roots, tingenone (C) and indomethacin on mice paw edema induced by intraplantar carrageenan injection (300 µg/paw). Each column represents the mean ± SEM of 4 to 6 mice.

The symbols denote the significance levels (one-way ANOVA followed by Bonferroni's test): *P<0.05; **P<0.01; ***P<0.001 when compared with the vehicle group.

naltrexone before the HEE treatment. The failure of naltrexone to reverse the antinociception of the HEE in both phases of the formalin test and the lack of anti-nociceptive effect of the HEE in the tail-flick test (data not shown), a thermal model that identifies centrally acting opioid analgesics (Le Bars et al., 2001), reveal that mechanisms other than the stimulation of the central and peripheral opioid receptors are involved. HEE may mediate antinociception by inhibiting the prostaglandin synthesis responsible for inflammatory pain in the second

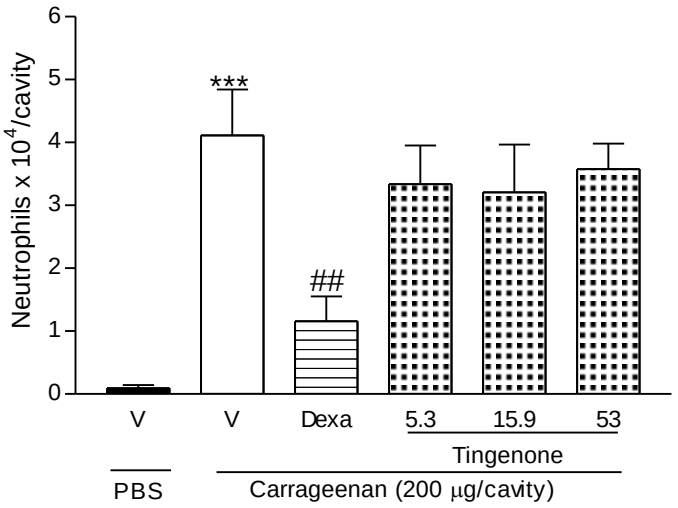

Figure 6. Effects of tingenone, from *Maytenus imbricata* roots, and dexamethasone (Dexa) on neutrophil recruitment induced by carrageenan (200 µg/cavity). Each column represents the mean ± SEM of four mice. The symbols denote the significance levels (one-way ANOVA followed by Bonferroni's test): *P<0.05; **P<0.01; ***P<0.001 when compared with the vehicle (V)/PBS group; #P<0.05; ##P<0.01 ###P<0.001 when compared with the vehicle (V)/carrageenan group.

phase. The ME and EAE produced antinociception in the second phase of the formalin test, but not in the tail flick test, suggesting that these extracts do not have central analgesic effects as morphine does.

The results of the inhibition of the second phase of the formalin test by the three extracts suggest a possible antiedematogenic effect. The anti-inflammatory effect was also evaluated using the carrageenan-induced mice paw edema test. In this model, inflammation is characterized by an early phase (1 to 2 h) with increased vascular permeability due to the release of histamine, serotonin and bradykinin, followed by a late phase (3 to 6 h) with intense edema induced by prostaglandins, named the "prostaglandin phase". This progression explains why the late phase is inhibited by NSAIDs, such as indomethacin (Di Rosa and Willoughby, 1971a; Di Rosa and Willoughby, 1971b). Oral pre-treatment with the HEE suppressed paw edema in the fourth hour after the injection of carrageenan; this effect may be related to COX inhibition, similar to the action of indomethacin (positive control). The EAE and ME presented a tendency (not statistically significant) to suppress the edematogenic response from the fourth hour onwards.

Tingenone presented a weak anti-inflammatory effect in both the carrageenan-induced paw edema and the pleurisy tests. The latter test is another model of inflammation characterized by two phases: the early phase (0 to 1 h), related to the production of histamine, 5-hydroxytryptamine, leukotrienes, platelet-activating factor and bradykinin, and the late phase (1 to 6 h), involving prostaglandin release and neutrophil infiltration (Di Rosa and Willoughby, 1971a; Cuzzocrea et al., 2000; Batinić-Haberle et al., 2009). The antinociceptive effect of tingenone was only observed in the second phase of the formalin test. The absence of antinociceptive effects in the tail-flick test (data not shown) and in the first phase of the formalin test suggests that tingenone has antinociceptive effects that are not related to an anti-inflammatory action. Moreover, treatment with the three extracts and tingenone did not affect motor performance, as observed in the rota rod test (data not shown).

The presence of flavonoids, triterpenes and steroids in *M. imbricata* is in agreement with the literature. Previous study indicated the presence of triterpenes in a hexane/ethyl ether (1:1) extract isolated from the roots (Rodrigues et al., 2012). Phytochemical investigation have shown that triterpenoids isolated from the *Maytenus* species exhibit potent inhibitory effects on prostaglandin E_2 (PGE_2) production in mice macrophages stimulated with a bacterial endotoxin (Reyes et al., 2006). Mattos et al. (2006) observed the anti-edematogenic effects of a steroid isolated from plants that was effective in reducing the edematogenic responses evoked by carrageenan. Moreover, flavonoids play an important role in various biological processes, such as antihepatotoxic, anti-allergic, anti-inflammatory, antiosteoporotic and antitumor activities (Di Carlo et al., 1999). Landolfi et al. (1984) reported that some flavonoids block both the COX and lipooxygenase pathways, inhibiting the production of inflammatory mediators such as prostaglandins and leukotrienes. Additionally, antinociceptive effects of triterpenes in formalin tests were reported (Lima et al., 2005; Gaertner et al., 1999).

According to our results, the medicinal plant *M. imbricata* has the potential to be used in the treatments of painful inflammatory diseases. It is also important to note that traditional healing preparations made from the plant may be more useful than purified drugs made from the plant.

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