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# Antinociceptive and anti-inflammatory properties of 7-epiclusianone, a prenylated benzophenone from *Garcinia brasiliensis*

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ABSTRACT

7-Epiclusianone, a natural prenylated benzophenone, was extracted from *Garcinia brasiliensis* Planch. & Triana (Clusiaceae), a native plant commonly known as bacupari and used in traditional Brazilian medicine for the treatment of inflammatory diseases. As a result of the wide spectrum of biological activities attributed to polyisoprenylated benzophenones, the aim of this study was to evaluate the analgesic and anti-inflammatory effects of 7-epiclusianone using two animal models. Carrageenan-induced paw oedema and peritonitis were used to investigate the anti-inflammatory activity of 7-epiclusianone in rats. The acetic acid-induced writhing, formalin and hot-plate tests were used to investigate its antinociceptive activity in mice. At test doses of 5, 10 and 15 mg/kg *p.o.*, 7-epiclusianone had an anti-inflammatory effect as demonstrated by the reduction of paw oedema induced by carrageenan and the inhibition of leukocyte recruitment into the peritoneal cavity. At the same doses, 7-epiclusianone inhibited nociception induced by an intraperitoneal injection of acetic acid, observed by the decrease in the number of writhing episodes. Additionally, 7-epiclusianone decreased licking time caused by a subplantar injection of formalin. Moreover, the hot plate test produced a significant increase in latency reaction, demonstrating an antinociceptive effect. The experimental data demonstrated that the polyisoprenylated benzophenone 7-epiclusianone has remarkable anti-inflammatory and antinociceptive activities.



## 1. Introduction

Pain is a common symptom of various inflammatory diseases and is the primary reason why patients pursue specialised treatment (Silva et al., 2010). Thus, there is a great demand for more effective anti-inflammatory drugs (Newman and Cragg, 2007). Because currently available anti-inflammatory drugs have considerable side effects that inhibit their clinical use, many studies are currently underway to develop new treatments for inflammatory diseases (Shukla et al., 2010). The search for alternatives to current treatments is necessary and will greatly benefit those afflicted with inflammatory diseases (Vasudevan et al., 2007). Natural products have been one of the most successful sources for the discovery of new therapeutic agents (Harvey, 2000).

The pharmacological study of polyisoprenylated benzophenones has been shown to be of interest due to the wide spectra of biological activities attributed to its derivates (Coelho et al., 2008). These compounds, isolated from plants of the Clusiaceae family, have been shown to have antioxidant, cytotoxic, antimicrobial, antiviral and anti-inflammatory properties (Acuña et al., 2009).

7-Epiclusianone, a natural prenylated benzophenone first isolated from the fruits of *Garcinia gardneriana* (Santos et al., 1999), was extracted from *Garcinia brasiliensis* Planch. & Triana (syn. *Rheedia brasiliensis*) (Clusiaceae). This native plant, commonly known as bacupari, has been used in traditional Brazilian medicine for the treatment of tumours, inflammation of the urinary tract, arthritis and pain (Corrêa, 1978). It has been shown that 7-epiclusianone exhibits activity against trypomastigotes of *Trypanosoma cruzi in vitro* (Almeida-Alves et al., 1999). Potent endothelium vasodilator effects (Cruz et al., 2006) and antianaphylactic (Neves et al., 2007), anti-HIV (Piccinelli et al., 2005), antimicrobial (Almeida et al., 2008; Murata et al., 2008, 2010a; Naldoni et al., 2009), antispasmodic (Coelho et al., 2008), antiproliferative (Murata et al., 2010b) and leishmanicidal activities have also been attributed to this benzophenone (Pereira et al., 2010).

While many studies are underway to determine the chemical composition and antinociceptive and anti-inflammatory activities of

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the leaves of *G. brasiliensis* (Corrêa et al., 2009; Santa-Cecília et al., 2011), the aim of this study was to evaluate the analgesic and anti-inflammatory proprieties of 7-epiclusianone isolated from this species using animal models.

## 2. Materials and methods

### 2.1. Plant material

The leaves of *G. brasiliensis* were collected from trees grown under controlled conditions at the herbarium of the University of Viçosa (latitude 20°45′14″ south and longitude 42°52′55″ west), Minas Gerais, Brazil, and a voucher specimen has been deposited (number VIC2604).

### 2.2. Extraction and isolation procedures

To obtain the extract, the leaves were dried, powdered (1 kg) and extracted with n-hexane in Soxhlet equipment for 24 h. The solvent was removed under reduced pressure and then dried with a spray dryer (BÜCHI Mini Spray Dryer B-290). The yield of the *G. brasiliensis* hexane extract (GbHE) was 8.5%. To isolate the bioactive compound, GbHE was chromatographed on a silica gel (230–400 mesh) column (8×100 cm) and eluted with crescent polarity mixtures of n-hexane/ethyl-acetate and ethyl-acetate/ethanol to give 25 fractions. These fractions were pooled into four groups according to their similarities after the analysis using thin layer chromatography (TLC) and compared to the standard 7-epiclusianone previously isolated from hexane extracts of the fruit of *G. brasiliensis*. Fractions 4–10 were rechromatographed on a silica gel (230–400 mesh) column (8×100 cm) and eluted with crescent polarity mixtures of n-hexane/ethyl-acetate and ethyl-acetate/ethanol to purify the prenylated benzophenone 7-epiclusianone (Fig. 1). Its structure was determined using spectroscopic techniques (IR, UV, MS and $^{1}H$ and $^{13}C$ NMR). The data were compared to those verified in a previous study investigating the chemical structure of this compound (Derogis et al., 2008; Santos et al., 1998, 1999).

### 2.3. Pharmacological procedures

#### 2.3.1. Animals

Adult male Wistar rats, weighing 180 to 220 g, and adult male Swiss mice, weighing 28 to 32 g, were obtained from the Central Animal Facility of the Federal University of Alfenas-MG and housed under controlled light (12:12 h light–dark cycle; lights on at 06:00 am) and temperature conditions (23±1 °C) with access to water and food *ad libitum*. The animals were allowed to habituate to the housing facilities for at least 1 week before the experiments began. All experiments were conducted in accordance with the Declaration of Helsinki on the welfare of experimental animals and with the approval of the Ethics Committee of the Federal University of Alfenas-MG (234/2009).

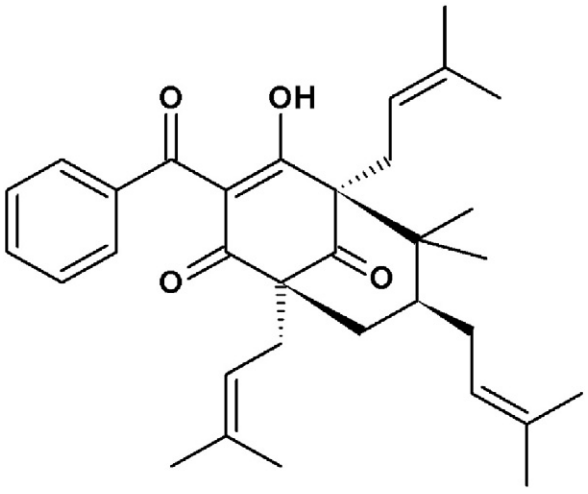


**Fig. 1.** Chemical structure of 7-epiclusianone, a polyprenylated benzophenone.

#### 2.3.2. Preparation of the test samples for the bioassay

7-Epiclusianone was dissolved in vehicle (soybean oil) and administered orally to animals in the test groups at doses of 5, 10 and 15 mg/kg. Animals in the control group received the same experimental handling as those in the test groups, except that the drug treatment was replaced by appropriate volumes of the vehicle. Indomethacin (10 mg/kg, *p.o.*) was diluted in Tris buffer (pH 8.5) and morphine sulphate (1 or 10 mg/kg, *i.p.*) was diluted in sterile saline (0.9% NaCl).

#### 2.3.3. Evaluation of anti-inflammatory activity in rats

*2.3.3.1. Carrageenan-induced rat paw oedema.* Pedal inflammation in rats was produced as described previously (Vinegar et al., 1969), following an overnight fast with free access to water. Paw oedema was measured using a plethysmometer (Model 7140, Ugo Basile, Italy). The basal volume of the right hind paw was determined before the administration of any drug. After determination of the basal volume, the animals (n=8 per group) were divided into experimental groups in such a way that the mean volumes of the different groups were similar. Vehicle, 7-epiclusianone or indomethacin was orally administered 1 h before the intraplantar (*i.pl.*) injection of carrageenan (1 mg, 100 µl). The paw volume was measured 1, 2, 3 and 4 h after the injection of the inflammatory stimulus. The results are presented as the paw volume (ml) variation normalised to the basal values.

*2.3.3.2. Peritonitis induced by lipopolysaccharide.* To assess the possible effect of the benzophenone on leukocyte recruitment to the peritoneal cavity, the animals (n=8 per group) were orally pre-treated with vehicle, 7-epiclusianone or indomethacin, and 30 min later, lipopolysaccharide (LPS) from *Escherichia coli* serotype 026:B6 dissolved in pyrogen-free sterile saline was administered at a dose of 500 µg/kg *i.p.* Four hours after the injection of LPS, rats were killed with an inhalatory overdose of halothane, and the cells from the peritoneal cavities were harvested by an injection of 10 ml of PBS containing 0.5% sodium citrate. The abdomens were gently massaged, and the blood-free cell suspension was carefully aspirated with a syringe. Abdominal washings were placed into plastic tubes, and total cell counts were performed immediately in a Neubauer chamber (Cunha et al., 1989).

#### 2.3.4. Evaluation of antinociceptive activity in mice

*2.3.4.1. Acetic acid-induced writhing in mice.* Acetic acid (0.6% v/v, 10 ml/kg) was injected into the peritoneal cavities of mice, which were placed in a large glass cylinder, and the intensity of their nociceptive behaviour was quantified by counting the total number of writhes occurring between 0 and 20 min after stimulus injection, as described previously (Collier et al., 1968). Oral treatment with vehicle, indomethacin or 7-epiclusianone was given 1 h prior to acetic acid injection (n=6 per group). Morphine was intraperitoneally administered 30 min before the test. The writhing response consisted of a contraction of the abdominal muscles together with a stretching of the hind limbs. The antinociceptive activity was expressed as the writhing scores over a period of 20 min.

*2.3.4.2. Formalin-induced nociception.* A formalin solution (5% in 0.9% saline) was injected (20 µl/paw) into the hind paw plantar surface (*i.pl.*), and the animals were individually placed in transparent observation chambers, as previously described (Santos and Calixto, 1997;

Vilela et al., 2009). Oral treatments (*p.o.*) with vehicle, indomethacin or 7-epiclusianone were given 1 h prior to formalin injection (n = 8 per group). Morphine was administrated (*i.p.*) 30 min before the test. The time spent licking the injected paw was recorded and expressed as the total licking time in the early phase (0–5 min) and late phase (20–30 min) after the formalin injection.

*2.3.4.3. Hot plate test.* The hot plate consisted of an electrically heated surface kept at a constant temperature of 50.0 ± 0.5 °C. Mice were placed on the heated surface within Plexiglas walls to constrain their locomotion on the plate, and the latency to a discomfort reaction (licking of the paws or jumping) was recorded 0, 30, 60 and 120 min after oral administration of vehicle, 7-epiclusianone or morphine (n = 8 per group), with a reaction time of zero minutes signifying the start of the test. A cut-off time of 20 s was chosen to indicate complete analgesia and avoid tissue injury. The latencies for paw licking or jumping were recorded for each animal.

Trying to elucidate the mechanism by which 7-epiclusianone induces antinociception, animals were pre-treated with naloxone 15 min before morphine (1 mg/kg) or 7-epiclusianone (10 mg/kg) treatment (Couto et al., 2011).

*2.3.5. Open-field test*

To discard the possible nonspecific effects of muscle relaxants or the sedative effects of the polyprenylated benzophenone, the motor performance of the mice was evaluated on the open-field apparatus (Archer, 1973). Groups of mice (n = 6) were treated with vehicle or 7-epiclusianone 1 h before the test. Each animal was placed in the centre of the open-field arena and allowed free ambulation for 5 min to observe the locomotion frequency (number of floor units the animal entered with all of its limbs) (Milano et al., 2008).

*2.4. Statistical analysis*

The data obtained were analysed using GraphPad software programme version 4.0 and expressed as the mean ± S.E.M. Statistically significant differences between groups were calculated by the application of an analysis of variance (ANOVA) followed by the Newman–Keuls test. *P*-values less than 0.05 ($P<0.05$) were considered significant.

## 3. Results

*3.1. Carrageenan-induced rat paw oedema*

The benzophenone significantly inhibited carrageenan-induced rat paw oedema ($F_{4,39} = 4.39$; $P = 0.0004$). The inhibitory values of oedema at 3 h post-carrageenan treatment were 57.7, 58.9 and 53.2% for 5, 10 and 15 mg/kg of the compound, respectively. Indomethacin (10 mg/kg) gave a percentage inhibition of 44.7% (Fig. 2). At 4 h post-carrageenan treatment, the inhibitory values of oedema were 67.6, 54.1 and 59.5% for 5, 10 and 15 mg/kg of the compound, respectively. Indomethacin (10 mg/kg) gave a percentage inhibition of 56.8% (Fig. 2).

*3.2. Peritonitis induced by lipopolysaccharide*

In agreement with previous studies, LPS-induced peritonitis was followed by a significant increase in the number of leukocytes in the peritoneal cavity of rats when compared to the vehicle-treated control group (Cunha et al., 1989). 7-Epiclusianone significantly inhibited leukocyte recruitment to the peritoneal cavity in rats ($F_{5,37} = 10.05$; $P<0.001$). The inhibitory values of leukocyte recruitment at 4 h post-LPS were 53.5 and 72.9% for 10 and 15 mg/kg of the compound, respectively. Indomethacin (10 mg/kg) gave a percent inhibition of 82.7% (Fig. 3).

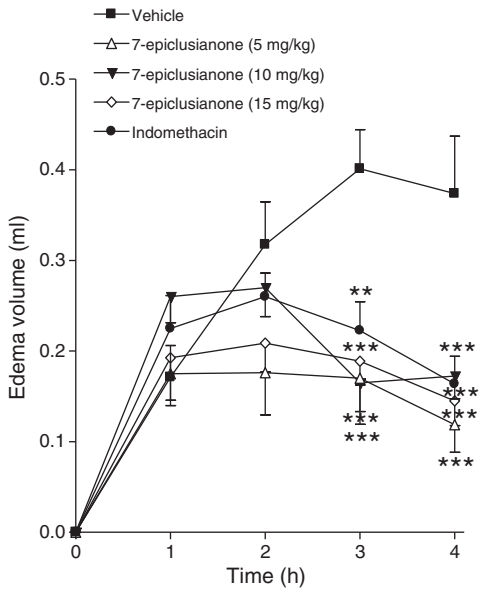


**Fig. 2.** Effects of the administration of 7-epiclusianone (*p.o.*), indomethacin (10 mg/kg, *p.o.*) or vehicle (10 ml/kg, *p.o.*) on rat paw oedema induced by intraplantar carrageenan injection (1 mg/paw). Each point represents the mean ± S.E.M. of eight animals per group. The asterisks denote the significance levels when compared with the control group: $^{**}P<0.01$, $^{***}P<0.001$.

*3.3. Acetic acid-induced writhing in mice*

The oral administration of 7-epiclusianone (5, 10 and 15 mg/kg) caused a significant reduction ($F_{5,41} = 37.45$; $P<0.001$) in the number of writhing episodes induced by acetic acid compared to the control by 16.9, 43.70 and 73.6%, respectively. Indomethacin and morphine produced 61.31 and 89.6% reductions in acetic acid-induced writhing movements, respectively, compared to the control (Fig. 4).

*3.4. Formalin test in mice*

The benzophenone at doses of 5 to 15 mg/kg *p.o.* had a significant antinociceptive activity compared to the control in both the early ($F_{5,63} = 19.86$; $P<0.001$) and late phases ($F_{5,63} = 8.18$; $P<0.001$) of

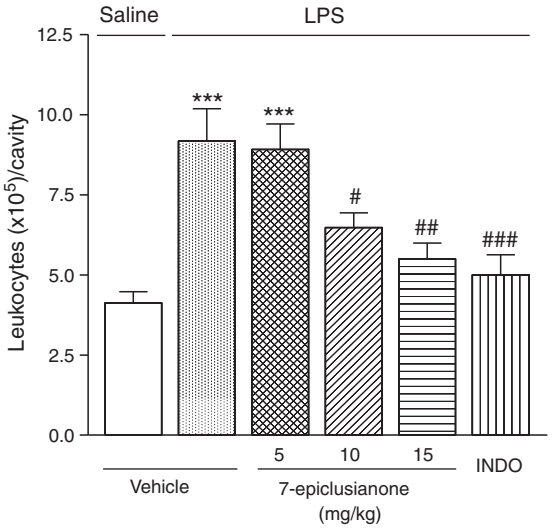


**Fig. 3.** Effects of the administration of 7-epiclusianone (*p.o.*), indomethacin (INDO) (10 mg/kg, *p.o.*) or vehicle (10 ml/kg, *p.o.*) on lipopolysaccharide-induced recruitment of leukocytes to the peritoneal cavity of rats. Each column represents the mean ± S.E.M. of eight animals per group. $^{***}P<0.001$ compared with the saline + vehicle group. $^{###}P<0.01$; $^{##}P<0.01$; $^{#}P<0.05$ compared with vehicle + LPS group.

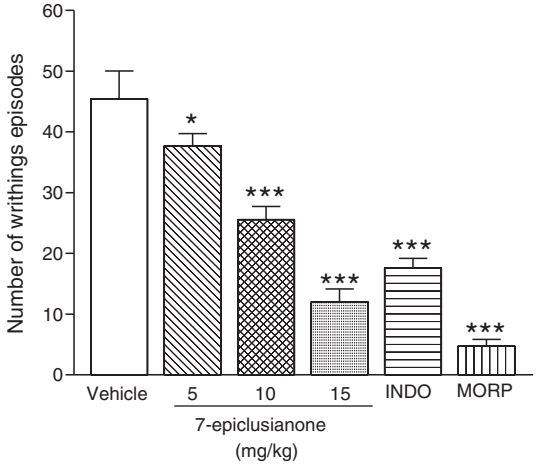


**Fig. 4.** Effects of 7-epiclusianone on acetic acid-induced writhing movements in mice. Animals were pretreated with 7-epiclusianone (*p.o.*), indomethacin (10 mg/kg *p.o.*), morphine (10 mg/kg *i.p.*) or vehicle (10 ml/kg, *p.o.*) prior to the administration of acetic acid (0.6%, *i.p.*). Each column represents the mean ± S.E.M. for six mice per group. The asterisks denote the significance levels when compared with the control group: $^{*}P<0.05$, $^{***}P<0.001$.

the formalin test. The reference drug, indomethacin, suppressed only the late phase of the formalin test, while morphine inhibited both phases of the painful stimulus (Fig. 5).

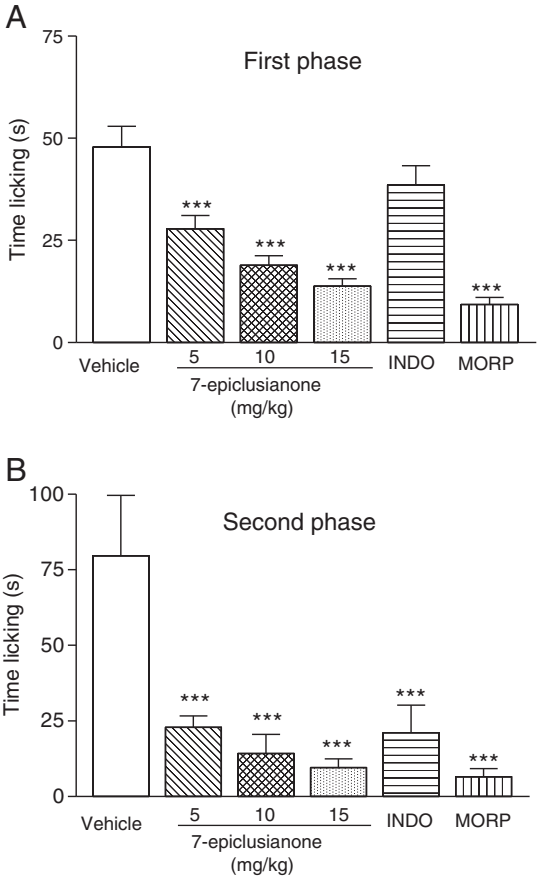


**Fig. 5.** Effects of 7-epiclusianone on the licking induced by a formalin injection in mice. Animals were pretreated with 7-epiclusianone (*p.o.*), indomethacin (INDO) (10 mg/kg, *p.o.*), morphine (MORP) (10 mg/kg, *i.p.*) or vehicle (10 ml/kg, *p.o.*) prior to formalin injection. The total time spent licking the hind paw was measured in the early (Panel A) and late (Panel B) phases after intraplantar injection of formalin. Each column represents the mean ± S.E.M. for eight mice per group. The asterisks denote the significance levels when compared with the control group: $^{***}P<0.001$.

### 3.5. Hot plate test in mice

7-Epiclusianone, administrated at doses of 5 to 15 mg/kg, increased the latency time compared to the control group at 30 ($F_{4,50}=6.11$; $P=0.005$) and 60 min ($F_{4,50}=4.49$; $P=0.0038$). Morphine, which was used as standard drug, also significantly increased the latency time (Fig. 6A). The naloxone completely blocked ($F_{4,34}=24.1$; $P<0.001$) the morphine antinociceptive effect and also blocked the antinociceptive activity of the 7-epiclusianone at 10 mg/kg dose (Fig. 6B).

### 3.6. Open-field test

Mice treated with the benzophenone at 5 to 15 mg/kg did not demonstrate a statistical reduction in the number of crossings and

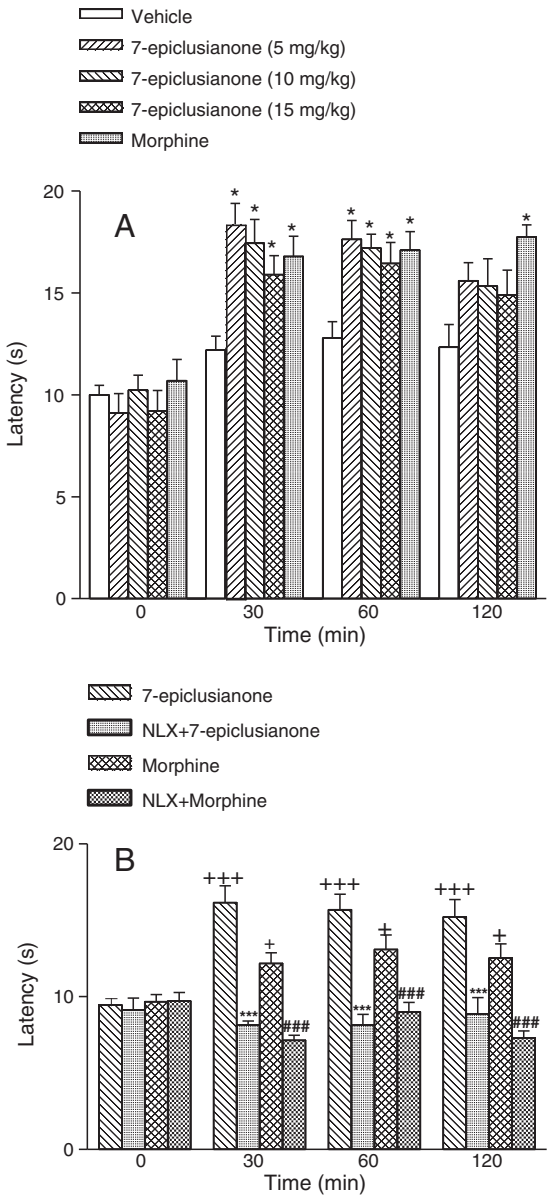


**Fig. 6.** Effects of 7-epiclusianone on the hot plate test. Panel A — animals were pretreated with vehicle (10 ml/kg, *p.o.*), 7-epiclusianone (*p.o.*) or morphine (10 mg/kg, *i.p.*) or prior to the tests at 50 °C. Panel B — animals were treated with vehicle + morphine (1 mg/kg), Vehicle + 7-epiclusianone (10 mg/kg), naloxone + morphine (NLX + morphine; 0.4 mg and 1 mg, respectively) or naloxone + 7-epiclusianone (NLX + 7-epiclusianone; 0.4 mg and 10 mg, respectively) prior to the tests at 50 °C. Each column represents the mean with S.E.M. for eight mice in each group. The symbols denote the significance levels: $^{*}P<0.05$ when compared with the time zero; $^{\#}P<0.05$ when compared with the vehicle + 7-epiclusianone group, $^{\dagger}P<0.05$ when compared with the morphine group.

rearings when compared to the control group in the open-field test (data not shown).

## 4. Discussion

Considering the studies already described and the wide spectra of biological activities of the polyisoprenylated benzophenones, we evaluated the anti-inflammatory and analgesic activity of 7-epiclusianone using animal models of pain behaviour.

For the screening of compounds possessing anti-inflammatory activity, carrageenan-induced paw oedema is a model widely employed and has frequently been used to assess the anti-oedematogenic effect of natural products (Mendes et al., 2010). Carrageenan has also been used as a noxious agent to induce experimental inflammation, which is discernible within 30 min after local injection into the rat paw (Ojewole, 2006). The development of oedema induced by carrageenan is a biphasic event. The early phase (1–2 h) is mainly mediated by histamine, serotonin and bradykinin. The late phase is sustained by the release of prostaglandins and nitric oxide, with a peak at 3 h, and is produced by inducible isoforms of cyclooxygenase (COX-2) and nitric oxide synthase (iNOS) (Brito and Antonio, 1998; Seibert et al., 1994). Previous oral treatment with this benzophenone was effective in reducing the oedematogenic response evoked by carrageenan in late phase. This reduction may be caused by inhibition of one or more intracellular signalling pathways involved in mediating the inflammatory response (Thomazzi et al., 2010).

Although leukocytes have a protective role in inflammation, tissue damage is a deleterious consequence of the intense migration of neutrophils, as observed in immune inflammatory diseases (Smiderle et al., 2008). To confirm the probable anti-inflammatory activity of 7-epiclusianone, a test using LPS-induced peritonitis was performed. Lipopolysaccharides bind to toll-like receptors (TLR4) present in phagocytic cells and promote the production of pro-inflammatory cytokines and leukocyte migration (Dantzer, 2009). In the peritonitis test, our results demonstrated that 7-epiclusianone significantly reduced leukocyte migration to the peritoneal cavity, showing that this compound has active anti-inflammatory properties.

The formalin test is a model that consists of two distinct phases. The first phase, or neurogenic phase (immediately after formalin injection), seems to be caused by the direct effect of formalin on sensory C-fibres. The second phase, or inflammatory phase (starting approximately 20 min after injection), is associated with the development of an inflammatory response and the release of nociceptive mediators (Abbott et al., 1995; Davidson and Carlton, 1998). It was reported that substance P and bradykinin participate in the appearance of the early phase responses and that histamine, serotonin, prostaglandin and bradykinin are involved in the late phase responses (Du et al., 2007). Centrally acting drugs, such as opioids, inhibit both phases equally, while peripherally acting drugs, such NSAIDs and corticosteroids, only inhibit the late phase (Shibata et al., 1989). Our results demonstrate that 7-epiclusianone shows analgesia in the early and late phases of the formalin test, suggesting that 7-epiclusianone acts both centrally and peripherally to reduce inflammatory pain.

A reduction of the late phase behavioural response to an *i.p.* formalin injection was observed, demonstrating the anti-inflammatory activity produced by 7-epiclusianone. The results obtained from carrageenan-induced rat paw oedema and peritonitis induced by lipopolysaccharide also confirmed this effect. Likewise, other benzophenones have been shown to possess anti-inflammatory activities. Garcinol modulates arachidonic acid metabolism in LPS-stimulated murine macrophages and suppresses iNOS and COX-2 expression and NO formation (Hong et al., 2006). Additionally, 3′4,5′,6-tetrahydroxy-*2-O-β-D*-xylosylbenzophenone and 3′4,5′,6-tetrahydroxy-2-O-(4-O-acetyl-β-D-Xylosyl) have been shown to suppress the oxidative burst of neutrophils (Demirkiran et al., 2009). Additionally, the benzophenone *N*-ethyl piperidine ether analogues have been shown to have a significant anti-inflammatory profile, mainly against the leukocyte infiltration measured in the carrageenan paw oedema test (Khanum et al., 2009).

Similar to other substances that act on the central nervous system (CNS), 7-epiclusianone inhibited both phases of the formalin test in a manner similar to that of morphine. Moreover, the results of this test are in agreement with those obtained from the hot plate test, confirming the central antinociceptive effect of the compound.

The acetic acid-induced writhing test is a visceral pain model widely used to screen potential analgesic substances. In this model, pain is generated indirectly via endogenous mediators, such as bradykinin, serotonin, histamine, substance P and prostaglandins, which stimulate peripheral nociceptive neurons that are sensitive to narcotic analgesics and non-steroidal anti-inflammatory drugs (NSAIDs) (Collier et al., 1968; Derardt et al., 1980). Moreover, these mediators are able to increase vascular permeability, reduce the threshold of nociception and stimulate the nerve terminals of nociceptive fibres (Martinez et al., 1999). Intraperitoneally injected acetic acid can also directly activate non-selective cation channels located at primary afferent pathways (Julius and Basbaum, 2001).

The results obtained in this study revealed that 7-epiclusianone significantly reduced acetic acid-induced writhing responses, suggesting that 7-epiclusianone may reduce the liberation of inflammatory mediators or block inflammation-promoting receptors. Neves et al. (2007) suggested that benzophenones might also block $Ca^{2+}$ influx and/or intracellular $Ca^{2+}$-dependent mechanisms, so this possibility must be also be considered.

The acetic acid-induced writhing test is a nonspecific model (*i.e.*, anticholinergic, tricyclic antidepressants, antihistamines and other agents show activity in this test) (Queiroz et al., 2010); thus, other tests, such as the formalin and hot-plate tests, need to be conducted before any conclusion can be made regarding the mechanism of antinociceptive activity (Zakaria et al., 2010).

The hot plate test specifically tests for central antinociception (Vilela et al., 2009) and measures complex responses to inflammation and nociception (Bhandare et al., 2010). The rapid effect with a maximum peak observed shortly after administration of 7-epiclusianone is similar to the time course of action of opioid agonists (*e.g.*, morphine, fentanyl and etorphine) that mediate analgesia by central mechanisms (Pinheiro et al., 2010) involving supraspinal components (Yaksh and Rudy, 1976, 1977; Yeung et al., 1977).

One of the main strategies in nociception studies has been the search for opioid analgesics acting at opioid receptors outside the central nervous system (CNS), with the prospect of avoiding centrally mediated side effects as tolerance and dependence (Benyhe, 1994; Vanegas and Tortorici, 2002). For the assessment of opioid system involvement in the analgesic activity the mice were pre-treated with an opioid antagonist, naloxone. In this study, naloxone prevented the antinociceptive effect of 7-epiclusianone in hot plate tests. Those results suggest that, at least part of the anti-hyperalgesic effect observed is due to involvement of this system (μ-opioid) since naloxone reverted the antinociceptive activity.

In the present study, we demonstrated the efficacy of 7-epiclusianone, a prenylated benzophenone isolated from the leaves of *G. brasiliensis*, in different anti-inflammatory and antinociceptive tests. These effects occurred at doses that evoked no visible modifications in the overall behaviour of the animals. However, further studies should be conducted to investigate the molecular mechanisms of action of this benzophenone and its participation in the pain inhibitory mechanisms of the CNS.

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