Anti-inflammatory and antinociceptive activities of *Campomanesia adamantium*

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ABSTRACT

Ethnopharmacological relevance: *Campomanesia* species are used in folk medicine as anti-inflammatory, anti-rheumatic, anti-diarrheal and hypocholesterolemic.
Aim of the study: The present study investigated the *in vivo* anti-inflammatory and antinociceptive properties of ethyl acetate (AE) and aqueous (Aq) extracts from leaves of *Campomanesia adamantium* and *in vitro* anti-inflammatory activity of AE and its isolated flavonols, myricitrin and myricetin.
Materials and methods: The antinociceptive activity of AE and Aq was evaluated using acetic acid-induced writhing and formalin methods. The *in vivo* anti-inflammatory effect of AE and Aq was evaluated using carrageenan-induced paw oedema in mice. AE, myricitrin and myricetin were evaluated for their abilities to modulate the production of NO, TNF-α and IL-10 in LPS/IFN-γ stimulated J774.A1 macrophages.
Results: It was found that orally administrated AE and Aq (125 and 250 mg/kg) inhibited carrageenan-induced paw oedema in mice. AE (125 and 250 mg/kg) and Aq (125 mg/kg) reduced the time to licking at the second phase of the formalin method *in vivo* in mice. AE (250 mg/kg) and Aq (125 mg/kg) also reduced the number of writhes. AE, myricitrin and myricetin inhibited NO (320 μg/mL and 6.25–100 μM, respectively) and TNF-α production by macrophages (320 μg/mL for AE, 100 μM for myricitrin and 25–100 μM for myricetin). AE (160 and 320 μg/mL), myricitrin (50 and 100 μM) and myricetin (25–100 μM) increased IL-10 production by macrophages.
Conclusions: The ethyl acetate and aqueous extracts from *Campomanesia adamantium* showed anti-nociceptive and anti-inflammatory effects supporting the use of the plant in folk medicine. The results suggest that anti-oedematogenic effect promoted by aqueous extract involves several anti-inflammatory mechanisms of action. The antinociceptive effect shown by aqueous extract can be due to the modulation of release of inflammatory mediators involved in nociception. The anti-inflammatory effects of AE and of its isolated flavonols may be attributed to inhibition of pro-inflammatory cytokines production, TNF-α and NO and to the increased of IL-10 production.

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1. Introduction

Campomanesia adamantium (Myrtaceae) is popularly known as gabiroba and used in folk medicine to treat inflammation and rheumatism (Lorenzi, 2000; Ballve Alice et al., 1995).

The inflammation process presents the signs of redness, heat, pain and oedema (Vane and Bolting, 1995) involves events such as enzymes activation, mediators release, fluid leakage, cell migration, tissue damage (Hayden et al., 2006) and can start by various stimuli such as tissue injury, viral or microbial infection, chemical or toxins irritation (Lin et al., 2008). Thus, macrophages play a central role in the inflammation process, since their activation leads to pro-inflammatory cytokines production such as tumour necrosis factor α (TNF-α) and interleukin 6 (IL-6), inflammatory mediators generated by inducible enzymes nitric oxide synthase (iNOS) and cyclooxygenase (COX-2) activation (Medzhitov and Janeway, 1997; Walsh, 2003; Kim et al., 2005). These mediators and cytokines induce the immune cells recruitment such as neutrophils and T lymphocytes (Walsh, 2003; Kim et al., 2005). The second phase of macrophages activation is responsible for preventing the tissue damage and involves the production of interleukin 10 (IL-10), an anti-inflammatory cytokine

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(Sabat et al., 2010). The aim of the present study was to evaluate the anti-inflammatory and antinociceptive effects of ethyl acetate and aqueous extracts from *Campomanesia adamantium* and to evaluate the ability of ethyl acetate extract and its isolated flavonols to modulate the production of NO, TNF-α and IL-10 in LPS/IFN-γ stimulated J774.A1 macrophages.

2. Materials and methods

2.1. General experimental procedures

Analytical high performance liquid chromatography (HPLC) was carried out on Waters Alliance 2695 equipped with vacuum degasser, quaternary pump, and diode array detector (DAD 2996), the analyses were performed on Shimadzu ODS column (250 mm × 4.6 mm × 5 μm). Semi-preparative HPLC was carried out on Shimadzu Liquid Chromatograph LC-6AD with photodiode array detector (SPD M20A). The analyses were performed on Shimadzu ODS column (250 mm × 20 mm × 5 μm). The chromatography columns (CC) were carried out on Polyamide (Fluka), Sephadex LH-20 (GE) and silica gel 60 Merck (0,063–0,200 mm), the fractions were monitored by thin-layer chromatography (TLC) on silica gel 60 G Merck coated plates. Tween-80 was obtained from U.S.P. Dimethylsulphoxide (DMSO) P.A., from Nuclear. Carrageenan, indomethacin, MTT (3-(4,5-dimethylthiazol)-2,5-diphenyl tetrazolium bromide), non-essential amino acids and sodium pyruvate and lipopolysaccharide (LPS) were purchased from Sigma. Interferon-γ (IFN-γ) was obtained from RD Systems and foetal bovine serum from LGC Biotecnologia. Digital caliper rule from Starret.

2.2. Plant material

Leaves of *Campomanesia adamantium* (Cambess.) O. Berg. were collected in Ouro Preto, MG, Brazil, in October, 2007, with permission of Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA-license no. 17021-4). The plant was identified by Dr. Marcos Eduardo Guerra Sobral of Departamento de Botânica of Universidade Federal de São João Del Rei. A voucher specimen was deposited at Herbarium of Instituto de Ciências Exatas e Biológicas of Universidade Federal de Ouro Preto, reference number OUPR25911.

2.3. Extraction, isolation and HPLC analysis

The leaves were dried at 40 °C, reduced to powder (688.0 g) and were submitted to exhaustive percolation with hexane, ethyl acetate and methanol. The solvents were evaporated under reduced pressure resulting in dried hexane (6.4 g), ethyl acetate (AE, 9.7 g) and methanol (85.7 g) extracts, respectively. AE (5.0 g) was fractionated by CC on Polyamide eluted with water, methanol, ethyl acetate and hexane, with gradual reduction of the polarity, resulting in eleven fractions (F1–F11). F5 [eluted with H_2O:MeOH (10:90)] was fractionated by CC on Sephadex LH-20 using methanol, resulting in ten fractions (F5.1–F5.10). F5.8 (eluted with MeOH) yielded solid identified with myricitrin (0.095 g, yellow solid, m.p. 190.4–191.3 °C). F5.10 (eluted with MeOH) was purified on semi-preparative HPLC with water (A)—methanol (B) gradient, 0–15 min (50%–100% B); 15–16 min (100% B) and 16–20 min (100%–50% B), resulting in quercetin isolation (0.006 g, yellow solid). The flow rate was of 8 mL/min, the column temperature was set at 25 °C, the volume of the sample injection was of 2 mL, and detection wavelength was in 254 nm. F7 (eluted with MeOH) was submitted on CC over silica gel eluted with hexane, dichloromethane, ethyl acetate and

methanol gradient resulting in five fractions (F7.1–F7.5). F7.5 [eluted with AcOEt:MeOH (80:20)] was purified on semi-preparative HPLC employing the same conditions described above resulting in the isolation of myricetin (0,012 g, yellow solid). Isolated compounds were characterized using spectroscopic techniques.

AE and its isolated compounds were individually analyzed by analytical HPLC. AE (5 mg) and 1 mg of myricitrin, quercetin and myricetin were dissolved in MeOH (1 mL). After injection of 25 μL of each sample, the gradient elution was carried out with mixture of water (A) and methanol (B). The elution was initiated with 80% A and 20% B, taking 55 min to reach 100% B, flow rate of 0.8 mL/min, wavelength detection 254 nm and column temperature at 25 °C. The isolated compounds were identified and compare using their time retention.

The leaves of *Campomanesia adamantium* (300.0 g) were extracted with 3 L of distilled water by percolation. 200 mL were lyophilized resulting in 500 mg of dried aqueous extract (Aq).

2.4. Animals

The experiments were conducted on male Swiss mice (25–35 g) supplied by Universidade Federal de Ouro Preto (UFOP). The animals received standard chow and water *ad libitum* with light/dark period of 12 h. All experimental procedures were approved by the Ethical Committee of Universidade Federal de Ouro Preto, Brazil (no. 2010/61 and 2012/45) and were carried out in accordance with international guidelines for the care and use of laboratory animals, published by the US National Institute of Health (NIH Publication, revised in 1985).

2.5. Preparation of test samples and drugs

Indomethacin and morphine were dissolved in distilled water and Tween-80 (95:5). Acetic acid and carrageenan were dissolved in distilled water just before use. AE was solubilized in Tween-80, DMSO and distilled water (1:1:8) and Aq was solubilized in distilled water. Morphine (10 mg/kg) and indomethacin (10 mg/kg) were orally administered (0.2 mL) and used as reference drugs. The control group received vehicle (Tween-80, DMSO and distilled water 1:1:8 for AE and distilled water for Aq).

2.6. Carrageenan-induced paw oedema assay

The anti-oedematogenic effect was evaluated by the carrageenan-induced paw oedema method in mice, according to previously described (Winter et al., 1962) with modifications. The animals were treated by oral route with vehicle, indomethacin (10 mg/kg) and both doses (125 and 250 mg/kg) of AE and Aq. Half an hour after administration of the various agents, oedema was induced by injection of carrageenan (20 μL, 0.1%, w/v) into the sub-plantar tissue of the right hind paw. Only needle introduction was performed into the left paw, corresponding damage induced by mechanical perforation. Paws thickness were measured with a caliper rule before and 1, 2, 3, 4, 5, and 6 h after carrageenan injection. Inflammatory swelling was expressed as thickness variation (Δ). Indomethacin was used as reference drug while control group received the vehicles that were used to dissolve AE and Aq. The group treated with vehicle was considered as maximum of inflammation and all others treatments were compared to this group.

2.7. Acetic acid-induced writhing method

This test was performed by using the modified method described by Koster et al. (1959). Animals were treated by oral

route with AE and Aq (125 and 250 mg/kg), indomethacin (10 mg/kg) or vehicle, 30 min before injection of 0.8% acetic acid solution (0.1 mL/10 g body weight, i.p.). The numbers of abdominal writhing were counted over a period of 30 min, soon after acetic acid administration. The antinociceptive effect was expressed as percentage of inhibition of abdominal writhing.

2.8. Formalin method

The antinociceptive effect was also carried out using the formalin method as previously described (Hunskaar and Hole, 1987) with modifications. The animals received by oral route AE and Aq (125 and 250 mg/kg), morphine or vehicle. Half an hour after administration of the various agents, 30 μL of formalin (1.5% in saline solution) were injected into the sub-plantar tissue of the left hind paw. The animals were placed individually into a glass funnel. The time spent licking the injected paw was registered for 30 min soon after formalin injection. Antinociceptive effect was indicated by the reduction of the mean time determined for the test groups compared to the control group. Morphine was used as reference drug.

2.9. Cell line and culture conditions

The murine macrophage cell line, J774.A1 was kindly provided by Dr. Luiz Carlos Crocco Affonso, (Laboratório de Imunoparasitologia–NUPEB–UFOP, MG, Brazil) and maintained in continuous culture in tissue culture flasks in RPMI-1640 supplemented with 100 U/mL of penicillin G, streptomycin sulphate (100 μg/mL), 2 mM of L-glutamine, 1 mM of sodium pyruvate, 1% non essential amino acids and 10% inactivated foetal bovine serum. Cells were grown in at 37 °C in a humidified 5% CO_2 atmosphere.

2.10. Macrophage viability

To evaluate the toxicity of AE, myricitrin and myricetin on J774 macrophages the MTT-assay was performed as previously described by Mosmann (1983). J774.A1 macrophages (1×10^6 cells/mL) were plated in 96-well plates and allowed to adhere at 37 °C in a humidified 5% CO_2 atmosphere for 2 h. Thereafter, the medium was replaced with fresh medium or medium containing increasing concentrations of AE, myricitrin and myricetin dissolved in DMSO (0.32% v/v). Following the incubation period for 24 h at 37 °C, the medium was replaced with fresh medium containing MTT (0.5 mg/mL). After 4 h of incubation, 100 μL of sodium dodecylsulphate 10% in hydrochloric acid 10 mM was added to each well and incubated overnight at 37 °C. The optical density was measured at 550 nm. The optical density in untreated cells was taken as 100% of viability.

2.11. NO production assay

NO production by J774.A1 cells was assayed by measuring the accumulation of nitrite in the culture medium using the Griess reaction (Green et al., 1982). J774.A1 cells were seeded on to a 48-well culture plate at density 1×10^6 cells/mL and allowed to adhere at 37 °C in a humidified 5% CO_2 atmosphere for 2 h. Thereafter, the medium was replaced with fresh medium or medium containing increasing non-cytotoxic concentrations of the AE (20–320 μg/mL), myricitrin and myricetin (6.25–100 μM) dissolved in DMSO (0.32% v/v). After 1 h at 37 °C, the cells were stimulated with LPS+IFN-γ (25 ng/mL+25 UI/mL, respectively) for 24 h at the same conditions. Following this incubation period, an aliquot (50 μL) of the supernatant was removed and combined with 100 μL of Griess reagent, followed by incubation at room temperature for 10 min and spectrophotometric measurement at

550 nm using a microplate reader (Molecular Devices). Nitrite concentrations were calculated using standard solutions of sodium nitrite in culture medium. Dexamethasone (5 μg/mL) was used as a reference standard.

2.12. Determination of TNF-α and IL-10 concentration

J774A.1 cells were seeded on to a 48-well culture plate at density 1×10^6 cells/mL and allowed to adhere at 37 °C for 2 h. After, the medium was replaced with fresh medium or medium containing increasing non-cytotoxic concentrations of the AE (20–320 μg/mL), myricitrin and myricetin (6.25–100 μM) dissolved in DMSO (0.32% v/v) and incubated for 1 h at 37 °C. The cells were stimulated with LPS+IFN-γ (25 ng/mL+25 UI/mL, respectively) for 24 h at the same conditions. Following this incubation period, the supernatant was used to estimate the levels of TNF-α and IL-10 by ELISA kits according to the manufacturers' instruction (ELISA kit, PeproTech, Brazil). Dexamethasone (5 μg/mL) was used as a reference standard.

2.13. Statistical analysis

In vitro results were obtained from three independent experiments in duplicate and are presented as mean ± S.E.M. In vivo results were presented as mean ± S.E.M from experiments performed with six animals per group. Differences among means were tested for statistical significance using one-way analysis of variance (ANOVA), the difference between treated groups and control group was evaluated by Dunnett's test. The difference between treated groups and reference drug group was evaluated by the Bonferroni test. The analyses were carried out using software PRISMA (Graph-Pad Software, Inc., San Diego, CA, version 5.01). $^*P < 0.05$ and $^{\#}P < 0.05$ were considered statistically significant.

3. Results

There are no reports about anti-inflammatory and analgesic activities of Campomanesia adamantium. In the present study the experiments were performed to demonstrate in vivo anti-inflammatory and antinociceptive activities of the ethyl acetate (AE) and aqueous (Aq) extracts from leaves of Campomanesia adamantium and to evaluate, whether AE and its isolated flavonoids have the ability to inhibit, in vitro, the production of the NO and TNF-α and to stimulate IL-10 release from LPS/IFN-γ stimulated J774.A1 macrophages.

3.1. Isolation of flavonoids from ethyl acetate extract.

Fractionation of AE yielded the pure previously known flavonoids named as myricitrin, quercetin and myricetin (Schmeda-Hirschmann, 1995). The compounds were identified by NMR spectroscopy (^{1}H and ^{13}C NMR, COSY, DEPT-135, HSQC and HMBC) and by comparison with spectral literature data (Agrawal, 1989; Güvenalp and Demirezer, 2005; Ceruks et al., 2007; Shen et al., 2009). It was first reported here the isolation of myricitrin from Campomanesia adamantium.

The fingerprint of AE (Fig. 1A) showed chromatographic profile with peaks with retention times of 20–30 min. Myricitrin (Fig. 1B), myricetin (Fig. 1C) and quercetin (Fig. 1D) were identified in the extract by comparing their retention times.
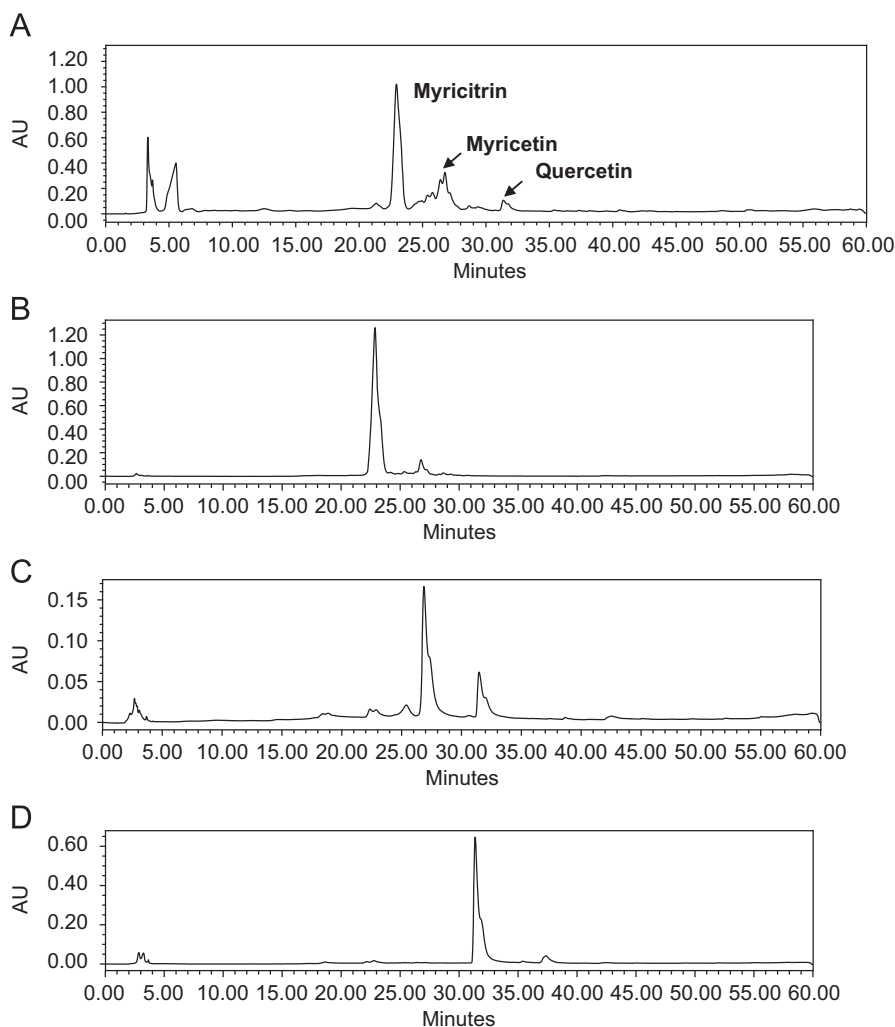
Fig. 1. Fingerprint of ethyl acetate extract (AE) (A). The isolated compounds were identified in the extract by comparing their retention times, myricitrin (B), myricetin (C) and quercetin (D). The chromatograms were obtained at a wavelength of 254 nm.

3.2. Effects of ethyl acetate and aqueous extracts on carrageenan-induced paw oedema

AE and Aq at doses of 125 and 250 mg/kg showed a significant reduction of the carrageenan-induced paw oedema in mice compared to control group. The effect of AE (Fig. 2A) began in the third hour after carrageenan administration and persisted throughout the experiment in a dose-dependent manner and similarly to indomethacin that also reduced significantly the oedema compared to group vehicle and compared to the left paws. The effect of Aq (Fig. 2B) started at the first hour after carrageenan administration at dose of 125 mg/kg. Aq was able to reduce significantly the oedema in both doses evaluated (125 and 250 mg/kg) from the second hour and remained throughout the experiment. Aq showed activity higher than indomethacin.

3.3. Effects of ethyl acetate and aqueous extracts on the acetic acid-induced writhing test

AE (Fig. 3A) at dose of 250 mg/kg significantly reduced number of acetic acid-induced writhing, similar to the indomethacin and at dose of 125 mg/kg did not produce any significant antinociceptive effect compared to control group. Aq was able to reduce significantly the acetic acid-induced writhing at dose of 125 mg/kg (Fig. 3B).

3.4. Effects of ethyl acetate and aqueous extracts on the formalin method

AE (Fig. 4A) and Aq (Fig. 4C) did not produce any significant antinociceptive effect in the first phase compared to control group. AE at doses of 125 and 250 mg/kg (Fig. 4B) and Aq at dose of 125 mg/kg (Fig. 4D) significantly reduced the licking time in the second phase of formalin test in mice. Morphine (10 mg/kg) produced significant antinociceptive activity in both phases of the formalin method (Fig. 4A, B, C and D). The antinociceptive effect of AE at both doses evaluated was similar to morphine at second phase.

3.5. Effects of AE and its isolated compounds on cell viability of J774.A1 macrophages

The AE 640 μg/mL induced a significant reduction of J774.A1 macrophage viability compared to untreated cell, indicating that the concentration was toxic. Myricitrin and myricetin did not show significant decrease in viability at any concentration evaluated (data not shown).

3.6. Effects of AE and its isolated compounds on NO production

The AE, myricitrin and myricetin were assessed for their inhibitory activity on NO production by LPS (25 ng/mL) and IFN-γ (25 UI/mL) stimulated J774.A1 macrophages. AE (Fig. 5A)
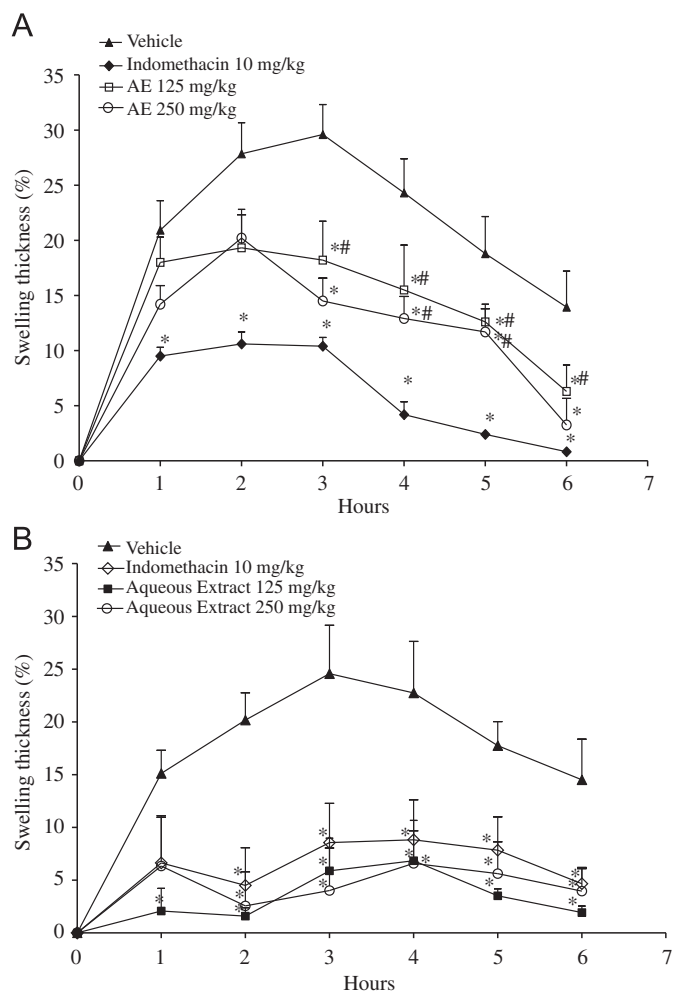
Fig. 2. Effect of the ethyl acetate (AE) (A) and aqueous (B) extracts of *Campomanesia adamantium* leaves on mice paw oedema induced by carrageenan. Results represent the mean ± S.E.M. of the percentage variation ($n=6$).*$P < 0.05$, when compared to the group treated with vehicle. #$P < 0.05$, when compared to the group treated with the reference drug.
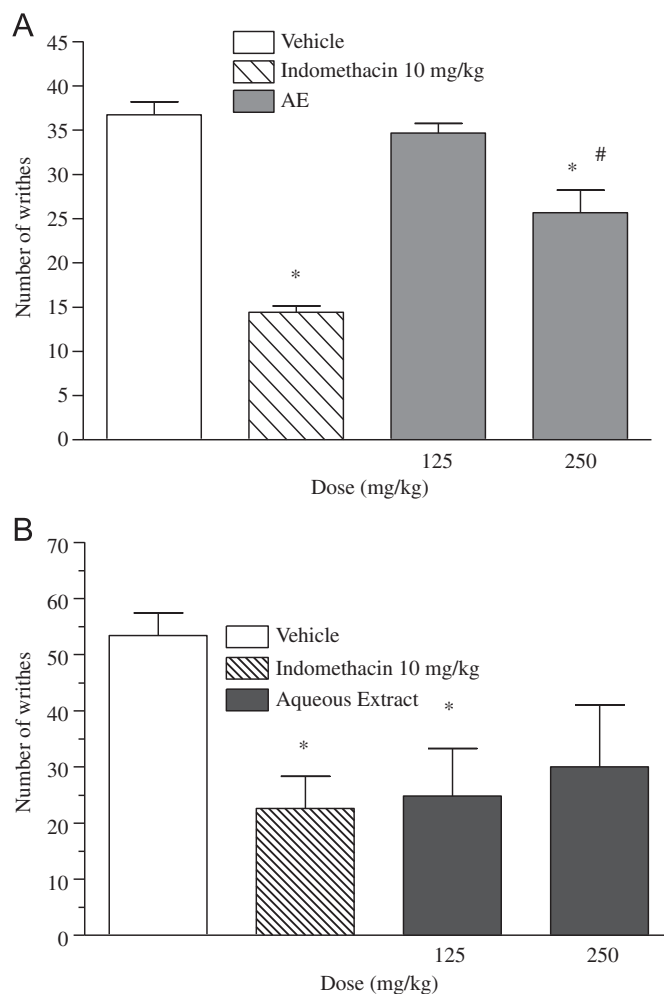

Fig. 3. Effect of ethyl acetate extract (AE) (A) and aqueous extracts (B) of *Campomanesia adamantium* leaves on writhing method induced by i.p. acetic acid. Results represent the mean ± S.E.M. of the number of writhes ($n=6$). *$P < 0.05$, when compared to the group treated with vehicle. #$P < 0.05$, when compared to the group treated with the reference drug.

at concentration of 320 µg/mL was able to inhibit significantly the NO production (Fig. 5). The compounds myricitrin (Fig. 5B) and myricetin (Fig. 5C) at all concentrations evaluated presented significant reduction on NO production by stimulated cells. In addition to the reduction promoted by myricitrin and myricetin at all concentrations did not differ significantly of effect promoted by dexamethasone on NO production by stimulated cells.

3.7. Effects of AE and its isolated compounds on TNF-α production

AE at concentration of 320 µg/mL was able to inhibit significantly the TNF-α production by stimulated cells (Fig. 6A). Myricitrin caused a significant reduction of TNF-α production at concentration of 100 µM (Fig. 6B). Myricetin at concentrations of 25, 50 and 100 µM promoted a significant reduction of TNF-α production induced by LPS/IFN-γ in J774.A1 cells (Fig. 6C). Additionally, it was observed that the reduction of TNF-α production promoted by AE, myricitrin and myricetin was similar to effect of dexamethasone.

3.8. Effects of AE and its isolated compounds on IL-10 production

A significant increase of IL-10 level was promoted by AE (Fig. 7A) at concentrations of 160 and 320 µg/mL, by myricitrin

(Fig. 7B) at concentrations of 25, 50 and 100 µM and by myricetin (Fig. 7C) at concentrations of 50 and 100 µM in LPS/IFN-γ stimulated J774.A1 cells. This production increase promoted by AE, myricitrin and myricetin were similar to the effect shown by dexamethasone, except for the lowest concentration of myricitrin (25 µM).

4. Discussion

Oedema is an essential feature of acute inflammation caused by increased vascular permeability, leading to leakage of transvascular fluid rich in proteins of high molecular weight (exudate) from the intravascular to the interstitial compartment (Aller et al., 2007). Studies have demonstrated that effect induced by carrageenan is a biphasic response with a first phase resulting from the rapid production of several inflammatory mediators such as histamine, serotonin and bradykinin, and a second phase by the release of prostaglandins and nitric oxide with peak at 3 h, produced by inducible isoforms of cyclooxygenase (COX-2) and nitric oxide synthase (iNOS), respectively (Seibert et al., 1994).

AE administration promoted a significant reduction of carrageenan-induced oedema at third hour after carrageenan administration. This result can be suggested that the effect
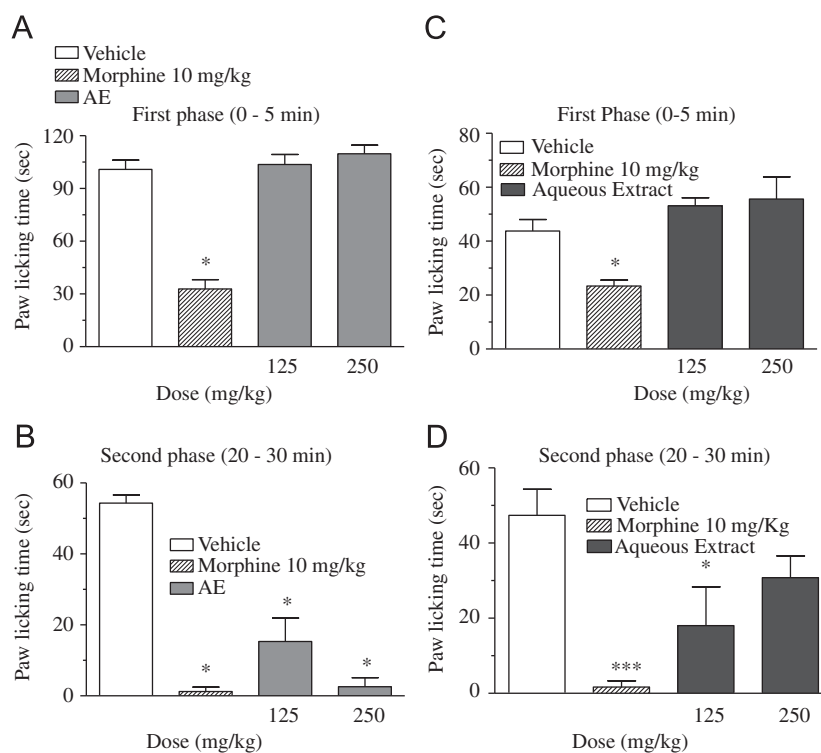
Fig. 4. Effect of ethyl acetate extract (AE) (A and B) and aqueous extracts (C and D) of *Campomanesia adamantium* leaves on formalin-induced paw licking or biting in mice. Results represent the mean $\pm$ S.E.M ($n=6$). *$P < 0.05$, when compared to the group treated with vehicle. ‡$P < 0.05$, when compared to the group treated with the reference drug.

anti-oedematogenic promoted by the extract is related to modulation of the enzymes responsible for the prostaglandins and NO production. AE was able to promote a significant reduction of NO production by stimulated cells that can be related to anti-oedematogenic effect observed. AE also significantly reduced the production of TNF-α, a pro-inflammatory cytokine that induces the production of PGE$_2$ (Perkins et al., 1997). Finally, AE promoted a increase of IL-10, cytokine that has an inhibitory effect on TNF-α and PGE2 production, fundamental condition for its anti-inflammatory activity *in vivo* (Mertz et al., 1994). Therefore, increased production of IL-10 demonstrated *in vitro* can be related to the anti-inflammatory effect observed in paw oedema, since this cytokine acts limiting and closing the inflammatory response (Sabat et al., 2010).

Aq was able to reduce the swelling at first hour and remained throughout the experiment. Therefore, the effect anti-oedematogenic promoted by Aq occurred during all phases of the inflammatory process, showing that this extract has several anti-inflammatory mechanisms of action. Aq showed higher activity that the indomethacin.

The acetic acid-induced writhing is a visceral pain model employed as a screening tool for the assessment of antinociceptive activity of new analgesic agents and represents a model of peripheral nociception (Souza et al., 2009). It has been suggested that acetic acid injection into peritoneal cavity leads to increased levels of cyclooxygenases (COX) and lipooxygenase (LOX) products in peritoneal fluids as well as the release of many inflammatory mediators such as bradykinin, substance P, TNF-α, IL-1β and IL-8, which eventually excites the primary afferent nociceptors entering dorsal horn of the central nervous system (Ikeda et al., 2001). The formalin injection produces two distinct phases, the first phase (0–5 min) corresponds to the neurogenic pain caused by the direct effect of formalin on the sensory C-fibres and the second phase (20–30 min), or the inflammatory pain, is associated with the development of an inflammatory response

and the release of nociceptives mediators. It is well known that drugs, such as morphine, that act primarily on the central nervous system inhibit both phases equally, while peripherally acting drugs, as anti-inflammatory and steroids, inhibit the late phase (Hunskaar and Hole, 1987). AE and Aq effects were evaluated by this method and by a second noxious model, the formalin method, to elucidate if the antinociceptive effect exerted by extracts demonstrated in writhing test is peripheral and/or central. AE and Aq showed significant effect at the acetic acid-induced writhing method and at the second phase of the formalin method, indicating that antinociceptive effect occurred to the peripheral level. Thus, it can be concluded that AE and Aq anti-nociceptive activities can be relate to the modulation of release of inflammatory mediators involved in nociception (Tjølsen et al., 1992). The antinociceptive effect promoted by AE may be due to the reduction of TNF-α levels and the increase of IL-10 production, as observed at the *in vitro* assays. The reduction of TNF-α may be responsible for a reduction of PGE$_2$ release, since the TNF-α induces this prostaglandin production (Perkins et al., 1997). Therefore the *in vitro* production modulation of these mediators may be related to the antinociceptive activity of AE shown here.

In this study it was isolated and characterized the flavonols myricitrin, quercetin and myricetin. In previous studies myricitrin promoted acute and chronic pain reduction in mice and its antinociceptive effect has been already attributed to the protein kinase inhibition, reducing NO production and anti-inflammatory activity (Meotti et al., 2006). Quercetin inhibited nociceptive behaviour in the acetic acid-induced writing and in both phases of formalin-induced pain methods (Filho et al., 2008). Quercetin also inhibited the inflammatory response induced by carrageenan in rats (Morikawa, et al., 2003). Myricetin showed significant antinociceptive effect in the acetic acid-induced writhing test and in the second phase of formalin method, and this reduction has already been associated to the PGE2 reduction in peritoneal fluid (Tong et al., 2009).
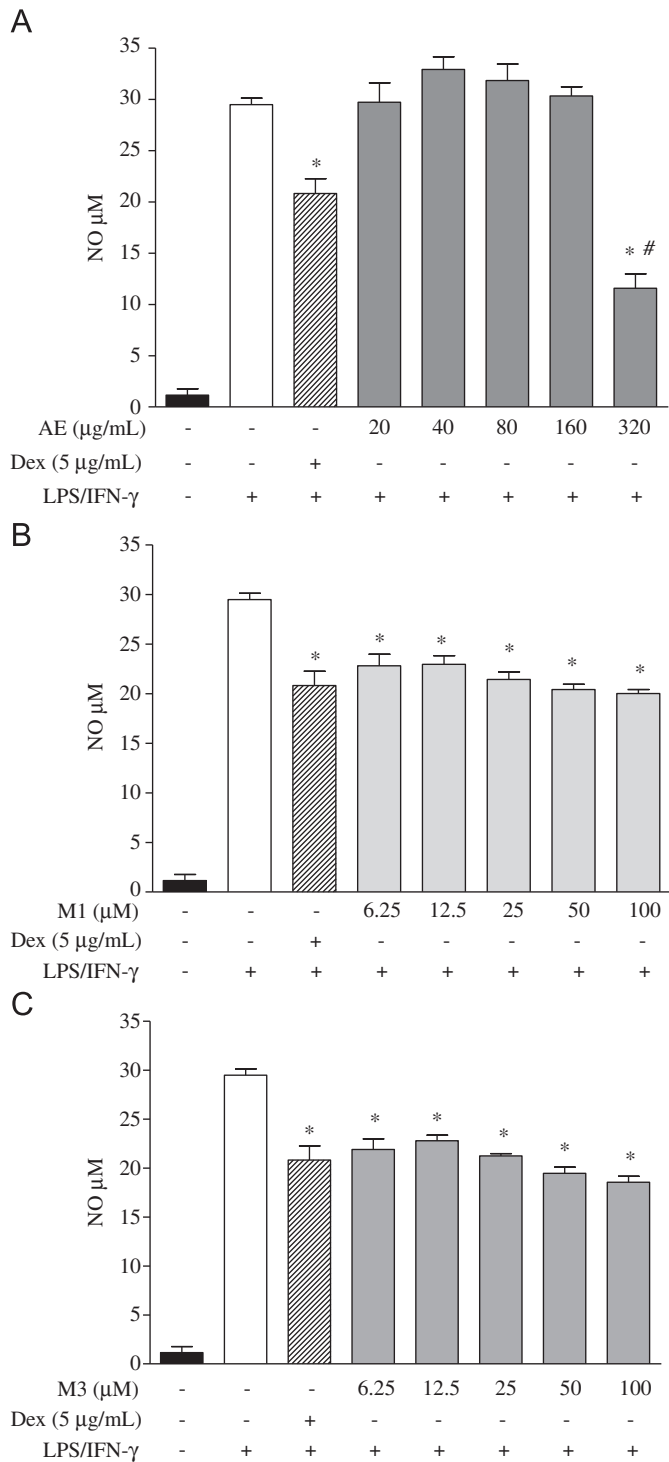
Fig. 5. Effect of ethyl acetate extract of *Campomanesia adamantium* leaves (AE) (A), and its isolated compounds myricitrin (M1) (B) and myricetin (M3) (C) on NO production by LPS/IFN-γ stimulated J774.A1 macrophages. The values shown are mean ± S.E.M of three independent experiments. *$P < 0.05$, when compared to the group treated with LPS/IFN-γ stimulated group (white bar). #$P < 0.05$, when compared to the group treated with the reference drug.
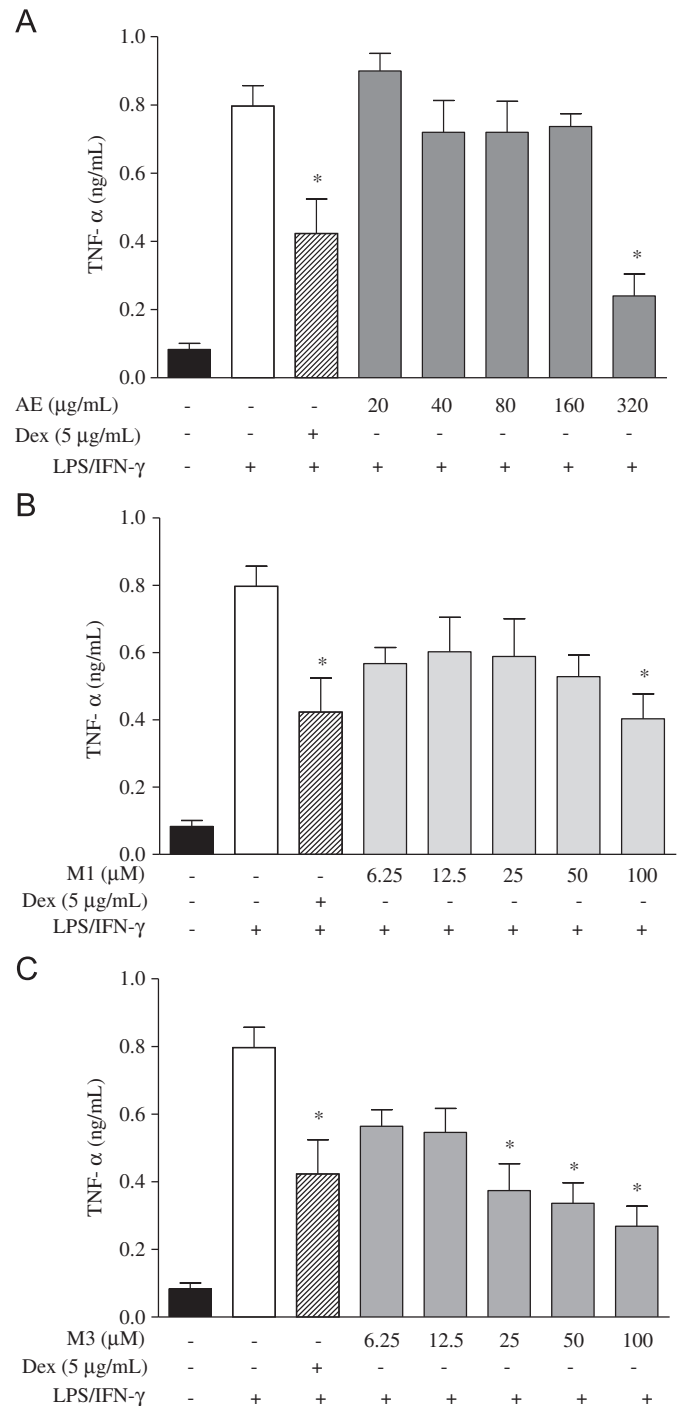

Fig. 6. Effect of ethyl acetate extract of *Campomanesia adamantium* leaves (AE) (A), and its isolated compounds myricitrin (M1) (B) and myricetin (M3) (C) on TNF-α production by LPS/IFN-γ stimulated J774.A1 macrophages. The values shown are mean ± S.E.M of three independent experiments. *$P < 0.05$, when compared to the group treated with LPS/IFN-γ stimulated group (white bar). #$P < 0.05$, when compared to the group treated with the reference drug.

In previous studies myricitrin, myricetin and quercetin were able to reduce NO and TNF-α production in cellular models of inflammation (Park et al. 2008; Shimosaki et al., 2011). In the present study, AE, myricitrin and myricetin exhibited potent inhibitory activity on NO and TNF-α production, and stimulatory ac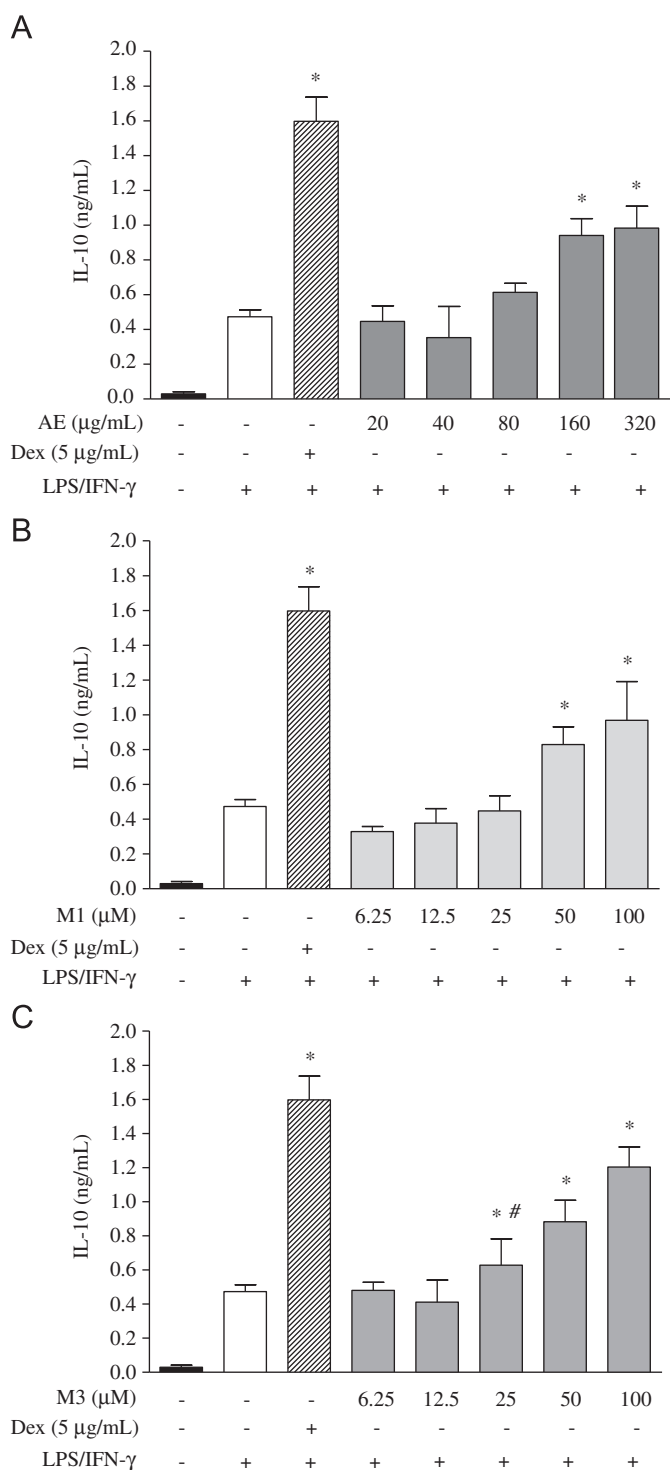tivity on IL-10 production. Therefore, the anti-inflammatory activity demonstrated by AE is related to the presence of flavonols myricitrin, myricetin and quercetin in this extract.

Myricetin showed inhibitory effect on TNF-α and stimulatory effect on IL-10 production at concentrations lower than myricitrin. Previous studies have reported that the reduced activity of glycosylated compounds has been attributed to increased hydrophilicity and/or steric hindrance by the further portion of sugar molecule, which can reduce the absorption/penetration of glycosylated compounds in cells (Kim et al., 1999).

A

B

C

Fig. 7. Effect of ethyl acetate extract of *Campomanesia adamantium* leaves (AE) (A), and its isolated compounds myricitrin (M1) (B) and myricetin (M3) (C) on IL-10 production by LPS/IFN-γ stimulated J774.A1 macrophages. The values shown are mean ± S.E.M of three independent experiments. *$P < 0.05$, when compared to the group treated with LPS/IFN-γ stimulated group (white bar). #$P < 0.05$, when compared to the group treated with reference drug.

5. Conclusions

The ethyl acetate and aqueous extracts from leaves of *Campomanesia adamantium* showed antinociceptive and anti-inflammatory activities supporting the use in folk medicine of the plant. Ethyl acetate extract, myricitrin and myricetin showed

immunomodulatory effects on NO, TNF-α and IL-10 in cell model of inflammation, and these can be the biochemical mechanisms that contribute to the antinociceptive and anti-inflammatory effects demonstrated on *in vivo* models to ethyl acetate extract. The anti-edematogenic effect promoted by aqueous extract occurred during all phases of the inflammatory process, showing that this extract has several anti-inflammatory mechanisms of action. The antinociceptive activity shown for aqueous extract can be due to the modulation of release of inflammatory mediators involved in nociception. More studies should be performed to further elucidate the mechanisms of action by which the ethyl acetate and aqueous extracts of *Campomanesia adamantium* leaves exert their anti-inflammatory and antinociceptive effects.

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