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Anti-inflammatory activity of *Lychnophora passerina*, Asteraceae (Brazilian "Arnica")

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ABSTRACT

Ethnopharmacological relevance: Lychnophora passerina (Asteraceae), popularly known as "arnica," is used to treat inflammation, pain, rheumatism, contusions, bruises and insect bites in Brazilian traditional medicine.

Materials and methods: The anti-inflammatory activity of crude ethanolic extract of aerial parts of *L. passerina* and its ethyl acetate and methanolic fractions had their abilities to modulate the production of NO, TNF-α and IL-10 inflammatory mediators in LPS/IFN-γ-stimulated J774.A1 macrophages evaluated. Moreover, the crude ethanolic extract and derived fractions were also *in vivo* assayed by carrageenan-induced paw oedema in mice.

Results: In vitro assays showed remarkable anti-inflammatory activity of *L. passerina* crude ethanolic extract (EE) and its ethyl acetate (A) and methanolic (M) fractions, through the inhibition of production of NO and TNF-α inflammatory mediators and induction of production of IL-10 anti-inflammatory cytokine. *In vivo* assays showed anti-inflammatory activity for EE 10% ointment, similar to the standard drug diclofenac gel. The A and M fraction ointments 20% presented anti-inflammatory activity.

Conclusion: The results obtained showed that possible anti-inflammatory effects of EE and its A and M fractions may be attributed to inhibition pro-inflammatory cytokines production, TNF-α and NO and to increased IL-10 production. EE, A and M ointments showed topical *in vivo* anti-inflammatory activity. The *in vivo* anti-inflammatory activity of EE of *L. passerina* may be related to synergistic effects of different substances in the crude extract. Therefore, traditional use of aerial parts of *L. passerina* in the inflammatory conditions could be beneficial to treat topical inflammatory conditions, as evidenced by the present study.

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1. Introduction

Inflammation is a natural host-defensive process of the innate immunity response. Bacterial and viral infection triggers the activation of numerous immune cells such as macrophages, monocytes, and neutrophils undergoing to cellular responses such as phagocytic uptake and production of inflammatory mediators, such as nitric oxide (NO), prostaglandin E_2 (PGE_2) and tumor necrosis factor (TNF)-α (Gautam and Jachak, 2009; Yu et al., 2010). NO is produced from L-arginine by the enzyme nitric oxide synthase (NOS) (Palmer et al., 1988). Large amounts of NO are produced by the enzyme inducible nitric oxide synthase (iNOS), enzyme involved in cellular overproduction of NO and active when a pathologic process is present, like inflammation or cancer, and many other conditions (Stichtenoth and Frolich, 1998). The TNF-α cytokine plays a crucial role in inflammation, stimulating the production of other cytokines and pro-inflammatory mediators (Verma et al., 2010). However, overproduction of TNF-α is related to development and progression of inflammatory process and autoimmune diseases (Williams et al., 2007). Unlike NO and TNF-α, the interleukin (IL)-10 is the most important cytokine presenting anti-inflammatory

Abbreviations: A, ethyl acetate fraction; DMSO, dimethyl sulfoxide; EE, ethanolic extract; H, hexanic fraction; IL-10, interleukin-10; INF-γ, interferon-γ; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; M, methanolic fraction; MTT, 3-(4,5-dimethyl-2-thiazyl)-2,5-diphenyl-2H-tetrazoliumbromide; NF-κB, nuclear factor-kB; NO, nitric oxide; NOS, nitric oxide synthase; PGE_2, prostaglandin E_2; RNA, ribonucleic acid; S.E.M., standard error of mean; TNF-α, tumor necrosis factor-α.

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properties. It is produced by activated immune cells, mainly monocytes/macrophages and regulates many different immune cells functions. In monocytes/macrophages, IL-10 diminishes the production of inflammatory mediators and inhibits antigen presentation (Sabat et al., 2010).

Lychnophora (Asteraceae) is endemic to campo rupestre habitats of Brazilian savanna (cerrado biome) and have a wide variety of biological activities including anti-inflammatory, antinociceptive and trypanocidal activity (Chiari et al., 1996; Oliveira et al., 1996; Ferraz Filha et al., 2006; Guzzo et al., 2008). The aerial parts of the *Lychnophora* species, including *Lychnophora passerina*, have been used in a folk medicine macerated in ethanol to treat inflammatory and pain conditions (Cerqueira et al., 1987).

Although the traditional use of *Lychnophora* species as anti-inflammatory agents is supported by scientific evidence (Gobbo-Neto et al., 2005; Ferraz Filha et al., 2006; Guzzo et al., 2008) there are few studies of its action mechanism. We reported previously that the ethanolic extract from aerial parts of the *L. passerina* exhibited *in vivo* topical anti-inflammatory activity (Guzzo et al., 2008). Based on these data, the objectives of the present work were to evaluate, whether the same ethanolic extract have the ability to inhibit, *in vitro*, the production of the NO and TNF-α and to stimulate IL-10 release from J774.A1 macrophages stimulated by LPS/IFN-γ. In addition, the ethanolic extracts and its fractions were also *in vivo* evaluated, using the carrageenan-induced paw oedema methods test in mice.

2. Materials and methods

2.1. Plant material

Aerial parts of *L. passerina* (Mart exDC.) Gardn were collected in Diamantina, Minas Gerais, Brazil, in September 2000. The plant botanical identification was realized by Dr. Júlio A. Lombardi, Departamento de Botânica, Instituto de Biociências de Rio Claro, UNESP, Rio Claro, SP, Brazil. A voucher specimen was deposited in the Herbarium of Instituto de Ciências Exatas e Biológicas of Universidade Federal de Ouro Preto – UFOP, under the number BHCB 53571.

2.2. Preparation of plant extract and fractions

Aerial parts (1 kg) of *L. passerina* were air-dried, ground and extracted with ethanol by percolation, at room temperature, for 14 days. The solvent was eliminated by evaporation under reduced pressure, resulting the dried crude ethanolic extract. The ethanolic extract (EE, 120.0 g) was submitted to filtration column chromatography on silica gel, eluted with hexane, ethyl acetate and methanol to yield the hexanic (H, 0.42 g), ethyl acetate (A, 55.0 g) and methanolic (M, 59.0 g) fractions, respectively.

2.3. Cell line and culture conditions

J774A.1 murine macrophage cell line was kindly provided by Dr. Affonso, L.C.C. (Laboratório de Imunoparasitologia – NUPEB – Universidade Federal de Ouro Preto, Minas Gerais, Brazil). The cells were maintained in RPMI supplemented with 100 U/ml of penicillin, 2 mM of L-glutamine, 100 U/ml of penicillin G, 1 mM of sodium pyruvate and 10% fetal bovine serum. Cells were grown at 37 °C in a humidified 5% CO_2 atmosphere.

2.4. Cell viability by MTT assay

To exclude the possible interference of the EE, A and M on cell viability the 3-(4,5-dimethyl-2-thiazyl)-2,5-diphenyl-2H-tetrazoliumbromide (MTT) assay was performed. J774.A1 (2.5×10^5 cells/well) macrophages were plated in 96-well plates and allowed to adhere at 37 °C in a 95% air and 5% CO_2 atmosphere for 2 h. Thereafter, the medium was replaced with fresh medium or medium containing increasing concentrations of the EE, A and M dissolved in DMSO for 24 h at 37 °C and 5% CO_2 in humidified air. After 24 h, MTT solution (dissolved in PBS) was added (final concentration of 0.5 mg/ml) to each well and incubated for 4 h in the same conditions. The medium was carefully discarded, and 100 μl of sodium dodecyl sulfate 10% in hydrochloric acid 10 mM was added to each well to solubilize the formazan. The optical density was measured at 550 nm. The end concentration of DMSO was adjusted to less than 0.1% for all treatments.

2.5. NO inhibition assay

J774A.1 cells were seeded onto a 24-well culture plate at density 2.5×10^5 cells per well with 500 μl of culture medium and incubated for 2 h. The cells were stimulated with LPS + IFN-γ (1 μg/ml + 10 UI/ml, respectively) for 1 h before the treatment with EE, A and M dissolved in DMSO at different non-cytotoxic concentrations (2.5–40 μg/ml) for 24 h at 37 °C and 5% CO_2 in humidified air. After 24 h, the presence of nitrite, a stable oxidized product of nitric oxide (NO), was determined in cell culture media using Griess reagent. Briefly, 50 μl of supernatant was removed and combined with 100 μl of Griess reagent in a 96-well plate, followed by incubation for 10 min at room temperature and spectrophotometric measurement at 550 nm using a microplate reader (Molecular Devices) as described by Green et al. (1982) and Verma et al. (2010). NO concentration was determined using comparison with a sodium nitrite standard curve. The final concentration of DMSO was adjusted to less than 0.1% for all treatments. Dexamethasone was used as a reference standard.

2.6. TNF-α inhibition assay and IL-10 stimulation assay

J774A.1 cells were seeded onto a 24-well culture plate at density 2.5×10^5 cells per well with 500 μl of culture medium and incubated for 2 h. The cells were stimulated with LPS + IFN-γ (1 μg/ml + 10 UI/ml, respectively) for 1 h before the treatment with EE, A and M dissolved in DMSO at different non-cytotoxic concentrations (2.5–40 μg/ml) for 24 h at 37 °C and 5% CO_2 in humidified air. After 24 h, the supernatant was collected and used to estimate the levels of TNF-α and IL-10 by specific ELISA kits according to the manufacturers' instruction (ELISA kit, PeproTech, Brazil), respectively. The final concentration of DMSO was adjusted to less than 0.1% for all treatments. Dexamethasone was used as a reference standard.

2.7. Preparation of ointments containing crude ethanolic extract and its fractions

EE, A and M were solubilized in Tween-80, DMSO and distilled water (1:1:8) and mixed with base ointment (lanoline/vaseline 70:30) for the 10% final concentration.

2.8. Animals

For *in vivo* assays were utilized male Swiss albino mice (30 ± 5 g), supplied by Animal House of Universidade Federal de Ouro Preto (UFOP). The animals received feed and water *ad libitum* with light/dark period of 12 h. The experimental protocols were approved, under the number 2010/59, by UFOP Ethical Committee of Animal Experimentation. The protocols were in accordance to the Guide for the Care and Use of Laboratory Animals, published

by the US National Institute of Health (NIH Publication, revised in 1985).

2.9. Carrageenan-induced paw oedema assay

The *in vivo* anti-inflammatory activity was determined by the carrageenan-induced paw oedema method in mice, according to previously described (Winter et al., 1962). The 10% EE, A and M ointments (three groups) were topically applied on right hind paws using a spatula, immediately after 0.1% carrageenan (Sigma–Aldrich) saline solution subplantar administration. Three more groups were established: (a) carrageenan, treated with base ointment, corresponding to 100% of inflammation (carrageenan group), (b) only needle introduction in the left paw, corresponding to activity induced by mechanical perforation (control group), and (c) carrageenan treated with standard drug diclofenac gel (Cataflan® Emulgel–Novartis, 11.6 mg/g). To ensure the ointment contact with paws bandages were used. To measure oedema variation a digital caliper rule (Starret) was used. The paws were measured before and 3 h after carrageenan administration, with or without treatments. The paw oedema was expressed in millimeters (mm) and was calculated as the percentage of variation between zero time and 3 h after carrageenan.

2.10. Statistical analysis

In vitro results were obtained from two independent experiments in duplicate and are presented as mean ± S.E.M. *In vivo* results were presented as means ± S.E.M from experiments performed with 8 animals per group. Statistical significance among groups was determined by ANOVA followed by Bonferroni test using software PRISMA (GraphPad Software, Inc., San Diego, CA, version 5.01). *P*-values $\leq$ 0.05 were taken to indicate statistical significance.

3. Results

L. passerina is used in the folk medicine as anti-inflammatory, analgesic and to treat rheumatism. In this study, the ethanol extract and its fractions were investigated for their *in vitro* and topical *in vivo* anti-inflammatory activity.

3.1. Effects of crude ethanolic extract and its fractions on cell viability of J774.A1 macrophages

The viability of the J774.A1 macrophages in the presence of EE, A and M were evaluated. The cytotoxicity evaluated to different concentrations of EE, A and M on the J774.A1 cells was negligible and did not show statistic difference when compared to the control. The exception occurred for the M fraction, since the concentration of 40 μg/ml showed cell viability lower than 90% (data not shown). Thus, EE, A and M fractions were *in vitro* assayed for anti-inflammatory activity using concentrations lower than 40 μg/ml.

3.2. Effects of crude ethanolic extract and its fractions on NO production

To evaluate the inhibitory effects of the EE, A and M on NO production by LPS/IFN-γ stimulated cells, they were treated with LPS (1 μg/ml) and IFN-γ (10 UI/ml) in the presence or absence of the EE, A and M for 24 h. The amount of nitrite, as an index of NO in culture medium, was measured with Griess reagent. Unstimulated J774.A1 cells secreted basal levels of NO, while LPS/IFN-γ stimulation resulted in NO production increase. EE and A were able to inhibit the NO production by LPS/IFN-γ stimulated J774.A1 cells in a concentration-dependent manner (Fig. 1A and B), at the higher evaluated concentrations: 20 μg/ml and 40 μg/ml for the EE, and 10 μg/ml, 20 μg/ml and 40 μg/ml for the A. M was not able to reduce the NO levels, in any evaluated concentration (Fig. 1C).

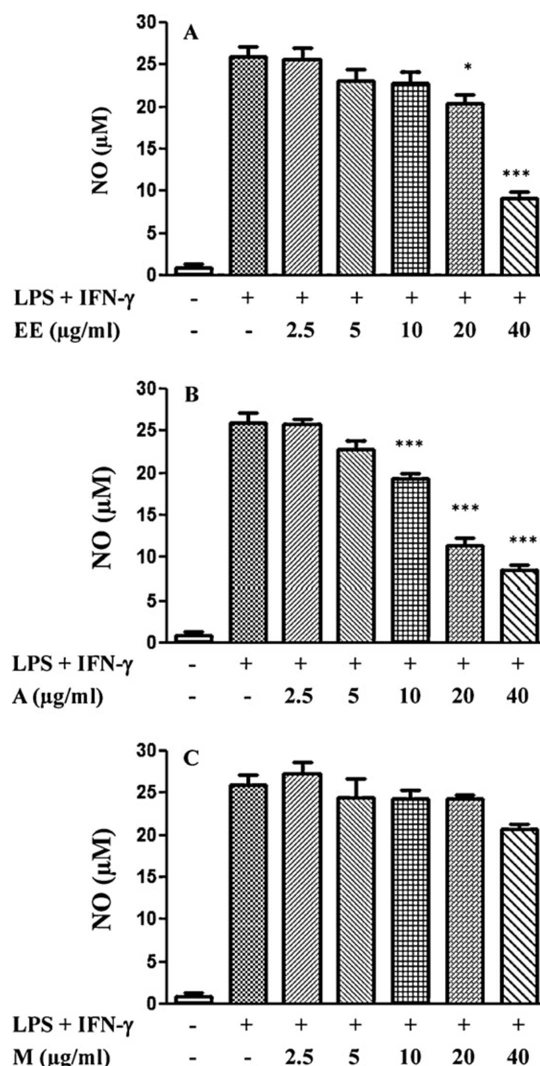

Fig. 1. (A) Effect of the ethanolic extract (EE), (B) effect of acetyl acetate fraction (A) and (C) effect of methanolic fraction (M) on NO production by LPS/IFN-γ stimulated J774.A1 macrophages. The cells were stimulated with LPS (1 μg/ml) and IFN-γ (10 UI/ml) for 1 h before the treatment with EE, A and M dissolved in DMSO at different non-cytotoxic concentrations (2.5–40 μg/ml) for 24 h. Supernatants were collected and nitrite (NO) concentration was determined by Griess reagent. Data are represented as mean ± S.E.M. values. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with LPS/IFN-γ-stimulated macrophages alone.

3.3. Effects of crude ethanolic extract and its fractions on TNF-α production

To determine the inhibitory effects of crude EE and A and M fractions of *L. passerina*, on LPS/IFN-γ-stimulated TNF-α production, J774.A1 macrophages were treated with LPS (1 μg/ml) and IFN-γ (10 UI/ml) in the presence or absence of different concentrations of EE, A and M. The macrophages stimulated only with LPS/IFN-γ exhibited an appreciable increase of TNF-α levels, while in the absence of any stimulus the cells secreted basal levels of TNF-α (Fig. 2). The strong inhibition of TNF-α production was observed for almost all evaluated concentrations of EE (Fig. 2A) and M (Fig. 2C) and a lower effect was observed for the A fraction (Fig. 2B). The EE at 5, 10, 20 and 40 μg/ml decreased the LPS/IFN-γ-stimulated TNF-α production. The M fraction was also active at the same concentrations. The A fraction

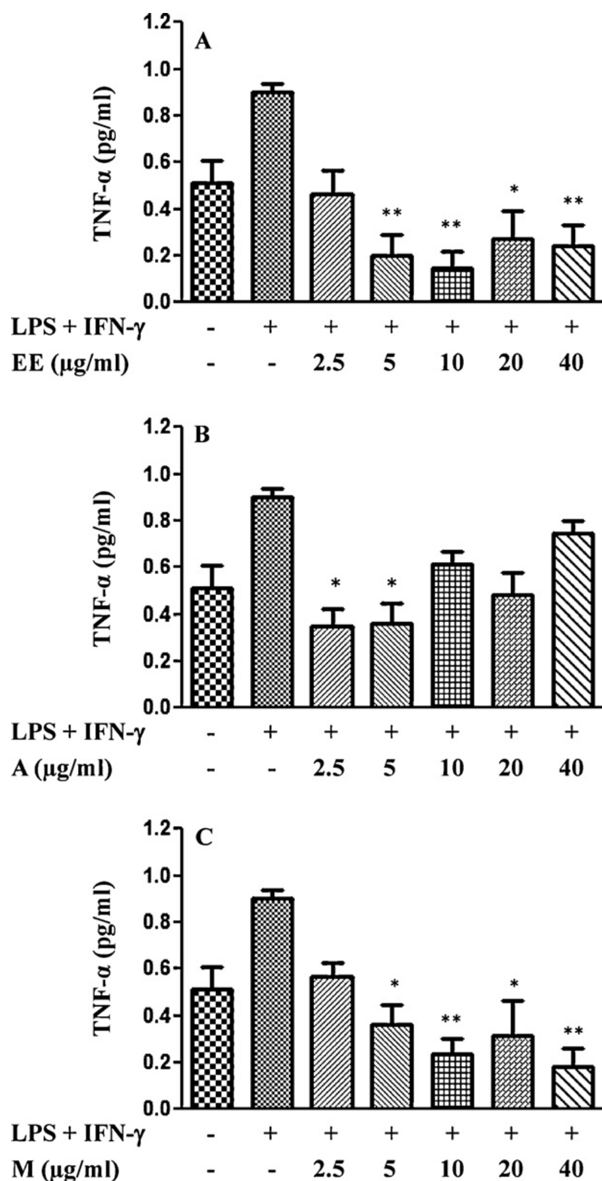

Fig. 2. (A) Effect of the ethanolic extract (EE), (B) effect of acetyl acetate fraction (A) and (C) effect of methanolic fraction (M) on TNF-α production by LPS/IFN-γ stimulated J774.A1 macrophages. The cells were stimulated with LPS (1 μg/ml) and IFN-γ (10 UI/ml) for 1 h before the treatment with EE, A and M dissolved in DMSO at different non-cytotoxic concentrations (2.5–40 μg/ml) for 24 h. The supernatants were collected and TNF-α concentration was determined by specific ELISA kit. Data are represented as mean ± S.E.M. values. *$P<0.05$, **$P<0.01$ and ***$P<0.001$ compared with LPS/IFN-γ-stimulated macrophages alone.

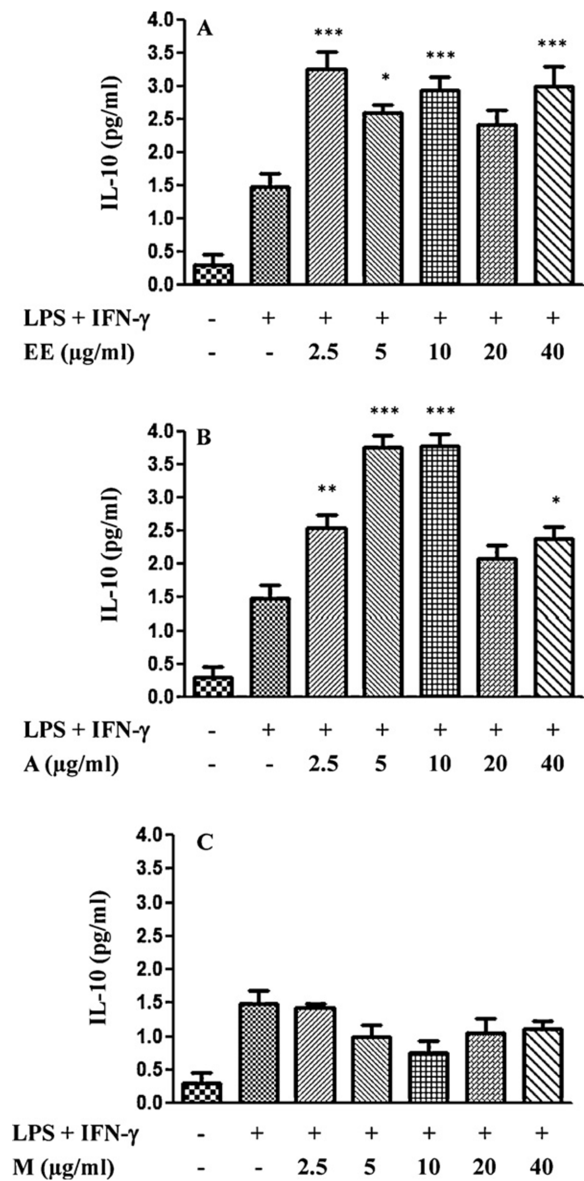

Fig. 3. (A) Effect of the ethanolic extract (EE), (B) effect of acetyl acetate fraction (A) and (C) effect of methanolic fraction (M) on IL-10 production by LPS/IFN-γ stimulated J774.A1 macrophages. The cells were stimulated with LPS (1 μg/ml) and IFN-γ (10 UI/ml) for 1 h before the treatment with EE, A and M dissolved in DMSO at different non-cytotoxic concentrations (2.5–40 μg/ml) for 24 h. The supernatants were collected and IL-10 concentration was determined by specific ELISA kit. Data are represented as mean ± S.E.M. values. *$P<0.05$, **$P<0.01$ and ***$P<0.001$ compared with LPS/IFN-γ-stimulated macrophages alone.

showed better inhibitory activity at concentrations of 2.5 μg/ml and 5 μg/ml.

3.4. Effects of crude ethanolic extract and its fractions on IL-10 production

The effects of EE and A and M of *L. passerina* on IL-10 production were investigated in J774.A1 macrophages stimulated with LPS (1 μg/ml) and IFN-γ (10 UI/ml) in the presence or absence of different concentrations. The cells stimulated with LPS/IFN-γ exhibited an increase of IL-10 production, while unstimulated J774.A1 cells secreted significant smaller basal levels of IL-10 (Fig. 3). EE increased the LPS/IFN-γ-induced IL-10 production in almost all evaluated concentrations, except for 20 μg/ml. A significantly increased the IL-10 folds, mainly at 2.5 μg/ml, 5 μg/ml and 10 μg/ml. M did not induce alteration of IL-10 production in all evaluated concentrations (Fig. 3C).

3.5. Effects of crude ethanolic extract and its fractions on carrageenan-induced paw oedema

The EE ointment at 10% significantly reduced the carrageenan-induced paw oedema (Table 1). The A and M ointments at 20%, significantly reduced the oedema. Diclofenac gel, used as standard anti-inflammatory drug, was able to reduce significantly the oedema. Moreover, the anti-inflammatory activity of EE ointment at 10%was similar to diclofenac gel.

Table 1
Effect of topically applied ointments of *L. passerina* ethanolic extract (EE) and its ethyl acetate (A) and methanolic (M) fractions evaluated using carrageenan-induced paw oedema.

Treatment after carrageenan	Swelling thickness (%)
Control condition (without carrageenan)	$2.7 \pm 0.35^{***}$
Base ointment	29.9 ± 1.86
Diclofenac gel	$6.6 \pm 1.22^{***}$
EE 10%	$12.6 \pm 2.61^{***}$
M 20%	$20.1 \pm 1.60^{*}$
A 20%	$16.9 \pm 1.27^{***}$

Results represent the mean ± S.E.M. of the percentage variation. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, when compared to the group treated with base ointment (ANOVA followed Bonferroni's test). The measurements were taken before and 3 h after administration of carrageenan.

4. Discussion

The present study assessed the anti-inflammatory potential *in vitro* of EE in J774.A1 macrophages stimulated by LPS (1 μg/ml) and IFN-γ (10 UI/ml). The assays quantified the release of pro-inflammatory mediators NO and TNF-α and the IL-10 production, an anti-inflammatory cytokine. The NO is produced by activated macrophages as a result of various stimuli, including TNF-α, IFN-γ and LPS, and it contributes to the pathological process of different acute and chronic inflammatory conditions (Nathan, 1992). The TNF-α also plays an important role in inflammation and may act on the monocytes and macrophages by an autocrine activity to increase diverse functional responses and to induce an over expression of inflammatory mediators. (Baugh and Bucala, 2001). IL-10 is an anti-inflammatory cytokine, well known for its inhibitory effects on TNF-α and inflammatory reactions. Its main activity is to inhibit the cytokine production by macrophages (Bortesi et al., 2009).

EE, A and M fractions presented inhibitory activity on NO and TNF-α production, by LPS/IFN-γ-induced J774.A1 macrophages. Moreover, EE was able to significantly increase the IL-10 production by J774.A1 macrophages activated with LPS/IFN-γ, the model of *in vitro* inflammation used in the present work. However, it is interesting to note that inhibition of TNF-α production and the induction of IL-10 production by A decreased at the highest concentrations evaluated. The lack or decreasing of plant extracts activity at higher concentrations is also frequently cited in the literature (Punturee et al., 2004; Hammer et al., 2008). Concerning to IL-10, this may be due to post-transcriptional effects of metabolites present in EE, since it is known that production of this anti-inflammatory cytokine may be regulated by post-transcriptional mechanisms in macrophages (Nemeth et al., 2005). Adittionally, M strongly inhibited the TNF-α production, but showed a weak inhibition of NO production and weak activity on the IL-10 production by LPS/IFN-γ-stimulated macrophages. The significant basal secretion of TNF-α, observed in the inhibition of production assay for this cytokine, has also been reported in other studies that showed greater production of this pro-inflammatory mediator by LPS/IFN-γ-stimulated J774.A1 macrophages and by the same cells stimulated only with LPS (Herath et al., 2003; Fan et al., 2010).

Previous phytochemical investigations of the aerial parts of *L. passerina* resulted in the isolation of the triterpenoids, sesquiterpenes, including sesquiterpene lactones, steroid and of the flavonoids (Bohlmann et al., 1981; Oliveira et al., 1996; Chicaro et al., 2004).

Several terpenoids have inhibitory properties on the production of inflammatory cytokines (Bremner and Heinrich, 2002; Ríos, 2010) and among the terpenoids present in plant kingdom, the sesquiterpene lactones are specially produced and accumulated by species of *Lychnophora* genus, as well as by other species of Asteraceae family (Bohlmann and Jakupovic, 1990). The sesquiterpene lactones goyazensolide and 15-desoxigoyazensolide showed pharmacological activities, including anti-inflammatory and trypanocidal activity (Chiari et al., 1996; Oliveira et al., 1996; Rüngeler et al., 1999). Several sesquiterpene lactones showed anti-inflammatory activity in cellular model of inflammation (Lyb et al., 1998; Rüngeler et al., 1999; Koo et al., 2001) and the action mechanism proposed has been the suppression of nuclear factor-kappa B (NF-κB) activation (Palladino et al., 2003; Ríos, 2010), which regulates the transcription of inflammatory cytokines and other molecules involved in inflammation (Cho et al., 2009; Lu et al., 2009). Thus, the inhibitory activity of NO and TNF-α production and induction of IL-10 production by A may be probably related to sesquiterpene lactones and triterpenoids presences and inhibition of the nuclear factor NF-κB activity.

Some flavonoids are able to inhibit TNF-α production, the iNOS expression and the NO production, as well as stimulation of the IL-10 expression, effects that have been associated to NF-κB activity inhibition (Herath et al., 2003; Comalada et al., 2006; Hämäläinen et al., 2007). However, in this study, M did not show significant activity on NO and IL-10 production, but inhibited the TNF-α secretion. Since TNF-α is produced early in the inflammatory process (Jin et al., 2003; Hammer et al., 2008) and IL-10 is produced later (Jin et al., 2003; Jung et al., 2004), the polar flavonoids present in M can act at the initial phase diminishing the production of pro-inflammatory cytokines, but was not able to increase the secretion of cytokines that act in the late phase of inflammation, such as the IL-10. EE inhibited NO and TNF-α and increased the IL-10 production by LPS/IFN-γ-stimulated macrophages. The EE fractionation was able to concentrate more active substances in the A fraction than in the M fraction, resulting better activity to A fraction. The EE, characterized by presence of terpenoids and flavonoids, can exert immunomodulatory activity by synergistic ways, resulting in a remarkable anti-inflammatory activity.

Since the topical application of alcoholic macerates of aerial parts of *L. passerina* is used in traditional medicine to treat inflammation and pain, this route of administration was chosen in order to evaluate the *in vivo* anti-inflammatory properties of EE, A and M. The *in vivo* anti-inflammatory activity for EE at 10% and A and M at 20% ointments was observed. These results confirmed the anti-inflammatory effects observed *in vitro*.

Therefore, the remarkable *in vivo* activity observed for EE may be attributed to synergism of several bioactive molecules. The activity of different substances on different inflammation targets, as well as the possibility to improve the bioavailability of molecules, may result in a grater activity of crude extracts (Ji et al., 2009), rather than semi purified fractions. There is growing evidence showing that the medicinal plants may exhibit synergistic combinations (Phillipson, 2003; Gilani and Rahman, 2005; Ma et al., 2009; Wagner and Ulrich-Merzenich, 2009; Graz et al., 2010).

5. Conclusion

The results obtained showed that possible anti-inflammatory effects of EE and its A and M fractions may be attributed to inhibition pro-inflammatory cytokines production, TNF-α and NO and to increased IL-10 production. EE, A and M ointments showed topical *in vivo* anti-inflammatory activity. The *in vivo* anti-inflammatory activity of EE of *L. passerina* may be related to synergistic effects of different substances in the crude extract. Therefore, traditional use of aerial parts of *L. passerina* in the inflammatory conditions could be beneficial to treat topical inflammatory conditions, as evidenced by the present study.

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