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RESEARCH ARTICLE

Different activities of *Schinus areira* L.: anti-inflammatory or pro-inflammatory effect

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Abstract

The anti-inflammatory drugs possess many serious side effects at doses commonly prescribed. It is really important to discover novel regulators of inflammation from natural sources with minimal adverse effects. *Schinus areira* L. is a plant native from South America and is used in folk medicine as an anti-inflammatory herb. For this study, the activity of aqueous extracts on inflammation and the effect on superoxide anion production in mice macrophages were assayed. Aqueous extracts were prepared by soaking herbs in cold water (cold extract), boiling water (infusion), and simmering water (decoction). Cold extract possess an anti-inflammatory activity. Decoction and infusion showed pro-inflammatory activity. Cold extract increased the production of superoxide anion. It has been proposed to use diverse methods to obtain extracts of *S. areira* L. with different effects. Cold extract, decoction, and infusion could be utilized as extracts or as pharmacological preparations for topical application.

Keywords: *Cold extract; decoction; inflammatory activity; infusion; Schinus areira L.*

Introduction

Inflammation is the complex biological response of vascular tissues to harmful stimuli, such as pathogens, damaged cells, and irritant agents.[1] It is a protective attempt by the organism to remove the injurious stimuli as well as to initiate the healing process for the tissue. Inflammation is not a synonym for infection.[2] Inflammation is the consequence of increased cell membrane permeability that may be initiated by changes to membrane lipids by reactive oxygen species. This results in capillary dilation causing redness and pain, and the increased vascular permeability allows plasma to escape into the surrounding tissue giving rise to edema. Inflammation is modulated by cytokines. Reactive oxygen species associated with inflammation are formed by the reduction of tissue oxygen, and include peroxide, superoxide anion, the hydroxyl radical, and singlet oxygen. Superoxide is biologically quite toxic and is deployed by the immune system to kill invading microorganisms.[3]

During the different phases of inflammation, several mediators such as histamine, serotonin, chemotactic factors, bradykinin, and prostaglandins are released. The first phase (acute phase) is characterized by local vasodilatation and increased capillary permeability resulting in an exudation of fluid from the blood into the interstitial space, the second one by infiltration of leukocytes into the inflamed tissues, and the third one by granuloma formation.[4] In this sense, phagocytes have long been recognized as fundamental components of the immune system in inflammatory process. In carrying out their protective task, phagocytes engulf and destroy infective organisms or degraded host cells without damaging themselves or other cells. Phagocyte cells such as polymorphonuclear leukocytes and macrophages respond to a variety of membrane stimulants

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with the production and extracellular release of a number of reactive oxygen and nitrogen species. This coordinated sequence of biochemical reaction, known as "oxidative burst," is initiated by an increase in oxygen uptake followed by the one-electron reduction of oxygen to superoxide anion using NADPH or NADH as the electron donor and catalyzed by a NADPH oxidase. Mutations in the gene coding for the NADPH oxidase cause an immunodeficiency syndrome called chronic granulomatous disease, characterized by extreme susceptibility to infection.[5]

In the absence of inflammation, wounds and infections would never heal and progressive destruction of the tissue would compromise the survival of the organism. However, an inflammation that runs unchecked can also lead to diseases.[2] Abnormalities associated with inflammation comprise a large unrelated group of disorders.[6]

On the other hand, there are two principal types of anti-inflammatory drugs: steroidal anti-inflammatory drugs (corticosteroids) that reduce inflammation by binding to cortisol receptors and non-steroidal anti-inflammatory drugs that inhibit cyclooxygenase enzyme. These drugs possess many serious side effects.[7] Besides, there are no drugs for the treatment of disease where the pro-inflammatory capacity is suppressed; therefore, it is important to develop novel natural drugs that can modulate inflammation process, also with minimal adverse effects. In this way, the study of the pharmacological activities of herbal extracts can also be used as a logical strategy for the search of new drugs.[8]

Schinus areira L. (Anacardiaceae) known as Aguará ibá guazú, Aguaribay, Molle, Gualeguay, Peppercorn, and Pepper-tree is a large spreading tree growing to a height of 12 m. It is native from South America and has been planted as a street tree in southern Europe.[9] In traditional medicine, *S. areira* was used for treating a variety of wounds and infections due to its antibacterial and antiseptic properties. It has also been used as an antidepressant and as diuretic. The major components of the essential oil of *S. areira* are limonene (28.6%), α-phellandrene (10.1%), sabinene (9.2%), and camphene (9.2%). The essential oils exhibited a high biotoxicity in a brine shrimp assay with *Artemia persimilis*.[10] Besides, it is shown that this plant inhibited bovine viral diarrhea virus replication.[11] Methanol extracts of *S. areira* L. showed cytotoxic activity against a human hepatocellular carcinoma cell line.[12] On the other hand, the plant is used in folk medicine as an anti-inflammatory agent[13] but there are no scientific reports to confirm this activity.

The aim of this study was to test the activity of different extracts obtained from *S. areira* L. on inflammation and to determine whether the observed effects are related to the methods used for preparing extracts.

Materials and methods

Preparation of plant extracts

Leaves and tender branches of *S. areira* L. were collected in San Luis, Argentina. The plant was identified in the herbarium of the National University of San Luis and number voucher was given. Leaves and tender branches were dried in a stove at 45°C for 5 days and reduced to fine dust.[14] From this material we prepared the following: Cold extract (CExt) was prepared by dissolving 5 g of dust in 100 mL of distilled water and allowing the solution to stand for 24 h at 4°C. The obtained yield was 18.14% (w/w). Infusion (Inf) was prepared by using 5 g of dust and 100 mL of boiling distilled water and allowing the solution to stand for 20 min. The yield was 22.14% (w/w). Decoction (Dec) was prepared by heating a solution of 5 g of dust and 100 mL of distilled water to boiling temperature and allowing it to stand for 20 min. The obtained yield was 21.01% (w/w). The extracts were filtered and centrifuged at 3500 rpm for 15 min. The supernatants were filter-sterilized through a 0.22 μm filter, lyophilized, and the aliquots were stored at –20°C until use.[14] Before assays, all reagents and extracts used were subjected to an assay based on the gelling properties of the amebocyte from the blood of the crab *Limulus polifemus* in the presence of an endotoxin. Limulus test for the detection of LPS contamination showed that endotoxin was either low (0.01 EU/mL) or undetectable.

Animals

Male and female Rockland mice (average weight 20 g) were used in this study. The animals were kept at a controlled temperature of 20 ± 2°C with a photoperiod cycle of 12 h light and 12 h darkness. The animals were fed *ad libitum* with Purina Chow and water. During the whole process, the animals were handled following the guidelines of care and experimental use of animals described in DHEW publication, NHI 80-23.

Groups of five animals were used. The first group was treated with 1× phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4); the second group was treated intraperitoneally with three doses of the CExt (133.3 mg/kg) in 1× PBS at intervals of 48 h; the third and fourth groups were treated with Inf and Dec, respectively, at conditions identical to those mentioned earlier.

Cell preparation

Groups of three mice were injected intraperitoneally with 1 mL of sterile 10% protease peptone (Difco, Cleveland, OH, USA) in PBS 3 days before cell collection. Peritoneal cells (PC) were harvested by sterile washes with 20 mL HBSS (Sigma, San Diego, CA, USA) supplemented

with 20 µg/mL gentamicin and heparin (50 U/mL) as described previously[15] and adjusted to 1×10^6 cells/mL. Macrophages were purified from PC by adherence onto 96-well flat-bottomed tissue culture plates in Dulbecco's modified Eagle's medium (DMEM; Sigma) with 20 µg/mL gentamicin and 5% heat-inactivated fetal calf serum (Gibco, Rockville, MD, USA). Non-adherent cells were removed after 2 h at 37°C and complete medium was added. In our hands, the adherent macrophage monolayer showed ±90% purity according to morphological analysis or non-specific esterase staining.[16]

Cell viability

Macrophage viability was determined by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) after 24 h of incubation. Briefly, cells (1×10^6 cells/mL), treated and untreated with extracts at different concentrations (1, 2, 4 mg/mL), were incubated with 100 µL RPMI 1640 containing 10 µL of 5 mg/mL MTT (Sigma). Untreated cells were used as the control of viability (100%) and the results were expressed as % of viability relative to control.[5]

Oxidative burst assay

Opsonized zymosan (OPZ) stimulates a robust respiratory burst and was used as a particulate stimulus. Zymosan A (Sigma) was opsonized with a 1:3 dilution of normal mice serum for 30 min at 37°C, washed three times, and resuspended to 1 mg/mL. For the assay, we used the protocol described by Schopf et al.[17] and the superoxide anion was evaluated by the reduction of nitro blue tetrazolium (NBT; Sigma) to formazan. In all these assays, macrophages were incubated with 1, 2, and 4 mg/mL CExt, Inf, or Dec for 90 min, 300 µL NBT-OPZ was added and left for another 60 min. The reaction was stopped with 1 N HCl (Tetrahedron, Buenos Aires, Argentina). Controls included untreated macrophages cultured with or without OPZ. Formazan was extracted with dioxane (Dorwill, Buenos Aires, Argentina) and the absorbance was measured using a microplate reader at 525 nm.[16]

Carrageenan-induced inflammation

The effect of *S. areira* L. extracts on carrageenan-induced inflammation was studied in groups of 10 mice. The first group received a subplantar injection of 0.2 mL PBS 1× (controls), second group received 0.2 mL CExt at 4 mg/mL corresponding to 133.3 mg/kg, and the third and fourth groups were injected with 0.2 mL of Inf or Dec, respectively, at conditions identical to those mentioned earlier. After 1 h, all the animals were inoculated by injecting a saline solution containing 1% γ-carrageenan (Sigma) into the right hind paw. Indomethacin was used as the reference drug (0.5 mg/mL). Groups of mice injected with CExt, Dec, and Inf without γ-carrageenan were also used. The volume of the paw was measured by plethysmometry (Kent Scientific Corporation, Litchfield, MA, USA) twice at 6 and 18 h, respectively. The results are expressed as % of inhibition of inflammation with respect to the controls.[18]

Ear edema in mice

Ear edema was induced in groups of 10 animals. The right ear of each mouse received a topical application of 2.5 µg of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Sigma) as 0.125 µg/µL acetone solution (10 µL to each side of the ear). CExt, Inf, or Dec (4 mg/ear) were dissolved in acetone or absolute ethanol and were applied topically immediately after TPA. The left ear, used as control, received the vehicle. Indomethacin (Sigma) was used as the reference drug (0.5 mg/ear). Groups of mice treated with CExt, Dec, and Inf without TPA were used. After 4 h, the animals were killed. Disks of 6 mm diameter were removed from each ear and their weights determined. The swelling was measured as the difference in weight between the punches from right and left ears, and expressed as an increase in the ear thickness.[19]

TNF-α, IL-6, and IL-10

Blood samples from animals were taken from their retro-ocular vein and serum was obtained as follows: blood samples were incubated at 37°C for 10 min and then centrifuged at 800 *g* for 10 min. Interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) were determined in the supernatant of macrophages cultured. IL-10, IL-6, and TNF-α were measured in serum. The cytokines were determined using commercial kits (mouse IL-6 ELISA kit; Pierce Biotechnology, Rockford, IL, USA); mouse TNF-α ELISA kit (Pierce Biotechnology), and IL-10 TiterZyme® EIA Kit (Assay Designs, Inc., Ann Arbor, MI, USA) in accordance with the manufacturer's recommendations.

Statistical analysis

Data are the average of duplicate samples performed by triplicate. The data were recorded as mean ± SEM. One-way analysis of variance was performed, followed by comparisons with Dunnett test. A $P \leq 0.05$ was considered statistically significant.[20]

Results

Cell viability

Figure 1 shows that the extracts did not significantly affect the macrophage viability after 24 h of incubation in used concentrations.

Oxidative burst assay

Figure 2 shows that only CExt at 2 and 4 mg/mL significantly increased the NBT reduction with respect to the control in the presence or absence of zymosan ($P \leq 0.05$). Inf and Dec did not produce a modification in the levels of NBT reduction with respect to the control in the concentrations tested.

Carrageenan-induced inflammation

The results in Figure 3 showed that CExt presented a significant anti-inflammatory activity with respect to the control. The % inflammatory inhibition increased to 72.22% when it was measured at 18 h ($P \leq 0.05$). Inf and Dec showed a significant pro-inflammatory activity ($P \leq 0.05$) with respect to the control. Dec presented the greatest pro-inflammatory activity (80%) ($P \leq 0.05$). CExt, Dec, and Inf without γ-carrageenan did not show differences with respect to the control.

Ear edema in mice

The results obtained in Figure 3 were confirmed by other methods, as shown in Table 1. Briefly, CExt possessed a significant anti-inflammatory activity ($P \leq 0.05$) with respect to control + TPA, while Inf and Dec showed pro-inflammatory activity ($P \leq 0.05$). Besides, Dec showed a higher pro-inflammatory activity than Inf ($P \leq 0.05$). CExt, Dec, and Inf without TPA did not show significant differences with respect to the control.

TNF-α, IL-6, and IL-10

Then, the effect of the extracts on TNF-α and IL-6 production in serum and in macrophages of mice

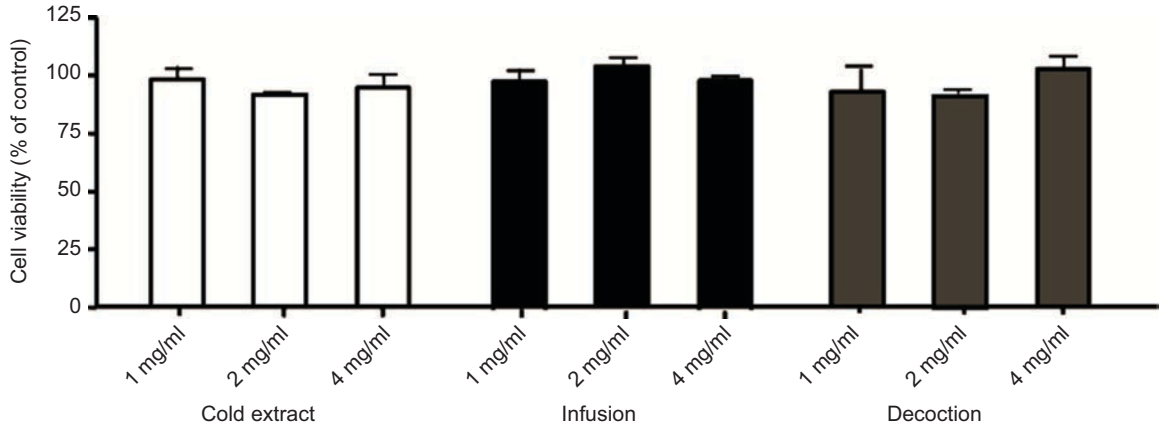

Figure 1. Effect of extracts (Dec, Inf, CExt) on the viability of macrophages (M). Cell viability was determined after an incubation time of 24 h, by MTT assay. Results were expressed as cell viability (% of control) and represented the mean ± SEM of three determinations performed by triplicate.

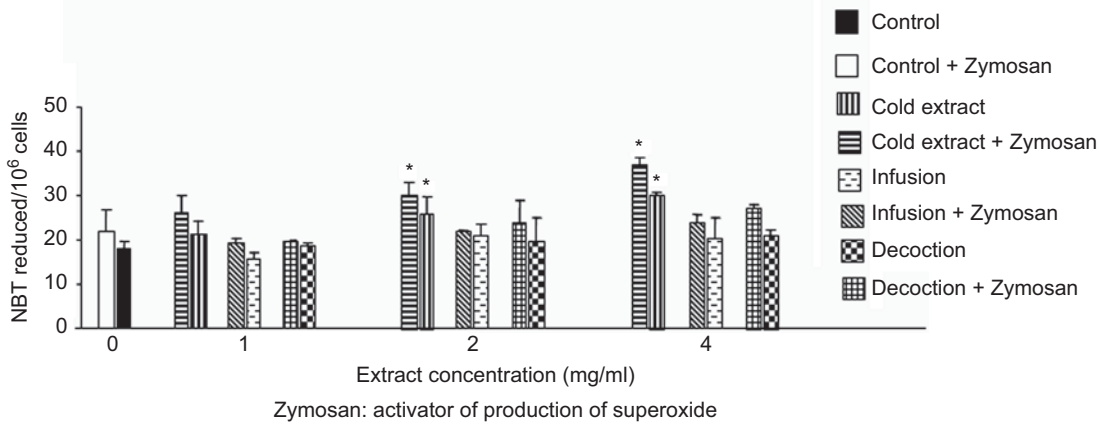

Figure 2. Effect of *Schinus areira* extracts on superoxide production in peritoneal macrophages. The cells were treated with medium, cold extract, infusion, or decoction for 90 min, and then were incubated with zymosan (1 mg/mL) and NBT for 60 min. The production of superoxide anion was assessed by the reduction of NBT to the insoluble formazan, measuring the absorbance at 525 nm. Results are means of three separate experiments. *$P \leq 0.05$ with respect to the control.

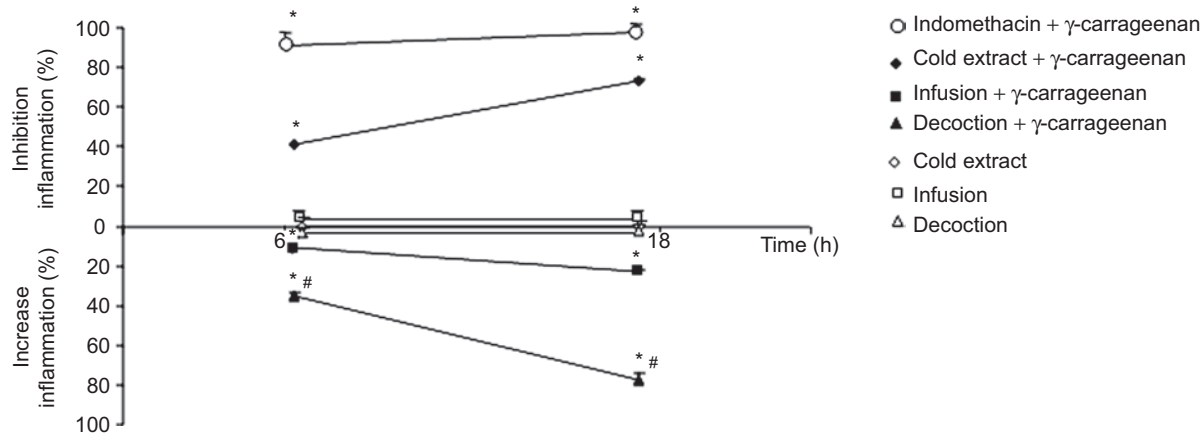

Figure 3. Effect of *Schinus areira* extracts on inflammation in mice. After treatment, inflammation was induced in groups of 10 mice with carrageenan. Inflammation was determined measuring the foot-pad swelling. Results are means of three separate experiments. *$P \leq 0.05$ with respect to control. #$P \leq 0.05$ respect to I.

Table 1. The topical anti-inflammatory activities of *S. areira* extracts were determined in TPA-induced mouse ear edema model.

Treatment	Ear weight (mg)	Increase (%)	Inhibition (%)
Control	0.77 ± 0.3	–	–
Control+TPA	19.1 ± 0.56	100 ± 3	0
Cold extract (4 mg/ear)+TPA	5 ± 0.5*		73.82 ± 4.2*
Cold extract (4 mg/ear)	0.78 ± 0.5	–	–
Infusion (4 mg/ear)+TPA	23.1 ± 3.1*	120.4 ± 1.5*	
Infusion (4 mg/ear)	0.77 ± 0.4	–	–
Decoction (4 mg/ear)+TPA	28.3 ± 2.8*#	146.5 ± 4*#	
Decoction (4 mg/ear)	0.78 ± 0.3	–	–
Indomethacin (1 mg/ear)	0.8 ± 0.23*		95.8 ± 2.2*

Results are means of three separate experiments. TPA, 12-*O*-tetradecanoylphorbol-13-acetate.
*$P < 0.05$ vs. control with TPA.
#$P < 0.05$ vs. I.

was assayed. It has been shown in Figure 4A and 4B that CExt significantly decreased the TNF-α ($P \leq 0.05$) and IL-6 ($P \leq 0.05$) levels in serum, while Inf and Dec significantly increased the levels of both the cytokines ($P \leq 0.05$). Also, in the supernatant of macrophages (M), CExt decreased the levels of TNF-α and IL-6 ($P \leq 0.05$) (Figure 5A and 5B), but Inf and Dec significantly increased them ($P \leq 0.05$). On the other hand, as CExt decreased the levels of the cytokines normally involved in inflammation response, it was thought that CExt could affect the cytokines normally implicated in anti-inflammatory effects. Therefore, the level of IL-10, a cytokine with a well-documented anti-inflammatory action, was measured in the serum extracts treated animals. It can be seen that while CExt significantly increased the IL-10 level in serum ($P \leq 0.01$), Inf and Dec did not modify it (Figure 4C).

Discussion

It is demonstrated in this study that CExt, Inf, and Dec obtained from *S. areira* did not show cell toxicity (Figure 1). On the other hand, CExt possesses an important anti-inflammatory activity (Figures 3–5 and Table 1). The mechanism by which the extract exerted the anti-inflammatory effect observed is not clear now, but we are realizing studies with different inhibitors to find the exact pathway involved in this action. However, there are several works that show the anti-inflammatory mechanism of some extracts and compounds from different plants. For example, crude ethanol extracts of *Kaempferia parviflora* possess anti-inflammatory activity mainly due to the inhibition of inducible nitric oxide synthase (iNOS) mRNA expression but partly through that of COX-2 mRNA in macrophage cells.[21] The ethanol extract of *Evodia rutaecarpa* and four bioactive components exhibited anti-inflammatory activities that could be partially explained by their different potentials for inhibiting NADPH oxidase–dependent reactive oxygen species and/or iNOS-dependent nitric oxide production in activated inflammatory cells.[22] A gum resin of *Boswellia serrata* showed that boswellic acids are specific non-redox inhibitors of leukotriene synthesis either interacting directly with 5-lipoxygenase or blocking its translocation.[23]

Although CExt did not have the same anti-inflammatory activity as that of indomethacin (anti-inflammatory reference drug), it exerted a significantly higher decrease in the percentage of inflammation induced in the control. This at last revealed that the extract could be an interesting source of new natural anti-inflammatory agents. On the other hand, Dec and Inf showed pro-inflammatory activity (Figures 3–5 and Table 1). In this sense, it is widely known

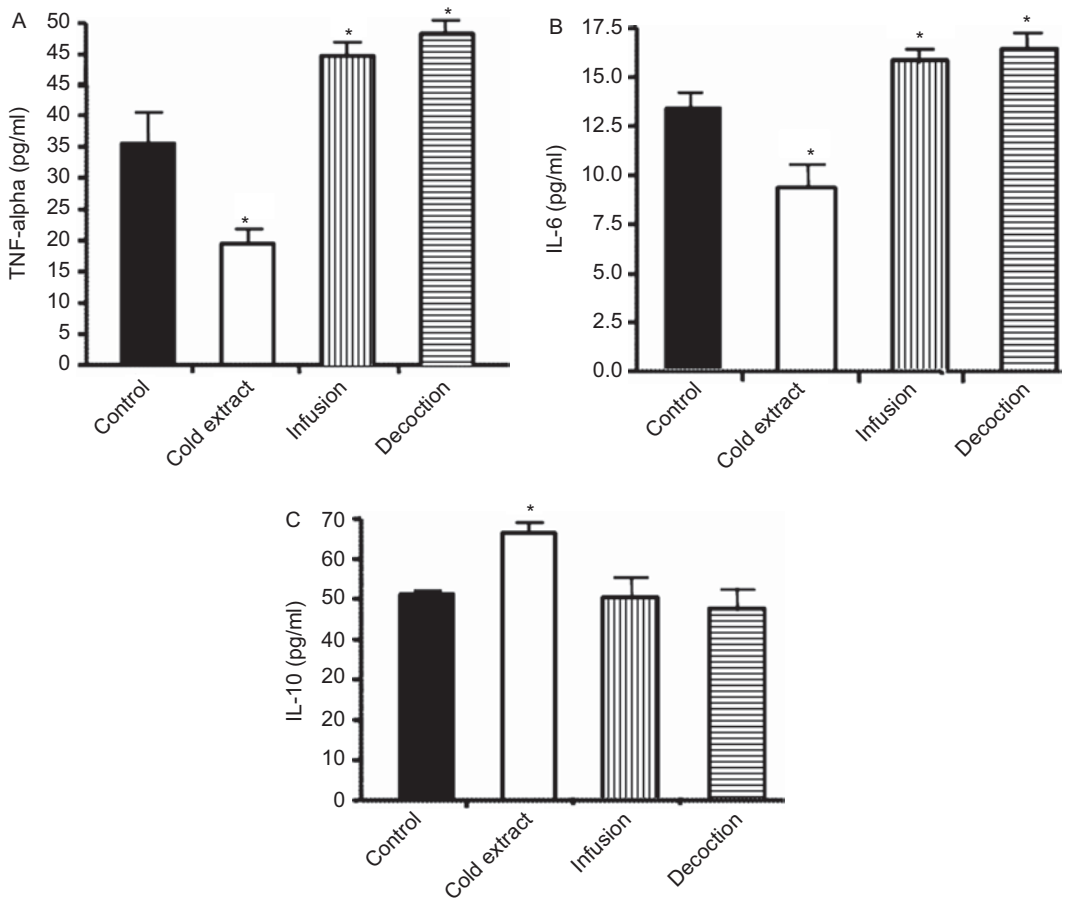

Figure 4. Effect of CExt, Inf, and Dec on the production of and (A) TNF-α, (B) IL-6, and (C) IL-10 in serum. Cytokines were determined in serum from treated animals. Basal values represent cytokines of untreated animals. Results represent the mean ± SEM of three experiments made by triplicate. *$P \leq 0.05$ represents the significant difference between basal and treated in accordance with Student's *t*-test.

that the mechanisms involved in inflammation can fail. Some defects may include leukocyte alterations by alcoholic or drug intoxication, diabetes, cancer; impair chemotaxis; delay in wound closure; and defective granulation.[24] Therefore, we may suggest the potential use of Dec or Inf as being pro-inflammatory in treating diseases in which inflammatory mechanisms are altered.

It is important to note that as the extracts were prepared using different methods, the compounds extracted in each extract could be different or are the same compounds but extracted in different quantities. It is known that different methods are used for the isolation of different compounds.[25] Furthermore, the extracts obtained by different methods do not have the same effects.[26] These observations show the importance of utilizing the correct methodology to prepare the extracts according to the effect that we wished to obtain. We did not find any previous studies about the correlation between the methodology employed for extracts preparation from *S. areira* and the effects exerted.

On the other hand, CExt increased the production of superoxide anion in mice macrophages at 2 and 4 mg/mL (Figure 2). This could suggest that CExt induces the respiratory burst and stimulates macrophages. Therefore, CExt could be used as an anti-inflammatory extract with stimulatory effect on the innate immune system. This fact suggests that CExt could exert a dual effect, not only by decreasing inflammation but also by exerting an immunomodulatory effect on the innate immunity.

According to the results obtained, not only must the nature of extract preparation be taken into account but also the mode of administration. In this work, the topical use of the extract has been proposed because the model used to induce inflammation allows the topical administration of drugs. In this sense, the concentration of the extracts at the site of action is greater than that in the plasma; therefore, we can consider that the major anti-inflammatory action was evaluated at the local level.

In conclusion, we propose the use of several methods to obtain extracts of *S. areira* L. with different effects. CExt could be used as an anti-inflammatory and macrophage enhancer agent. Inf and Dec can be used as pro-inflammatory extracts. The next step is to evaluate

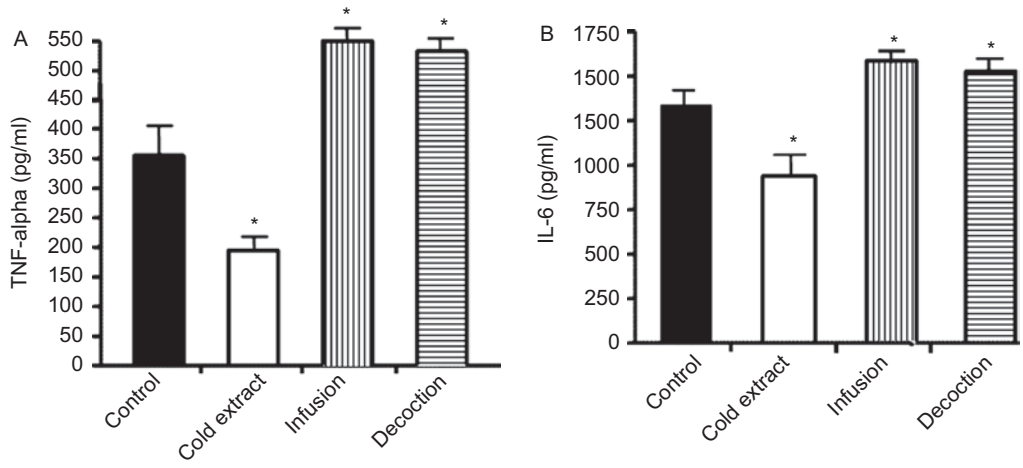

Figure 5. Effect of CExt, Inf, and Dec on the production of (A) TNF-α and (B) IL-6 in supernatants of treated and untreated macrophages. Results represent the mean ± SEM of three experiments made by triplicate. $*P \leq 0.05$ represents the significant difference between basal and treated in accordance with Student's *t*-test.

the toxicity of the extracts and identify the compounds related to these effects in order to progress in a future possible application of *S. areira* to treat several disorders in humans.

Declaration of interest

This work was supported by funds provided by Project 9601 UNSL. Roberto Davicino is a postdoctoral fellow of CONICET. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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