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Full Length Research Paper

Anti-inflammatory activity of *Salvia officinalis* L.

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This study evaluate the effects of sage hydroalcoholic extract (SE) and sage essential oil (SO) on the inflammatory response using an experimental model of acute inflammation and a leukocyte migration assay. In the carrageenan-induced pleurisy test, SE did not reduce the exudate volume and leukocyte migration to the pleura, but SE exerted a topical anti-inflammatory effect by significantly inhibiting croton oil-induced ear edema. All SO doses tested significantly inhibited leukocyte chemotaxis induced by casein and reduced the number of rolling, adhesion, and leukocytes migration to spermatic fascia after inflammatory stimulus. Our data demonstrated that SO has anti-inflammatory activity.

Key words: *Salvia officinalis*, essential oil, anti-inflammatory, medicinal plants.

INTRODUCTION

Salvia officinalis L. is a spice, popularly known as sage that belongs to the Lamiaceae family (Cunha et al., 2003). In folk medicine, the leaves are mainly used, but the flowers and stems have also been used in infusions and alcoholic extracts for various therapeutic purposes. Its use is indicated in the treatment of dyspepsia, pharyngitis, oropharyngeal infection, stomatitis, gingivitis (Velickovic et al., 2003), glossitis, and dysmenorrhea. In addition to these medicinal purposes, sage is also commonly used as a condiment in food preparations, especially in Mediterranean cuisine (Cunha et al., 2003). The major constituents identified in sage are terpenoids, the most commonly found of which are α- and β-thujone, camphor, 1,8-cineole, humulene, borneol acetate, limonene, viridiflorol, caryophyllene manool, muruleno, and ferruginol, each with established biological activity (Velickovic et al., 2003). Although studies have reported the biological activity of *Salvia officinalis* or its individual components, few data have demonstrated the anti-inflammatory activity of this spice (Shipochliev et al., 1981; Leal-Cardoso and Fonteles, 1999; Santos and Rao, 2000; Peana et al., 2002; Hamburger et al., 2003).

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EXPERIMENTAL DESIGN

Plant material

The fresh aerial parts of *Salvia officinalis L.* were collected from the Prof[a] Irenice Silva Medicinal Plant Garden on the campus of the State University of Maringá, Paraná, Brazil, identified, and authenticated. Voucher specimen was deposited in the Herbarium of the Department of Botany, State University of Maringá (no. 10.592).

Preparation of the crude hydroalcoholic extract and essential oil of Salvia officinalis

Salvia hydroalcoholic extract was extracted from the dry aerial parts of *Salvia officinalis* (120 g) that were macerated with ethanol and water at a ratio of 9:1 for 30 days. After vacuum filtration, the extract was concentrated in a rotary evaporator (R114, Schutt Labrtechnike) to eliminate the ethanol and then lyophilized (LDC2M, Christ). Final weight of the extract was 5.8 g.

Sage essential oil was extracted by conventional steam distillation using a Clevenger-type apparatus for 2 h. The essential oil was kept at 4°C in dark vials and then used in the tests. The essential oil of *Salvia officinalis* L. yielded 0.4% v/w.

Animals

Male Wistar rats weighing 200 to 240 g, and male Swiss mice weighing 25 to 30 g, were provided by the Central Animal House of the State University of Maringa. The animals were housed at 22 ± 2°C under a 12 h/12 h light/dark cycle. Prior to the experiments, the animals were fasted overnight with water provided *ad libitum*. The experimental protocols were approved by the Ethical Committee in Animal Experimentation of the State University of Maringa (CEAE/UEM 066/2010).

Anti-inflammatory activity of SE and SO

Carrageenan-induced pleurisy in rats

The test was performed according to Vinegar et al. (1973). The groups of rats were pretreated with SE (200, 400, or 800 mg/kg, p.o.) or indomethacin (5 mg/kg, p.o.) as the standard drug or water (0.1 ml, p.o.) as the control. Thirty minutes later, all of the animals received an intrapleural injection of carrageenan (200 μg per animal). Four hours later, the pleural exudate was collected, the volume was determined, and the pleural cavity was washed with 1.0 ml of saline that contained heparin (10 IU/ml). The number of migrating leukocytes in the exudate was determined with a Neubauer chamber. The results are expressed as the mean ± SEM pleural exudate volume and number of migrated cells.

Topical ear inflammatory edema

Cutaneous inflammation was induced by the application of 5% croton oil (10 µl) solubilized in acetone (vehicle) in the inner surface of the right ear in mice. The left ear received an equal volume of vehicle. Sage hydroalcoholic extract (0.315, 0.625, 1.25, 2.5, or 5 mg/ear), dexamethasone (0.1 mg/ear), or vehicle was applied topically to the right ear 1 h before croton oil application. Four hours after the inflammatory stimulus, 7 mm diameter plugs were removed from the treated and untreated ears (n = 10). The edematous response was measured as the weight difference between the two plugs. Ear edema is expressed as the increase in ear thickness caused by inflammatory challenge, measured using an electronic micrometer (Digimess) before and after the induction of the inflammatory response. The micrometer was applied near to the tip of the ear just distal to the cartilaginous ridges, and the thickness was determined in micrometers. To minimize variations in this technique, a single investigator was used for measurement throughout the experiments. The data are expressed as mean ± SEM.

Myeloperoxidase activity

Myeloperoxidase (MPO) activity was assayed in the supernatant of homogenates of the ear sections from untreated controls and animals treated with SE (0.315, 0.625, 1.25, 2.5, or 5 mg/ear) or 0.1 mg dexamethasone (Bradley et al., 1982). The ears were placed in 50 mm potassium phosphate buffer (pH 6.0) that contained 0.5% hexadecyl trimethyl ammonium bromide (Sigma, St. Louis, MO, USA; 1 ml/50 mg tissue) in a Potter homogenizer. The homogenate was shaken in a vortex-mixer and centrifuged for 5 min. Ten microliters of the supernatant were added to each well of a 96-well microplate in triplicate. Two hundred microliters of a buffer solution that contained 16.7 mg o-dianisidine dihydrochloride (Sigma), 90 ml double-distilled water, 10 ml potassium phosphate buffer, and 50 µl of 1% H_2O_2 were added. The enzyme reaction was stopped by the addition of ethyl acetate. Enzyme activity was determined by measuring absorbance at 460 nm in an enzyme-linked immunosorbent assay reader.

In vivo leukocyte migration assessed by intravital microscopy

The rolling and adhesion of leukocytes to the endothelium were evaluated in the internal spermatic fascia of rats 2 h after carrageenan injection (100 μg) in the wall of the scrotal chamber. Sage essential oil (5, 10, or 25 mg/kg), indomethacin (5 mg/kg), or saline (0.9%) was administered orally 30 min before the carrageenan injection to different groups of rats. The animals were anesthetized with chloral hydrate (600 mg/kg, s.c.) and maintained on a special board thermostatically controlled at 37ºC with a transparent platform for transillumination of the tissue on which the spermatic fascia was exposed and fixed for microscopic analysis *in situ*. The preparation was kept moist and warm with Ringer Locke's solution (pH 7.2-7.4) that contained 1% gelatin. The vessels selected for the study were postcapillary venules with a diameter of 15-25 μm. The number of rolling and adherent leukocytes was determined at 10 min intervals. The leukocytes were considered to adhere to the venular endothelium if they remained stationary for more than 30 s. In another series of experiments, the number of leukocytes that migrated to an area of 2,500 μm^2 of connective tissue adjacent to postcapillary venules 4 h after carrageenan injection was determined. The number of cells was determined from an image recorded using four different fields for each animal, and the average value was calculated.

In vitro chemotaxis assay

To evaluate the effects SO on chemotaxis, neutrophils were obtained from the peritoneal cavity of rats 4 h after an injection of 200 µl of a carrageenan solution (1 mg/cavity, i.p). The cell number was adjusted to 1×10^6 cells/ml in Hank's Buffered Salt Solution (HBSS). The chemotaxis assay was performed using a modified Boyden's chamber with an 8-µm-pore cellulose nitrate filter (150 µm thickness, Nucleopore, Pleasanton, CA, USA). The chemoattractant casein (50 mg/ml) and negative control (HBSS) were placed in the lower chamber. A purified neutrophil suspension (1×10^6 cells/ml) that was pretreated for 30 min with SO (10^{-4}, 10^{-3}, or 10^{-2} µl/ml) was then placed in the upper chamber. The plate was incubated at 37°C with 5% CO_2 for 1 h. Subsequently, the filter was removed, fixed, and stained with Harris's hematoxylin. Leukocytes that migrated through the membrane were counted under a light microscope on at least five randomly selected fields. The results are expressed as the mean number of neutrophils per field and represent triplicate measurements from three separate experiments.

Statistical analysis

The data are expressed as the mean ± SEM for each group. The results were statistically analyzed using one-way variance analysis (ANOVA) followed by Tukey's *post hoc* test. Differences were considered significant when $p < 0.05$.

RESULTS AND DISCUSSION

Plants represent one of the most important sources of substances with biological activity that has applications in ethno-medicine (Pavan-Fruehalf, 2000). Many studies have evaluated natural products as possible anti-inflammatory agents. However, few studies have focused

Table 1. Exudate volume and leukocytes number after 4 h carrageenan injection (200 μg per pleural cavity) in the pleurisy test.

Groups	Exudate volume (ml)	Leukocytes (cells/mm^3) × 10^3
Control	0.90 ± 0.06	58.85 ± 4.37
Indomethacin 5 mg/kg	0.43 ± 0.02*	27.56 ± 2.65*
SE 200 mg/kg	0.82 ± 0.09	53.0 ± 4.72
SE 400 mg/kg	0.80 ± 0.08	59.62 ± 2.10
SE 800 mg/kg	0.82 ± 0.06	56.87 ± 4.76

Data are mean ± S.E.M. n = 8-10 rats per group. *p < 0.05 compared to the control group (ANOVA, Tukey's test).

on this property of *Salvia officinalis*. In the present study, we demonstrated the effect of SE in experimental models of acute inflammation (that is, carrageenan-induced pleurisy and croton oil-induced ear edema) and the effect of SO on leukocyte migration using an *in vitro* chemotaxis assay and intravital microscopy.

The acute inflammatory process is one of the most important protection mechanisms against invading pathogens and other stimuli. It is typically characterized by redness, swelling, pain, and heat. The activation of leukocyte cells (Aderem and Ulevitch, 2000) induces the secretion of many pro-inflammatory mediators, such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), eicosanoids, nitric oxide (NO; (Nantel et al., 1999) and prostaglandins (PGs). In an uncontrolled response, leukocyte migration could be a main aggravating factor because this cell type also releases proteolytic enzymes and reactive metabolites of oxygen and nitrogen, which could be toxic and cause tissue damage (Ramos et al., 2003). Inhibition of the synthesis or release of these mediators with steroids and nonsteroidal anti-inflammatory drugs has been shown to be beneficial in the treatment of inflammatory disorders (Bogdan, 2001). However, these agents lead to adverse effects, such as gastrointestinal discomfort, inhibition of platelet aggregation, liver and kidney toxicity (Batlouni, 2010). Therefore, the search for natural products with fewer side effects has been increasingly important. Under such conditions, pharmacological interventions with drugs that are able to control the inflammatory response may present an interesting therapeutic possibility.

Carrageenan induces the development of inflammatory edema characterized by an initial stage (1 to 2 h) that is dependent on the release of histamine, serotonin, and bradykinin, followed by a later stage (3 to 4 h) that is maintained principally by the release of kinins, lysozymes, and prostanoids (Niemegeers et al., 1964; Crunkhorn and Meacock, 1971). Eicosanoids promote the chemotaxis of neutrophils and induce the biosynthesis of elastase, collagenase, and other compounds. As a consequence, an increase in vascular permeability and hydrostatic pressure occurs, resulting in edema and the migration of neutrophils to the damaged tissue (Havsteen, 2002).

The pleurisy test is used to screen for anti-inflammatory drugs, and the accumulation of exudate in the pleural cavity and leukocyte migration can be evaluated (Vinegar et al., 1973). In the pleurisy test, 200, 400, and 800 mg/kg SE did not reduce inflammatory exudate or leukocyte migration (Table 1). The groups treated with indomethacin exhibited only a decrease in inflammatory edema.

To demonstrate the topical effect of SE *in vivo*, we evaluated ear inflammatory edema induced by croton oil. Croton oil is an irritant agent that causes cell damage and activates phospholipase A_2, which releases arachidonic acid, a precursor of prostaglandins and leukotrienes. These inflammatory mediators are involved in edema formation and leukocyte migration. After 4 h, croton oil application to the right ears of mice induced an evident inflammatory response characterized by the formation of tissue edema and increased MPO activity in the injured tissue. As expected, dexamethasone treatment significantly reduced ear edema by 39.59% (Figure 1A). Sage hydroalcoholic extract at doses of 0.625, 1.25, 2.5, and 5 mg/ear had a topical anti-inflammatory effect, significantly inhibiting edema by 28.19% 33.09, 33.57 and 34.06%, respectively. These data indicate that SE has an anti-edematogenic effect when administered topically, similar to dexamethasone.

The enzyme MPO is found in the azurophilic granules of neutrophils and other cells of myeloid origin. It is considered a marker of polymorphonuclear leukocyte influx into inflamed tissue. In addition to fluid extravasation inhibition, a reduction in MPO activity was observed, indicating the indirect inhibition of cellular influx. Therefore, MPO inhibition may result in an anti-inflammatory effect. Sage hydroalcoholic extract at doses of 0.625, 1.25, 2.5, and 5 mg/ear significantly inhibited MPO activity by 35.79, 54.68, 51.20 and 45.96%, respectively. Dexamethasone (0.1 mg/ear) inhibited ear edema by 67.05% (Figure 1B). Our data showed that SEat the doses tested did not inhibit inflammatory edema or leukocyte migration in the pleura. Instead, it exerted anti-edematogenic and anti-migration effects *in vivo* when topically administered. Based on our data and reports of the anti-inflammatory activity of Lamiaceae species, the effect of SE on leukocyte migration was evaluated.

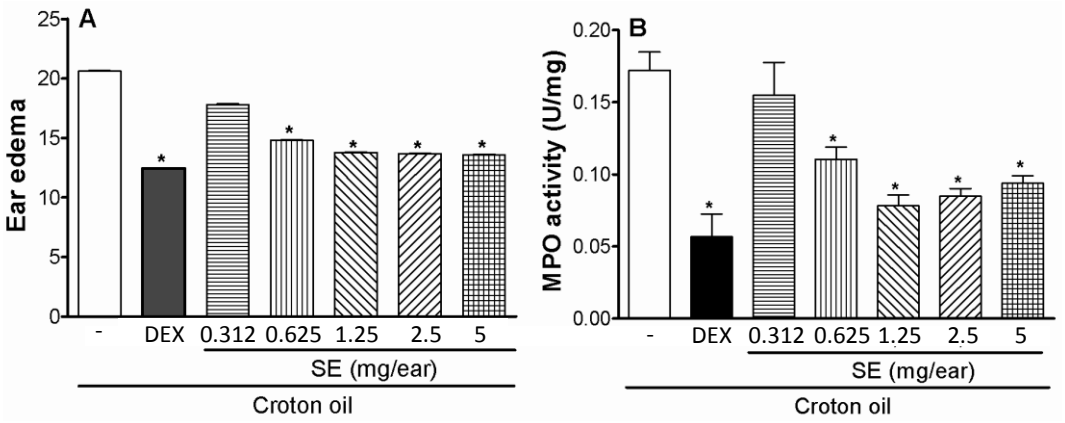

Figure 1. SE inhibited ear edema and myeloperoxidase activity (MPO) by croton oil in mice. The animals (n = 9) were treated topically with SE or dexamethasone (Dex) 1 h before croton oil application (10 µg/ear). Dex (0.1 mg/ear) was used as anti-inflammatory drugs (positive control). The right ears received only the vehicle (basal). Data are mean ± S.E.M. weight of the ears (**A**) or MPO activity (**B**), 4 hours after application of croton oil, $^{\#}p < 0.05$, compared to the control group (croton oil) (ANOVA, Tukey's test).

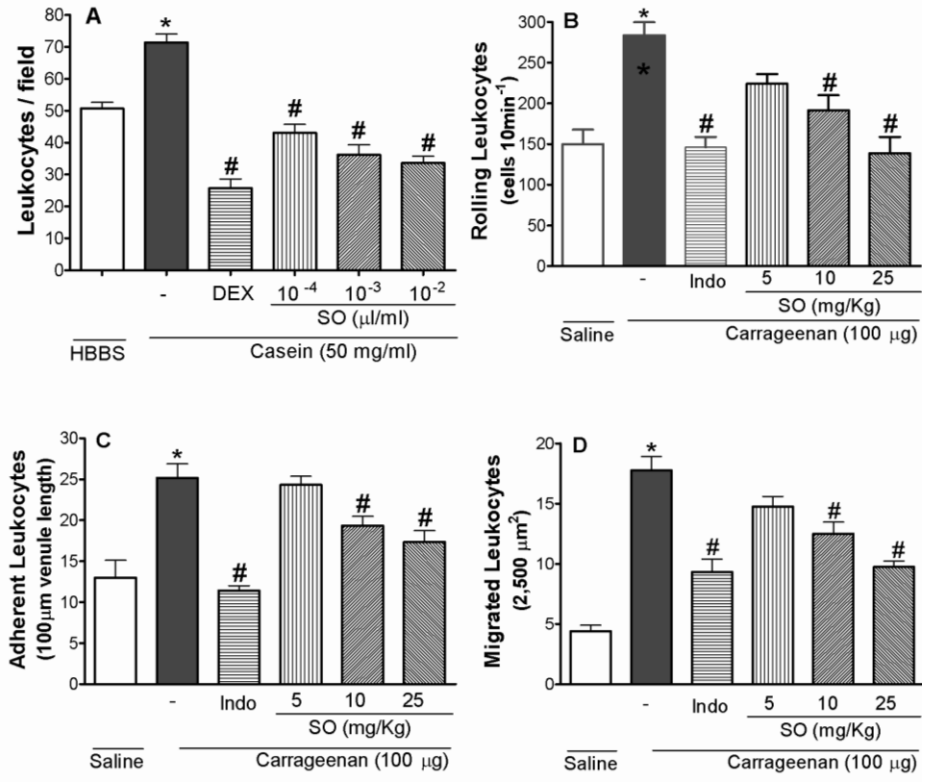

Figure 2. SO inhibited *in vitro* chemotaxis and *in vivo* leukocytes migration**.** (A) Chemotaxis of leukocytes obtained from carrageenan-induced peritonitis (200 µg/cavity) and stimulated with casein (50 mg/ml) after 30 min of SO (10^{-4}, 10^{-3} or 10^{-2} µl/ml) or dexamethasone (DEX, 10^{-5} M) administration. Values are means ± S.E.M (n = 5) and are representative of three independent experiments. *P < 0.05 versus vehicle, #P < 0.05 versus group of leukocytes stimulated with casein. (B to D) SO pre-treatment (5, 10 or 25 mg/kg, orally) or indomethacin (IND, 5 mg/kg) 30 min before carrageenan injection (100 µg/scrotal). Number of rolling (B), adherent leukocytes (C) during 10 min periods after 2 h and leukocytes migrated (D) after 4 h of inflammatory stimulus. Values are means ± S.E.M of 5 to 7 animals for each group. *P < 0.05 versus vehicle, #P < 0.05 versus carrageenan-injected group.

To investigate the effect of SO on leukocyte migration, an *in vitro* chemotaxis assay was performed. Different concentration of SO (10^{-4}, 10^{-3}, and 10^{-2} µl/ml) were tested and leukocyte viability was not affected. Incubation with 10^{-4}, 10^{-3}, and 10^{-2} µl/ml SO significantly reduced neutrophil migration toward the casein stimulus by 39.44 ± 1.02, 49.06 ± 1.79 and 52.67 ± 2.06%, respectively (Figure 2A). These results are consistent with other *in vivo* studies that used an intravital microscopy technique. Indeed, SE inhibited carrageenan-induced leukocyte migration *in vivo*. We observed a marked increase in the number of rolling and adherent cells after carrageenan injection into the scrotum (Figure 2B and C) compared with saline-pretreated rats. The reduction of the number of migrated leukocytes induced by 10 and 25 mg/kg SO may reflect the decreased number of rolling and adherent cells (Figure 2B to D).

Our data are similar to previous studies, in which the anti-inflammatory activity of plant extracts and essential oils inhibited inflammatory edema and/or chemotaxis (Siani et al., 1999; Vendruscolo et al., 2006; Takaki et al., 2008; Melo et al., 2011; Nogueira de Melo et al., 2011). Terpene compounds found in herbs of the Lamiaceae family exhibit anti-inflammatory activity (Alcaraz and Jiménez, 1988; Amabeoku et al., 2001; Bispo et al., 2001; Maleki et al., 2001). Moreover, the effects of natural products on leukocyte-endothelial interactions have been reported using an intravital microscopy technique (Smith, 1993; Nogueira de Melo et al., 2011). Thus, the present study showed that SO exerts a significant inhibitory effect on the number of migrated cells to perivascular tissue. The inhibition of leukocyte migration could be partially related to a reduction of the expression of adhesion molecules, prostanoids synthesis, cytokine release and other mediators.

The SO constituents were monoterpene ketones, characterized as α-thujone (90%) and β-thujone (6%). Thujone reduces the production of proinflammatory cytokines, such as TNF-α, IL-1β, IL-6, granulocyte-monocyte colony-stimulating factor (Siveen and Kuttan, 2011) and the anti-inflammatory activity of this oil could be related to its constituents.

Thus, as already described in the literature, *Salvia officinalis* L. has anti-inflammatory effects that are partially attributable to its essential oil and constituents. The present study contributes to the growing number of studies of the anti-inflammatory effects of medicinal plants. Overall, our data support the hypothesis that the inhibitory effect of SO on leukocyte migration contributes to its anti-inflammatory action.

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