Journal of Applied Pharmaceutical Science

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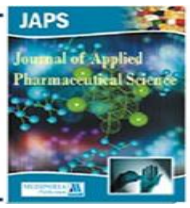

ISSN: 2231-3354
Received on: 05-09-2011
Revised on: 12-09-2011
Accepted on: 15-09-2011

Anti-inflammatory activity of fractionated extracts of *Salvia officinalis*

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ABSTRACT

This work is focused on the evaluation of the anti anti-inflammatory activity of fractionated extracts of *Salvia officinalis* in experimental model of peritonitis. The extract of *Salvia officinalis* was produced by maceration with ethanol and fractionated by increasing the polarity of the solvents from hexane to methanol in a thin layer chromatographic column with Silica Gel 60 as stationary phase. The acute induced-peritonitis assay was used as model of inflammation, were the fractionated extracts of *S. officinalis* were administered subcutaneously. After 4h of induction of inflammation the number of total circulating leukocytes was not increased in animals that received the methanol extract. After the induction of inflammation, all animals except those treated with the fraction of ethyl acetate and methanol showed an increased number of circulating neutrophils. The results obtained suggesting the occurrence of inhibition of the total leukocytes recruitment in the circulating blood after the induced-inflammatory process by ethyl acetate and methanolic extracts of *S. officinalis* in a single dose of 25 $\mu g.kg^{-1}$. It was concluded that the extracts of *S. officinalis* could be used as anti-inflammatory agent.

***Key words*:** *Salvia officinalis*, anti-inflammatory, fractionated extracts, peritonitis.

INTRODUCTION

Salvia is a genus belonging to the family Lamiaceae presenting approximately 900 species. Amongst these species, *Salvia officinalis* has been extensively used as a medicinal plant in treating many diseases (Cuvelier et al., 1994; Baricevic and Bartol, 2000; Zupko et al., 2001; Capasso et al., 2004; Ren et al., 2004; Kamatou et al., 2008). *Salvia officinalis* is from Europe, however, nowadays, is widespread throughout the world (Schhultz et al., 1998). *Salvia* species are reported to have anti-inflammatory properties and their local use as medicinal herbs includes the treatment of body wounds. The *in vitro* anti-inflammatory activity of essential oils and solvent extracts of *Salvia* species was evaluated using the 5-lipoxygenase assay (Kamatou et al., 2005; Kamatou et al., 2006a). Essential oils exhibited better anti-inflammatory activity when compared to the solvent extracts. With the exception of *Salvia radula*, solvent extracts displayed poor ability to inhibit the enzyme. However, it was interesting to note that in contrast to the poor activity recorded for the essential oil of *Salvia radula*, the solvent extract was fractionally more potent (Kamatou et al., 2006a).

Baylac and Racine (2003) found that the essential oil of *Salvia stenophylla* has the potential to inhibit the *in vitro* 5-lipoxygenase enzyme. Kamatou et al. (2006a) also found that *Salvia stenophylla* displayed anti-inflammatory activity. Some differences in the activity were found between the two studies, although the same method was used. This could be attributed to variation in chemical composition of the two essential oils.

Peana et al. (2002) demonstrated that linalool and its ester linalyl acetate exhibited anti-inflammatory activity. These two compounds were found in *Salvia dolomitica* (16.6 and 19.6%, respectively) (Kamatou et al., 2006b) and may have partly contributed to the anti-inflammatory activity.

It was recently demonstrated by Baricevic et al. (2001) that the leaves of *Salvia officinalis* obtained from four plant populations of different origins were investigated for their topical anti-inflammatory properties. The n-hexane and the chloroform extracts dose-dependently inhibited the Croton oil-induced ear edema in mice, the chloroform extracts being the most active. By contrast, the methanol extracts showed a very low effect and the essential oil was inactive. Ursolic acid was found to be responsible of the anti-inflammatory activity after chemical and pharmacological investigation.

A relevant aspect to be taken into account when dealing with medicinal plants is the chemical composition variation due to the geographic localization, harvest time, climate conditions, cultivation handling, age of vegetable material, period and storing conditions, among others (Mossi et al. 2009). For example, significant differences in chemical composition was verified in extracts of *maté* plants with different age of leaves, age of plants, cultivated with diverse fertilization types and exposed to a variety of light intensity (Esmelindro et al., 2004). Also, Mossi et al. (2009) verified that the concentration of tannins were highest at populations of *Maytenus ilicifolia* located at the region with the highest average annual temperature.

In this sense, the main objective of this work was to evaluate the anti-inflammatory effects of *Salvia officinalis* leaves. The ethanolic extracts were concentrated until obtain a dry crude extract that was fractionated in a thin-layer chromatography column. The column was eluted by increasing the polarity grade of the solvents, conform: hexane, ether, ethyl acetate and methanol. The individual fractions were used to evaluate the anti-inflammatory activity on acute induced-peritonitis in rats.

MATERIAL AND METHODS

Plant materials and extraction

Leaves of *S. officinalis* were collected in Erechim in June 2009, dried at room temperature, crushed and stored under nitrogen atmosphere prior to the analysis. The sample containing 92 g was submitted to extraction with ethanol at room temperature. After this step the extracts were filtered using cotton filter and the solvent removed under vacuum at 30°C until obtain dry crude extract.

After the extraction, the dry crude extract was fractionated using a thin layer chromatographic column with Silica Gel 60 as stationary phase. The column was eluted using 300 mL of hexane, 600 mL of ethyl ether, 300 mL of ethyl acetate and 300 mL of methanol. The order of elution of solvents was determined in a manner that the polarity of the solvent was increasing from hexane to methanol. After the fractionation, the solvents were removed under vacuum at 30°C until obtain dry extract of the respective solvent.

Chemical analysis

The fractionated extracts were analyzed in a gas-chromatograph coupled with a mass selective detector (GC/MSD, Shimadzu QP5050A), using a capillary column DB5 (30m, 0.25mm, 25μm). Column temperature was programmed 60°C/2min, 8°C/min to 130°C, 3°C/min to 180°C/25min, 20°C/min to 300°C/20min. Helium was the carrier gas and the injection port and detector temperatures were 290°C and 300°C, respectively. The sample (1 μL of 40.000 $mg.L^{-1}$ in CH_2Cl_2) components were identified by matching their mass spectra with those of Wiley library database. Triplicate measurements were performed for each sample.

Animals

Thirty adult male Wistar rats (3 months old, 180-220g) were obtained from the Laboratory of Animal Experimentation from URI - Campus de Erechim, Brazil. The animals were kept in a room under controlled humidity (50 ± 5%) and temperature (22 ± 2 °C) and subjected to a 12h light cycle with free access to food and water. All the procedures used in the present study comply with the guidelines on animal care of the URI Ethics Committee on the use of Animals.

Anti-inflammatory evaluation

The anti-inflammatory activity of the fractionated extracts of *Salvia officinalis* was evaluated using 30 animals, where the control was composed of six animals and the treated divided in four groups of four animals each ones. All the animals were submitted to blood collection (orbital plexus) before the acute induced-peritonitis assay by the injection of 10 mL of oyster glycogen (1%) dissolved in of phosphate-buffered saline (PBS) 10 mM, 7.4 pH under light Zoletil 50® anesthesia (Oktar et al., 2004). For the treated group, it was administered a single dose of 25 μg/kg of the respective fraction of the extract through subcutaneous injection, while the control group received the vehicle only (PBS).

Four hours later, rats were re-anesthetized for blood collection (orbital plexus), and then sacrificed in CO_2 chamber, with cells in the peritoneum removed by washing with 30 mL of PBS containing 1000 $U.L^{-1}$ heparin. The peritoneal exudates at 4h contained >98% neutrophils. The suspension was centrifuged at 2000 rpm for 10 min and the erytrocytes were destroyed by lysis buffer containing 0.15 M NH_4Cl. Cells were re-suspended in ice-cold PBS, and the cell counting was performed using a light microscope. None of the treatments altered the cell viability, which was >95%. The assays were carried out in duplicate.

Statistical analysis

The values were expressed as the mean value ± standard error. One-way analysis of variance (ANOVA) followed by Tukey test was applied for the statistical evaluations of the results

obtained in the anti-inflammatory tests. Values of $p<0.05$ were regarded as significant.

RESULTS

Extraction yield and chemical characterization of the extracts

The extraction of the leaf of *S. officinalis* resulted in 6.2 g of crude dry extract that correspond to a yielding of 6.7 wt%. After the fractionation of the crude extracts with hexane, ether, ethyl acetate and methanol was obtained 0.01, 0.21, 0.16 and 0.117 g of extracts, respectively. The chemical analysis by GC/MS of the fractionated extracts showed significant alterations in the composition of the fractions. In the hexanic extracts were identified 10 compounds, amongst them the caryophyllene, caryophyllene oxide, phenanthrene and hydrocarbons. The etheric extracts presented 16 compounds, being characterized by volatile compounds present in essential oils such as camphor, borneol, bornyl acetate, 2-tridecenal, caryophyllene oxide, veridiflorol, humuladienone, palmitic acid and 2-hexadecen-1-ol. The main compounds found in the ethyl acetate extracts were 2-cyclopenten-1-one, 2-hexadecen-1-ol, and torulosol. In the methanolic extract was not possible identify and quantify the compounds due to low resolution of chromatography peaks.

Anti-inflammatory evaluation

The acute induced-inflammatory is a fast response to a harmful agent responsible to induce the defense mechanism of the body (leukocytes and antibody) to the lesion place. The defense mechanisms are present and move according to the bloodstream. In an inflammatory process the blood vessels suffer some alterations to facilitate the movement of the leukocytes and the antibody to the lesion place, whose rate depends on the level of inflammation. Leukocyte recruitment to the site of inflammation is a fundamental event in the inflammatory process. Acute peritonitis promoted by oyster glycogen greatly induces leukocyte recruitment, including neutrophils, to the peritoneal cavity (Castelucci et al., 2007).

The results obtained regarding the evaluation of the anti-inflammatory activity of the fractioned extracts of *S. officinalis* are presented at Fig. 1.

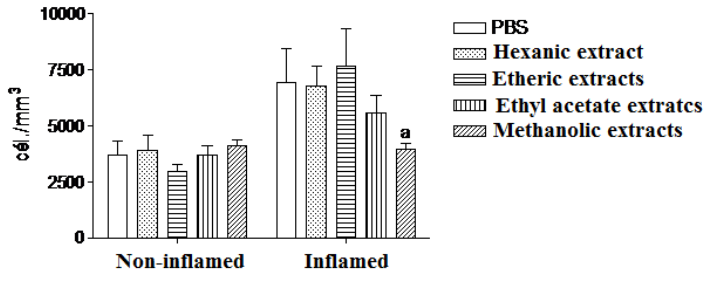

Fig. 1. Total leukocytes in the circulating blood for the inflamed and non-inflamed groups treated with PBS (control) and fractionated extracts of *S. officinalis*. [a]$p<0.05$ when compared with control.

Before the induction of the inflammation the animals presented a level of $4x10^3$ leukocytes.mm^{-3}. After 4 hours of induction the control group increasing the count of leukocytes, showing that the inflammation model using oster glycogen was efficient. For the treated group with the hexanic and etheric extracts *of S. officinalis* was verified to increase the number of leukocytes in the circulating blood, while that for the treated group with ethyl acetate and methanolic extracts the number of leukocytes in the circulating blood was very similar to that obtained for the animals before the induced-inflammatory process. This result suggests the occurrence of inhibition of the total leukocytes recruitment in the circulating blood after the induced-inflammatory process by ethyl acetate and methanolic extracts of *S. officinalis* in a single dose of 25 µg.kg^{-1}. This result was confirmed by the statistical analysis that indicated significant differences ($p<0.05$) among the treated group with methanolic extracts and other extracts fractions. Although there was not verified significant statistical differences ($p<0.05$) among the treated group with ethyl acetate extracts and other extracts fractions it is seen a reduction in the count of the total leukocytes in the circulating blood.

Fig. 2 presents the subtypes of circulating leukocytes (neutrophils, lymphocytes and monocytes) before and after the inflammation for the control and treated groups with fractionated extracts of *S. officinalis*. After the inflammation the animals of control group and that treated with hexanic, etheric and ethyl acetate extracts increased the number of neutrophils in comparison with the count before the inflammation ($p<0.05$).

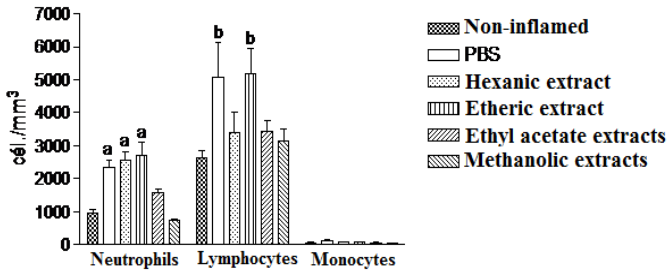

Fig. 2. Subtypes of circulating leukocytes (neutrophils, lymphocytes and monocytes) before and after the inflammatory induction for the control and treated groups with fractionated extracts of *S. officinalis*. [a]$p<0.05$ and [b]$p<0.001$ when compared with non-inflamed.

However, animals in the group treated with methanolic extracts fraction did not increase the number of neutrophils after the inflammatory process, confirming the previous results that showed the anti-inflammatory activity of methanolic extract, since an increase in the number of neutrophils in the circulating blood is indicative of an inflammatory process, because these cells are the defense mechanisms. The statistical analysis there are no significant differences ($p<0.05$) amongst the control and treated groups with methanolic and ethyl acetate extracts.

The levels of lymphocytes in the circulating blood increased for the control and etheric fractions of *S. officinalis*, showing statistically significant differences ($p<0.001$). For the other groups was verified similar count that for the animals before the induced-inflammation. The levels of monocytes in the circulating blood was similar o the value obtained for the animals before the induced-inflammation for both treated and control groups.

Fig. 3 presents the results obtained regarding the number of total leukocytes in the inflammatory exudates for the control and treated groups. As can be seen, were not verified significant statistical differences amongst the groups, indicating that the cell recruitment to the peritoneum was not influenced by the different fractions of *S. officinalis.* Fig. 4 presents the results regarding the number of mononuclear and polymorphonuclear cells in the exudates, where was verified that polymorphonuclear cells were higher than mononuclear ones of the peritoneum exudates for all groups, characterizing the inflammatory process.

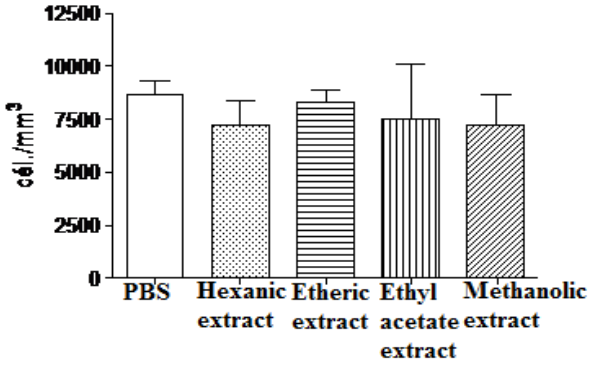

Fig. 3. Total leukocytes in the inflammatory exudates for the control and treated groups.

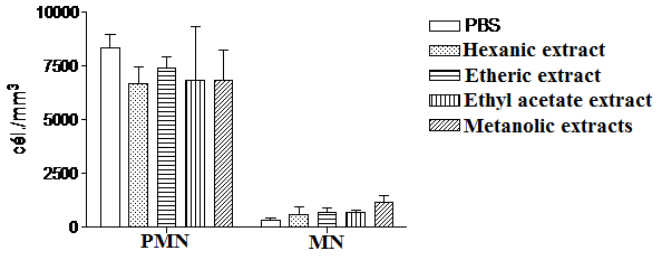

Fig. 4. Number of mononuclear (MN) and polymorphonuclear (PMN) cells in the exudates for the control and treated groups.

DISCUSSION

The anti-inflammatory activities of some compounds identified in the fractionated extracts of *S. officinalis* already were reported in the literature. Caryophyllene is one of the chemical compounds that contribute to the spiciness of black pepper. Gertsch et al. (2008) showed that caryophyllene selectively bind to the cannabinoid receptor type-2 (CB_2) and to exert significant cannabimimetic anti-inflammatory effects in mice. Since the widespread plant natural product caryophyllene is an FDA approved food additive and ingested daily with food it is the first dietary cannabinoid. The caryophyllene and caryophyllene oxide are compounds found in propolis oil, which present anti-inflammatory activity (Sousa et. al., 2006). 2-Cyclopenten-1-one was reported as inhibitor of the molecule of the intercellular adhesion (ICAM-1), with therapeutic relevance to prevent the restenosis after balloon angioplasty (Ianaro et al., 2003).

Some of the anti-inflammatory and antioxidant activities of *S. officinalis* can be attributed to its phenolic compounds content (Mayer et al., 2009). Among the principal properties that may account for the potential health benefits of phenolic compounds, especially flavonoids, is their antioxidant activity, which is efficient in trapping superoxide anion, hydroxyl, peroxyl and alcohoxyl radicals (Masaki et al., 1995; Repetto et al., 2002). Some flavonoids have been shown to increase the mucosal content of prostaglandins and mucus in gastric mucosa, showing cytoprotective effects. Several of them prevent gastric mucosal lesions produced by various experimental ulcer models and protect the gastric mucosa against different necrotic agents (Repetto et al., 2002; Zayachkivska et al., 2005). Earlier studies also demonstrated the presence of carnosol, ursolic acid, oleanolic acid and rosmarinic acid in the leaves of *S. officinalis* (Topçu, 2006). Some properties of these compounds have already been described, such as their antioxidant activity (Lu and Foo, 2001). The anti-inflammatory and occasionally pro-inflammatory activity of ursolic acid (Baricevic et al., 2001; Ikeda et al., 2008), the inhibition of lipid peroxidation by carnosol and also by rosmarinic acid have already been described too.

Baricevic et al. (2001) reported different chromatographic profiles in the analysis of *n*-hexane, chloroform and methanol extracts. In particular, the *n*-hexane (with carnosol as the main band) and the methanol extracts (with prevalent rosmarinic acid and caffeic acid) were characterized by chromatographic lanes in the upper and in the lower part of the plate, respectively, whereas the chloroform extracts showed the presence of several bands along the entire developing path. The chloroform extracts revealed a pronounced band, corresponding to ursolic and oleanolic acid. This band was observed also in fractions I and II obtained from the chloroform extract of sample 1, and, less pronounced, in the *n*-hexane extracts.

In this work was verified that the methanolic extracts of *S. officinalis* presented anti-inflammatory activity, although it was not identified volatiles or semi-volatiles compounds by GC-MS. This activity could be due to the fact that methanolic extracts were more concentrated with polar compounds as rosmarinic, ursolic, caffeic and oleanolic acids, since other solvents firstly extracted volatiles compounds, because the ethanolic extract was fractionated by increasing the polarity of the solvents from hexane to methanol. Durling et al. (2007) extracted phenolics compounds from *S. officinalis* using ethanol:water as solvent and obtained 14.9% of yielding, of which 6.9% was rosmarinic acid, 10.6% was carnosic compounds and 7.3% of essential oil. All these compounds are related to anti-inflammatory activity of the extracts.

CONCLUSIONS

The data obtained in this study showed that the ether extract is the richest constituents. The methanol extract inhibited the recruitment of total leukocytes to the circulating blood. The number of circulating neutrophils was also reduced by the methanol extract, while that the cell recruitment to the peritoneum was not altered by any of the extracts. The methanolic extract of *S. officinalis* showed anti-inflammatory activity at a dose of 25 µg/kg, since inhibited the recruitment of leukocytes from circulating blood

to the lesion site. We have concluded that the constituents of methanolic fraction act synergistically in the inflammatory process.

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