ANTINOCICEPTIVE AND ANTI-INFLAMMATORY EFFECTS OF THE METHANOL EXTRACT OF *CALEA ZACATECHICHI* LEAVES AND ITS FRACTIONS

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Summary

The methanol extract of *Calea zacatechichi* Schlecht. (Compositae/Asteraceae) at doses of 25 and 50 mg/Kg produced antinociceptive activity in the test of acetic acid writhing in a doses-response manner, and also inhibited carrageenan-induced edema in the rat paw at doses of 150 and 300 mg/Kg. The methanol extract was then partitioned with chloroform. Further separation of the chloroform fraction (FCHL) was carried out by column and thin layer chromatography to obtain the subfractions SF7 and SF74 using bioguided fractionation. Those subfractions were more effective than methanol extract on the test of acetic acid writhing 30 mg/Kg of SF7 diminished the writhing response at the same level than 10 mg/Kg indomethacin. On the other hand, 50 mg/Kg of the FCHL showed a higher inhibitory effect on carraggenan-induced paw edema in the rat than methanol extract. In the chemical test done for SF74, we found flavonoids which inhibited (*in vitro*) the production of PGE_2 by macrophages stimulated by LPS to a similar extent than indomethacin (10 μg/mL). *Conclusion:* This study shows that *C. zacatechichi* leaves possess antinociceptive and anti-inflammatory activity, which seem to depend on its capacity to prevent the production of PGE_2. These results strongly support the use of *C. zacatechichi* in herbal medicine.

Key words: *Calea zacatechichi,* antinociceptive, anti-inflammatory activity, carrageenan, indometacin, prostaglandin E_2

Introduction

The inflammatory reaction occurs in response to a variety of injurious stimuli, it is a complex pathophysiological process, initiated by cells already present in all tissues mainly resident macrophages, mast cells, platelets, lymphocytes and other leucocytes, at the onset of injuries these cells undergo activation and release inflammatory mediators responsible for the clinical signs of inflammation. Increased permeability of the blood vessels is result of exudation (leakage), of plasma proteins and fluid into the tissues (edema), which manifests itself as swelling (tumor) (1). Pain is a reaction caused by the release of bradykinin, substance P and prostaglandins (2). Redness and heat are caused by vasodilation, which reduces blood pressure and increases circulation (3). Macrophages play a crucial role in modulating and maintaining the inflammatory response through the release of compounds like nitric oxide, proinflammatory cytokines and prostanoids (4, 1). Cyclooxygenase-2 (COX-2) is a highly inducible enzyme that is expressed in the course of inflammation and other kinds of cellular stress, and accounts for the important synthesis of prostanoids that occurs in several pathophysiological situations such as septic shock and local inflammation of target tissues (5, 6).

Plants are well known as an important source of many biologically active compounds. In recent years, active principles with anti-inflammatory activity of varied chemical structures have been isolated from plants (7). The research carried out on plants with alleged medicinal use as pain relievers and anti-inflammatory agents opens the possibility of obtaining new useful drugs (8).

Calea zacatechichi Schlecht. (Compositae/Asteraceae), in Mexico is called "prodigiosa" it is used for many medical purposes, especially as treatment for various inflammatory diseases such as rheumatism, edema and respiratory tract disorders. Different parts of the plant are administered for cold fever or painful limbs (9). *C. zacatechichi* (*CZ*) contains many compounds such as chromones, flavones, acetylenic compounds, flavonoids and sesquiterpene lactones (10,11,12, 13). Has been reported by (14), that the ethanol extract of *CZ,* inhibit the nuclear factor kappa B (NF-ķB), which induced enzymes that mediated inflammatory processes. At the same time, in our previous research we have shown that the aqueous extract of this plant inhibited the plantar edema produced with carrageenan and abdominal writhes produced by intraperitoneal administration of 0.6% acetic acid (15).

Therefore, the present study investigated the antinociceptive and anti-inflammatory effects of the methanol extract and its chloroform fractions from *CZ* using *in vivo* experimental models i.e., carrageenan-induced hind paw edema for anti-inflammatory activity and the acetic acid abdominal writing test for analgesic activity, and also investigated using *in vitro* model, if the chloroform fractions affected the production of prostaglandin E by peritoneal macrophages.

Methods

Animals

Adult male Wistar rats weighing between 250 and 300 g and Swiss albino mice weighing between 20 and 25 g were used throughout the experiments and kept at the animal facilities of The Iztacala Faculty. They were housed at $24 \pm 0.5^{\circ}$C under a 12:12 h light/dark cycle and with free access to rat chow and water. Animals were randomly divided into groups of six animals each. All procedures were conducted in accordance to institutional ethical guidelines and to the Mexican Official Norm NOM-062-ZOO-1999, regarding technical specifications for production, care and use of laboratory animals (16).

Plant material and preparation

The leaves of *CZ* were bought in the Sonora's market, a place in Mexico City where medicinal plants are sold. The plant's botanical identity was verified at the herbarium of the Botanical Department at Iztacala Faculty. The specimen deposited with the voucher number 26901 was authenticated by Edith López Villafranco, Biologist in charge of the Herbarium.

Preparation of the methanol extract and its chloroform fractions

The leaves were dried at room temperature and then powdered with a mechanical grinder and stored in airtight containers, and then 100 g of powdered leaves were refluxed with 70% methanol solution for 8 h in water bath. The solution was filtered and vacuum dried. Each 100 g of powdered leaves yielded 39 g of lyophilized powder. The methanol extract (50 g) was suspended in 1000 mL mixture of water /MeOH (9:1), and then fractionated with chloroform in a separated funnel. The combined chloroform fraction was then evaporated to dryness in a rotary evaporator (yield: 0.063%). Further fractionation of chloroform fraction was carried out by column chromatography using silica gel G 60 (Merck), with 2 cm diameter and 50 cm long column and eluting it with a mixture of chloroform/ethyl acetate (6:1). Eight subfractions (SF) were collected, and then the solvents were evaporated and all the subfractions were assayed for their analgesic activity, and because only SF7 showed antinociceptive activity, this was further separated by thin layer chromatography using a mixture of chloroform/methanol (5:1) as the mobile phase. The flavonoid acacetin (purchased from Fluka. St. Gallen, Switzerland) was used as standard drug in order to establish if it correspond to some spot of the last fractionating. All subfractions were assayed for their analgesic activity, and only subfraction four of SF7 (called herein SF74) showed an appropriate analgesic activity.

Phytochemical analysis

Qualitative chemical tests were carried out on the fraction SF7 and subfraction SF74 of the chloroform fraction for detection of alkaloids, cardiac glycosides, anthraquinones, saponins flavonoids, sterols and triterpenes (17,18).Thin layer chromatography of the chloroform fraction and subfraction with biological activity was performed on silica gel G 60 plates (Merck), with ethyl acetate/methanol/water, 100:16.5:13.5 (v/v) or benzene/methanol 4:1 (v/v); the chromatograms were observed with ultraviolet lamp at 254-365 nm to detect the separated fractions, which were marked and eluted from the plate. Chemical tests were performed on the eluted fractions.

Acetic acid-induced writhing in mice

Abdominal writhes induced by the intraperitoneal injection of 60 mg/Kg acetic acid were carried out according to the procedures described previously (19). Briefly, acetic acid solution in saline (0.6% v/v, 10 mL/Kg) was intraperitoneally injected in mice. Thirteen minutes before the injection of the acetic acid, the methanol extract (25 and 50 mg/Kg), the chloroform fraction (50 mg/Kg) or subfractions F7 and F74 (30 mg/Kg) of *CZ* were administered p.o., both suspended in a mixture 0.3% Tween 80 and 1% dimethyl sulfoxide (DMSO) solution in water. Indomethacin (Sigma, St Louis MO, 10 mg/Kg) was administered as a reference drug. The writhing response consists of a contraction of abdominal muscles together with a stretching of the hind limbs. The total number of abdominal writhes was counted up 20 min after the acetic acid administration.

Carrageenan-induced paw edema in the rat

Edema was induced according to the method described by (20). Briefly, 0.1 mL of 1% carrageenan (type II, Sigma, St Louis, MO) in sterile saline solution was injected into the subplantar tissue of the right hind paw and the same volume of saline solution into the left hind paw (control group). The paw volume was measured before injection of carrageenan or saline solution by the mercury displacement method, and the time course of edema formation was followed. 60 min before the carrageenan injection the groups of rats were administered orally with methanol extract (150 and 300 mg/Kg), the FCHL (50 mg/Kg), indomethacin (10 mg/Kg) was administered as a reference drug, and the control group received the mixture of 0.3% Tween 80 and 1% DMSO in water solution. The volume increased of the inflamed paw was estimated by subtracting the volume of the contralateral paw (control). The anti-inflammatory effect of drugs was evaluated as the degree of edema inhibition.

Production of PGE$_2$ by lipopolysaccharide-activated macrophages

Rats were intraperitoneally injected with 1 mL thioglycolate (3% w/v), and five days later they were killed by cervical dislocation and intraperitoneally injected with 30 mL Hanks solution and after a soft abdominal massage, the peritoneal fluid was carefully aspirated to avoid blood contamination by hemorrhage and kept at 4°C to prevent the adhesion of macrophages to the plastic wall. After centrifuging at 400 x g for 10 min at 4°C, the cell pellet was washed twice with 45 mL ice-cold PBS and adjusted to 1×10^6 cells/mL. Cell viability (greater than 95%) was confirmed by the trypan blue exclusion assay. 1×10^5 cells were incubated in 200 µL sterile RPMI 1640 medium supplemented with 10 % fetal calf serum (FCS), using tissue plates of 96 wells. After incubation for 2 h at 37°C in 5% CO$_2$, non adherent cells were removed by extensive washing with PBS. Cells were incubated in the same medium containing 1µg/mL *Escherichia coli* lipopolysaccharide (LPS; Sigma St. Louis MO) with or without test compounds at 37°C for 24 h. Culture supernatants were used for determining the PGE$_2$ produced by using ELISA kits (Cayman Chem.). The assays were performed according to the manufacturer's instructions.

Statistical analysis

Results are presented as the mean ± S.E.M. of experiments carried out on 6 animals per group. Differences among groups were evaluated by analysis of variance (one-way ANOVA) followed by the Tukey post hoc test. $P < 0.05$ was considered to be statistically significant.

Results

Phytochemical analysis
The phytochemical analysis of the SF7 showed the presence of sterols, triterpenoids, sesquiterpene lactones, and flavonoid chalones, and SF74 contains flavonoids with Rf of 0.63, which was different to acacetin, whose Rf was 0.79.

Acetic acid-induced writhing response in mice
Methanol extract at doses of 25 and 50 mg/Kg inhibited the number of abdominal writhes in a dose dependent response (49.7%, and 66.5% respectively), indomethacin (10 mg/Kg) also inhibited the writhes in 89% (Figure 1). Chloroform fraction (FCHL) (50 mg/Kg) showed an inhibition in the abdominal writhes of 54%, while 30 mg/kg of its subfractions SF7 and SF74 caused a higher inhibition of the writhes (72% for SF7, and 90.2% for the fraction SF74) (Figure 2). Other subfractions of the chloroform fraction were also evaluated, but none showed a significant decrease in the number of writhings (data not shown).
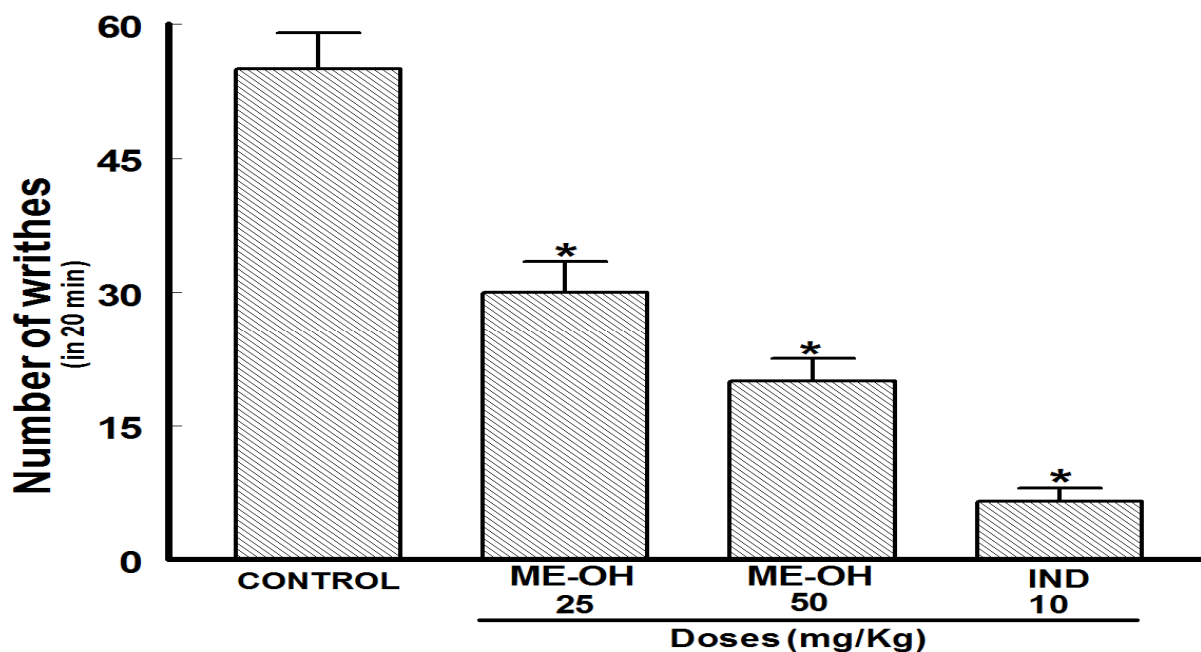

Fig.1. Effect of the methanol extract of *CZ* on the total number of writhes induced by acetic acid in mice. The extract methanol (ME-OH) and indomethacin (IND) The control group only received vehicle. Values are the mean $\pm$ S.E.M. of the number of animals used (n=6), *p<0.05.
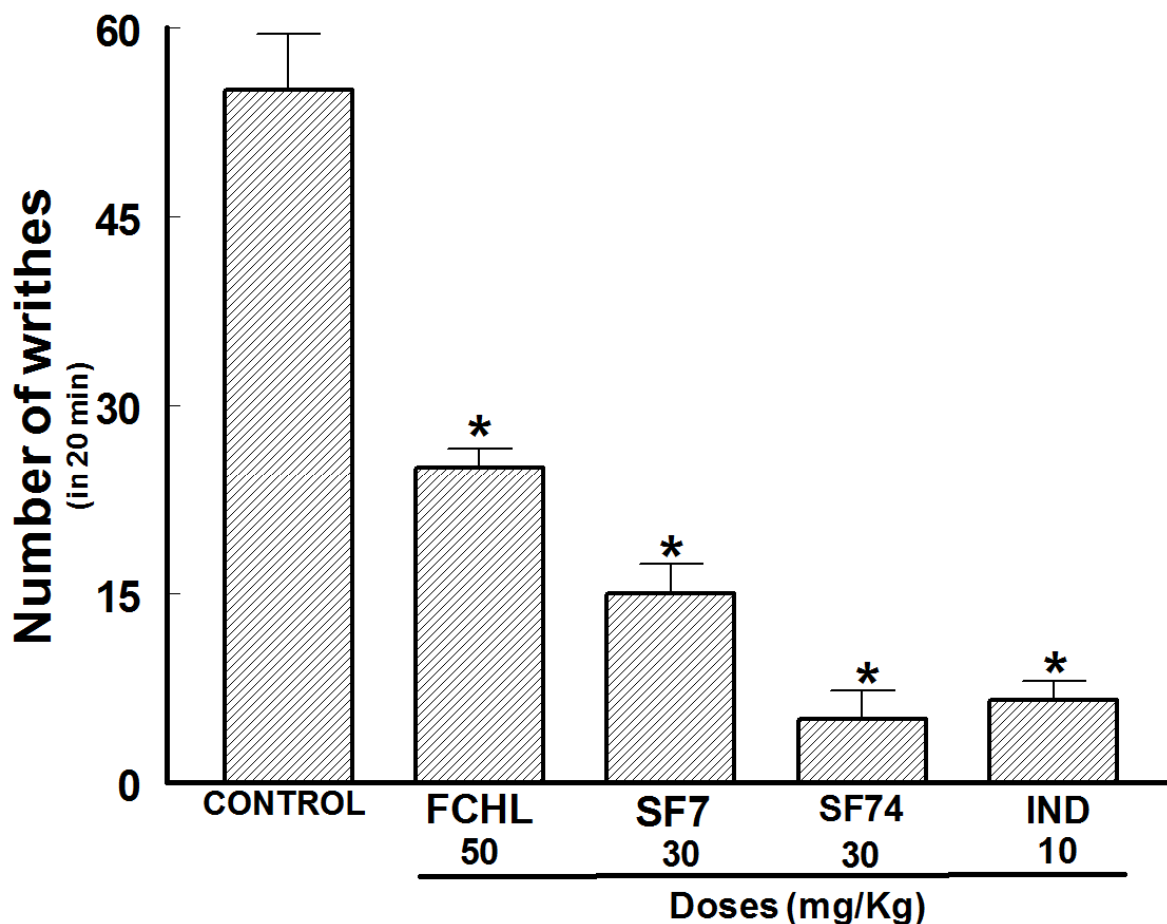
Fig.2. Effect of the chloroform fraction (FCHL) and its subfractions obtained from *CZ* on the total number of writhes induced by acetic acid in mice. Further separation by column chromatography of the FCHL gave rise the subfraction seven (SF7), and from even further fractionation of SF7, subfraction SF74 was obtained. The control group only received vehicle. Values are the mean $\pm$ S.E.M. for the number of animals used (n=6), *p<0.05

Carrageenan-induced paw edema in the rat
The edema induced by the carrageenan injected subcutaneously into the hind paws in rats was followed for 8 h, the maximum effect was observed four hours after its injection (360 ± 30 µL), (Figure 3). Carrageenan administered concomitant with the extract methanol at doses of 150 and 300 mg/kg decreased paw volume (271 ± 33 µL and 210 ± 20 µL, respectively) (Figure 3). The chloroform fraction at a dose of 50 mg/kg, also decreased the edema in 280 ± 9 µL), indometacin (10 mg/kg) showed better effect in decreased paw volume (102.74 ± 20 µL).
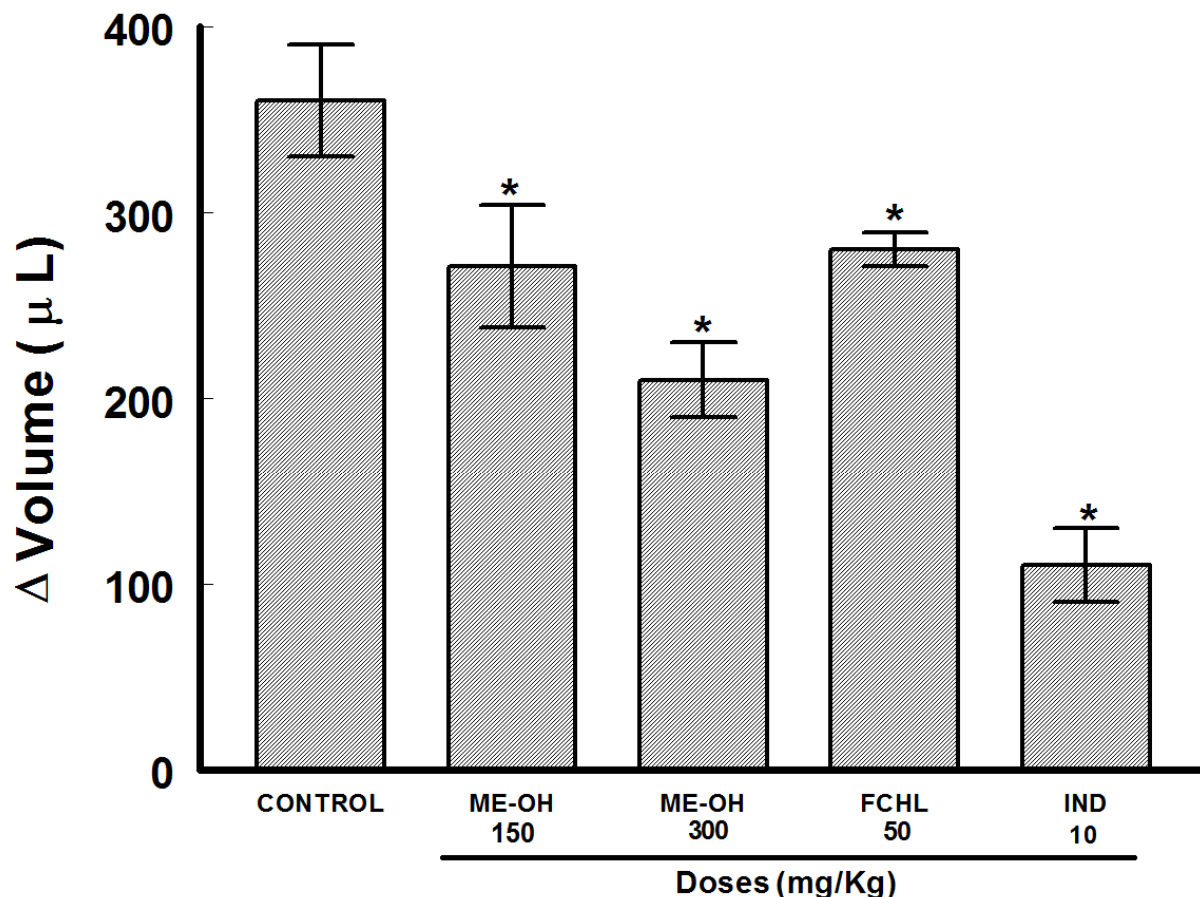
Fig.3. Effect of the methanol extract (ME-OH) and FCHL fraction on the edema induced by intraplantar injection of carrageenan (100 μg in 0.1 mL) in rats. The control group received only the vehicle. Values are the mean $\pm$ S.E.M. of the number of animals used (n=6). *p<0.05 when compared with the control group.

Production of PGE_2 in lipopolysaccharide-activated macrophages
Incubation of macrophages with 1μg/mL *Escherichia coli* lipopolysaccharide (LPS) at 37°C for 24 hours significantly increased the production of PGE_2 compared to the control group (0.492 $\pm$ 0.038 ng/mL vs 0.136 $\pm$ 0.013 ng/mL). When macrophages were incubated with the same amount of LPS in the presence of 2 mg/mL of subfractions SF7 and SF74, only SF74 significantly decreased the PGE_2 production (0.114 $\pm$ 0. 03 ng/mL). In fact, considering that the concentration of PGE_2 in the conditioned medium with indomethacin was diminished to 0.317 $\pm$ 0.022 ng/mL, it means that the capacity to inhibit PGE_2 production by SF74 at concentration of 2 mg/mL was similar than 10 μg/mL indomethacin (Figure 4).
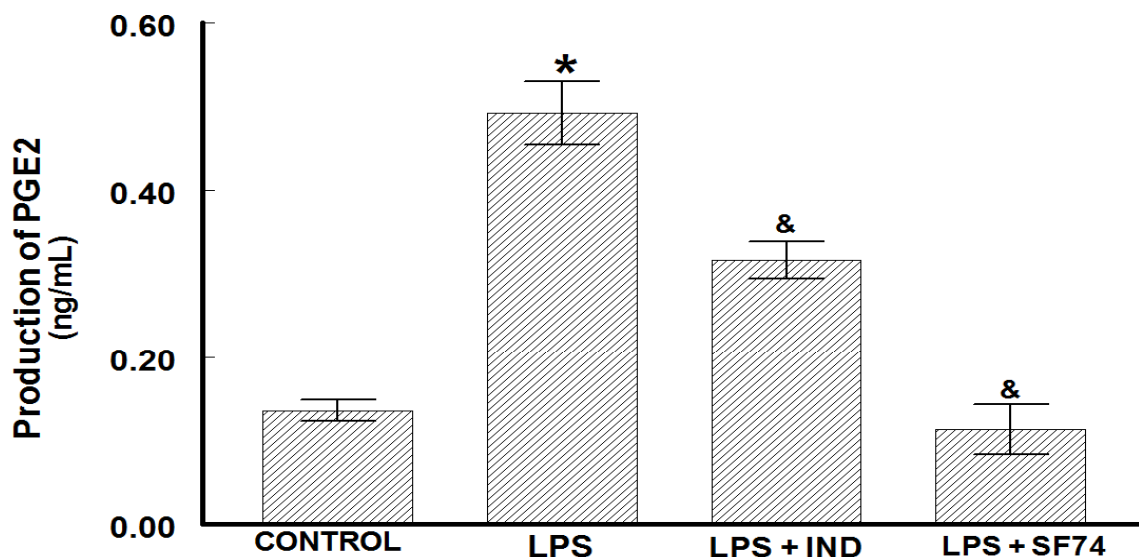
Fig.4. *In vitro* inhibitory effect of subfraction SF74 on the production of PGE_2 Isolated peritoneal macrophages from rats were incubated at 37^oC for 24 h with 1 µg/mL of lipopolysaccharide (LPS) in the presence of IND (10 µg/mL) or the subfraction SF74 (2 mg/mL). Bars represent the mean ± S.E.M. (n=6). *$p<0.05$ when compared vs. control (0.1% of DMSO). &$p<0.05$ when compared vs. LPS treated cells.

Discussion

In previous experiments with the aqueous extract of *CZ* we have shown that this extract has anti-inflammatory and antinociceptive effects. Considering the ethnomedical uses of *CZ* leaves, the present study was carried out to evaluate the antinociceptive and anti-inflammatory effects of the methanol extract of *CZ* and its chloroform fractions, as well as to identify the active subfraction from the chloroform fraction for further isolation of the active principles.

Acetic acid injection into the peritoneal cavity causes an increase of serotonin, histamine and prostaglandins such as PGE_2 in peritoneal fluids, which causes inflammation and pain (21,22). The methanol extract and chloroform fraction and the subfractions SF7 and SF74 obtained from the latter, showed significant inhibition of the number of abdominal writhes, observing that, lower

doses of the chloroform fraction had similar or greater antinociceptive effect than the corresponding methanol fraction, which indicates that the active compounds of the extract may be distributed principally in the chloroform fraction and it was getting a further enrichment of the active principle by subfractioning this fraction. A marked antinociceptive effect was observed in subfractions SF7 and SF74, which inhibit the abdominal writhes at comparable degree to that of indomethacin, which is a standard drug widely used by its antinociceptive activity, mediated by the inhibition of prostaglandin biosynthesis.

On the other hand, paw edema formation induced by the injection of carrageenan, is the result of a synergism between inflammatory mediators that increase blood flow and microvascular permeability (23). The carrageenan-induced rat paw edema is characterized by an early phase (1 h) caused by the release of 5-hydroxytryptamine, histamine and bradykinin followed by a late phase (2 h) mainly sustained by prostaglandin release which causes edema dependent on mobilization of neutrophils (24, 25). In this model, the methanol extract showed a maximal inhibitory effect on edema formation at 4 h after intraplantar injection of carrageenan suggesting that the extract may be affecting the synthesis and/or release of mediators during the second phase of the response. The findings are in line with our observation on the ability of the *CZ* to block the production of PGE_2. The results also indicate that only the SF74 isolated from the chloroform subfraction SF7, was an effective inhibitor of LPS-induced PGE_2 production in rat peritoneal macrophage. PGE_2, a well studied chemical mediator of the inflammatory process is produced by activated cells and tissues following activation of COX-2, and is released by macrophages. PGE_2 increases vascular permeability along with other vasoactive components such as histamine, bradykinin or NO, resulting in edema, pain and hyperalgesia at the site of inflammation. In this study, the increase in PGE_2 production following stimulation by LPS was attenuated by the SF74, indicating that antinociceptive and anti-inflammatory potential can be due to the inhibition of PGE_2 production and suggesting that the SF74 has cyclooxygenase inhibitory properties. Inducible COX-2 is expressed by activated macrophages and other cells at the site of inflammation. Therefore, the inhibition of COX-2 can provide relief from symptoms of inflammation. Sesquiterpene lactones and flavonoids are active compounds present in a great variety of medicinal plants (26, 27,28) Sesquiterpene lactones presents in the ethanol extract of *CZ*, were able to inhibit the nuclear factor kappa B (NF-κB) (14), which is involved in the induction of enzymes that mediate inflammatory processes. Other secondary metabolites present in *CZ* are the flavonoids, of which acacetin, isolated from others medicinal plants, has been demonstrated to inhibit the activity of cyclooxygenase and lipoxygenase (28). However, phytochemical tests carried out on subfraction SF74 showed that it contains flavonoids, but not identified acacetin, its Rf was different of the SF74, more studies are required in order to establish if SF74 contains flavonoids structurally related with acacetin and if it is responsible of the antinociceptive effect of SF74.

In conclusion, based on the results of this study, *CZ* leaves have similar compounds with flavonoids that exhibited anti-inflammatory and analgesic activity. We isolated partially the subfraction SF74, which showed an inhibitory effect on the PGE_2 production by macrophages stimulated with LPS. Further purification and characterization of the components responsible of these activities is also important and remains to be clarified in further studies.

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