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ORIGINAL ARTICLE

Anti-Inflammatory and Antinociceptive Activities of *Salvia keerlii*

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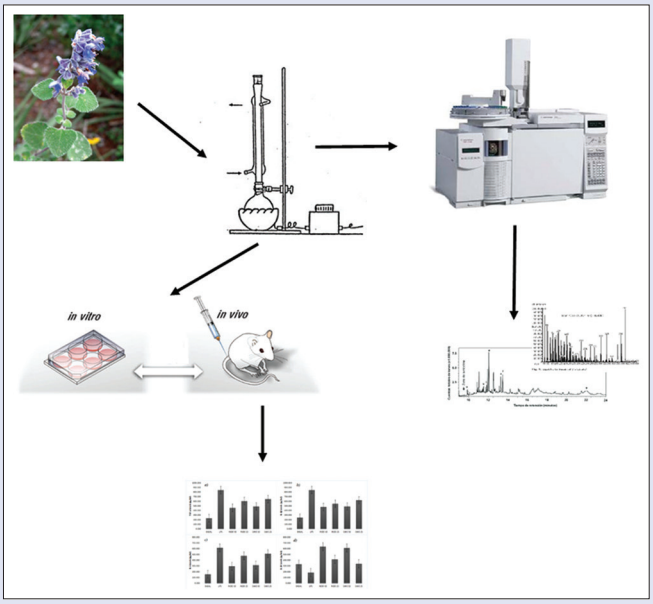
ABSTRACT

Background: Inflammation is a response to an attack on the body and is treated with several drugs that cause severe side effects. An alternative treatment source is plants, such as *Salvia keerlii* (SAKE) Benth. **Objective:** The objective is to study the determination of the anti-inflammatory and antinociceptive properties and chemical composition of the chloroform extract of SAKE. **Materials and Methods:** The plant was defatted with hexane, and then, chloroform and methanol extracts were obtained by heating, and the solvent was evaporated. The chemical composition of the extract was analyzed using gas chromatography–mass spectrometer. Total phenolic was determined using Folin–Ciocalteu reagent, and total flavonoid was determined by $AlCl_3$ assay. The acute and chronic anti-inflammatory effects of SAKE were evaluated on ear edema induced with 12-O-tetradecanoylphorbol-13-acetate, and the antinociceptive activity was determined. The effects of SAKE on protein denaturation and membrane stabilization were determined. The levels of nitric oxide, tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1β, IL-6, and IL-10 were measured in J774A.1 macrophages stimulated with lipopolysaccharide. **Results:** SAKE at 2 mg/ear inhibited ear edema by 84.96% in the acute assay, whereas 100 and 200 mg/kg (p. o.) SAKE decreased inflammation in the chronic assay. In macrophages, SAKE decreased the levels of TNF-α (1.7-fold), IL-1β (1.7-fold), and IL-6 (1.9-fold) but increased the levels of IL-10 (1.9-fold). SAKE inhibited protein denaturation (IC_{50} = 8.68 μg/mL). The inhibition of writhing obtained with SAKE at doses of 10, 50, 100, and 200 mg/kg was 28.73%, 46.48%, 56.1%, and 63.05%, respectively. **Conclusions:** SAKE has *in vitro* and *in vivo* anti-inflammatory and antinociceptive effects.

Key words: Anti-inflammatory, antinociceptive, *Lamiaceae, Salvia keerlii*

SUMMARY

- The chloroform extract of *Salvia keerlii* had anti-inflammatory and antinociceptive activities in models *in vivo* and *in vitro*.



Abbreviations used: SAKE: Chloroform extract of *Salvia keerlii*; TPA: 12-O tetradecanoylphorbol-13-acetate; MTT: 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide; LPS: lipopolysaccharide; IND: indomethacin; GC-MS: gas chromatography-mass spectrometer; GAE: galic acid equivalents; QE: quercetin equivalent; UAM-X: Universidad Autónoma Metropolitana-Xochimilco; PVP: Polyvinylpyrrolidone; NO: nitric oxide; PBS: phosphate-buffered saline; NCSS: National Council for the Social Studies; ATCC: American Type Culture Collection.

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INTRODUCTION

Inflammation is a response to protect the body against physical trauma, noxious chemicals, bacteria, and viruses. Inflammation is a defense reaction caused by an injury and is beneficial in short-term processes. Nevertheless, chronic inflammation might lead to many diseases such as cancer, rheumatoid arthritis, and diabetes.[1-3] There are effective treatments for inflammation. However, the commonly used drugs exert undesirable side effects, which have led to the search for new compounds with anti-inflammatory activity. Plants are a possible source to obtain new compounds with pharmacological effects. The genus *Salvia*, which comprises more than 900 species, belongs to the *Lamiaceae* family.

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Species of this genus have shown many biological activities.[4] *Salvia keerlii* (SAKE) Benth. is a plant used in the state of San Luis Potosí for the traditional treatment of bruises or twists. This plant is a perennial shrub that is wild-growing and reaches up to 2.5 m in height. Its leaves are oval-lanceolate with light gray coloration, and its flowers are purple, scented, and grow in whorls in small inflorescences. Blooming occurs from summer to autumn. SAKE grows in the states of Durango, Guanajuato, Hidalgo, Oaxaca, Puebla, Querétaro, San Luís Potosí, and Zacatecas. The three following neoclerodane diterpenes have been isolated from this plant: Kerlin and kerlinolide,[5] as well as kerlinic acid.[6]

Herein, we describe the chemical composition of the chloroform extract of SAKE, as well as its *in vitro* and *in vivo* anti-inflammatory effects, antinociceptive activity, and possible anti-inflammatory mechanism of action.

MATERIALS AND METHODS

Plant material

Aerial parts of SAKE were collected in the community of Las Comadres, municipality of Guadalcazar, San Luis Potosí State (Mexico) in September 2016. The plant was classified in the Isidro Fabela Herbarium of the Universidad Autónoma de San Luís Potosí (voucher number SPLM43012). José García Pérez contributed to the classification of plant material.

Chemicals

N-O-Bis (trimethylsilyl) trifluoroacetamide (10%), polyvinylpyrrolidone (PVP), dry pyridine, 12-O-tetradecanoylphorbol-13-acetate (TPA), and indomethacin (IND) were purchased from Sigma-Aldrich. Immunoenzymatic kits for the quantification of interleukin (IL)-1β, IL-6, IL-10, and tumor necrosis factor-alpha (TNF-α) were purchased from PeproTech. All other reagents were of high chemical purity.

Plant extract preparation

The chloroform and methanol extracts of SAKE were prepared as follows. Dried aerial parts (350 g) of SAKE were powdered and defatted in 3.0 L of hexane under reflux for 4 h, and the extracts were obtained with 3 L of chloroform or methanol which was refluxed for 4 h. Then, each solvent was evaporated in a rotatory evaporator at reduced pressure.

Derivatization

A mixture of 10 mg of SAKE, 1 mL of dry pyridine, and 100 µL of bis (trimethylsilyl) trifluoroacetamide with 10% trimethylsilyl chloride was heated (100°C) for 10 min at 150 watts in a microwave oven (CEM Discover).

Salvia keerlii analysis

Analysis of the chemical composition of SAKE was performed with a gas chromatography–mass spectrometer (GC-MS) Agilent Technologies, model 6890N connected to a mass selective detector (model 5973) with a DB-5HT capillary column (30 m length, 0.25 mm internal diameter, and 0.10 µm film thickness). The injector temperature was set at 320°C. The temperature program was used as follows: the initial oven temperature (100°C) was held for 3 min and then increased at a rate of 10°C/min–320°C. This temperature was held constant for 5 min. The splitless ratio was 1:100, and the injector temperature was predetermined at 320°C. The spectrum was determined at 70 eV. The compounds were identified by comparing their mass spectra to those reported in the Wiley14.1/NIST11 library.

Total phenolic assay

The total phenolic content of SAKE was determined by the Folin–Ciocalteu assay.[7] A solution of 100 µL of SAKE (0.1 mg/mL), 800 µL of deionized water, and 100 µL of Folin–Ciocalteu reagent was incubated for 8 min in the dark, followed by the addition of 50 µL of 7.5% (w/v) Na_2CO_3. The mixture was allowed to stand at room temperature for 60 min in the dark, and the absorbance was measured at 760 nm. The calibration curve was obtained with gallic acid (0–200 µg/mL). The total phenolic content was expressed as mg of gallic acid equivalents (GAE)/g dry weight. The tests were performed in triplicate.

Total flavonoids assay

The total flavonoid content of SAKE was determined by the $AlCl_3$ colorimetric method.[8] Specifically, 500 µL of SAKE (I mg/mL ethanol) was mixed with an equal volume of 2% $AlCl_3$ in ethanol. The absorbance was measured at 420 nm. The total flavonoid content was calculated from a quercetin calibration curve (10–60 µg/mL), and the result was expressed as mg quercetin equivalents (QE)/g dry weight. The assay was performed in triplicate.

Experimental animals

Male CD-1 mice (25–30 g) from the animal facility of Universidad Autónoma Metropolitana-Xochimilco (UAM-X) were used. The animals were housed at 20°C–22°C, with relative humidity from 50% to 55%, and under light-dark cycles of 12:12 h. Water and food (Lab Diet) were supplied *ad libitum*. All experimental protocols were approved by the Research Bioethics Committee of UAM-X. All tests were conducted in accordance with the current procedure for the care of experimental animals established by the Official Mexican Norm (NOM-062-ZOO-1999).

Anti-inflammatory evaluation

12-O-tetradecanoylphorbol-13-acetate-induced mouse ear edema

The procedure for TPA-induced mouse ear edema has been described by Carlson *et al.*[9] Groups of 8 animals were used. The right ear of each mouse received a topical application of 2.5 µL of TPA in 25 µL of acetone. Thirty minutes after the application of TPA, the following treatments were administered at a dose of 2 mg/ear, as follows: methanol or chloroform extracts of SAKE, vehicle group (PVP, in a 1:4 ratio), and IND. After 6 h of TPA administration, the animals were euthanized, and 6 mm plugs of the central segment of the two ears of each animal were weighed. The percentage of edema inhibition was determined as follows:

$$\% \text{ inhibition} = \frac{(W - Wo)}{(W' - W'o)} \times 100$$

Where W = TPA + treatment; Wo = vehicle-treated ear; W' = right ear with TPA, without treatment; and W'o = left ear with vehicle, without treatment.

Evaluation of anti-inflammatory activity on multiple application of TPA-induced ear mouse edema.

Groups of 8 mice per group were used. Thirty min before the administration of 2.5 µg of TPA/ear dissolved in 25 µL of acetone, animals were orally administered the following test treatments: vehicle (PVP, in a 1:4 ratio), 5 mg/kg IND (8 mg/kg), or 12.5, 25, 50, 100, and 200 mg/kg SAKE.

The treatments and TPA were administered on days 1, 3, 5, 7, and 9 of the experiment. All animals were sacrificed on the final day by cervical

dislocation, and 6 mm diameter discs were obtained from both ears. The percent inhibition of the edema was determined with the same formula as described above.[10]

Antinociceptive activity assay

The acetic acid-induced writhing method was carried out following the protocol of Berkenkopf and Weichman to evaluate the antinociceptive activity of SAKE.[11] Groups of 8 mice were administered the following test samples: vehicle (PVP), naproxen (100 mg/kg), or SAKE (10, 50, 100, and 200 mg/kg). Thirty minutes later, mice received 1% acetic acid (10 mL/kg) to induce pain. After acetic acid administration, writhing count was started and continued over a period of 30 min.

$$\% \text{ inhibition} = 100 - \left(\frac{\text{ACT} \times 100}{\text{ACV}} \right)$$

Where ACT = abdominal constrictions of test; ACV = abdominal constrictions of vehicle.

Acute toxicity assessment

The methodology of Lorke was followed for testing the acute toxicity of chemical products. SAKE was prepared with PVP in a ratio of 1:4. Groups of 5 mice per group were used. The groups were divided as follows: the control group was administered PVP. Doses of 625, 1250, 2500, and 5000 mg/kg SAKE were administered. The animals were sacrificed 72 h after the administration, and some biopsies were conducted to identify possible visual signs of toxicity.[12]

Cell culture

Murine macrophages J774A.1, obtained from the American Type Culture Collection (ATCC), were maintained with Dulbecco's Modified Eagle's Medium supplemented with 10% heat-inactivated fetal bovine serum and antibiotics at 37°C under CO_2 (5%).

Cell viability assay

Murine macrophages J774A.1 were seeded at a density of 5000 cells per well in a 96-well plate and treated with various concentrations of SAKE (3.125, 6.25, 12.5, 25, 50, 100, and 200 μg/mL) for 24 h. The cells without treatment were considered as the negative control. At the end of the experiment, 10 μL of a solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (5 mg/mL) was added, and the plate was placed at 37°C for 4 h. Subsequently, the medium was discarded, and 100 μL of dimethylsulfoxide was added to dissolve the formazan crystals. The microplate was read in an enzyme-linked immunosorbent assay reader at 540 nm. The experiment was carried out in triplicate.

Determination of cytokines and nitric oxide levels

Macrophages J774A.1 were grown at 5×10^5 per well in a 96-well plate and treated with 25 and 50 μg/mL SAKE and 50 μM IND (equivalent to 17 μg/mL) and incubated for 2 h. Then, 5 μg/mL lipopolysaccharide (LPS) was added to each well, and the cells were incubated for 24 h. Subsequently, the supernatants were collected and stored at −70°C until further analysis. The concentrations of IL-1β, IL-6, IL-10, and TNF-α in the supernatants were determined using a commercial immunoenzymatic kit (PeproTech) according to the manufacturer's instructions. The absorbance was recorded using a microplate reader at 405 nm. For the nitric oxide (NO) assay, a volume of 100 μL of treated culture supernatant was mixed with 100 μL of Griess reagent and incubated at 37°C for 30 min, and the absorbance was recorded at 540 nm.[13]

In vitro anti-arthritic activity

This activity was determined using the bovine serum protein denaturation method.[14] Briefly, 0.05 ml of SAKE (25, 50, 100, 200, 500, 750, and 1000 μg/mL), diclofenac sodium (25, 50, 100, 200, 500, 750, and 1000 μg/mL), or distilled water (control) was added to 0.45 mL of bovine serum albumin (5% w/v aqueous solution). The samples stood at 37°C for 20 min, and the temperature was then increased to 57°C for 3 min. After cooling, 2.5 mL of phosphate-buffered saline (PBS) was added to the solutions. The absorbance was measured using a UV-Visible spectrophotometer at 560 nm. The control represents 100% protein denaturation. The results were compared with diclofenac sodium. The percentage of inhibition of protein denaturation can be estimated as follows:

$$\% \text{ inhibition} = 100 - \left(\frac{\text{Optical density of Test} - \text{Optical density of Control}}{\text{Optical density of Test}} \times 100 \right)$$

Membrane stabilization property

Blood from healthy human volunteers who did not consume nonsteroidal anti-inflammatory drugs for 2 weeks was obtained. A suspension of 5% human erythrocytes was prepared with Alsever's solution and centrifuged at 3000 rpm. Seven different concentrations of SAKE (0.1–200 μg/mL) were prepared in PBS. Diclofenac was used as a reference drug at the same concentrations, whereas distilled water and PBS were used as negative controls.[15]

Then, the reaction was incubated for 30 min at 37°C, the samples were centrifuged, and the supernatants were read at 540 nm. Hemolysis was calculated using the following equation:

$$\% \text{ Hemolysis} = \frac{(\text{As} - \text{Anc})}{(\text{Apc} - \text{Anc})} \times 100$$

Where As = Absorbance of the sample, Anc = Absorbance of the negative control, and Apc = Absorbance of the positive control.

Statistical analysis

The results are expressed as the means ± standard error of means, and statistical analyses were performed using ANOVA followed by Dunnett's test. The statistical significance was set to $P < 0.05$ using the statistical program National Council for the Social Studies (NCSS) 2004.

RESULTS

Chemical composition of *Salvia keerlii*

SAKE was derivatized by silylation, and its chemical composition was determined by GC-MS. A total of 18 compounds were identified and represented 99% of the extract [Table 1]. The main identified compounds were pentadecanoic acid (22.66%), myristic acid (17.55%), methyl dehydroabietate (12.35%), and ursolic acid (11.37%).

The total phenolic content of SAKE was determined to be 9 mg GAE/g dry weight, and its flavonoid content was 3.7 mg QE/g dry weight.

Anti-inflammatory activity *in vivo*

The anti-inflammatory activity of the chloroform and methanol extracts of SAKE was determined on TPA-induced mouse ear edema at doses of 2 mg/ear for each extract [Table 2]. The methanol extract showed low anti-inflammatory activity (38.58%), whereas the chloroform extract significantly diminished ($P < 0.05$) the ear edema by 84.96%, which was higher than the effect obtained with IND (64.11%). Therefore, further experiments were conducted only with the chloroform extract of SAKE.

In chronic ear edema induced by multiple applications of TPA, SAKE showed a dose-dependent anti-inflammatory effect. SAKE at 100 mg/kg

Table 1: Chemical composition of *Salvia keerlii*

Name of compound	Retention time (%)	Kovalts index
Myristic acid	15.483 (17.55)	1788
Oleic acid	17.032 (9.20)	2194
(E)-9-Octadecenoic acid	17.088 (3.89)	2194
Pentadecanoic acid	17.250 (22.66)	1888
Eicosapentaenoic acid	17.531 (0.42)	2425
3-Hydroxyestran-17-one, (3α,5β)-	18.333 (0.30)	2113
9,12-Octadecadiynoic acid	18.598 (0.54)	2221
Methyl dehydroabietate	18.644 (12.35)	2271
2-Hydroxyphenethyl alcohol	18.791 (0.34)	1475
(Z)-5,8,11-Eicosatrienoic acid	18.891 (8.56)	2409
Arachidonic acid	19.569 (0.95)	2417
(3β,5α)-Cholestan-3-ol, 2-methylene-	19.681 (1.01)	2652
(Z)-13-Retinoic acid	19.747 (5.37)	2371
Tetradecane, 2,6,10-trimethyl-	25.025 (0.78)	1519
10,12-Tricosadiynoic acid	25.925 (0.51)	2718
2-Oleoylglycerol	28.519 (0.55)	2788
Ursolic acid	28.840 (11.37)	3306
Oleanolic acid	29.530 (2.90)	3242

Table 2: Anti-inflammatory activity of the chloroform and methanol extracts of *Salvia keerlii* in ear edema induced with 12-O-tetradecanoylphorbol-13-acetate in mice

Treatment	Dose (mg/ear)	Inhibition percentage
Vehicle	-	0
Indomethacin	2	64.11±1.82*
Methanol	2	38.58±7.3
Chloroform	2	84.96±3.92

The values are expressed as mean±SEM (n=8). *P<0.05 versus vehicle.
SEM: Standard error of mean

Table 3: Anti-inflammatory activity of *Salvia keerlii* in ear edema induced by multiple applications of 12-O-tetradecanoylphorbol-13-acetate in mice

Treatment	Dose (mg/kg)	Inhibition percentage
Vehicle	-	0
Indomethacin	8	58.41±1.25*
SAKE	12.5	NA
	25	16.35±2.63
	50	40.31±4.62
	100	56.57±3.16*
	200	59.69±4.82*

The values are expressed as mean±SEM (n=8). *P<0.05 versus vehicle. NA: No activity; SEM: Standard error of mean; SAKE: *Salvia keerlii*

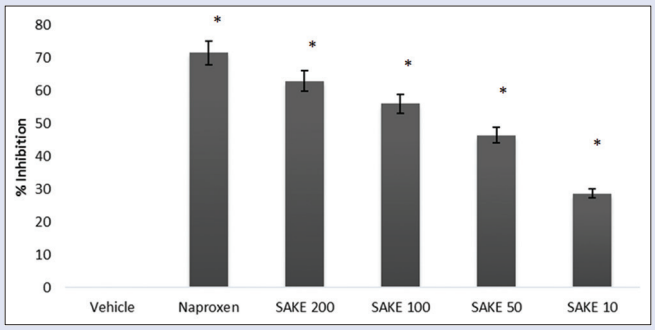

Figure 1: Effect of *Salvia keerlii* (10, 50, 100, and 200 mg/kg) and naproxen as control (100 mg/kg) on the intraperitoneal administration of acetic acid. Values are expressed as mean ± standard error of mean (n = 8). * P < 0.05 versus vehicle

(56.57%) and 200 mg/kg (59.69%) showed an anti-inflammatory effect comparable to that obtained with 8 mg/kg IND (58.41%) [Table 3]. The effective dose 50 (ED_{50}) calculated for SAKE was 82.55 mg/kg.

Antinociceptive activity assay

SAKE showed antinociceptive effects (ED_{50} = 74.11 mg/kg) in a dose-dependent manner by reducing acetic acid-induced writhing in mice. The percentage inhibition of writhing by SAKE at the doses of 10, 50, 100, and 200 mg/kg was 28.73%, 46.48%, 56.1%, and 63.05%, respectively, whereas 100 mg/kg naproxen, the positive control, showed the antinociceptive activity of 71.64% [Figure 1].

Acute oral toxicity of *Salvia keerlii*

Groups of 5 mice were administered 625, 1250, 2500, and 5000 mg/kg doses and observed for 72 h. The mice administered 2500 and 5000 mg/kg doses presented somnolence after 10 min of administration and remained in a catatonic state for 3 h. All mice administered with 5000 mg/kg SAKE died after 24 h of treatment. Mice administered with SAKE at doses of 625 and 1250 mg/kg showed somnolence after 45 min posttreatment, and this effect lasted 95 min. The other groups of mice were sacrificed 72 h posttreatment. Mice administered a dose of 2500 mg/kg presented irritation in the stomach and changes in the tone of the kidneys. These damages were also observed in a lower grade in the 1250 mg/kg SAKE groups. In contrast, mice treated with 625 mg/kg SAKE showed no damage to the liver, heart, stomach, lung, or kidney. The LD_{50} calculated for SAKE was 3393 mg/kg.

Cell viability in macrophages

The effect of SAKE on the cell viability of J774A.1 macrophages was evaluated using the MTT assay. The results showed that SAKE at concentrations of 3.125–50 μg/mL did not affect cell viability [Figure 2]. Therefore, the concentrations of 25 and 50 μg/mL were used in subsequent experiments. The IC_{50} calculated for SAKE was 147.85 μg/mL.

Levels of pro- and anti-inflammatory cytokines

The action of SAKE and IND on the production of proinflammatory and anti-inflammatory cytokines was studied [Figure 3]. The results showed that compared to the control group, SAKE administration at 25 μg/mL did not alter the levels of IL-6 but slightly diminished those of IL-1β and TNF-α. However, SAKE at 50 μg/mL reduced the levels of TNF-α (1.7-fold), IL-1β (1.7-fold), and IL-6 (1.9-fold), and the findings were

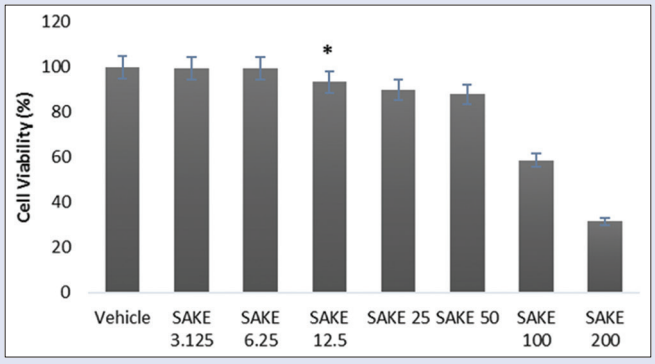

Figure 2: Effect on the cell viability of macrophages treated with *Salvia keerlii* at 3.125, 6.25, 12.5, 25, 50, 100, and 200 μg/mL. Results are expressed as the percentage of surviving cell relative to control cell. The results are the mean of three determinations ± standard error of mean. *P < 0.05 versus vehicle

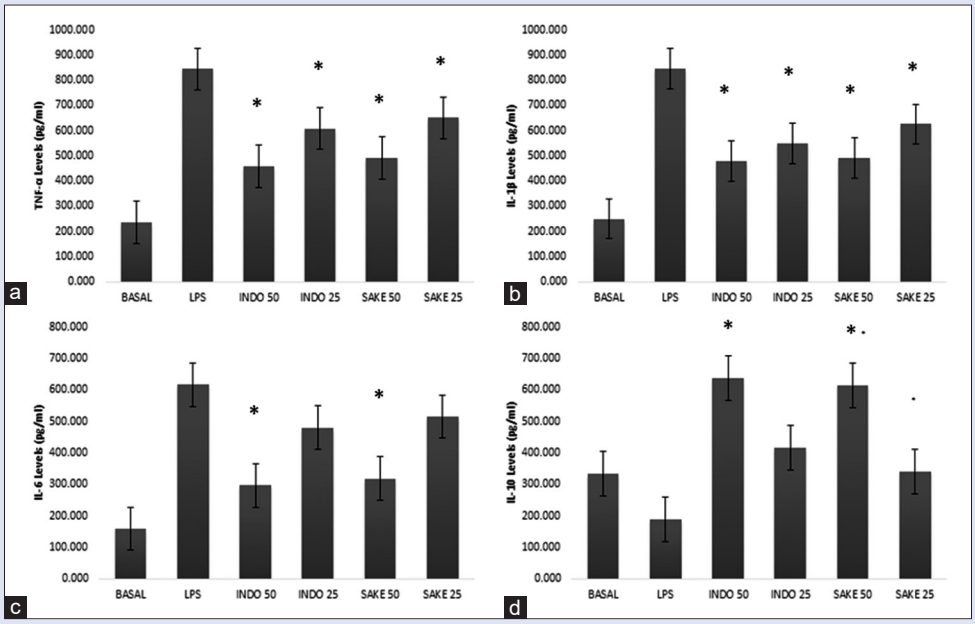

Figure 3: Effects of *Salvia keerlii* on the levels of (a) tumor necrosis factor-α, (b) interleukin-1β, (c) interleukin-6, and (d) interleukin-10 in macrophages stimulated with lipopolysaccharide. The concentration was determined by enzyme-linked immunosorbent assay. The results are the mean of three determinations ± standard error of mean. * $P < 0.05$ versus vehicle

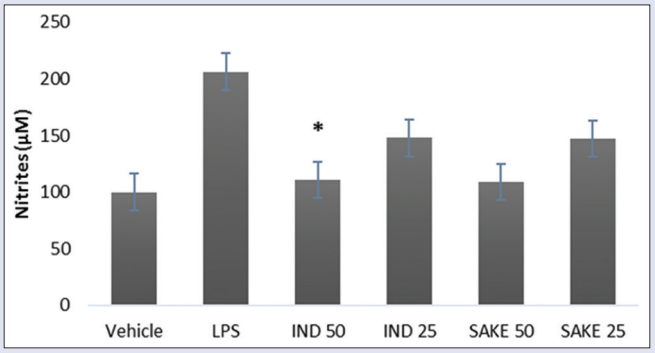

Figure 4: Effects of *Salvia keerlii* at concentrations of 25 and 50 μg/mL on the production of nitric oxide in lipopolysaccharide-stimulated macrophages. The values are the mean ± standard error of mean of three independent experiments. * $P < 0.05$ versus vehicle

similar to those found with IND. SAKE at 50 μg/mL increased IL-10 production (1.9-fold) in comparison with the control group.

Inhibitory effects of *Salvia keerlii* on nitric oxide production

When cells were exposed to 5 μg/mL LPS for 24 h, the NO concentration increased markedly [Figure 4]. SAKE at 25 and 50 μg/mL inhibited the production of NO by 28.59% and 47.12%, respectively. This effect was similar to that obtained with IND at the same concentrations (28.29% and 46.29%, respectively).

Anti-arthritic activity *in vitro*

The results of the *in vitro* anti-arthritic activity of SAKE on the inhibition of protein denaturation are shown in Table 4. SAKE exhibited a significant anti-arthritic activity in a concentration-dependent manner. SAKE at 500, 750, and 1000 μg/mL inhibited the protein denaturation by 68.23%, 72.86%, and 79.01%, respectively. Diclofenac, at the same concentrations, inhibited the protein denaturation by 84.05%, 89.50%, and 92.94%, respectively. The activity shown by SAKE (IC_{50} = 40.73 μg/mL) was lower than that obtained with diclofenac (IC_{50} = 19.43 μg/mL).

Membrane stabilization property

SAKE tested at 25, 50, and 100 μg/mL showed 75.85%, 81.15%, and 86.50%, respectively, of protection in the membrane stabilization [Table 5]. The activity of SAKE (IC_{50} = 8.68 μg/mL) was comparable with that obtained with diclofenac (IC_{50} = 5.19 μg/mL). Diclofenac tested at 25, 50, 100, and 200 μg/mL showed 89.7%, 91.63%, 92.20%, and 94.85%, respectively, of protection on membrane stabilization.

DISCUSSION

In this study, we determined the total phenolic and flavonoid content in SAKE using the Folin–Ciocalteu method and the $AlCl_3$ colorimetric method, respectively. The results showed that SAKE contains both types of secondary metabolites. In the GC-MS analysis of SAKE [Table 1], no phenolic or flavonoids were found, which could be because phenols and flavonoids with high molecular weight are very difficult to derivatize and cannot be determined by this method.

SAKE significantly inhibited (84.96%) acute TPA-induced ear edema in mice with higher activity than that obtained with IND; for this reason, the study was carried out with the chloroform extract. The topical application of TPA produces inflammation mediated by protein kinase C, the stimulation of phospholipase A_2, and cyclooxygenase.[16] Inhibition of the production of phospholipase A_2 and protein kinase C could be involved in the anti-inflammatory effect shown by SAKE.

SAKE was also tested at different doses on chronic TPA-induced edema. In this model, the infiltration of inflammatory cells, such as polymorphonuclear leukocytes, and epidermal hyperplasia occur,[17] which increase the size of the ear edema. SAKE decreased ear edema induced by multiple applications of TPA, which suggests that this extract diminishes cellular infiltration.

Table 4: Effect of *Salvia keerlii* on the *in vitro* anti-antiarthritic activity on bovine serum protein denaturation method

Group	Concentration (µg/mL)	Absorbance	Percentage denaturation	Percentage inhibition of denaturation
Control	-	0.029	100	0
Diclofenac	1000	0.0312	7.05	92.94
	750	0.0324	10.49	89.50
	500	0.0345	15.94	84.05
	200	0.0384	24.47	75.52
	100	0.0401	27.68	72.31
	50	0.0496	41.53	58.46
	25	0.0501	42.11	57.88
SAKE	1000	0.0367	20.98	79.01
	750	0.0398	27.13	72.86
	500	0.0425	31.76	68.23
	200	0.043	32.55	67.44
	100	0.0501	42.11	57.88
	50	0.054	46.29	53.70
	25	0.064	54.68	45.31

SAKE: *Salvia keerlii*

Table 5: Percent human red blood cell membrane stabilization of *Salvia keerlii*

Group	Concentration (µg/mL)	Percentage membrane stabilization
Control	-	0
Diclofenac	200	94.85
	100	92.20
	50	91.63
	25	89.70
	12.5	85.84
	1	29.47
	0.1	4.77
SAKE	200	61.50
	100	86.50
	50	81.15
	25	75.85
	12.5	70.01
	1	12.74
	0.1	1.47

SAKE: *Salvia keerlii*

The acetic acid-induced writhing test is selective for the initial screening of peripheral antinociceptive agents. Intraperitoneal administration with acetic acid induces nociception by the release of proinflammatory mediators such as prostaglandins, bradykinin, histamine, and substance *P* in the peritoneal cavity, which cause vascular permeability, resulting in abdominal contraction and elongation of limbs.[18] SAKE (ED_{50} = 74.11 mg/kg) showed a similar potency in the inhibition of nociception compared to that reported with naproxen (ED_{50} = 33.7 mg/kg).[19] The results suggest that the antinociceptive activity of SAKE could be due to the inhibition of prostaglandin synthesis.

LPS is a membrane component of Gram-negative bacteria that promotes the production of proinflammatory cytokines and NO in macrophages.[20] NO is a signaling molecule with multiple physiological effects, such as vasodilation, host defense, inflammation, and blood clotting.[21] Overproduction of NO during inflammation can activate nuclear factor-kappa B and induce the expression of proinflammatory mediators. The increased levels of NO by inducible NO synthase can result in tissue damage.[22] Thus, inhibition of NO production in macrophages is a therapeutic strategy for inflammation. In this study, SAKE significantly inhibited NO production in LPS-stimulated macrophages.

Cytokines play an important role in the inflammatory process. TNF-α is one of the main proinflammatory cytokines involved in acute inflammation,[23] and it regulates other proinflammatory cytokines such as IL-6, which is involved in the induction and prolongation of early inflammation.[24,25] IL-1β is a proinflammatory IL that plays an important role in the progression of pain,[26] whereas IL-10 is an anti-inflammatory cytokine that has a central role in the reduction of inflammation, promoting tissue protection.[27] Thus, it is highly desirable to find agents that inhibit the production of proinflammatory cytokines but increase the levels of anti-inflammatory cytokines such as IL-10. These new drugs might be effective in the treatment of inflammatory diseases.

One of the causes of rheumatoid arthritis is the denaturation of protein, which results in the formation of auto-antigens.[28] The mechanism of protein denaturation most likely involves alterations in the electrostatic hydrogen-bonding, hydrophobic, and disulfide interactions. We found that SAKE inhibited heat-induced protein denaturation, which suggests that this extract might have anti-arthritic activity.

The hemolytic effect of hypotonic solution is related to the accumulation of fluid within the cell, resulting in the breakage of the cell membrane. Lipid peroxidation by free radicals is another type of injury to human red blood cell membrane. Compounds with membrane-stabilizing properties should offer protection to the cell membrane.[29] Moreover, compounds with membrane-stabilizing properties can interfere with the release of phospholipases by decreasing the levels of inflammatory mediators.[15] SAKE exhibited a membrane stabilization effect by inhibiting the induced lysis of the erythrocyte membrane. This effect and the anti-inflammatory activity of SAKE on ear edema induced by TPA suggest that its anti-inflammatory activity may be associated with the inhibition of phospholipase release.

CONCLUSIONS

SAKE has anti-inflammatory activity in *in vivo* and *in vitro* models. SAKE diminishes the concentration of proinflammatory cytokines and increases the production of the anti-inflammatory cytokine IL-10 in macrophages stimulated with LPS. SAKE also inhibits protein denaturation and protects against membrane lysis. In addition, SAKE has an antinociceptive effect. These results suggest that SAKE could be used in the treatment of inflammatory conditions such as rheumatoid arthritis.

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Conflicts of interest

There are no conflicts of interest.

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