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*

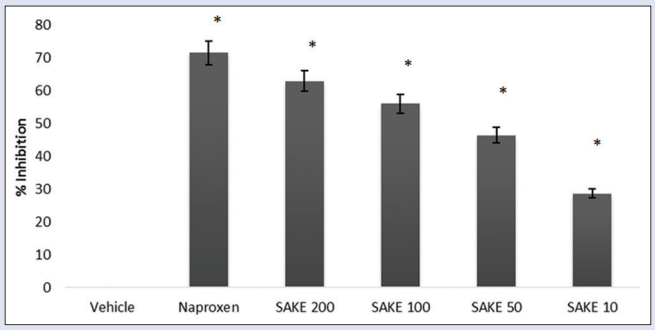

Figure 1: Effect of *Salvia keerlii* (10, 50, 100, and 200 mg/kg) and naproxen as control (100 mg/kg) on the intraperitoneal administration of acetic acid. Values are expressed as mean ± standard error of mean (n = 8). * P < 0.05 versus vehicle

(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

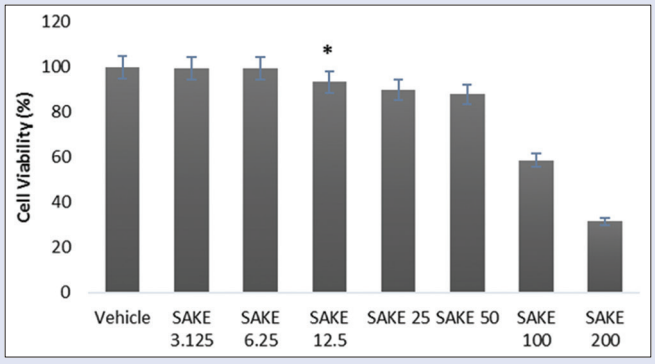

Figure 2: Effect on the cell viability of macrophages treated with *Salvia keerlii* at 3.125, 6.25, 12.5, 25, 50, 100, and 200 μg/mL. Results are expressed as the percentage of surviving cell relative to control cell. The results are the mean of three determinations ± standard error of mean. *P < 0.05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

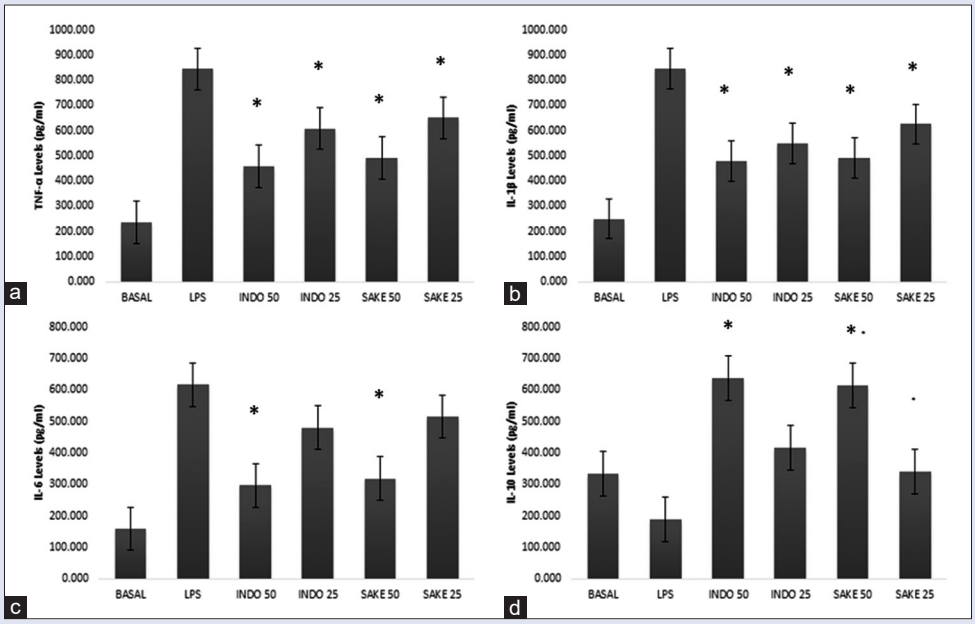

Figure 3: Effects of *Salvia keerlii* on the levels of (a) tumor necrosis factor-α, (b) interleukin-1β, (c) interleukin-6, and (d) interleukin-10 in macrophages stimulated with lipopolysaccharide. The concentration was determined by enzyme-linked immunosorbent assay. The results are the mean of three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

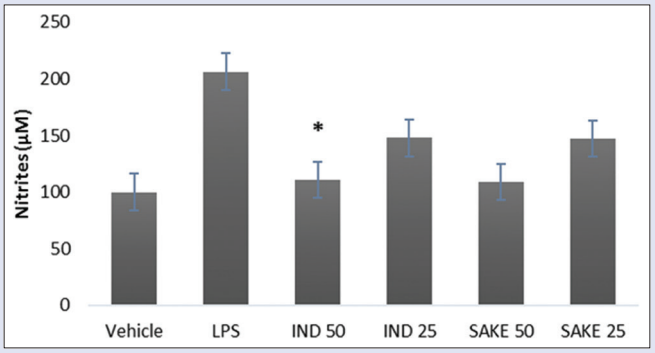

Figure 4: Effects of *Salvia keerlii* at concentrations of 25 and 50 μg/mL on the production of nitric oxide in lipopolysaccharide-stimulated macrophages. The values are the mean ± standard error of mean of three independent experiments. * $P < 0.05$ versus vehicle

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.

Acknowledgements

Serrano R was supported with a Master Fellowship Number 302033 from CONACYT.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Ljung T, Lundberg S, Varsanyi M, Johansson C, Schmidt PT, Herulf M, *et al.* Rectal nitric oxide as biomarker in the treatment of inflammatory bowel disease: Responders versus nonresponders. World J Gastroenterol 2006;12:3386-92.
2. Guo LY, Hung TM, Bae KH, Shin EM, Zhou HY, Hong YN, *et al.* Anti-inflammatory effects of schisandrin isolated from the fruit of *Schisandra Chinensis* Baill. Eur J Pharmacol 2008;591:293-9.
3. Mueller MM. Inflammation in epithelial skin tumours: Old stories and new ideas. Eur J Cancer 2006;42:735-44.
4. Rivera D, Obón C, Cano F. The botany, history and traditional uses of three lobed sage (*Salvia fruticosa* Miller) (Labiatae). Econ Bot 1994;48:190-5.
5. Esquivel B, Mendez A, Ortega A, Soriano-Garcia M, Toscano A, Rodriguez-Hahn L. Neo-clerodane-type diterpenoids from *Salvia keerlii*. Phytochemistry 1985;24:1769-72.
6. Rodríguez-Hahn L, García A, Esquivel B, Cárdenas J. Structure of kerlinic acid from *Salvia keerlii*. Chemical correlation with melisodoric acid. Can J Chem 1987;65:2687-90.
7. Singleton VL, Rossi JA. Colorimetry of total phenolic with phosphomolybdic – Phosphotungistic acid reagents. Am J Enol Vitic 1965;161:144-58.
8. Palacios-Espinosa JF, Arroyo-García O, García-Valencia G, Linares E, Bye R, Romero I, *et al.* Evidence of the anti-*Helicobacter pylori*, gastroprotective and anti-inflammatory activities of *Cuphea aequipetala* infusion. J Ethnopharmacol 2014;151:990-8.
9. Carlson RP, O'Neill-Davis L, Chang J, Lewis AJ. Modulation of mouse ear edema by cyclooxygenase and lipoxygenase inhibitors and other pharmacologic agents. Agents Actions 1985;17:197-204.
10. Xian YF, Hu Z, Ip SP, Chen JN, Su ZR, Lai XP, *et al.* Comparison of the anti-inflammatory effects of *Sinapis alba* and *Brassica juncea* in mouse models of inflammation. Phytomedicine 2018;50:196-204.
11. Berkenkopf JW, Weichman BM. Production of prostacyclin in mice following intraperitoneal injection of acetic acid, phenylbenzoquinone and zymosan: Its role in the writhing response. Prostaglandins 1988;36:693-709.
12. Lorke D. A new approach to practical acute toxicity testing. Arch Toxicol 1983;54:275-87.
13. Sun J, Zhang X, Broderick M, Fein H. Measurement of nitric oxide production in biological systems by using Griess reaction assay. Sensors 2003;3:276-84.
14. Rahman H, Eswaraiah MC, Dutta AM. *In vitro* anti-inflammatory and anti-arthritic activity of *Oryza sativa* var. Joha rice (an aromatic indigenous rice of Assam). Am Eurasian J Agric Environ Sci 2015;15:115-21.
15. Rahman H, Eswaraiah MC, Vakati K. *In vitro* studies suggest probable mechanism of eucalyptus oil for anti-inflammatory and anti-arthritic activity. Int J Phyto Pharm 2012;2:81-3.
16. Ashendel CL, Boutwell RK. Direct measurement of specific binding of highly lipophilic phorbol diester to mouse epidermal membranes using cold acetone. Biochem Biophys Res Commun 1981;99:543-9.
17. Stanley PL, Steiner S, Havens M, Tramposch KM. Mouse skin inflammation induced by multiple topical applications of 12-O-tetradecanoylphorbol-13-acetate. Skin Pharmacol 1991;4:262-71.
18. Duarte ID, Nakamura M, Ferreira SH. Participation of the sympathetic system in acetic acid-induced writhing in mice. Braz J Med Biol Res 1988;21:341-3.
19. Alonso-Castro AJ, González-Chávez MM, Zapata-Morales JR, Verdinez-Portales AK, Sánchez-Recillas A, Ortiz-Andrade R, *et al.* Antinociceptive activity of ent-dihydrotucumanoic acid isolated from *Gymnosperma glutinosum* spreng less. Drug Dev Res 2017;78:340-8.
20. Kanno S, Shouji A, Tomizawa A, Hiura T, Osanai Y, Ujibe M, *et al.* Inhibitory effect of naringin on lipopolysaccharide (LPS)-induced endotoxin shock in mice and nitric oxide production in RAW 264.7 macrophages. Life Sci 2006;78:673-81.
21. Posadas I, Terencio MC, Guillén I, Ferrándiz ML, Coloma J, Payá M, *et al.* Co-regulation between cyclo-oxygenase-2 and inducible nitric oxide synthase expression in the time-course of murine inflammation. Naunyn Schmiedebergs Arch Pharmacol 2000;361:98-106.
22. Lee JK. Anti-inflammatory effects of eriodictyol in lipopolysaccharide-stimulated raw 264.7 murine macrophages. Arch Pharm Res 2011;34:671-9.
23. Parameswaran N, Patial S. Tumor necrosis factor-α signaling in macrophages. Crit Rev Eukaryot Gene Expr 2010;20:87-103.
24. Gabay C. Interleukin-6 and chronic inflammation. Arthritis Res Ther 2006;8 Suppl 2:S3.
25. Sharma JN, Al-Omran A, Parvathy SS. Role of nitric oxide in inflammatory diseases. Inflammopharmacology 2007;15:252-9.
26. Ren K, Torres R. Role of interleukin-1beta during pain and inflammation. Brain Res Rev 2009;60:57-64.
27. Iyer SS, Cheng G. Role of interleukin 10 transcriptional regulation in inflammation and autoimmune disease. Crit Rev Immunol 2012;32:23-63.
28. Gopal TK, Sheela T, Chamundeeswari D, Reddy CU. Investigation of *in vitro* anti-oxidant, anti-inflammatory and anti-arthritic activity of aerial parts of *Securinega leucopyrus* (Willd) Muell. Indian J Res Pharma Biotech 2013;1:371-8.
29. Hossain MM, Kabir MS, Hasanat A, Kabir MI, Chowdhury TA, Kibria AS. Investigation of *in vitro* anti-arthritic and membrane stabilizing activity of ethanol extracts of three Bangladeshi plants. Pharma Innov 2015;4 (1, Part B):76.