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Original Article

Potential anti-inflammatory activity of the *Salvia fruticosa* Mill. essential oil

[Potencial actividad antiinflamatoria del aceite esencial de *Salvia fruticosa* Mill.]

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Abstract

Context: *Salvia fruticosa*, known as Greek sage, is a perennial shrub indigenous to the eastern Mediterranean. Historically, its leaves have been prized for their medicinal properties, particularly in traditional remedies for anti-inflammatory, antimicrobial, and digestive ailments.

Aims: To evaluate the anti-inflammatory properties and possible mechanisms of action of *Salvia fruticosa* essential oil (EOSF).

Methods: The essential oil was obtained from the aerial parts of the plant using hydrodistillation and then analyzed using gas chromatography and mass spectrometry. The anti-inflammatory effects of the essential oil were examined using established inflammation models, specifically carrageenan-induced paw edema and peritonitis. To investigate the anti-inflammatory effects of the essential oils, widely recognized inflammation models were employed, specifically carrageenan-induced paw edema and peritonitis. In addition, the essential oil's antioxidant activity was evaluated by measuring its ability to scavenge nitric oxide radicals and inhibit lipid peroxidation.

Results: The primary constituents of the EOSF were found to be 1,8-cineol (eucalyptol) (45.5%), β-caryophyllene (9.2%), and β-pinene (6.5%). The results of the study demonstrated that EOSF displayed a significant reduction in edema, peritonitis, myeloperoxidase activity, and NOx-peritoneal lavage concentration induced by carrageenan. Moreover, the essential oil exhibited notable inhibition of nitric oxide radical production stimulated by sodium nitroprusside. Additionally, EOSF demonstrated the ability to prevent lipid peroxidation caused by Fe^{2+} or Fe^{2+} plus H_2O_2.

Conclusions: The findings indicate that EOSF possesses anti-inflammatory properties, potentially attributed to its antioxidant capacity.

Keywords: anti-inflammatory; essential oil; rats; *Salvia fruticosa*.

Resumen

Contexto: La *Salvia fruticosa*, conocida como salvia griega, es un arbusto perenne autóctono del Mediterráneo oriental. Históricamente, sus hojas han sido apreciadas por sus propiedades medicinales, sobre todo en remedios tradicionales para dolencias anti-inflamatorias, antimicrobianas y digestivas.

Objetivos: Evaluar las propiedades antiinflamatorias y los posibles mecanismos de acción del aceite esencial de *Salvia fruticosa* (EOSF).

Métodos: El aceite esencial se obtuvo de las partes aéreas de la planta mediante hidrodestilación y luego se analizó mediante cromatografía de gases y espectrometría de masas. Los efectos antiinflamatorios del aceite esencial se examinaron utilizando modelos de inflamación establecidos, concretamente el edema de la pata y la peritonitis inducidos por carragenina. Para investigar los efectos antiinflamatorios de los aceites esenciales, se emplearon modelos de inflamación ampliamente reconocidos, concretamente el edema de pata y la peritonitis inducidos por carragenina. Además, se evaluó la actividad antioxidante de los aceites esenciales midiendo su capacidad para eliminar los radicales de óxido nítrico e inhibir la peroxidación lipídica.

Resultados: Los componentes primarios del EOSF fueron 1,8-cineol (eucaliptol) (45,5%), β-cariofileno (9,2%) y β-pineno (6,5%). Los resultados del estudio demostraron que el EOSF presentaba una reducción significativa del edema, la peritonitis, la actividad mieloperoxidasa y la concentración de NOx en el lavado peritoneal inducidos por la carragenina. Además, el aceite esencial mostró una notable inhibición de la producción de radicales de óxido nítrico estimulada por nitroprusiato sódico. Además, el EOSF demostró la capacidad de prevenir la peroxidación lipídica causada por Fe^{2+} o Fe^{2+} más H_2O_2.

Conclusiones: Los resultados indican que el EOSF posee propiedades anti-inflamatorias, atribuidas potencialmente a su capacidad antioxidante.

Palabras Clave: aceite esencial; antiinflamatorio; ratas; *Salvia fruticosa*.

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Abbreviations: COX: cyclooxygenase; EO: essential oils; EOSF: *Salvia fruticosa* essential oil; GC/MS: gas chromatography and mass spectrometry; H_2O_2: hydrogen peroxide; IL-6: interleukin-6; MDA: malondialdehyde; MPO: myeloperoxidase; NF-κB: nuclear factor *kappa*-light-chain-enhancer of activated B cells; NO: nitric oxide; NOx: nitrate/nitrite; PGs: prostaglandins; PMN: leukocytes and polymorphonuclear cells; ROS: reactive oxygen species; TBARS: thiobarbituric-acid reactive substances; TNF-α: tumor necrosis factor-alpha.

INTRODUCTION

The inflammatory process encompasses a range of pathological and physiological activities (Park et al., 2017). One notable aspect of this response is the migration of leukocytes from the bloodstream to the affected tissues, which occurs through a series of sequential steps. Inflammation and oxidative processes are closely interconnected, as they share similar pathways (Kunsch and Medford, 1999). Disruptions in these mechanisms can contribute to the development of various diseases. Furthermore, elevated levels of free radicals have been observed in different pathological conditions like cancer and ischemic disorders (Harput et al., 2012). Consequently, there is a global interest among researchers in exploring indigenous remedies and their potential effects and benefits. In the context of ethnopharmacological studies, the investigation of natural sources plays a crucial role in the search for new therapeutic agents. Additionally, essential oils (EO), which are naturally occurring in plants and primarily composed of monoterpenes, have gained widespread use in the treatment of inflammation and pain (Miguel, 2010).

Over the past few decades, there has been an increasing demand for safe and natural bioactive compounds in the field of medicine. This demand stems from the desire to avoid harmful synthetic and chemical substances in medications (Evans, 2009). As a result, there has been a surge of interest in the discovery of new, effective, and non-toxic compounds. Aromatic and medicinal plants, like the species of *Salvia*, have gained recognition for their significant therapeutic potential in treating a wide range of ailments (Michel et al., 2020). These include conditions such as pain, epilepsy, colds, bronchitis, tuberculosis, hemorrhage, and menstrual disorders. Out of the approximately 900 species of *Salvia*, only a select few hold commercial significance (Kamatou et al., 2008). One notable species is *Salvia fruticosa* Miller, which belongs to the *Lamiaceae* family and is native to the Mediterranean region (Gürdal and Kültür, 2013). This particular species, commonly known as East Mediterranean sage, is more commonly imported to the United States compared to *Salvia officinalis* (Farhat et al., 2001). In Jordan, it has been recognized as the most extensively utilized medicinal plant since ancient times (Flamini et al., 2007). In traditional medicine, herbalists often employ the aerial parts of *S. fruticosa*, either by consuming infusions internally to alleviate symptoms of colds, inflammation of the mouth and throat, coughs, and abdominal pain or by applying it externally (Boukhary et al., 2016). Extensive research has been conducted on the EO derived from *S. fruticosa*, resulting in the identification of over 100 volatile compounds in various sage species. These compounds primarily fall into the categories of monoterpenes, sesquiterpenes, diterpenes, and non-isoprenoid compounds, with thujone, camphor, and 1,8-cineole typically being the most dominant ones (Arikat et al., 2004). These compounds have been demonstrated to exert potent anti-inflammatory effects by multiple mechanisms. One of the primary anti-inflammatory mechanisms of these compounds involves the inhibition of nitric oxide (NO) production. NO is a critical mediator in inflammation, and its excessive production has been linked to various inflammatory diseases (Baricevic et al., 2001). Furthermore, compounds in *S. fruticosa* may also modulate the cyclooxygenase (COX) pathway. COX enzymes are pivotal in the synthesis of proinflammatory prostaglandins. By targeting these enzymes, *S. fruticosa* can effectively reduce inflammation (Yesil-Celiktas et al., 2010). Moreover, the flavonoids present in *S. fruticosa* can attenuate the expression of proinflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) and interleukins. These cytokines play central roles in amplifying and perpetuating inflammatory responses (Lima et al., 2005).

Furthermore, several studies have revealed that EO extracted from *S. fruticosa* (EOSF) possess pharmacological properties such as antibacterial, antioxidant, anticholinesterase, and antiproliferative activities (Pirintsos et al., 2020). Although extensive research has been conducted on the chemical compositions and antibacterial, antioxidant, anticholinesterase, and antiproliferative effects of EOSF. Despite the known anti-inflammatory mechanisms demonstrated by *S. fruticosa* , such as inhibiting NO production, modulating the COX pathway, and attenuating the expression of proinflammatory cytokines, the holistic anti-inflammatory effects of the EOSF have not been comprehensively investigated. This study seeks to bridge the existing knowledge gap by evaluating the anti-inflammatory activity of the essential oil extracted from *S. fruticosa's* aerial parts, employing classical inflammation models to discern the underlying mechanisms.

MATERIAL AND METHODS

Chemical and reagents

Tween 80, dexamethasone, carrageenan, phosphate buffer, hexadecyl-trimethylammonium bromide, o-dianisidine dihydrochloride, hydrogen peroxide (H_2O_2), Griess reagent, $FeSO_4$, trichloroacetic acid, ascorbic acid, malondialdehyde, and sodium nitroprusside were purchased from Sigma-Aldrich, UK. Diff-3 stain kit (SP300) was purchased from GCC Diagnostics, UK.

Plant material

In March 2021, the aerial parts portions of *S. fruticosa* were gathered from North Amman, Jordan (GPS coordinate; 32.0189° N, 35.8819° E). A botanist named Jamil Salam authenticated the plant material. To ensure future reference, a voucher specimen was sent to the Hashemite University herbarium in Zarqa, Jordan, and was assigned the Herbarium number HU.No. 5525. To extract the EOSF, 700 g of dried aerial parts of *S. fruticosa* underwent hydrodistillation (Charles and Simon, 1990). The extraction process employed a Clevenger-type apparatus with durations of 1, 2, and 4 hours. The highest EO yield (0.3%) was achieved after 4 hours (Alqudah et al., 2023).

Determination of essential oil composition

The EOSF was subjected to gas chromatography analysis using a Trace GC ULTRA gas chromatograph equipped with an FID detector (Nexis, Shimadzu, UK) (Tsujino and Kuwata, 1993). The chromatograph was outfitted with a VB-5 column (30 m × 0.25 mm × 0.25 µm) composed of methylpolysiloxane with 5% phenyl, and a split injection method was employed. For the mass spectrometry analysis, a Polaris Q MS mass spectrometer (Illinois, UK) with an ion-trap at 70 eV was utilized. The column temperature was programmed to start at 40°C for 2 minutes and then ramped up to 180°C at a rate of 4°C/min. Helium gas was used as the carrier gas, maintaining a constant flow rate of 1.4 mL/min. The volatile constituents of the essential oil were identified by comparing their mass spectra with those in the NIST (National Institute of Standards and Technology) library through automated processes. Quantitative data were obtained by measuring peak areas using a flame ionization detector (FID), and the areas were normalized with the assistance of an internal standard, ethyl octanoate. The analysis of the essential oil samples was performed in triplicate.

Experimental animals

Male albino rats, aged 6-8 weeks and weighing between 110 and 180 grams, were employed in this study. The rats were obtained from the animal house at the Hashemite University and were accommodated in a controlled environment with unrestricted access to food and water. A consistent 12-hour light/dark cycle was maintained. To allow the rats to adapt to the laboratory conditions, a minimum acclimation period of 2 hours was provided before commencing the experiments. Each rat was used only once to ensure the integrity of the data. All animal experiments conducted during the study adhered to the regulations and guidelines of the Ethics Committee for Animal Experiments at the Hashemite University, Jordan (IRB number: 13/7/2019/2020, 12/01/2020). The care and handling of the animals followed the ethical standards outlined by the International Association for the Study of Pain concerning the appropriate and ethical utilization of animals in pain research. Procedures that may cause more than momentary or slight pain or distress to animals should be performed with appropriate sedation, analgesia, or anesthesia, unless the procedure was justified for scientific reasons.

Acute toxicity test

The OECD Guidelines for Acute Oral Toxicity, utilizing the AOT425statPgm, version 1.0 (OECD, 2020), guided the limit dose test, which evaluated the increments of 1000 mg/kg for EOSF. From a group of 30 rats, 5 females were chosen using systematic random sampling. The weight variation among these rats did not surpass ±10% of the average starting weight of the entire group. Before being given a dose, the rats were not fed for a night. Each rat, one at a time, received a 1000 mg/kg EOSF, via a stomach feeding tube. After each dosage, the initial 5 minutes were monitored for any signs of throwing up. After this, each rat was placed in individual metabolic cages. In the initial 4 hours' post-dose, monitoring took place every 15 minutes, then every 30 minutes for the following 6 hours, and daily over the next 38 hours for short-term results. Monitoring continued for a full 14 days to observe long-term effects, including potential fatalities. Any behavioral changes, possibly due to the oral toxicity, were noted for each subject.

Assessment of *Salvia fruticosa* essential oil anti-inflammatory effect through carrageenan-induced rat paw edema model

To investigate the *in vivo* anti-inflammatory properties of EOSF, an experiment was conducted using male Sprague-Dawley rats 8 weeks old and 130‑200 g weight (Rathod et al., 2023). Before the experiment, the rats underwent a 24-hour fasting period, during

which they had unrestricted access to water. A total of six groups were formed, with each group consisting of six rats.

Group 1 served as the negative control and received the vehicle, which was administered orally as 0.2% Tween 80. Groups 2 to 5 received different concentrations of EOSF orally at doses of 10 mg/kg, 31.6 mg/kg, 100 mg/kg, and 316 mg/kg, respectively. Group 6, the positive control, received subcutaneous injections of dexamethasone at a concentration of 2 mg/kg. To assess the anti-inflammatory effect of EOSF, a rat paw edema model induced by carrageenan was employed. One hour after administering the desired concentration of EOSF or the control substances, acute paw edema was induced by injecting 0.1 ml of a 1% freshly prepared carrageenan suspension in normal saline into the right hind paw of each rat. The volume of the paw was measured before (0 h) and at 1, 2, 3, 4, 5, and 6-hour intervals following the carrageenan injection. This measurement was performed using a water displacement method with a plethysmometer (type 7140 Ugo Basile, Italy) (Choi et al., 2005). The anti-inflammatory action of EOSF was calculated as a percentage using the formula [1].

$$\text{Inhibition (\%)} = \frac{\text{paw final volume - initial volume}}{\text{initial volume}} \times 100 \qquad [1]$$

Leukocyte migration into the peritoneal cavity

A total of six male Sprague-Dawley rats 8 weeks old and 130‑200 g weight rats per group were used in the experiment (Marques et al., 2021). After one hour of oral administration of EOSF at concentrations of 10, 31.6, 100, and 316 mg/kg, rats were intraperitoneally injected with carrageenan (1% 250 µL, i.p.). Dexamethasone (2 mg/kg, s.c.) and a vehicle solution (0.2% Tween 80 orally) were used as positive and negative controls, respectively. After four hours of carrageenan injection, the rats were euthanized, and 4 mL of saline containing ethene-1,1-disulfonyl difluoride (1 mM) were injected into the peritoneal cavity to collect peritoneal lavage fluid. The resulting cell suspension was chilled on ice and centrifuged at 1500 rpm for 10 minutes. The supernatant was discarded, and the cell pellets were resuspended in 1 mL of saline. The total cell count (number of cells/mL) was determined using a Neubauer chamber, and the cells were stained with Diff-3 stain to differentiate between different types of leukocytes, including polymorphonuclear and mononuclear cells.

Measurement of myeloperoxidase activity

To assess the ability of EOSF to inhibit myeloperoxidase (MPO) enzyme activity, the paw tissue of the rats was homogenized in a mixture of 50 mM phosphate buffer (pH 6.0) and 0.5% hexadecyltrimethylammonium bromide at the end of the 4-hour edema measurement (Luo et al., 2020). The resulting supernatants were then combined with o-dianisidine dihydrochloride (0.167 mg/mL, in 50 mM phosphate buffer) and 0.005% hydrogen peroxide. The absorbance of the samples was measured at 460 nm using a spectrophotometer. The results were expressed as units of MPO (UMPO) per milligram of paw tissue. One UMPO represents the amount of enzyme that degrades 1 µmol of H_2O_2 per minute. This measurement provides an indication of the MPO enzyme activity and allows for the evaluation of the inhibitory effect of EOSF on this enzyme.

Total nitrate/nitrite (NOx) concentration

To explore the potential anti-inflammatory mechanisms of EOSF, rats were given intraperitoneal injections of carrageenan (1%, 250 µL) one hour after oral administration of EOSF at a dose of 316 mg/kg. As positive controls, some rats received subcutaneous injections of dexamethasone at a dose of 2 mg/kg, while others were given oral administration of the vehicle (0.2% Tween 80) as negative controls. After six hours of carrageenan injection, the concentration of NOx in the peritoneal lavage was measured spectrophotometrically at 546 nm using the Griess reagent (Valentim-Silva et al., 2022). The Griess reagent consists of 5% H_3PO_4, 2% sulphanilamide, and 0.1% napthylethylenediamine dihydrochloride (Bradley et al., 1982). A standard curve was constructed using known concentrations of sodium nitrite, which allows for the quantification of NOx levels in the samples. This measurement helps assess the impact of EOSF on the production of nitric oxide, which plays a crucial role in the inflammatory response.

Determination of lipid peroxidation *in vitro*

To evaluate the antioxidant capacity and free radical scavenging effect of EOSF, the inhibition of lipid peroxidation induced by $FeSO_4$ (0.145 mM) or a combination of $FeSO_4$ (0.145 mM) and H_2O_2 (0.4 M) was measured. The level of oxidized lipids was determined by quantifying the amount of thiobarbituric-acid reactive substances (TBARS) following the method described by Kizil et al. (2010). Rat liver was isolated and homogenized in a phosphate buffer (0.1% w/v in 50 mM phosphate buffer, pH 7.4). The homogenate was then incubated with different concentrations of EOSF (0.001, 0.01, 0.1, 1, 10, 100, 316 µg/mL), along with either 100 µL of 0.145 mM $FeSO_4$ or 100 µL of 0.145 mM $FeSO_4$ combined with 0.4 M H_2O_2. The mixture was incubated at 37°C for 1 hour. After incubation, 0.3 mL of the samples were mixed

with 0.6 mL of trichloroacetic acid (20% v/v) and centrifuged at 10,000 g at 4°C for 15 minutes. Subsequently, 0.5 mL of thiobarbituric acid (0.67% w/v) was added to the supernatant, and the mixture was heated at 100°C for 30 minutes. The samples were transferred to a 96-well plate in triplicate, and the absorbance was measured at 532 nm using a spectrophotometer. As a positive control, the same range of concentrations of ascorbic acid was included in the experiment. A standard curve was created by serial dilution of freshly prepared malondialdehyde (MDA), which was used to determine the TBARS concentrations in the samples.

Assessment of nitric oxide radical scavenging activity

The quantification of NO radical scavenging activity was conducted using spectrophotometry (Haida and Hakiman, 2019). Different concentrations of EOSF (0.001, 0.01, 0.1, 1, 10, 100, 316 µg/mL) were added to individual test tubes. Subsequently, 0.5 mL of sodium nitroprusside in phosphate buffer saline (10 mM) was added to each tube. All the tubes were then incubated at 37ºC for 60 minutes. Following that, an equal volume of freshly prepared Griess reagent, consisting of 5% H_3PO_4, 2% sulphanilamide, and 0.1% napthylethylenediamine dihydrochloride, was added to each tube. A control sample without the plant EO, prepared in the same manner as the test samples, was used for comparison. As a positive control, the same concentration range of ascorbic acid was utilized. Afterwards, 150 µL of the reaction mixture from each tube was transferred in triplicate to the wells of a 96-well plate, and the absorbance was measured at 546 nm. NO scavenging activity was calculated using the formula [2].

$$\text{Scavenging activity (\%)} = \left(1 - \frac{[A_{sample}}{A_{control}]}\right) \times 100 \qquad [2]$$

Where: A_{sample}: The absorbance values obtained from the samples containing plant EO. $A_{control}$: The absorbance values obtained from the control samples without any plant EO.

Statistical analysis

The data obtained was expressed as mean ± SEM and analyzed statistically using GraphPad Prism 6. Statistical analysis involved performing either one-way analysis of variance (ANOVA) or two-way ANOVA, followed by a Tukey post hoc test. A significance level of $p<0.05$ was used to determine statistical significance.

RESULTS

Chemical characterization of essential oil

Table 1 and Fig. 1S display the analysis and chemical compositions of the EOSF. The EO obtained from the plant contained a total of 19 compounds, which accounted for 99.5% of the total oil content. The predominant compounds detected were 1,8-cineol (eucalyptol) (45.5%), β-caryophyllene (9.2%), and β-pinene (6.5%).

Acute toxicity of plant essential oil

No deaths were recorded after the administration of 1000 mg/kg of EOSF, based on the short and long-term outcomes of the dose tolerance testing. Nevertheless, some behavioural signs of toxicity were observed, including tachypnoea, irritation, and restlessness. In addition, severe depression, abnormal gait, ataxia, increased respiration and decreased activity.

S. fruticosa essential oil reduces edema in carrageenan-induced rat paw edema model

Following the administration of EOSF, dexamethasone, and the vehicle, rat paw edema was induced by injecting a 0.1 mL 1% freshly prepared carrageenan suspension. Over time, an increase in paw edema was observed. However, the positive control dexamethasone (2 mg/kg) and EOSF significantly reduced rat paw edema at doses of 31.6, 100, and 316 mg/kg between 2 to 6 hours after inducing carrageenan edema, in comparison to the vehicle control. This reduction was found to be statistically significant at all-time points (Fig. 1, n = 6, $p<0.05$). The group treated with 316 mg/kg of EOSF exhibited a 65 ± 3.7% reduction in edema, while the group treated with 2 mg/kg dexamethasone showed a 76 ± 5.2% reduction, both compared to the vehicle control group, 6 hours after inducing carrageenan edema (Fig. 1, n = 6, $p<0.05$). However, the group treated with a dose of 10 mg/kg failed to reverse the edema induced by carrageenan in comparison to the vehicle control at all tested time points.

S. fruticosa essential oil reduces leukocyte migration in carrageenin-induced peritonitis model

The peritonitis induced by carrageenan was evaluated by analyzing the migration of mononuclear leukocytes (lymphocytes, monocytes, and macrophages) and polymorphonuclear leukocytes (neutrophils, eosinophils, and basophils) into the peritoneal cavity. Upon intraperitoneal injection of carrageenan, there was an influx of leukocytes into the peritoneal cavity over a 4-hour period. The positive control dexamethasone (2 mg/kg) significantly affected the migration

Table 1. Essential oil composition of *Salvia fruticosa* identified by GC/MS

No.	Substances	RI (Literature)	RI (exp.)	% Composition
1	α–Pinene	933	950	5.5
2	Camphene	947	963	3.5
3	β–Pinene	980	990	6.5
4	β–Myrcene	991	995	4.2
5	α–Terpinene	1014	1018	1.2
6	1,8-Cineol (eucalyptol)	1027	1030	45.5
7	γ-Terpinene	1057	1065	1.5
8	α-Thujone	1105	1110	0.6
9	β-Thujone	1115	1120	1.2
10	Camphor	1145	1150	5.1
11	Terpinen-4-ol	1174	1180	0.8
12	α-Terpineol	1201	1215	4.2
13	α-Terpinyl acetate	1333	1339	0.5
14	α-Copaene	1378	1390	0.2
15	β–Caryophyllene	1416	1420	9.2
16	allo-Aromadendrene	1438	1447	3.6
17	α-Humulene	1454	1460	0.6
18	β-Selinene	1488	1495	0.4
19	Viridiflorol	1592	1603	5.2
				99.5

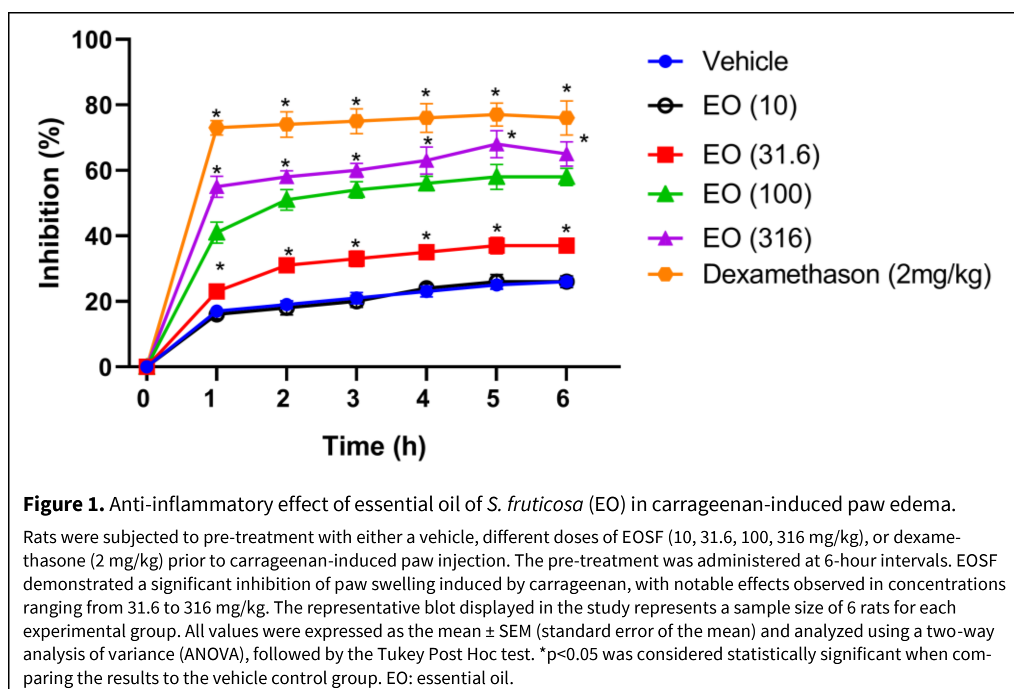

Figure 1. Anti-inflammatory effect of essential oil of *S. fruticosa* (EO) in carrageenan-induced paw edema.

Rats were subjected to pre-treatment with either a vehicle, different doses of EOSF (10, 31.6, 100, 316 mg/kg), or dexamethasone (2 mg/kg) prior to carrageenan-induced paw injection. The pre-treatment was administered at 6-hour intervals. EOSF demonstrated a significant inhibition of paw swelling induced by carrageenan, with notable effects observed in concentrations ranging from 31.6 to 316 mg/kg. The representative blot displayed in the study represents a sample size of 6 rats for each experimental group. All values were expressed as the mean ± SEM (standard error of the mean) and analyzed using a two-way analysis of variance (ANOVA), followed by the Tukey Post Hoc test. *$p<0.05$ was considered statistically significant when comparing the results to the vehicle control group. EO: essential oil.

of leukocytes and polymorphonuclear (PMN) cells, as supported by statistical analysis (Fig. 2, n = 6, $p<0.05$). Likewise, the groups treated with EOSF at doses of 31.6, 100, and 316 mg/kg exhibited a significant reduction in both total PMN cell migration (Fig. 2, n = 6, $p<0.05$) compared to the vehicle control. However,

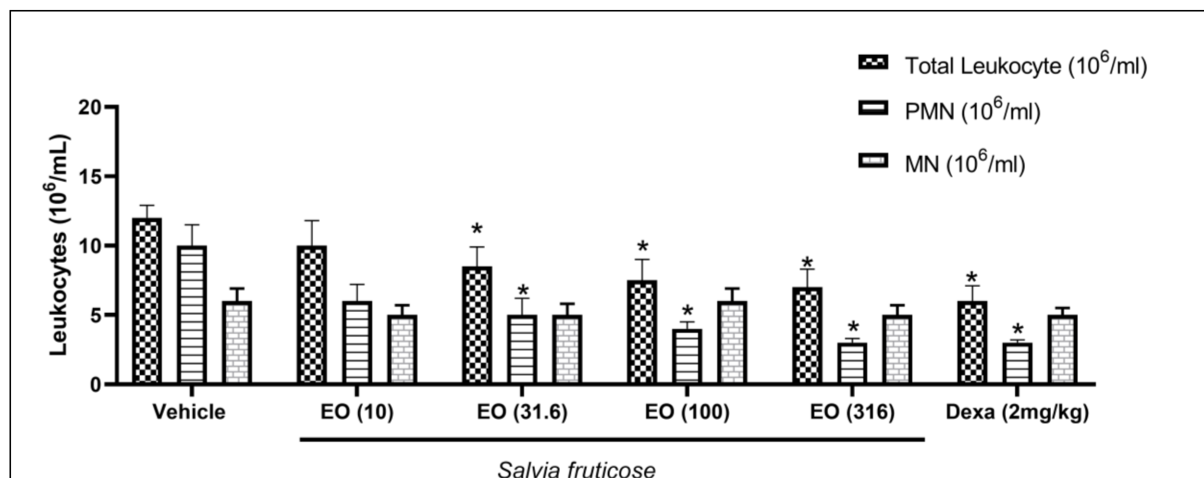

Figure 2. Anti-inflammatory effect of essential oil of *S. fruticosa* (EO) treatment on leukocyte migration.

Rats were administered different oral doses of EOSF (10, 31.6, 100, 316 mg/kg) or a vehicle containing 0.2% Tween 80, one hour prior to receiving an intraperitoneal injection of carrageenan (1%, 250 µL). Each experimental group consisted of six rats. Following the injection, intraperitoneal lavage was performed to collect inflammatory cells. These cells were subsequently evaluated to assess the anti-inflammatory effect of EOSF using a Neubauer Chamber for cell counting and Diff-3 Stain for leukocyte differentiation. All values were expressed as the mean ± SEM and analyzed using one-way analysis of variance (ANOVA), followed by the Tukey Post Hoc test. *p<0.05 was considered statistically significant when comparing the EOSF-treated groups to the vehicle control group. EO: essential oil.

Table 2. Anti-inflammatory effect of EOSF on the production of Myeloperoxidase (MPO) following carrageenan-induced paw injection in rats was examined.

Treatment	Dose (mg/kg)	UMPO/mg tissue
Vehicle	—	6.32 ± 0.60
EO	10	4.5 ± 0.50
EO	31.6	1.7 ± 0.30 *
EO	100	0.94 ± 0.20 *
EO	316	0.61 ± 0.13 *
Dexamethasone	2	0.42 ± 0.10 *

Data represent mean ± SD (n = 6). *p<0.05 was considered statistically significant when comparing the EOSF-treated groups to the vehicle control group.

neither the doses of the plant essential oil nor dexamethasone influenced the migration of mononuclear cells. These findings indicate that EOSF demonstrates anti-inflammatory effects based on the presented results.

S. fruticosa essential oil reduces myeloperoxidase activity in carrageenan-induced rat paw edema model

MPO, also known as myeloperoxidase, is an enzyme released during the degranulation of monocytes and neutrophils. Its primary role is to facilitate the oxidation of various cellular and biological structures (Frangie and Daher, 2022). Furthermore, MPO is implicated in the development of inflammation, cardiovascular diseases, and immune-mediated conditions. The significant anti-inflammatory effect of EOSF has been observed through the inhibition of MPO activity. This effect became apparent after 4 hours of carrageenin induction. Notably, concentrations of EOSF tested at 31.6, 100, and 316 mg/kg exhibited a considerable reduction in MPO activity compared to the vehicle-treated control group (Table 2, n = 6, p<0.05). Additionally, these concentrations demonstrated anti-inflammatory effects comparable to those of the reference drug dexamethasone (Table 2, n = 6, p<0.05). Conversely, the dose of 10 mg/kg of EOSF did not elicit any significant alteration in MPO activity when compared to the control group (Table 2, n = 6). Consequently, EOSF displays promising potential as an antioxidant and anti-inflammatory agent by inhibiting the activation of inflammatory cells.

Table 3. The impact of EOSF on the total concentration of nitric oxide (NOx) in peritoneal lavage following the administration of carrageenan.

Treatment	Lavage NOx – concentration
Vehicle	8.3 ± 1.8 mM
EO 316 mg/kg	4.4 ± 0.6 µM *
Dexamethasone	3.3 ± 0.4 µM *

Data represent mean ± SD (n = 6). *p<0.05 was considered statistically significant when comparing the EOSF-treated groups to the vehicle control group.

Table 4. Effect of EOSF on lipid peroxidation induced by Fe^{2+} and the scavenging activity against NO radicals.

Sample (µg/mL)	NO scavenging activity	TBARS ($FeSO_4$)	TBARS ($FeSO_4 + H_2O_2$)
Vehicle	42.2 ± 2.5	46.3 ± 1.1	67.4 ± 1.6
EO 0.001	45.1 ± 4.1	45.2 ± 1.2	69.5 ± 1.5
EO 0.01	50.2 ± 2.2*	51.6 ± 0.9*	75.4 ± 1.2*
EO 0.1	52.2 ± 3.3*	60.2 ± 1.5*	80.5 ± 2.3*
EO 1	54.3 ± 2.1*	65.7 ± 1.8*	81.3 ± 1.8*
EO 10	56.4 ± 5.2*	67.3 ± 1.1*	84.7 ± 2.3*
EO 100	61.3 ± 2.4*	73.6 ± 1.2*	85.2 ± 1.1*
EO 316	63.1 ± 3.6	74.1 ± 0.6*	88.8 ± 1.2*
Ascorbic acid 0.001	70.2 ± 2.4*	44.2 ± 1.1*	65.4 ± 0.7*
Ascorbic acid 0.01	75.3 ± 3.7*	52.6 ± 1.2*	71.3 ± 1.1*
Ascorbic acid 0.1	77.5 ± 3.3*	58.5 ± 2.3*	72.8 ± 1.2*
Ascorbic acid 1	82.6 ± 2.4*	63.6 ± 2.1*	72.3 ± 1.3*
Ascorbic acid 10	84.4 ± 1.3*	72.2 ± 2.4*	75.2 ± 1.1*
Ascorbic acid 100	86.2 ± 2.1*	76.9 ± 2.2*	77.4 ± 1.2*
Ascorbic acid 316	88.1 ± 3.1*	74.4 ± 2.1*	81.3 ± 1.1*

Data represent mean ± SD (n = 6). *p<0.05 was considered statistically significant when comparing the EOSF-treated groups to the vehicle control group.

Anti-inflammatory effects of *S. fruticosa* essential oil through inhibition of peritoneal lavage NOx-generation

Upon pretreating rats with EOSF at a dose of 316 mg/kg before intraperitoneal injection of carrageenan, a notable reduction in the concentration of total NOx was observed in comparison to the control group treated with a vehicle (as shown in Table 3, n = 6, p<0.05). Similarly, pretreating rats with dexamethasone also resulted in a significant decrease in NOx concentration, indicating that EOSF exhibits an anti-inflammatory effect (Table 3, n = 6, p<0.05).

Antioxidant activity of *S. fruticosa* essential oil

Antioxidants play a vital role in preventing oxidative damage caused by reactive oxygen species (ROS) to cellular DNA, lipids, and proteins. ROS, such as hydrogen peroxide and nitric oxide, can cause cellular damage through oxidative processes. Lipid peroxidation, initiated by free radicals, can lead to the destruction of cell membranes and eventual cell death. Therefore, the use of antioxidants to scavenge free radicals can help prevent lipid oxidation. The antioxidant activity of EOSF was assessed by comparing its ability to scavenge NO with that of ascorbic acid, which was used as a standard. The study revealed that both EOSF and ascorbic acid exhibited dose-dependent increases in scavenging capacity within the tested concentration range. EOSF demonstrated significant inhibition of NO generation from sodium nitroprusside at concentrations ranging from 0.01 to 100 µg/mL, compared to the vehicle control (Table 4, n = 6, p<0.05). The highest inhibition was observed with the highest concentration of ascorbic acid, 100 µg/mL

(Table 4, n = 6, p<0.05). To assess lipid peroxidation, the level of TBARS was measured. This measurement is based on the reaction between thiobarbituric acid and MDA, a breakdown product of lipid hydroperoxides. In this study, the effect of EOSF on lipid peroxidation induced by $FeSO_4$ (Fe^{2+}) in liver homogenate was evaluated. The results demonstrated that EOSF exhibited concentration-dependent inhibition of lipid peroxidation. All tested concentrations of EOSF (ranging from 0.01 to 100 µg/mL), as well as ascorbic acid, significantly inhibited the generation of TBARS compared to the vehicle control (Table 4, n = 6, p<0.05). This indicates that EOSF has a concentration-dependent inhibitory effect on lipid peroxidation induced by $FeSO_4$, similar to the effect observed with ascorbic acid.

DISCUSSION

By conducting experiments on well-established acute inflammatory models like paw edema and peritonitis, the anti-inflammatory properties of the EO extracted from the plant were evaluated. These research results indicate that EOSF significantly diminishes the inflammatory response in rat models.

The carrageenan-induced rat/mouse model has gained popularity as a means to assess the anti-inflammatory and antinociceptive effects of natural products, as well as to investigate the underlying mechanisms of inflammation. Carrageenan, a potent chemical, is utilized in this model to induce the release of inflammatory and proinflammatory mediators, including histamine, bradykinin, TNF-α, leukotrienes, and prostaglandins (PGs) (Ndrepepa, 2019).

The process of edema development in the hind paw of rodents following carrageenan injection is characterized by two distinct phases, each involving the activation of different mediators that contribute to the inflammatory response. In the early phase, which lasts approximately three hours, the initial mediators detected include histamine, serotonin, and 5-HT. In the late phase of inflammation, sustained levels of TNF-α, leukotrienes, and bradykinin are observed, accompanied by increased levels of NO and PGs (Li et al., 2021). These elevated levels of NO and PGs are associated with heightened vascular permeability in the injected area. It is worth noting that conventional anti-inflammatory drugs available in the market primarily target the late phase of inflammation (Brigantiand Picardo, 2003).

In our investigation, we observed a substantial and time-dependent edema in rats following the injection of carrageenan into their hind paw, with the edema reaching its peak at 4 hours. Additionally, a significant increase in levels of MPO at the 4-hour mark was noted, which serves as an indicator of neutrophil infiltration in the hind paw tissue. These findings indicate not only swelling caused by excessive plasma extravasation but also a significant influx of leukocytes in the injected area. However, when the rodents were treated with the EO derived from the plant, we observed a noteworthy reduction in both the edema and the elevated levels of MPO. These results suggest that the essential oil exerts its effects by suppressing vascular permeability (edema) and inhibiting the influx of leukocytes into the affected area.

To further validate the impact of the plant essential oil on the migration of inflammatory cells, specifically neutrophils, to the site of injury, we conducted experiments using an animal model of carrageenan-induced peritonitis. As expected, a significant infiltration of leukocytes was observed after 4 hours of carrageenan injection into the peritoneal cavity of rats. However, EOSF demonstrated remarkable inhibition of both total PMN cell migration, comparable to the effects observed with a 2 mg/kg dosage of dexamethasone. Interestingly, neither the plant EO at any of the tested doses nor dexamethasone had any impact on mononuclear cell migration. These findings confirm the anti-inflammatory properties of EOSF. It has been reported that carrageenan induces peritonitis by increasing the levels of PGs, leukotrienes, and ROS, which subsequently promote vasodilation, exudation, and recruitment of leukocytes (Barung et al., 2021). The inhibition of leukocyte and PMN migration by EOSF may be attributed to its ability to suppress the production of PGs, leukotrienes, and ROS, thereby attenuating the inflammatory response.

MPO is commonly employed as a marker to evaluate neutrophil infiltration, inflammation, and oxidative stress *in vivo* (Gunter et al., 2016). In this study, treatment with EOSF at doses of 31.6, 100, and 316 mg/kg resulted in a significant reduction in MPO activity within the inflamed tissues. This indicates that EOSF treatment may alleviate oxidative stress. Therefore, an additional potential mechanism underlying the anti-inflammatory effects of EOSF could be attributed to its antioxidative activity. In line with the anti-inflammatory effects of EOSF, rats that received a pre-treatment of 316 mg/kg of EOSF before the intraperitoneal injection of carrageenan demonstrated a notable decrease in the total concentration of NOx, which includes NO_2 and NO_3.

NO is a small molecule produced by various cells in mammals and plays crucial roles in signaling pathways within several physiological systems, including blood pressure regulation, neurotransmission, smooth muscle relaxation, and defense mechanisms (Barth et al., 2016). While NO is involved in

important functions, it can also act as a toxic agent due to its free radical properties. Elevated levels of NO have been implicated in various diseases, including cancer, diabetes, and inflammation. During the inflammatory process, there is a release and sustained presence of high levels of NO in injured tissues, contributing to inflammation in joints, lungs, and the gastrointestinal tract. Hence, plant extracts that can scavenge NO or inhibit its production may hold therapeutic potential in managing inflammatory diseases (Pace et al., 2017). In this particular study, it was observed that EOSF significantly inhibits the generation of NO radicals derived from sodium nitroprusside.

Free radicals can have detrimental effects on various biological molecules, including nucleic acids, proteins, and lipids. One such process affected by free radicals is lipid peroxidation, which is a chain reaction targeting unsaturated lipids in cell membranes. This process can result in significant changes in the biochemical properties of biomolecules and contribute to the development of various pathological conditions (Patil et al., 2019). In our study, it was observed that EOSF displayed a concentration-dependent inhibition of lipid peroxidation induced by $FeSO_4$ (Fe^{2+}) in liver homogenate. This indicates that EOSF has the ability to prevent or reduce the oxidative degradation of unsaturated lipids present in cell membranes. Furthermore, EOSF exhibited antioxidant properties by scavenging NO radicals and inhibiting lipid peroxidation in the conducted assays. These findings suggest that EOSF has the potential to act as an antioxidant agent, capable of mitigating the harmful effects of free radicals on biological molecules.

It is important to highlight that the primary constituents of EO may contribute to their biological effects. One such constituent is 1,8-cineole (also known as eucalyptol), which makes up 45.5% of the EOSF. 1,8-Cineole has anti-inflammatory and antioxidant effects mainly via the regulation of NF-κB and Nrf2, and was used for the treatment of respiratory diseases and cardiovascular (Cai et al., 2020). Another significant component of EOSF is β-caryophyllene, which constitutes 9.2% of the EO. Studies have shown that trans-β-caryophyllene possesses the ability to diminish acute inflammation and paw edema in rats induced with carrageenan (Dahham et al., 2016). Additionally, experimental investigations have demonstrated that trans-β-caryophyllene can alleviate chronic inflammation and oxidative stress by reducing the levels of proinflammatory mediators, including TNF-α, interleukin 1β, interleukin-6 (IL-6), and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Scandiffio et al., 2020). β-Pinene, accounting for 6.5 % of EOSF, is a notable component that has demonstrated anti-inflammatory activity through the suppression of MAPKs and the NF-κB pathway in mouse peritoneal macrophages (Kim et al., 2015). Finally, experimental investigations have demonstrated that α-terpineol had an inhibitory effect on IL-6 formation (Held et al., 2007).

Lastly, thujone, a compound found primarily in the essential oil of wormwood (*Artemisia absinthium*), is best known for its presence in the alcoholic beverage absinthe. Research has shown that thujone can affect the nervous system, and in higher doses, it can lead to convulsions. The European Food Safety Authority has established maximum levels of thujone content in foods and beverages is up to 35 mg/kg (Anon, 2010). The toxicity of thujone in rats, as with many substances, is often determined through studies that examine the LD_{50}. The oral LD_{50} for thujone in rats has been found to be approximately 45 mg/kg of body weight (Deiml et al., 2004). In this study, α-thunjone and β-thunjone were found in EOSF at 0.6 and 1.2%, respectively. At the dose used in assessing the acute toxicity of EOSF (1000 mg/kg), the toxicity of thujone most probably did not appear in the rats based on the percentages of thujone from the total amount of essential oil.

CONCLUSION

The present study shows evidence supporting the anti-inflammatory properties of EOSF. Furthermore, the antioxidant activity observed in the essential oil may play a role in its anti-inflammatory effects. The observed anti-inflammatory activity in our study may be attributed to certain major compounds present in EOSF. However, it is also plausible that the activity of these components is influenced by other major and minor constituents.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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AUTHOR CONTRIBUTION:

Contribution	Alqudah A	Qnais E	Gammoh O	Bseiso Y	Wedyan M
Concepts or ideas	x	x			
Design			x		
Definition of intellectual content	x				
Literature search					x
Experimental studies				x	
Data acquisition			x		x
Data analysis	x	x			
Statistical analysis			x	x	
Manuscript preparation	x	x			
Manuscript editing			x		x
Manuscript review	x	x	x	x	x

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Supplementary data

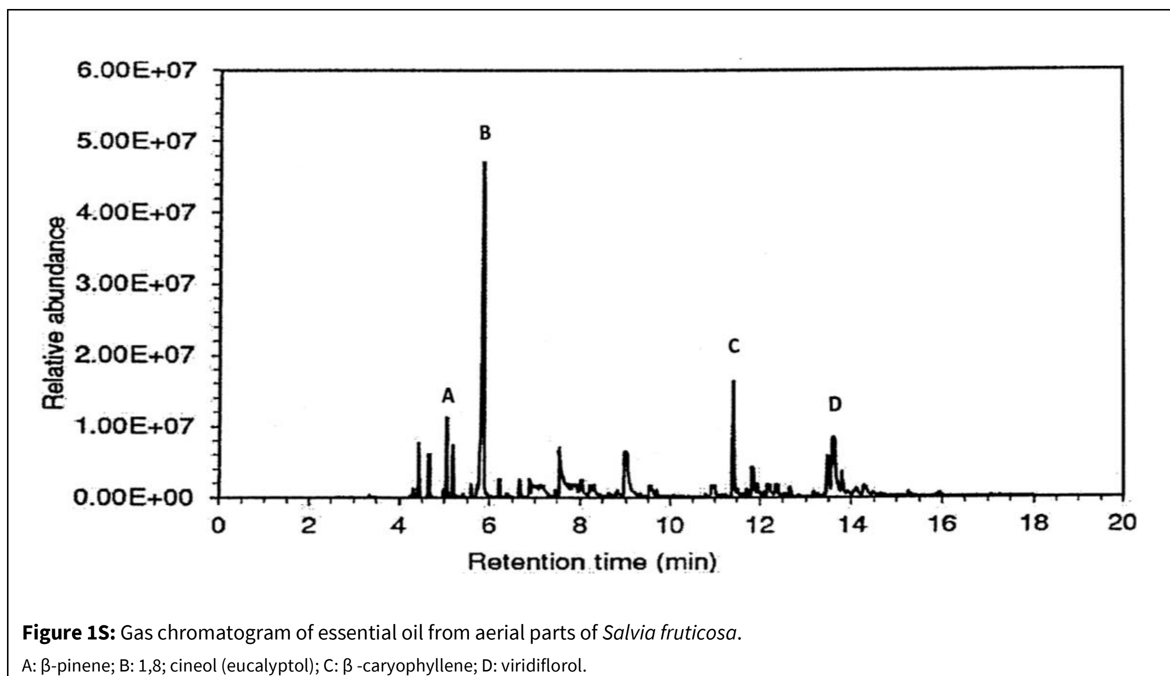

Figure 1S: Gas chromatogram of essential oil from aerial parts of *Salvia fruticosa*.

A: β-pinene; B: 1,8; cineol (eucalyptol); C: β -caryophyllene; D: viridiflorol.