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ORIGINAL ARTICLE

Salvia argentea L. extract inhibits the production of NO, and pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α), alleviates the inflammatory response of LPS-induced macrophages cells, and reduces the CRP level on carrageenan-induced paw edema

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Abstract

Salvia argentea L. (Lamiaceae) is a medicinal plant originating from the Mediterranean region and has been used since ancient times for the treatment of various diseases. This study aimed to determine the phytochemical composition of *S. argentea* L. ethanol extract and to evaluate its in vitro and in vivo anti-inflammatory activity and its acute oral toxicity. The chemical constituents of the ethanol extract prepared from the aerial parts of the plant were identified using HPLC. The in vitro anti-inflammatory activity of the extract was evaluated in LPS-stimulated murine macrophage RAW 264.7 cells and the human monocytic cell line THP-1 by measuring the levels of nitric oxide (NO), pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α). Acute toxicity of the extract was assessed in accordance with OECD guideline no 423. In vivo anti-inflammatory activity was evaluated based on the inhibition of 1% carrageenan-induced paw edema in rats. Serum CRP levels as an inflammatory marker, were measured via ELISA. Histological and immunohistochemical assessments were performed to identify tissue changes in the paw. HPLC profiling revealed that the extract contained rosmarinic acid (11.334 µg/mg dry extract), and salvigenin (2.74 µg/mg of dry extract) as major compounds. The extract significantly inhibited the production of NO, IL-1β, IL-6, and TNF-α without affecting cell viability. In vivo, the extract treatment exhibited a dose-dependent reduction in paw edema and serum CRP levels, along with notable histological improvements. Administration of the extract resulted in dose-dependent decreases of NF-κB expressions in the paw tissues. No signs of acute toxicity were observed (oral $LD_{50} > 2000$ mg/kg). These findings suggest that *S. argentea* L. ethanol extract possesses significant anti-inflammatory potential supporting its possible development as a natural therapeutic agent for inflammatory disorders.

Keywords Acute toxicity · Anti-inflammatory effect · Ethanol extract · Phytochemistry · Pro-inflammatory cytokines · *Salvia argentea* L.

Abbreviations

CRP	C-reactive protein
IFN-γ	Interferon-gamma
IL-1β	Interleukin 1-beta
IL-6	Interleukin 6
iNOS/NOS2	Nitric oxide synthase

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LPS	Lipopolysaccharides
NF-κB	Nuclear factor κB
NO	Nitric oxide
NSAIDs	Non-steroidal anti-inflammatory drugs
TNF-α	Tumor necrose factor alpha

Introduction

Inflammation is a normal physiological response to infection, injury, or disease. It is primarily mediated by immune cells and involves a complex cascade of events (Hu et al. 2016). The inflammatory process is typically accompanied by increased microvascular permeability, which facilitates the migration of immune cells to the site of inflammation, where they eliminate pathogens or foreign substances (Makanjuola et al. 2018). Macrophages play a central role in the mammalian immune response by providing rapid defense against invading pathogens (Szliszka et al. 2011). Upon activation either by interferon-gamma (IFN-γ) or bacterial lipopolysaccharides (LPS) macrophages release signals that trigger the production of various pro-inflammatory cytokines, such as prostaglandin E2, interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α), as well as inflammatory mediators like nitric oxide (NO) and reactive oxygen species (ROS) (Szliszka et al. 2013). Persistent macrophage activation results in increased nitric oxide (NO) production, which triggers the inflammatory response. This process is primarily driven by the enzyme inducible nitric oxide synthase (iNOS/NOS2). Excessive NO production via iNOS has been implicated in the pathogenesis of various inflammatory diseases, including cancer, pulmonary fibrosis, atherosclerosis, and rheumatoid arthritis (Szliszka et al. 2011; Sittisart and Chitsomboon 2014).

Current anti-inflammatory treatments largely rely on glucocorticoids, non-steroidal anti-inflammatory drugs (NSAIDs), and immunosuppressants. Although widely used to relieve pain and manage chronic inflammation, these drugs are often associated with limited efficacy or severe side effects (Wirth et al. 2024). As a result, there is a growing need for novel anti-inflammatory agents. Medicinal plants, many of which have been traditionally used to treat and prevent chronic inflammatory conditions, are considered promising sources of such bioactive compounds (Akram et al. 2015).

The genus *Salvia* L., commonly known as sage, belongs to the family Lamiaceae, a diverse botanical family comprising over 252 genera and approximately 7200 species (Ibrahim 2012). The name "*Salvia*" is derived from the Latin word "salveo", meaning "to save or to heal" (Baran et al. 2008). In alignment with its name, various *Salvia* species have long been used in traditional medicine to treat respiratory and gastrointestinal disorders and are also known for their hemostatic properties (Pieroni et al. 2004; Hachem et al. 2020; Nazım et al. 2020). In addition to their traditional uses, numerous studies have reported that extracts and isolated compounds from *Salvia* species exhibit a wide range of biological activities, including antioxidant, antimicrobial, cytotoxic, anticholinesterase, antidiabetic, neuroprotective, spasmolytic, and anti-inflammatory effects (Ibrahim 2012; Bălăşoiu et al. 2023). Phytochemical investigations on different *Salvia* species have revealed that they are rich sources of phenolics, flavonoids and their glycosides, as well as diterpenes and triterpenes, all of which contribute to their diverse pharmacological properties (Yang et al. 1996; Nakiboğlu 2002; Nikolova et al. 2006; Ben-Farhat et al. 2013a, 2015; Mirzaei et al. 2019; Hachem et al. 2020; Zare et al. 2020).

Salvia argentea L. is a Mediterranean plant species native to southern Europe (including Spain, Portugal, Italy, Sicily, Malta, and Greece), northwestern Africa (north of Algeria, Morocco, and Tunisia), and the westernmost region of Asia (Turkey) (Hachem et al. 2020). Traditionally, the leaves of this plant have been used in folk medicine for the treatment of respiratory tract infections, as well as for their hemostatic and wound-healing properties (Pieroni et al. 2004; Hachem et al. 2020; Nazım et al. 2020). Despite its widespread traditional use, only a limited number of studies have focused on the biological activities of *S. argentea* L., which have primarily reported its antioxidant, antimicrobial, cytotoxic, and anticholinesterase properties (Salah et al. 2006; Stagos et al. 2012; Ben-Farhat et al. 2013a, 2013b; Orhan et al. 2013; Bechkri et al. 2019; Benabdesslem et al. 2020; Lakhal et al. 2023). Although *Salvia* species often share similar therapeutic properties, *S. argentea* L. distinguishes itself by its dense covering of glandular hairs, which release abundant volatile oils and sticky secretions believed to contribute to its pharmacological effects (Baran et al. 2008). Phytochemical analyses of its essential oil have identified sesquiterpenes such as α-humulene, viridiflorol, and manool as major constituents (Couladis et al. 2001; Taârit Rayouf et al. 2012; Ben-Farhat et al. 2013b; Riccobono et al. 2016). Previous investigations into its phenolic profile have reported the presence of several phenolic acids (rosmarinic, gallic, caffeic, chlorogenic, ferulic, and p-hydroxybenzoic acids), flavonoids (naringenin, luteolin, apigenin and their glycosides), phenolic diterpenes, diterpenes, and triterpene saponins (Michavila et al. 1986; Bruno et al. 1987; Ben-Farhat et al. 2013b; Lakhal et al. 2014; Bechkri et al. 2019; Lakhal et al. 2023).

However, no studies to date have explored the molecular mechanisms or the potential bioactive compounds

responsible for the anti-inflammatory activity of *S. argentea* L. from Turkey. Therefore, in this study, we investigated the anti-inflammatory potential of *S. argentea* L. ethanol extract in LPS-stimulated macrophage cells and in a carrageenan-induced paw edema model in rats, along with identifying its possible active constituents.

Materials and methods

Reagents and chemicals

The murine macrophage cell line (RAW 264.7, ATCC TIB-71™) and the human monocytic leukemia cell line (THP-1, ATCC TIB-202™) were obtained from the American Type Culture Collection (ATCC, USA). Cell culture media, including Dulbecco's Modified Eagle's Medium (DMEM), RPMI-1640 medium, fetal bovine serum (FBS), penicillin/streptomycin, dimethyl sulfoxide (DMSO), trypsin–EDTA (0.25%), and phosphate-buffered saline (PBS), were purchased from Gibco (USA). Lipopolysaccharide (LPS), MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], indomethacin, carrageenan, ethanol, xylene, hematoxylin, and eosin were obtained from Sigma-Aldrich (St. Louis, MO, USA). HPLC standard compounds, including chlorogenic acid, p-hydroxybenzoic acid, caffeic acid, coumaric acid, rutin, luteolin-7-glucoside, apigenin-7-glucoside, rosmarinic acid, luteolin, naringenin, apigenin, and salvigenin, were also purchased from Sigma-Aldrich. Ethanol, acetonitrile, and formic acid for chromatographic analysis were obtained from Merck (Germany). ELISA kits specific to rat IL-1β, IL-6, and TNF-α were purchased from RayBiotech (Peachtree Corners, GA, USA). All other reagents and chemicals used in the study were of analytical grade. Ultrapure water was produced using a Milli-Q Water Purification System (Direct-Q 8 UV, Millipore, USA).

Plant material and extract preparation procedure

The aerial parts of *S. argentea* L. were collected during June 2018 from Yapagilar village, Usak Province, Turkey (38°37′10″N 29°30′43″E). The plant material was authenticated by Prof. Dr. Sura Baykan, and a voucher specimen was deposited at the Herbarium of Ege University, Faculty of Pharmacy, Department of Pharmaceutical Botany, Izmir, Turkey (IZEF no: 6679).

Dried aerial parts of the plant (25 g) were ground into a fine powder and extracted with 600 mL of 85% ethanol using an ultrasonic water bath. The extract was concentrated under reduced pressure at 40 °C using a rotary evaporator to obtain the crude *S. argentea* L. ethanol extract. The extraction yield was calculated as 37.4% (w/w).

High-performance liquid chromatography (HPLC) analysis

Preparation of the standard solutions

A stock solution of the standard compounds was prepared in ethanol at a concentration of 500 µg/mL. Calibration standards were prepared by diluting the stock solution to final concentrations of 0.5, 1.0, 2.5, 5.0, 10.0, 25.0, and 50.0 µg/mL. All standard solutions were stored at 4 °C until analysis.

Sample preparation

A portion of the extract (0.2 g) was placed into an Eppendorf tube, and 1.5 mL of ethanol was added. The mixture was vortexed for 10 min to ensure complete dissolution. The solution was then filtered through a 0.45 µm PTFE syringe filter (Chrom Fil), diluted 1:10 with the mobile phase, and injected into the HPLC system for analysis.

Equipment and chromatographic conditions

HPLC analysis of the extract was carried out using a Shimadzu LC-20 HPLC system equipped with degasser unit (DGU-20A SR), a quaternary pump (LC-20AT), auto injector (SIL-20AC HT), column oven (CTO-10AS VP), communications bus module (CBM-20A) and a PDA detector (SPD-M20A). The system was operated by Shimadzu LC solution software. The chromatographic separation was carried out on a reversed-phase C18 column (ACE 5 C18—250 × 4.6 mm i.d., 5 µm particle size). The mobile phase consisted of acetonitrile (A) and water containing 5% formic acid (B). The detection wavelength was set at 280 nm, and the column oven was maintained at 25 °C. Aliquot of 20 µL of each standard and sample solution was injected into the system and eluted by the following program at the flow rate of 1.0 mL/min: 0 min, 95% B; 10 min, 85% B; 30 min, 75% B; 35 min, 70% B; 50 min, 45% B; 55 min, 10% B; 57 min, 0% B and then it was kept at 95% B until the 67 min (Ben Farhat et al. 2013b).

Limit of detection (LOD) and Limit of quantitation (LOQ)

The LOD and LOQ for each compound were calculated based on the standard calibration curves, using the following equations:

$$\mathrm{LOD} = 3.3\sigma/\mathrm{S} \text{ and } \mathrm{LOQ} = 10\sigma/\mathrm{S}$$

where, σ is the standard deviation of the response and S is the slope of the corresponding calibration curve.

LOD and LOQ levels were found to be 0.105–0.351 µg/mL for chlorgenic acid; 0.119–0.395 µg/mL for p-hydroxy benzoic acid; 0.070–0.234 µg/mL for caffeic acid; 0.051–0.171 µg/mL for coumaric acid; 0.238–0.792 µg/mL for rutin; 0.197–0.657 µg/mL for luteoline-7-glucoside; 0.144–0.479 µg/mL for apigenine-7-glucoside; 0.073–0.242 µg/mL for rosmarinic acid; 0.224–0.745 µg/mL for leutolin; 0.090–0.300 µg/mL for naringin; 0.099–0.329 µg/mL for apigenin and 0.069–0.230 µg/mL for salvigenin, respectively.

In vitro anti-inflammatory activity

Cell culture and cell viability assay

RAW 264.7 cells were cultured at a concentration of 1×10^6 cells/mL and incubated for 48 h. The cells were maintained in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) supplemented with 10% fetal bovine serum (FBS) and 0.1% penicillin/streptomycin. THP-1 cells were cultured at a concentration of 1×10^5 cells/mL and incubated for 24 h in RPMI-1640 medium supplemented with 10% FBS and 0.1% penicillin/streptomycin. All cells were incubated in a 95% humidified atmosphere of 5% CO_2 at 37 °C.

The cytotoxic effects of the extract were evaluated by MTT assay. For the assay, cells were seeded at an initial concentration of 1×10^5 cells/mL in 96 well plates. After 24 h, various concentrations of *S. argentea* L. extract (0.5, 5, 50 and 100 µg/mg) were added to the cells. Following a 48-h incubation, 20 µL of MTT solution (from a 2.5 mg/mL stock) was added to each well and incubated for 4 h. The resulting formazan crystals were dissolved in 150 µL of dimethyl sulfoxide (DMSO), and the optical density (OD) was measured at 570 nm using a microplate reader (ThermoFisher, Scientific, USA). The cytotoxic effects of the extract were then analyzed based on these measurements.

Determination of NO scavenging activity and pro-inflammatory cytokine expressions

NO production, resulting from LPS-induced stimulation of iNOS, was measured in the supernatant of RAW 264.7 cells using the Griess reaction, as previously described by Stuehr and Nathan (1989). Cells were seeded into 96-well plates at a concentration of 1×10^5 cells/mL and incubated for 24 h. After incubation, cells were treated with various concentrations of the extract and incubated for 1 h. Subsequently, LPS was added at a final concentration of 1 µg/mL, and the cells were incubated for an additional 18 h. After the incubation period, the culture medium was collected, and Griess reagent [0.1% N-(1-naphthyl)-ethylenediamine and 1% sulfanilamide in 5% phosphoric acid] was added to the supernatant. The mixture was incubated at room temperature for 10 min in the dark. The absorbance was measured at 540 nm using a microplate reader (Thermo Fisher Scientific, USA), and nitrite concentrations were calculated based on a sodium nitrite standard curve.

For the evaluation of pro-inflammatory cytokine expression, THP-1 cells were cultured in 6-well plates at a concentration of 1×10^5 cells/mL and incubated for 24 h. Following a 48-h treatment with 15 ng/mL phorbol 12-myristate 13-acetate (PMA), the cells were washed once with culture medium and fresh medium was added. Cells were then treated with *S. argentea* L. extract in the presence of 5.0 µg/mL of LPS and incubated for 24 h. Extract concentrations were selected based on prior cytotoxicity results. The level of pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) in the supernatant were quantified using commercial ELISA kits, following the manufacturer's instructions (RayBio, Peachtree Corners, GA, USA).

Animals

The experimental procedures were approved by the Ege University, Local Ethical Committee for Animal Experiment (Date: 26.02.2020, No: 2020-022). All procedures were conducted in accordance with the ARRIVE Guideline 2.0 (Percie du Sert et al. 2020). Healthy adult BALB/c mice (weighing 20–25 g) were used for the acute toxicity test, and Wistar rats (weighing 250–280 g) were used for anti-inflammatory study. The animals were obtained from Ege University Laboratory Animal Application and Research Center and housed in groups of six per cage in stainless steel cages under controlled environmental conditions (22.0 ± 2.0 °C, relative humidity 45–65%) with a 12-h light/dark cycle at Ege University ARGEFAR. Throughout the study, the animals were fed with standard laboratory feed and water, ad libitum. They were observed daily, and body weights were recorded before and during the study period.

Single dose toxicity test

To determine potential single-dose acute toxicity of *S. argentea L.* extract, 12 female BALB/c mice (8–10 weeks old, weighing 20–25 g) were used in accordance with OECD Guideline 423 Acute Oral Toxicity (Acute Toxic Class Method) (OECD 2002). Initially, a limit dose of 2000 mg/kg body weight (bw) of *S. argentea* L. extract was administered orally by gavage to three mice. The animals were monitored for signs of toxicity and mortality for the first 30 min and again at 24 h post administration. Since no mortality was observed, the same dose was administered to an additional three mice for confirmation test. All animals were monitored daily for 14 days for clinical signs

of toxicity, including death, changes in general appearance, behavior, water and food consumption, and body weight loss.

At the end of the study, blood samples (100–150 µL) were collected via cardiac puncture under ketamine + xylazine anesthesia (50 + 10 mg/kg ip). Major organs (brain, heart, lung, liver, kidney, and spleen) were excised and weighed. Relative organ weights (organ to total body weight ratio) were calculated according to Lazic et al. (2020). Biochemical analyses were performed on serum samples using a Fujifilm FUJI DRI-CHE M NX500V IC device with a Comprehensive S Panel kit including 11 parameters (Total protein-TP, Albumin-ALB, Globulin-GLOB, Glucose-GLU, Alanine aminotransferase-ALT, Alkaline phosphatase-ALP, Total bilirubin- TBIL, Total cholesterol-TCHOL, Creatinine-CRE, Blood urea nitrogen-BUN, Calcium-Ca). An additional six mice were used as controls and received a single dose of 2000 mg/kg bw of saline via gavage. The same monitoring and experimental procedures were applied to the control group at the end of the study.

In vivo anti-inflammatory study

The anti-inflammatory activity was evaluated using the carrageenan-induced paw edema model in rats, as described by Winter et al. (1962). To induce paw edema, 100 µL of 1% carrageenan solution in isotonic saline was injected intraplantarly into the right hind paw of each rat. The contralateral paw served as a control and was injected with 0.1 mL physiological saline (0.9% NaCl). *S. argentea* L. extract was administered orally at doses of 50, 75 and 100 mg/kg, 1 h prior to carrageenan injection. Paw edema was measured using a digital water plethysmometer (UGO Basile, Italy) at baseline (0 h) and at 1, 2, 3, 4, and 5 h after carrageenan administration.

As a positive control, the anti-inflammatory test was repeated with oral administration of indomethacin (10 mg/kg body weight; Sigma Chemical Co., St. Louis, USA). The changes in paw edema volume were calculated for each group using the following equation:

$$Pawedema(\%) = \frac{(Vt - Vo)}{Vo} \times 100$$

where; Vo: the basal volume (measured before the injection), and (Vt): the pathological volume (measured at 0, 1, 2, 3, 4, and 5 h).

The percentage of anti-inflammatory effect for each group was calculated using the following equation:

$$anti\text{-}inflammatory\ effect\ (\%) = \left[1 - \frac{paw\ change\ for\ given\ time\ of\ treated\ group}{paw\ change\ for\ given\ time\ of\ control\ group}\right] \times 100$$

After the final measurement (at the 5th hour), blood samples (1 mL) were collected from all rats via cardiac puncture under ketamine + xylazine anesthesia (50 + 10 mg/kg, ip) for the measurement of C-reactive protein (CRP). Additionally, paw tissues were excised for histological and immunohistochemical evaluation.

CRP measurement

CRP levels were measured using an ELISA kit (Boster Biological Technology, CA, USA; Catalog No: EK0978), following the manufacturer's instructions.

Histological and immunohistochemical (IHC) assessment

Paw tissues were collected into Falcon tubes and washed with phosphate-buffered saline (PBS). The tissues were then fixed in 10% buffered formalin overnight. Following fixation, samples were dehydrated through a graded ethanol series (70%, 80%, 96%, and 100%) and allowed to air-dry. Tissues were cleared in xylene for 30 min until they became transparent and subsequently embedded in paraffin. Paraffin blocks were sectioned at 5 µm thickness, mounted onto glass slides, and dried overnight at 37 °C. The sections were then deparaffinized, rehydrated, and stained with hematoxylin and eosin (H&E). Histological evaluation was performed under a light microscope (Olympus BX51, Tokyo, Japan) and images were captured using an Olympus C-5050 digital camera. To detect the level of injury, sections were examined for dermal edema, inflammatory cells infiltration and connective tissue thickness. Histological scoring was performed using the following scale: (−) uninjured, (+) low injury, (++) mild injury, (+++) moderate injury, (++++) severe injury.

For IHC analysis, tissue sections were incubated with 3% hydrogen peroxide (H_2O_2; Sigma-Aldrich) for 10 min to quench endogenous peroxidase activity, followed by blocking with Super Block for 20 min at room temperature. Sections were then incubated overnight at 4 °C in a humid chamber with a primary antibody against Nuclear factor κB (NFκB) (bs-10037R, Bioss, USA) diluted 1:100. The following day, sections were incubated with a biotinylated secondary antibody, followed by streptavidin conjugated to horseradish peroxidase (HRP), each for 30 min. Immunoreactivity was visualized using a DAB chromogen + substrate solution [UltraTek HRP Anti-Polyvalent (DAB) Staining System, ScyTec Inc.] and counterstaining was performed with Mayer's hematoxylin (Sigma-Aldrich). Stained slides were examined and photographed.

Statistical analysis

Statistical analysis of the in vitro anti-inflammatory study data was performed using GraphPad Prism version 9. For the in vivo studies, data were analyzed using SPSS software version 25.0 (IBM Corp., Armonk, New York, USA). The normality of the data distribution was assessed using the Kolmogorov–Smirnov test. For the single-dose toxicity test, comparisons between groups were made using Student's *t*-test. In the carrageenan-induced paw edema model, comparisons among groups were performed using one-way analysis of variance (ANOVA) followed by the least significant difference (LSD) post hoc test. Data are presented as mean ± standard deviation (SD), and a p value of ≤0.05 was considered statistically significant.

Results

Phytochemical analysis

The retention times and concentrations (µg/mg of dry extract) of identified phenolic and flavonoid compounds in the ethanol extract of *S. argentea* L. are presented in Table 1. Rosmarinic acid (11.334 µg/mg of dry extract) and salvigenin (2.74 µg/mg of dry extract) were identified as the major compounds in the extract. HPLC chromatograms of the authentic standard compounds mixture and the plant extract are shown in Fig. 1.

Cell viability

Cell viability assays were performed using four different concentrations (0.5, 5, 50, and 100 µg/mL) of the ethanol extract of *S. argentea* L. At concentrations up to 50 µg/mL, ≥75% cell viability was observed in both RAW 264.7 and THP-1 cells, and no cytotoxic effects were detected (IC_{50}: not determined [ND]) (Figs. 2A, 3A). Based on these results, the same extract concentrations were selected for use in the in vitro anti-inflammatory assays.

NO and pro-inflammatory cytokines

The effects of *S. argentea* L extract on LPS-induced NO production in RAW264.7 cells were investigated at concentrations of 0.5, 5 and 50 µg/mL. As shown in Fig. 2B, NO production was significantly increased in LPS-stimulated cells compared to unstimulated controls. However, treatment with the extract significantly inhibited NO production in a dose-dependent manner ($R = 0.9991$; $p < 0.0001$) compared to the LPS-induced vehicle control group.

To assess the extract's effect on pro-inflammatory cytokine production, an ELISA assay was performed to measure IL-1β, IL-6, and TNF-α levels in the supernatants of LPS-stimulated THP-1 cells. As illustrated in Fig. 3B–D, stimulation with LPS markedly increased the production of all three cytokines compared to the unstimulated control. Treatment with the extract significantly reduced IL-1β production in a concentration-dependent manner ($p < 0.0001$). In addition, IL-6 and TNF-α levels were significantly decreased by the 50 µg/mL concentration of the extract ($p < 0.05$), whereas no significant changes were observed at lower concentrations. The 100 µg/mL concentration was excluded from the assay due to observed cytotoxicity in previous tests. These findings suggest that *S. argentea* L. exhibits anti-inflammatory activity by modulating the production of pro-inflammatory cytokines.

Single dose toxicity

During the acute toxicity test, no mortality or adverse effects such as changes in general appearance, behavior,

Table 1 Phenolic and flavonoid contents of the ethanol extract of *S. argentea* L

	Retention time	Identified compound	Concentration (µg/mg of dry extract)
Phenolic compounds	9.067	Chlorogenic acid	0.665
	9.450	p-hydroxy benzoic acid	0.123
	11.146	Caffeic acid	0.736
	15.100	Coumaric acid	0.282
	24.566	**Rosmarinic acid**	**11.334**
Flavonoids	16.640	Rutin	0.007
	17.546	Luteoline-7-glucoside	0.190
	22.493	Apigenine-7-glucoside	0.200
	32.152	Leutolin	0.320
	39.003	Naringen	0.097
	40.415	Apigenin	0.111
	54.161	**Salvigenin**	**2.740**

Bold font indicates major compounds detected in the extract

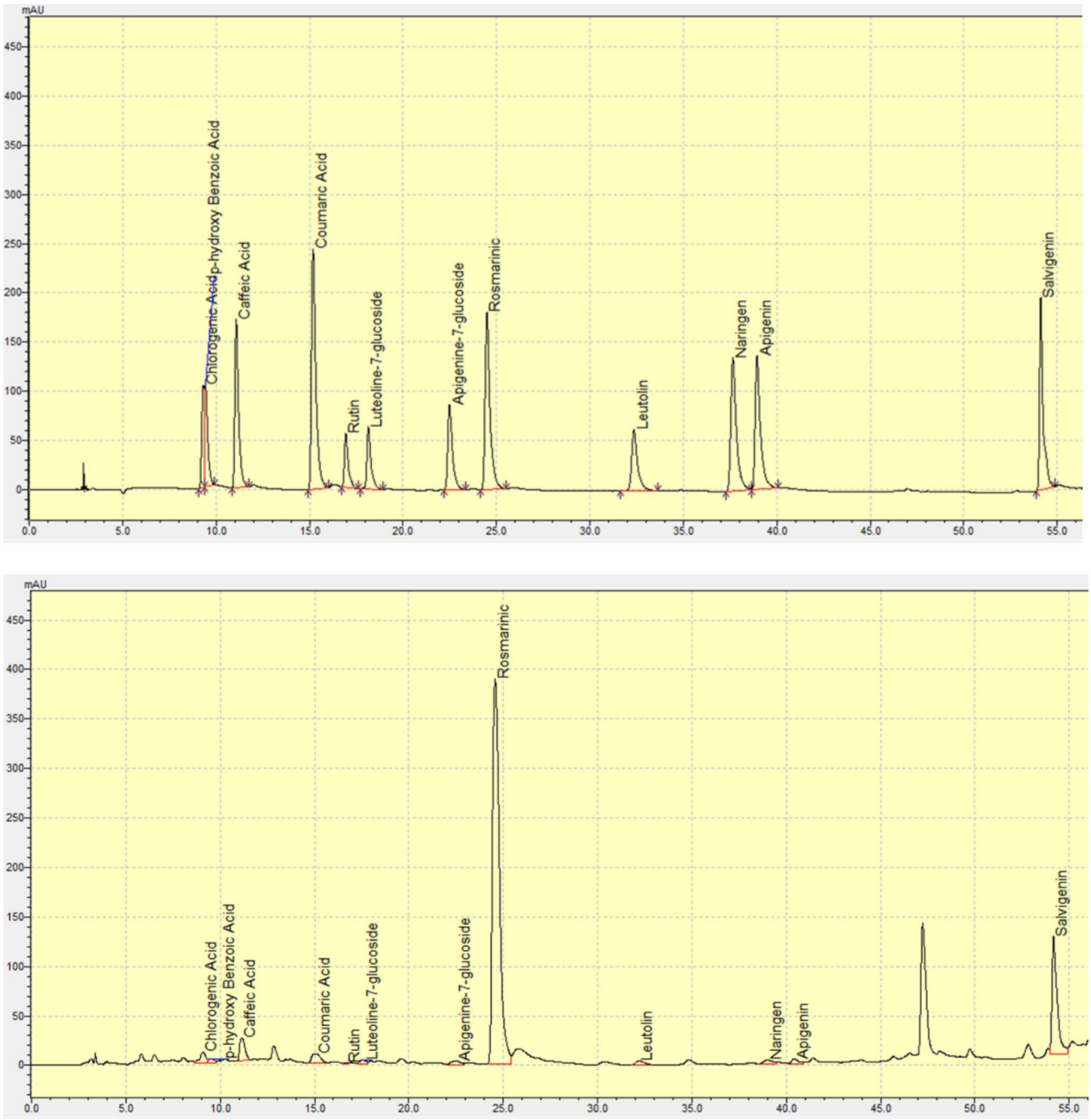

Fig. 1 HPLC chromatograms of 50.0 µg/mL standard mix solution of polyphenolics (above); HPLC chromatograms of ethanol extract of *S. argentea* L. (below)

or body weight loss were observed in the treatment groups. The acute oral LD_{50} value of *S. argentea* L. ethanol extract was >2000 mg/kg. Body weights and percentage weight gain by group are presented in Table 2. No statistically significant differences in body weight were found between the treatment and control groups. At the end of the study, absolute and relative organ (brain, heart, lung, liver, kidney and spleen) weights are shown in Table 3. No statistically significant differences in relative organ weights were observed between the treatment and control groups. Blood biochemical parameters indicated that the general health status of the treated animals remained within normal ranges (Table 4). Additionally, there were no observable differences in water or food consumption between the groups (data not shown).

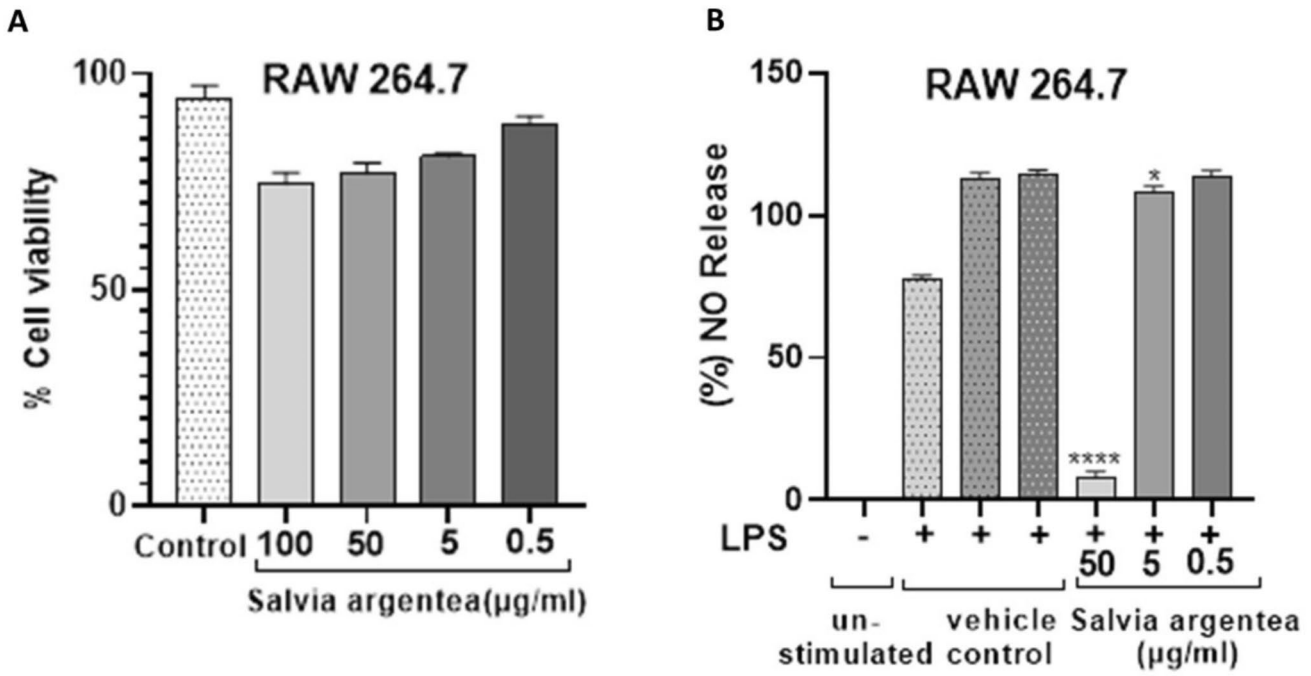

Fig. 2 **A** Effect of *S. argentea* L. ethanol extract on viability of RAW 264 cells. Cells were treated with various concentrations of the extract for 48 h. **B** Effects of *S. argentea* L. extract on NO production in LPS stimulated RAW 264.7 cells. Data are presented as mean ± SEM of three independent experiments. $*p < 0.05$, $****p < 0.0001$ compared with LPS induced control cells

Carrageenan-induced paw edema

Oral administration of *S. argentea* L. ethanol extract at doses of 50, 75, and 100 mg/kg body weight exhibited significantly greater anti-inflammatory activity compared to the control group ($p < 0.05$; Fig. 4). In the control group, paw edema peaked at 3 h post-carrageenan injection, whereas in the extract-treated groups, a reduction in paw swelling was observed as early as 2 h, indicating a recovery trend. The percentage of anti-inflammatory inhibition in the extract-treated groups compared to the control is presented in Table 5. The highest inhibitory effect was recorded at the 100 mg/kg dose, with an inhibition rate of 67.19%, while indomethacin (10 mg/kg) showed an inhibition rate of 75.13%.

In addition, ELISA analysis revealed a significant ($p < 0.05$) increase in C-reactive protein (CRP) levels in the carrageenan-injected control group. Treatment with *S. argentea* L. extract resulted in a dose-dependent reduction in CRP levels, with the most pronounced effect observed in the 100 mg/kg treatment group (Fig. 5).

Microscopic examination of H&E-stained paw sections from the physiological saline-injected control group showed a normal histological structure of the epidermis, dermis, and hypodermis layers (Fig. 6A). In contrast, intraplantar injection of 1% carrageenan induced severe inflammation, marked edema, intense inflammatory cell infiltration in the dermis and hypodermis layers, and pronounced degeneration of collagen fibers within the fibrous connective tissue of the dermis layer (Fig. 6B). Treatment with *S. argentea* L. extract reduced carrageenan-induced edema and tissue damage in a dose-dependent manner. At 50 mg/kg, moderate inflammation, edema, and inflammatory cell infiltration were observed, along with a noticeable reduction in collagen fiber degeneration in the dermis (Fig. 6C). At the 75 mg/kg dose, inflammation, edema, and inflammatory cell infiltration were significantly reduced, and collagen fibers in the fibrous connective tissue appeared normal (Fig. 6D). At the highest dose of 100 mg/kg, the histological structure of the epidermis, dermis, and hypodermis layers resembled that of the indomethacin-treated group, displaying nearly normal tissue morphology (Fig. 6E, F). Histological scores for paw tissue in each experimental group are presented in Table 6.

The effect of *S. argentea* L. extract on the protein expressions of NF-κB in paw tissues was evaluated by IHC staining. Compared to the saline-injected control group, the carrageenan-injected group showed a significant increase in paw NF-κB expressions at 5 h post-injection. However, oral treatment of *S. argentea* L. extract prior to carrageenan injection resulted in a dose-dependent reduction in NF-κB levels relative to the carrageenan-induced control group (Fig. 7). Similarly, treatment with indomethacin (10 mg/kg) significantly decreased NF-κB concentrations.

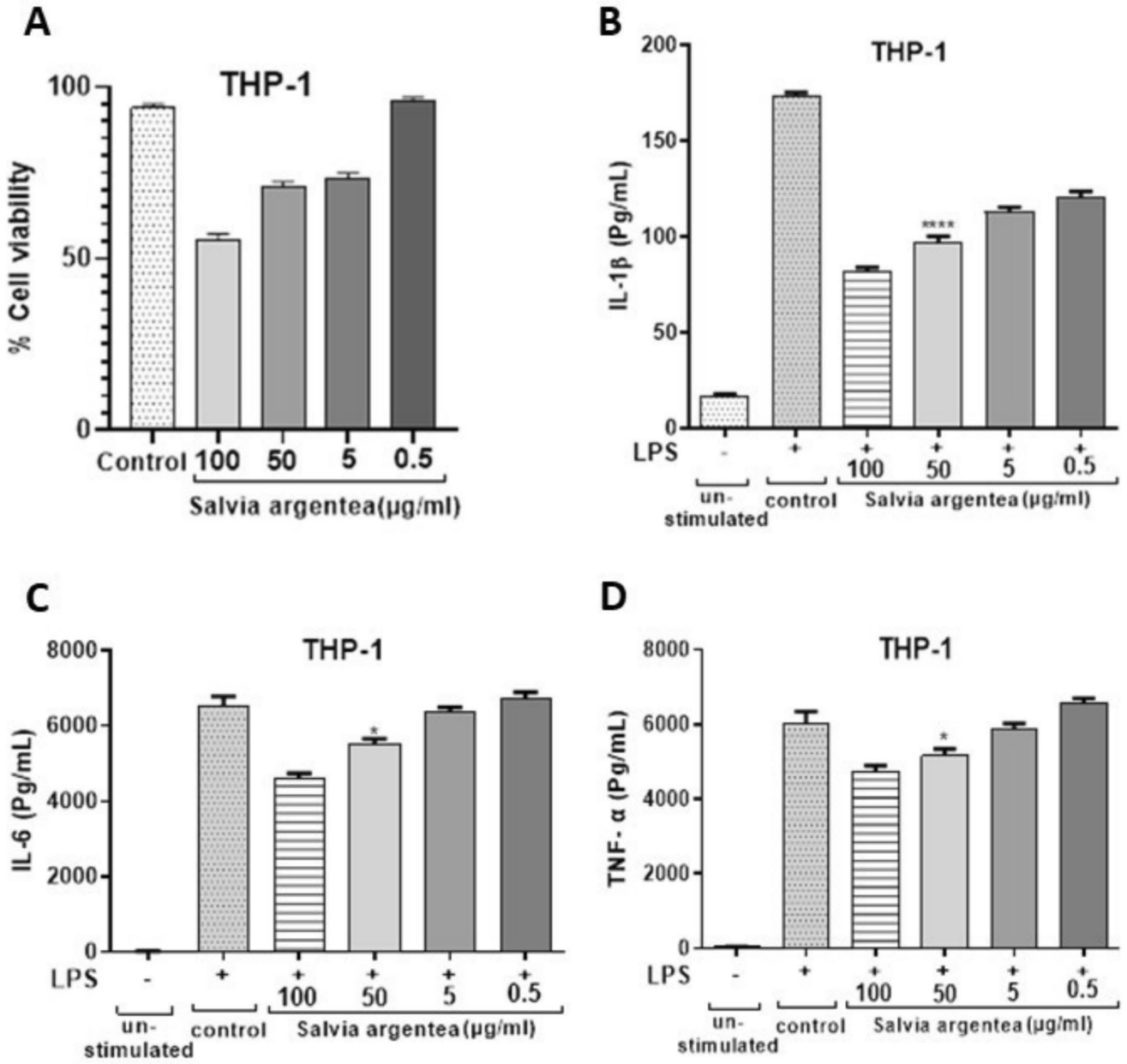

Fig. 3 **A** Effect of *S. argentea* L. ethanol extract on viability of THP-1 cells. Cells were treated with various concentrations of the extract for 48 h. **B–D** Inhibitory effect of *S. argentea* L. ethanol extract on LPS-stimulated pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) produced in THP-1 cells. Data are presented as mean ± SEM of three independent experiments. $*p < 0.05$, $****p < 0.0001$ compared with LPS induced control cells

Table 2 Total body weight values of mice after a single dose toxicity study

	Total body weight (grams) (mean ± SD)			
	Day 0	Day 7	Day 14	Body weight gain (%)
Control	22.94 ± 1.68	23.53 ± 1.26	23.96 ± 1.85	4.42 ± 0.41
S.argentea L.	23.53 ± 0.65	23.32 ± 0.32	24.38 ± 0.48	3.64 ± 0.93
P	0.601	0.794	0.721	0.256

Table 3 Organ weights of mice after a single dose toxicity study

	Organ weights and relative organ weights (grams) (mean ± SD)					
	Brain	Heart	Lung	Liver	Spleen	Kidney
Organ weights						
Control	0.40 ± 0.01	0.19 ± 0.02	0.25 ± 0.04	2.18 ± 0.07	0.16 ± 0.02	0.56 ± 0.01
S.argentea L.	0.41 ± 0.02	0.16 ± 0.01	0.20 ± 0.02	2.02 ± 0.13	0.13 ± 0.03	0.52 ± 0.03
P	0.442	0.101	0.074	0.116	0.192	0.289
Relative organ weights						
Control	0.012 ± 0.001	0.005 ± 0.002	0.006 ± 0.002	0.071 ± 0.009	0.004 ± 0.001	0.02 ± 0.001
S.argentea L.	0.015 ± 0.003	0.006 ± 0.003	0.008 ± 0.001	0.078 ± 0.008	0.005 ± 0.001	0.02 ± 0.002
P	0.577	0.334	0.208	0.115	0.528	0.264

Immunoreactivity scores for the marker in the paw tissues are shown in Table 6.

Discussion

Due to the well-documented side effects of non-steroidal anti-inflammatory drugs (NSAIDs), particularly on the gastrointestinal and renal systems, there is ongoing interest in developing new anti-inflammatory agents and in screening medicinal plants for potential activity. Plant-derived phytochemicals are widely recognized as promising sources of anti-inflammatory compounds (Gonfa et al. 2023). In this study, the ethanol extract of *S. argentea* L., a plant used traditionally for medicinal purposes, was evaluated for its anti-inflammatory potential. For the first time, both in vivo and in vitro assays confirmed the extract's anti-inflammatory activity.

The in vitro anti-inflammatory activity of *S. argentea* L. was investigated using LPS induced RAW264.7 and THP-1 macrophages models. Treatment of LPS-stimulated cells with the extract significantly inhibited NO production in a dose-dependent manner compared to the LPS control group. Similarly, the extract significantly suppressed the production of IL-1β in LPS-stimulated THP-1 human monocytic cells in a concentration-dependent manner. In addition, the levels of IL-6 and TNF-α were significantly reduced following treatment with *S. argentea* L. ethanol extract. It is known that upon activation of RAW264.7 or THP-1 cells by different stimulus including bacterial LPS, the cells release a broad range of pro-inflammatory cytokines (such as TNF-α, IL-1β, and IL-6), inflammatory mediators (e.g., NO and prostaglandins), and reactive oxygen species (ROS), all of which contribute to the development and progression of inflammatory diseases (Wadleigh et al. 2000). NO is a signaling molecule that plays a key role in the pathogenesis of inflammation. It is considered as a pro-inflammatory mediator that induces inflammation due to over production in abnormal situations (Sharma et al. 2007). NF-κB as a transcription factor is a master regulator of the inflammatory response. It regulates the transcription of a wide variety of genes that are important to the inflammatory response including chemokines, cytokines, and adhesion molecules (Hobbs et al. 2018). NF-κB mediates the induction of pro-inflammatory cytokines, such as TNF-α, IL-1 β and IL-6 in LPS-stimulated macrophages (Lawrence 2009; Guo et al. 2024). In resting cells, NF-κB resides in the cytoplasm, bound non-covalently to its inhibitory protein IκB. Upon stimulation, IκB is phosphorylated and subsequently degraded, allowing NF-κB to translocate into the nucleus, where it activates the transcription of pro-inflammatory genes, including those involved in NO production (Mao et al. 2025). One of the genes upregulated by NF-κB during an inflammatory response is the inducible nitric oxide synthase (iNOS/NOS2), that produces NO, a highly reactive free radical with important second messenger functions involving the mediation of inflammatory events. Increased expression of iNOS and concomitant NO levels have been reported in several inflammatory diseases. Macrophages are the primary producers of NO and are one of the dominant cell types to display NF-κB activation in inflammatory diseases (Jones et al. 2007; Liu et al. 2017). Therefore, the suppression of

Table 4 Effect of *S. argentea* L. extract on biochemical parameters of mice (ort ± SD)

	Group	
	Control	*S. argentea* L.
Total protein (g/dL)	4.9 ± 0.31	6.18 ± 0.40
ALB (g/dL)	2.6 ± 0.14	4.18 ± 0.37
GLOB (g/dL)	2.2 ± 0.17	2.00 ± 0.22
GLU (mg/dL)	178.0 ± 10.0	142.4 ± 31.6
ALT (U/L)	55.0 ± 3.96	50.6 ± 12.1
ALP (U/L)	81.2 ± 4.70	92.8 ± 8.38
TBIL (mg/dL)	1.1 ± 0.31	0.42 ± 0.19
TCHOL (mg/dL)	90 ± 6.89	59.6 ± 7.57
CRE (mg/dL)	90 ± 6.89	0.56 ± 0.07
BUN (mg/dL)	17.5 ± 1.29	20.9 ± 2.01
Ca (mg/dL)	8.9 ± 1.03	10.0 ± 0.49

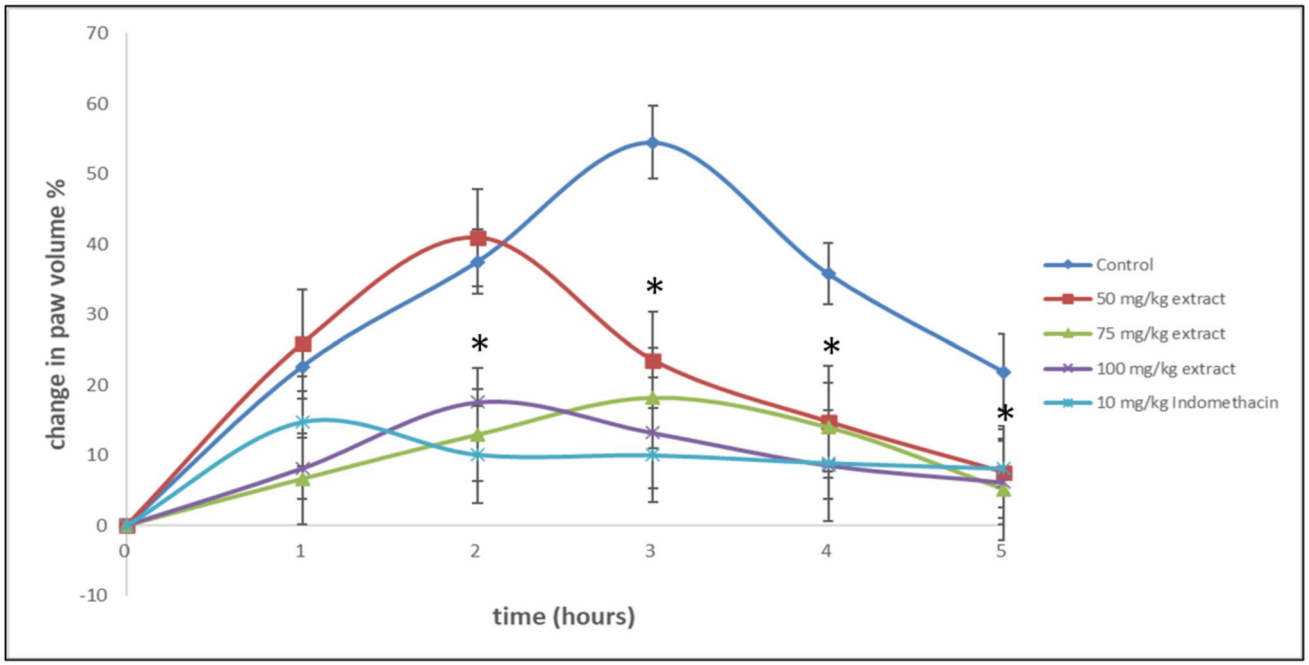

Fig. 4 Effect of *S. argentea* L. ethanol extract on the carrageenan-induced paw edema formation. The results are expressed as a percentage change from control value; each point represents the mean ± SEM of six rats per group. *$p < 0.05$ compared with the control group at respective time points

Table 5 Percent of anti-inflammatory activity in experimental groups according to control group (n = 6)

Group	Dose (mg/kg)	Paw volume (mL) (mean ± SD)		Anti-inflammatory effect (%)
		0 h	3 h	
Control (Carrageenan, 1%)	–	3.47 ± 0.04	5.36 ± 0.16	–
S. argentea L.	50	3.69 ± 0.13	4.63 ± 0.10	55.56
	75	3.77 ± 0.07	4.52 ± 0.23	60.31
	100	3.78 ± 0.07	4.40 ± 0.14	67.19
Indomethacin	10	3.74 ± 0.08	4.21 ± 0.09	75.13

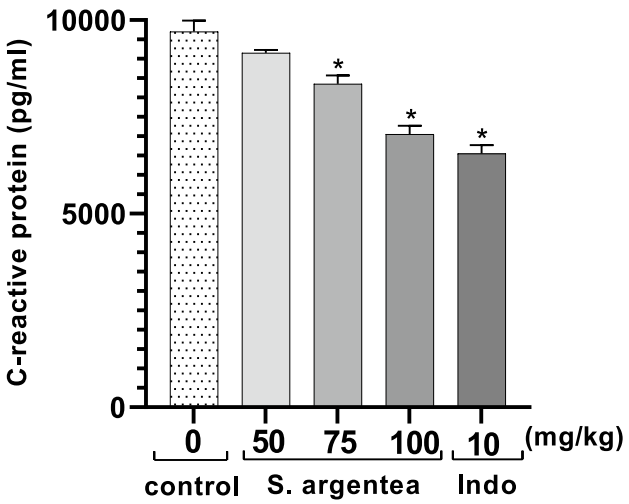

Fig. 5 Effect of *S. argentea* L. on the CRP level in rats. Data are expressed as mean ± SEM. *$p < 0.05$ compared with the control group.

pro-inflammatory cytokine expression and NF-κB signaling represents a promising strategy for the development of novel anti-inflammatory agents. Our findings demonstrated that *S. argentea* L. extract effectively inhibited LPS-induced inflammatory cytokine and NO production in a concentration-dependent manner.

Previous studies have reported that *Salvia* species are rich in polyphenolic and flavonoid compounds, which are known contribute to their antioxidant and anti-inflammatory activities (Ben-Farhat et al. 2013a, 2013b; Akram et al. 2015). In this study, twelve phenolic compounds including five phenolic acids and seven flavonoids were identified in the ethanol extract of *S. argentea* L. using HPLC analysis. Among them, rosmarinic acid (11.334 µg/mg of dry extract) and salvigenin (2.74 µg/mg of dry extract) were identified as the major constituents. These findings are consistent with previous reports on *Salvia* species collected from various countries (Adzet et al. 1988; Lu and Foo 2002; Nikolova et al. 2006; Ben-Farhat et al. 2013b). Rosmarinic acid is commonly found in members of the Lamiaceae family

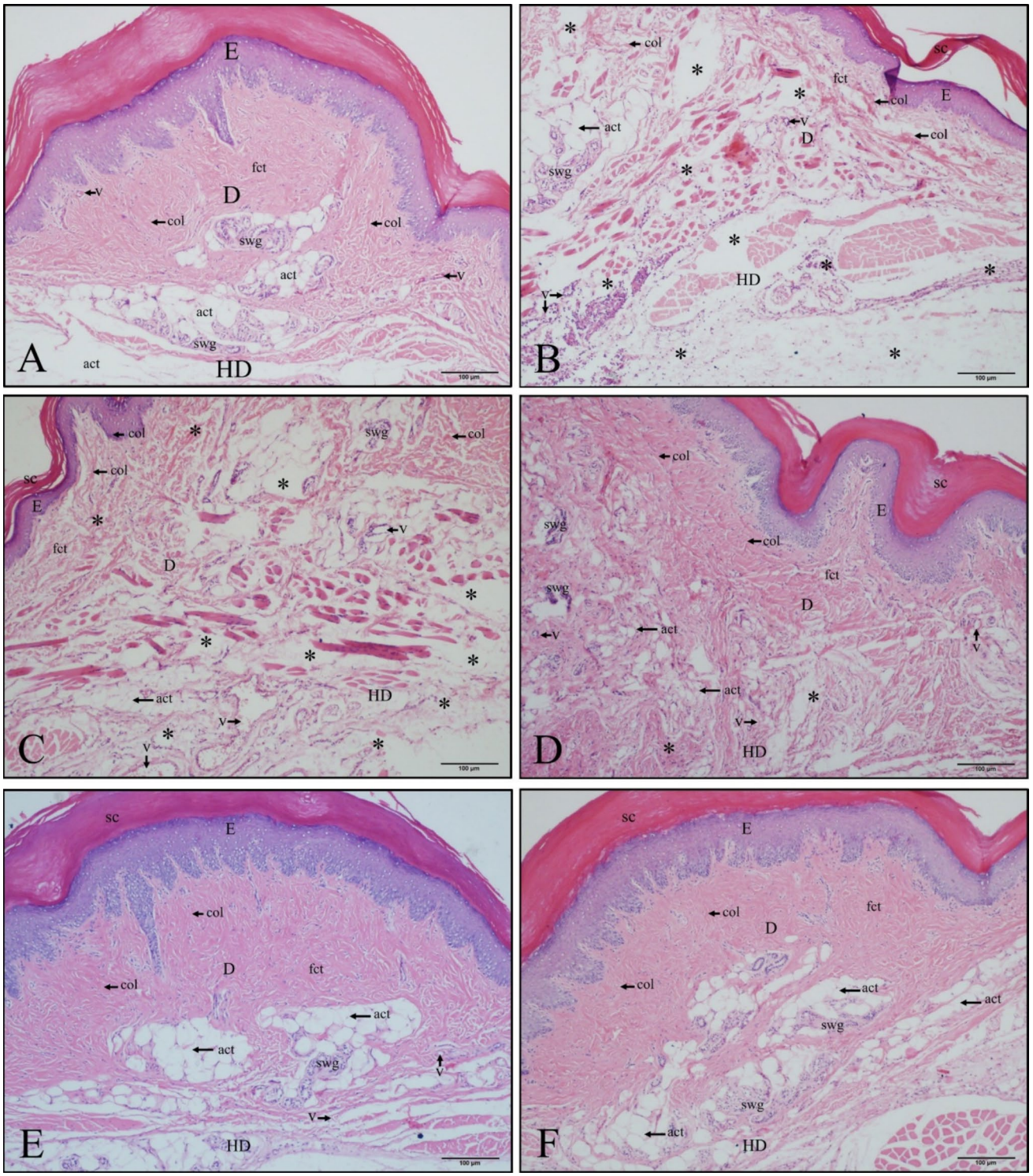

Fig. 6 Histological analysis of rat paw tissue (H&E dying, 10×). **A** Physiological saline (NaCl, 0.9%): epidermis (E), dermis (D) and hypodermis (HD) layers have normal histological structure. **B** Carrageenan treatment: while severe inflammation, edema and intense inflammatory cell infiltration (*) are observed in the dermis (D) and hypodermis (HD) layers, quite severe collagen fiber (col) degeneration is observed in the fibrous connective tissue (fct) of the dermis (D) layer. **C** *S. argentea* L. treatment (50 mg/kg.): while moderate inflammation, edema and inflammatory cell infiltration (*) are observed, a decrease in collagen fiber (col) degeneration is observed in the fibrous connective tissue (fct) of the dermis (D) layer. **D** *S. argentea* L. treatment (75 mg/kg.): inflammation, edema and inflammatory cells (*) were significantly reduced. The collagen fibers (col) in the fibrous connective tissue (fct) of the dermis (D) layer were evaluated as normal. **E** *S. argentea* L. treatment (100 mg/kg): epidermis (E), dermis (D) and hypodermis (HD) layers have normal histological structure. **F** Indomethacin treatment (10 mg/kg): all layers appear normal. sc: stratum corneum, swg: sweat gland, act: adipose connective tissue, v: vessel

(Akkol et al. 2008), while salvigenin has also been reported as one of the most abundant flavonoids in *Salvia* species (Nikolova et al. 2006). Other identified compounds, such as naringenin, apigenin, and luteolin glycosides, have also been previously reported in studies on *Salvia* species (Ben-Farhat et al. 2013b; Lakhal et al. 2023). Choi et al. (2018) demonstrated that rosmarinic acid and salvianolic acid C, isolated from *Salvia miltiorrhiza* roots, exhibit potent anti-inflammatory activity through the inhibition of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). Similarly, phytochemical studies on *Salvia bicolor* revealed high flavonoid content, particularly apigenin, luteolin-7-O-glucoside, and salvigenin, which were associated with strong antioxidant, anti-inflammatory, and analgesic effects (Ibrahim 2012). Luteolin has been shown to inhibit the production of TNF-α, IL-6, iNOS, and COX-2 in a dose-dependent manner in mouse alveolar macrophage MH-S and peripheral RAW 264.7 macrophages, primarily through suppression of redox-dependent NF-κB and AP-1 activation (Chen et al. 2007). Similarly, apigenin exhibits anti-inflammatory effects by inhibiting NF-κB activation, thereby reducing the release of inflammatory mediators (Xu et al. 2008). Rosmarinic acid, identified as a major constituent of *S. argentea* L., has demonstrated anti-inflammatory activity through modulation of the NF-κB signaling pathway in both in vitro and in vivo models (Luo et al. 2020). Salvigenin has also been reported to exert anti-inflammatory effects and inhibit nitric oxide (NO) production in LPS-stimulated RAW 264.7 murine macrophages (Kuo et al. 2011). The molecular mechanisms underlying the anti-inflammatory actions of various phytochemicals in *Salvia* species have been well-documented, revealing that many of these compounds exert their effects through the NF-κB and activator protein 1 (AP-1) pathways. Therefore, the anti-inflammatory effects of *S. argentea* L. extract observed in this study are likely attributable to the presence of these bioactive compounds, which may act through same molecular mechanisms. NF-κB and AP-1 are key transcription factors involved in the regulation of numerous physiological and pathological processes, particularly inflammation. NF-κB activation is mediated by the inducible phosphorylation and subsequent degradation of its inhibitor, IκB, via IκB kinase (IKK) activity. In contrast, AP-1 is transcriptionally regulated through the action of serum response factors (SRFs) and ternary complex factors (TCFs). Despite their distinct regulatory mechanisms, NF-κB and AP-1 pathways exhibit crosstalk, often mediated by mitogen-activated protein kinases (MAPKs). MAPK signaling not only induces AP-1 activity but has also been shown to activate IKKs in response to stimuli such as TNF-α, thereby contributing to NF-κB activation. This coordinated activation allows for potential synergistic regulation of gene expression. For instance, NF-κB activation can enhance the transcription of Elk-1 and c-Fos, leading to increased AP-1 activity. Consequently, MAPK signaling cascades may serve as a central axis in the co-regulation of NF-κB and AP-1, amplifying the inflammatory response (Fujioka et al. 2004).

The anti-inflammatory potential of *S. argentea* L. ethanol extract was evaluated in vivo using a carrageenan-induced paw edema model in rats. The results demonstrated a dose-dependent inhibition of paw swelling following carrageenan injection, along with notable improvements in the histopathological features of paw tissues. These effects are likely attributed to the extract's strong antioxidant capacity, evidenced by its DPPH-scavenging activity, and its rich phenolic and flavonoid content (Ben-Farhat et al. 2013b). Notably, Mansourabadi et al. (2015) reported that salvigenin isolated from *Salvia officinalis*, exhibited dose-dependent analgesic effects in the carrageenan-induced paw edema model in rats, supporting its potential in managing inflammation and acute pain. Likewise, rosmarinic acid has demonstrated significant anti-inflammatory activity in the same in vivo model through inhibition of NF-κB signaling and matrix metalloproteinase-9 expression (Rocha et al. 2015). The inflammatory response induced by carrageenan is characterized

Table 6 Histological and immunohistochemical scoring of paw tissues in all experimental groups

Group	Dose (mg/kg)	Histological lesions			NFκB expression
		Dermal edema	Inflammatory cells infiltration	Connective tissue thickness	
Saline (0.9% NaCl)	0.1 mL	+	−	−	−
Carrageenan (1%)	0.1 mL	++++	++++	++++	+++
S. argentea L.	50	+++	+++	++	++
	75	++	+	+	+
	100	+	−	−	−
Indomethacin	10	+	−	−	−

Histological scoring: (−) uninjured; (+) low injury; (++) mild injury; (+++) moderate injury; (++++) severe injury. Immunoreactivity scores for NFκB: −: no immunoreactivity, +: mild intensity expression; ++: moderate intensity expression; +++: very intensity expression

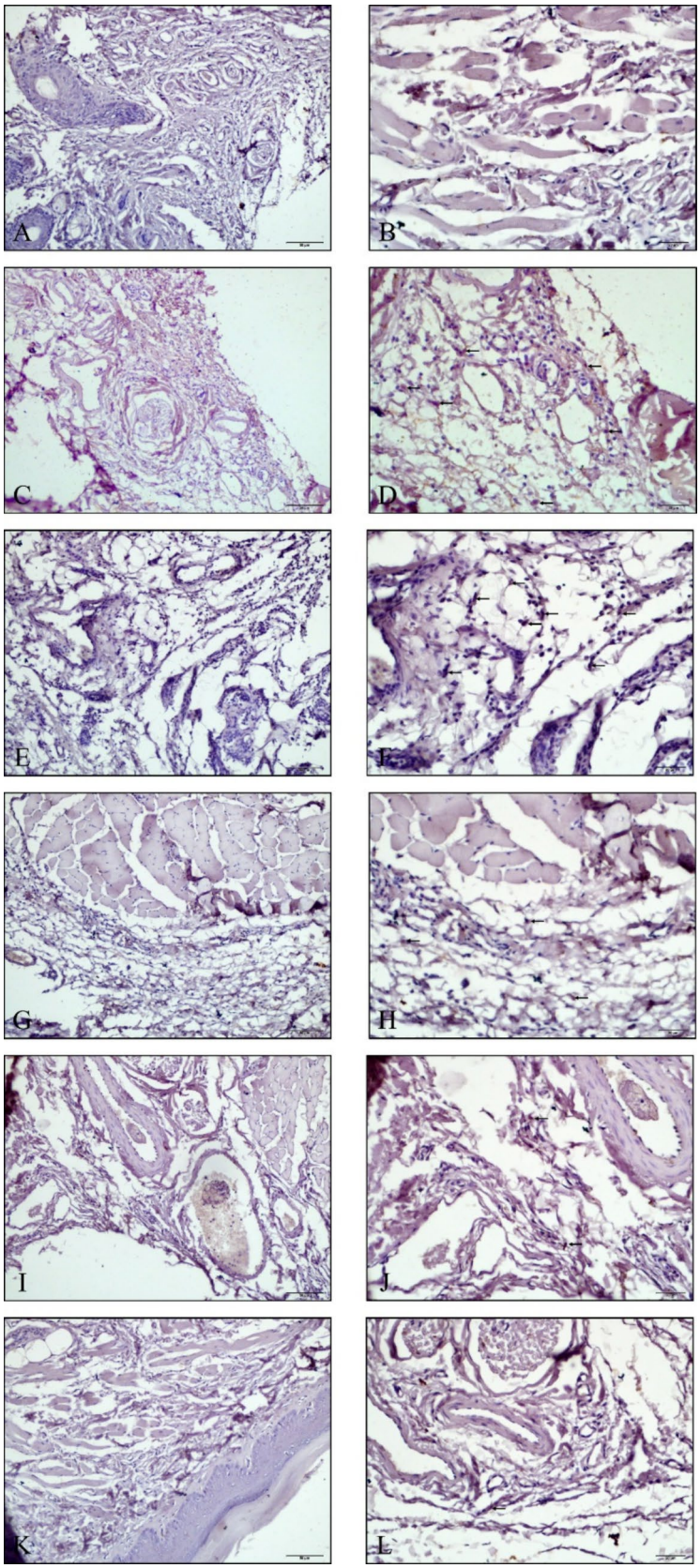

Fig. 7 Histological section of rat paw tissue (NF-κB immunohistochemistry staining). **A, B** Physiological saline (NaCl, 0.9%); **C, D** Carrageenan treatment; **E, F** *S. argentea* L. treatment (50 mg/kg.); **G, H** *S. argentea* L. treatment (75 mg/kg.); **I, J** *S. argentea* L. treatment (100 mg/kg); **K, L** Indomethacin treatment (10 mg/kg). Arrow: NF-κB expressions. Magnification: **A**, **C**, **E**, **G**, **I**, **K** ×20; **B**, **D**, **F**, **H**, **J**, **L** ×40

as an acute and local inflammatory response elicited by the injection of carrageenan, characterized by the development of paw edema and mediated initially by histamine, serotonin, and bradykinin, followed by prostaglandins and various cytokines such as IL-1β, IL-6, IL-10, and TNF-α (Mossa et al. 1995; Posadas et al. 2004). This model effectively simulates the complex physiological events involved in acute inflammation. Acute inflammation represents a rapid, transient response of the innate immune system to harmful stimuli, including microbial invasion or physical injury. It functions as a frontline defense mechanism aimed at eliminating pathogens, minimizing tissue damage, clearing necrotic debris, and initiating repair processes to restore tissue homeostasis (Mao et al. 2025). The inflammatory cascade is initiated by pattern recognition receptors (PRRs) located on resident immune cells, particularly macrophages, which upon activation, trigger the release of pro-inflammatory cytokines. Although tightly regulated, acute inflammation, if unresolved or excessively prolonged, can transition into chronic inflammation, thereby contributing to the development of various pathological conditions. It is known that NF-κB plays a central regulatory role in this process, orchestrating the expression of key inflammatory mediators and genes involved in immune response (Mao et al. 2025).

In our in vivo model, treatment with *S. argentea* L. extract significantly reduced the levels of C-reactive protein (CRP) compared to the control group. CRP levels are known to increase dramatically in response to injury, infection, and inflammation. It is the principal downstream mediator of the acute-phase response following an inflammatory event and is primarily synthesized by IL-6-dependent hepatic biosynthesis. Evidence suggests that CRP is not only just a marker of inflammation but also plays an active role in the inflammatory process (Sproston and Ashworth 2018). Elevated serum CRP levels are associated with monocyte/macrophage infiltration and act as downstream mediators of the acute-phase response, promoting the release of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α (Del Giudice and Gangestad 2018; Olson et al. 2023; Rizo-Téllez et al. 2023). Moreover, accumulating evidence suggests that elevated levels of CRP can activate the NF-κB signaling pathway, thereby contributing to the amplification of the inflammatory response (Agrawal et al. 2003; Verma et al. 2003; Sheng et al. 2009). In the present study, the significant reduction in CRP levels observed in the extract-treated groups is likely indicative of the anti-inflammatory effects exerted by the bioactive constituents of *S. argentea* L. The dose-dependent decrease in NF-κB expression further supports this effect. Notably, rosmarinic acid, a major component of the extract, is known for its ability to inhibit NF-κB activation, suggesting that the extract may suppress CRP-induced NF-κB signaling. This suppression could explain the observed reductions in inflammatory markers and the histopathological improvements, reinforcing the potential of the extract in modulating acute inflammatory responses.

Conclusion

In conclusion, our findings suggest that *S. argentea* L. extract exhibits anti-inflammatory effects by inhibiting the production of nitric oxide (NO) and pro-inflammatory cytokines, including IL-1β, IL-6, and TNF-α, as well as by reducing C-reactive protein (CRP) levels. This study highlights the presence of bioactive compounds in *S. argentea* L. and provides scientific support for its traditional therapeutic use. The proposed mechanism of action is likely related to the suppression of key inflammatory mediators. Therefore, the extract may be a promising candidate for the effective management of inflammatory responses. To our knowledge, this is the first study to report on the anti-inflammatory activity of *S. argentea* L.

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Author contribution Almonther Alhamedi: Writing–original draft, Investigation, Formal analysis, Visualization. Tugce Demiroz Akbulut: Writing–original draft, Investigation. Sura Baykan: Conceptualization, Investigation. Barış Gümüştaş: Data curation, Formal analysis, Visualization. Ebru Şancı: Investigation, Formal analysis. Karrar Ali Mohammed Hasan Alsakini: Data curation, Formal analysis, Visualization. Ayşe Nalbantsoy: Investigation, Supervision. Aylin Buhur: Data curation, Formal analysis, Visualization. Altuğ Yavaşoğlu: Writing – original draft, Data curation, Visualization. N. Ülkü Karabay Yavaşoğlu: Writing – review & editing, Project administration, Conceptualization, Methodology, Data curation, Software, Funding acquisition.

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Data availability All data was generated in-house, and no paper mill was used. All authors agree to be accountable for all aspects of work ensuring integrity and accuracy.

Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical approval The animal study protocol was approved by the Ege University, Local Ethical Committee for Animal Experiment (Date: 26.02.2020, No: 2020-022).

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