**Effect of ethanolic extract of* Stachys pilifera *Benth on subacute experimental models of inflammation and some underlying mechanisms**

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**Abstract**

**Background and purpose:** This study was designed to evaluate the anti-inflammatory activities of *S. pilifera* (HESP) in two sub-acute models of inflammation and clarified some possible mechanisms.

**Experimental approach:** Colorimetric methods were used to determine total phenol and flavonoid contents. Carrageenan or formalin-induced rat paw edema (seven days) and multiple application TPA-induced ear edema in mice (9 days) were used. The concentration of IL-1 and TNF-α were measured in the inflamed paw, as well as MDA levels in the serum and liver. Histopathological studies and *in vitro* anti-inflammatory effects of the extract were also studied using heat- or hypotonicity-induced hemolysis in RBC humans.

**Findings/Results:** Total phenol and flavonoid contents of HESP were 101.35 ± 2.96 mg GAE/g extract and 660.79 ± 10.06 mg RE g extract, respectively. Oral (100 and 200 mg/kg) and topical applications (5 mg/ear) of HESP significantly inhibited formalin-induced paw edema and multiple TPA-induced ear edema. The extract also significantly decreased the serum and liver levels of MDA in the carrageenan and formalin tests. The elevated levels of TNF-α and IL-1β in the carrageenan-injected paw were not affected by HESP. The extract (50-800 µg/mL) inhibited heat- or hypotonicity-induced hemolysis. Histopathological examination of the inflamed tissues revealed that HESP inhibited congestion and leukocyte infiltration.

**Conclusion and implications:** The findings confirmed the potent anti-inflammatory effects of *S. pilifera* in two sub-acute inflammation models and suggested that these properties were not related to IL-1 and TNF-α, but could be attributed to inhibition of lipid peroxidation, membrane stabilization, and inhibition of leukocyte penetration.

**Keywords:** Anti-inflammatory; IL-1beta; *Stachys pilifera* Benth; TNF-alpha; TPA.

**INTRODUCTION**

Inflammation is a natural healing process for controlling pathogenic agents that cause swelling, redness, heat, and pain. The pathophysiology of inflammation is complex and initiates in response to various harmful factors, such as infections, chemicals, toxins, injury, trauma, or any other tissue damage (1). Various studies have been reported that inflammation helps to eliminate harmful factors and repairs tissue damage, but persistent inflammation can lead to a variety of chronic diseases including diabetes, cardiovascular disease, tumor, Alzheimer's disease, etc. (2,3).
Nowadays, chemical drugs such as various glucocorticoids and nonsteroidal anti-inflammatory drugs (NSAIDs) are usually prescribed for treatment or management of inflammation, but serious complications such as gastrointestinal bleeding, liver and kidney dysfunction, osteoporosis, and skin reactions to some extent limit their use (4). Therefore, research on complementary medicine, especially herbal medicine, has often attracted the attention of researchers as a cost-effective treatment with lower side effects (5-6).

Stachys pilifera Benth (S. pilifera) is a shrub, perennial, and aromatic, belonging to the Labiatae family, which is often grown in high mountain regions (7). In Iranian folk medicine, the leaves of the plant are used in the management of viral, gastrointestinal, rheumatoid, and inflammatory problems (8). The antimicrobial, antitumor, and antioxidant properties of this plant have also been reported in previous documents (8,9). A study by Firuzi et al. confirmed the inhibitory effects of hydroalcoholic extract of S. pilifera Benth, against neurotoxin-induced apoptosis (10). Hepatoprotective, nephroprotective, and antiproliferative effects of the plant have also been reported in other studies (11-13).

Our previous study confirmed the anti-inflammatory effects of S. pilifera in some acute animal models of inflammation (14). The results of that study showed that S. pilifera inhibited acute inflammation in two animal models, including carrageenan-induced paw edema in rats and 2-O-tetradecanoylphorbol-13-acetate (TPA)-induced ear edema in mice. In that study, possible anti-inflammatory mechanisms of the plant, as well as the effect of the plant in subacute and chronic models of inflammation, were not investigated. Therefore, the main aim of the present study was to evaluate the anti-inflammatory effects of S. pilifera in two subacute animal models of inflammation, including formaldehyde-induced arthritis in rats and multiple TPA application-induced mouse ear edema as well as some possible mechanisms involved in these effects.

**MATERIALS AND METHODS**

**Chemicals**

Carrageenan λ (Fluka Chemical, Switzerland) was prepared in isotonic saline solution (1% w/v). Formalin, trichloroacetic acid (TCA), a standard solution of malondialdehyde (MDA), aprotinin A, and thiobarbituric acid (TBA) were purchased from Merck Co. (Darmstadt, Germany). Ethylenediaminetetraacetic acid (EDTA), bovine serum albumin (BSA), phenylmethylsulfonyl fluoride, aprotinin A, benzethonium chloride, tween 20, and TPA were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). To measure biochemical parameters, including interleukin (IL)-1β and tumor necrosis factor (TNF)-α, corresponding kits were purchased from ALPCO, USA and R & D Company, USA, respectively.

**Extract preparation**

The aerial parts of S. pilifera were collected from the suburbs of Yasuj, Iran. This plant was identified by Dr. A. Jafari from the Department of Botany, Natural Resources and Animal Husbandry Research Center, Yasuj University, Yasuj, Iran, where a specimen (Voucher No. 1897) was stored. The collected plant was dried in the shade away from sunlight.

The dried plant powder (200 g) was soaked in 1000 mL of EtOH-H2O (7:3) and then kept in an incubator at 37 °C for 48 h (13). At the next step, the mixture was filtered and ethanol was completely evaporated by a rotary evaporator at 46 °C, under vacuum conditions (Hyedolph, type: Heizbad Hei-VAP, Germany) (14). Then, the concentrated extract was dried at room temperature and was stored for the next experiments. The average yield ratio of the hydroethanolic extract was about 20.5%.

**Experimental animals**

In the present study, adult healthy Wistar male rats (200-250 g), and healthy adult male mice (NMRI, 25-35 g) were used. The animals were purchased from the Pasteur Institute of Iran, Tehran, Iran. The animals were kept in standard plastic cages, with free access to standard food and water, at a temperature of 21 ± 3 °C, with 12/12-h dark/light (17). All studies on laboratory animals were approved by the Ethics Committee of Yasuj University of Medical Sciences (Ethical code: IR-YUMS.REC1394.28) and conducted following the Principles of Laboratory Animal Care (NIH Publication No. 86-23).
Determination of total phenol content in ethanolic extract

The Folin-Ciocalteu method was used to measure the total phenol content of HESP (18). First, 5 mL of Folin-Ciocalteu was mixed with 50 μL of the extract (200 μg/mL), and then 10 mL of a saturated solution of Na₂CO₃ was added. The mixture was vortexed for 15 s and incubated for approximately 30 min in a dark place. Subsequently, the optical absorption of the tubes was read at 760 nm, using a Cecil UV/Vis spectrophotometer. Also, for creating the standard curve, gallic acid was prepared at different concentrations. The experiments were repeated three times and the mean was reported. The total phenol content of the HESP (expressed in mg of gallic acid per gram of the extract) was calculated based on the standard curve of gallic acid using the following equation:

\[ \text{Absorbance} = 0.0008 \times \text{gallic acid (mg)} \]

Determination of total flavonoid content of the ethanolic extract

The flavonoid content of the extract was measured, based on a colorimetric assay of aluminum chloride (19), with minor modification. A 250 μL of the extract was added to 150 μL of a 5% NaNO₂ solution. The solution was incubated for 6 min at room temperature. Then, 300 μL of a 10% solution of AlCl₃ was added to the mixture and again incubated for 5 min. Subsequently, 1500 μL of 1 M NaOH was added to the solution and the final volume of the solution was brought to 5000 μL by adding distilled water. After 15 min of incubation, the absorbance of the solution was read at 510 nm. The tests were repeated three times and the mean was reported. Flavonoid content was calculated and reported as the equivalent of one milligram quercetin per gram of the extract, using standard curves.

Carrageenan-induced paw edema

A similar technique was used for inducing inflammation as previously described (20,21). The animals were randomly divided into four groups of the normal saline (carrageenan, 10 mL/kg, p.o), extract (100 and 200 mg/kg, p.o), and indomethacin group (10 mg/kg, p.o) (12). The inflammation was induced by subplantar injection of 0.1 mL of carrageenan 1% (w/v) suspension in the right hind paw. The paw thickness from the anterior to posterior surfaces was recorded, using a digital caliper (Mitutoyo, Japan), just before the injection of carrageenan, and 4 h after carrageenan injection. After measuring the paw thickness, the blood samples were collected to measure MDA. Finally, the animals were euthanized with excess ether and injected carrageenan paw tissue was collected to assess TNF-α and IL-1β levels.

Formaldehyde-induced arthritis in rats

The rats were randomly divided into four groups of six, including normal saline (10 mL/kg, p.o), HESP (100 and 200 mg/kg, p.o), and indomethacin (10 mg/kg, p.o.) groups. The inflammation was induced in all animals, through subplantar injection of 0.1 mL freshly prepared 2% formalin in the right hind paw (22). A reminder dose of formalin was given on the third day. The paw thickness was measured daily for seven days. On the seventh day, the rats were euthanized and the inflamed paws were collected and fixed in 10% formalin for histological examination. The animal livers were also dissected to measure the MDA levels.

Mouse ear edema induced by multiple topical applications of TPA

The mice were randomly grouped into three groups six. Inflammation was induced by multiple topical applications of TPA (2.5 μg/20 μL acetone), on both sides of the mouse right ear, every other day (23). The animals received the vehicle (acetone), HESP (5 mg/ear dissolved in acetone), and dexamethasone (0.05 mg/ear, as a standard drug) topically twice daily, once in the morning immediately after TPA administration, and six hours later for four consecutive days. On the fifth day, the compounds were used only in the morning. Six hours after the last treatment, the animals were euthanized by ether and each animal's ear (6 mm in diameter) was punched and weighed. The punched pieces were fixed in 10% formalin for further histological examination. The level of edema was reported based on weight gain (mg) of the punched piece from the inflamed ear, compared to the weight of the healthy earpiece.
Human red blood cell membrane stabilization tests

Preparation of erythrocyte suspension

Fresh blood (10 mL) was collected from a human subject (without the use of anti-inflammatory drugs for two weeks before the study). The blood collected was subsequently centrifuged for 10 min at 3000 rpm and the supernatant was carefully removed. The residual packed erythrocytes were washed in 0.9% w/v normal saline. Steps of washing and centrifugation were repeated until obtaining a clean supernatant. Then, erythrocytes were re-dissolved by isotonic phosphate-buffered saline (PBS) solution, as a 10% v/v suspension.

Heat-induced hemolysis

Five hundred μL of the extract (50, 100, 200, 400, and 800 μg/mL) was added to the same volume of RBC (10%) and the solution was warmed at 56 °C for 30 min. After centrifuging the solution (2500 rpm for 10 min), the supernatant was gathered and absorption was recorded at 560 nm (24).

Hypotonicity-induced hemolysis

Five mL of either hypotonic sodium phosphate buffer solution (50 mM NaCl in 10 mM sodium phosphate buffer, pH 7.4) or hypotonic buffered solution of the extract (50, 100, 200, 400, and 800 μg/mL) were added to 0.5 mL RBC suspension (10%). Then, the solution was incubated for 30 min at room temperature. After that, the mixture was centrifuged for 10 min at 3000 rpm and the absorption of the supernatant was measured at 540 nm (24).

Measurement of the IL-1β and TNF-α concentration in carrageenan-treated rat paws

Four h after subplantar injection of carrageenan, rats assigned for the evaluation of cytokines were euthanized, and the paw subcutaneous tissue was removed. The tissue samples were weighed, frozen in liquid nitrogen, and stored at -70 °C, until further measurement of TNF-α and IL-1β levels. Levels of TNF-α and IL-1β in the inflamed paws were measured using enzyme-linked immunosorbent assay (ELISA), as previously described elsewhere (18). The skin tissue was homogenized in PBS (pH = 7.4), containing 0.4 M NaCl buffer, 0.5% BSA, 0.05% Tween20, 0.1 mM benzethonium chloride, 0.1 mM phenylmethylsulfonyl fluoride, 10 mM EDTA, and aprotinin A. The homogenates were centrifuged at 12000 g for 30 min at 4 °C, and then the supernatant was used to measure TNF-α and IL-1β using ELISA.

Assessment of serum and liver MDA levels

MDA concentration was measured in the serum of carrageenan-treated rats and the liver formalin-treated rats (25) according to our previous study.

The liver tissue was homogenized with normal saline to produce a 10% (w/v) liver homogeneous. Homogenates were centrifuged for 30 min at 18000 g. A total of 0.5 mL of homogenate was mixed with 2 mL of TBA-TCA reagent. The mixture was heated (15 min), then cooled (10 min), and centrifuged for 15 min at 2000 g. The absorbance was read at 532 nm and presented as μmol/g wet tissue.

To evaluate serum MDA level, 0.5 mL serum was combined with 2 mL TCA-TBA-HCl solution. The mixture was then heated (15 min) and centrifuged (2500 g, 15 min). The absorption of supernatant was read at 535 nm against a blank. The concentration of serum MDA was expressed as nmol/mL.

Histopathological study

After euthanizing of animals, three samples of the inflamed paws (formalin test) and ears (TPA test) were randomly collected and fixed in 10% formaldehyde solution for one week. Then, the fixed tissue was molded using paraffin, and sliced with 3-4 μm thickness. The slices were placed on glass slides, stained with hematoxylin and eosin to assess the histopathological alterations such as infiltration of leukocytes and subepidermal edema in all experimental groups.

Statistical analysis of data

The data are reported as mean ± SEM. The Kolmogorov-Smirnov test was performed to ensure the normality of the distribution, the differences between the test groups and the
control group were analyzed using one-way ANOVA, and subsequently, Tukey’s post hoc test was performed using SPSS 16 software. $P < 0.05$ was considered statistically significant in all comparisons.

**RESULTS**

**Total phenolic and flavonoid contents**

The total phenol content of HESP was about 101.35 ± 2.96 mg GAE/g extract and the flavonoid content of the extract was 660.79 ± 10.06 mg RE/g extract. The results are reported as mean ± SEM, of three independent optical absorption readings.

**Carrageenan-induced paw edema**

The full data of carrageenan test reported in our previous work (14), as shown in Fig. 1, sub-plantar injection of carrageenan-induced significant paw edema, 1 and 4 h after the carrageenan injection. Oral administration of the plant extract (100 and 200 mg/kg), 45 min before sub-plantar carrageenan challenges noticeably inhibited the paw inflammation, when compared to the saline group ($P < 0.001$).

**Formaldehyde induced arthritis in rats**

As shown in Fig. 2, sub-plantar injection of formalin-induced a significant paw inflammation, over 7 days. Oral administration of the plant extract (100 and 200 mg/kg), 45 min before sub-plantar injection of formalin considerably inhibited the formalin-induced paw inflammation when compared to the formalin group ($P < 0.001$). As expected, indomethacin (10 mg/kg), as a positive control significantly decreased paw inflammation, compared with the control group ($P < 0.001$).

**Mouse-ear edema induced by multiple topical applications of TPA**

As shown in Fig. 3, topical administration of the HESP (5 mg/ear) simultaneously with TPA reduced inflammation of the ear. Statistical analysis of the data showed that the dose of 5 mg/ear of the extract of the plant significantly inhibited ear edema, compared with the TPA group ($P < 0.001$). As expected, dexamethasone, as a positive control (0.05 mg/ear) also significantly decreased ear inflammation ($P < 0.001$).
Fig. 3. Effect of HESP on multiple topical applications of TPA-induced ear edema in mice. HESP (5 mg/ear), and dexamethasone (0.05 mg/ear) were topically administered twice daily, once in the morning immediately after using TPA, and for the second time 6 h after TPA, for four sequential days. The data were expressed as mean ± SEM. ***P < 0.001 indicates significant differences compared to the TPA group. HESP, Hydroalcoholic extract of Stachys pilifera; TPA, 2-O-tetradecanoylphorbol-13-acetate.

Fig. 4. Effect of HESP on IL-1β concentration in rat paws. HESP (100 and 200 mg/kg), indomethacin (10 mg/kg), and normal saline were orally administered 45 min before sub-plantar injection of carrageenan. The data were expressed as mean ± SEM. ***P < 0.001 indicates significant differences compared to the saline group. HESP, Hydroalcoholic extract of Stachys pilifera; IL-1β, interleukin-1β.

Table 1. Effect of HESP on heat- and hypotonicity-induced hemolysis in human red blood cells. The data expressed as mean ± SEM. *P < 0.05, **P < 0.01 and ***P < 0.001 indicate significant differences compared to the hydrocortisone group.

| Groups          | Concentrations (µg/mL) | Inhibition of hemolysis (%) |
|-----------------|------------------------|----------------------------|
|                 |                        | Heat                      | Hypotonicity               |
|                 |                        | 50                        | 28 ± 1.16**                | 8 ± 0.54                   |
|                 |                        | 100                       | 43 ± 0.83***               | 13 ± 0.09*                 |
|                 |                        | 200                       | 62 ± 0.72***               | 16 ± 1.018*                |
|                 |                        | 400                       | 75 ± 1.23***               | 10 ± 0.58*                 |
|                 |                        | 800                       | 89 ± 1.94***               | 2 ± 0.01                   |
| Hydrocortisone  | 200                    | 12 ± 0.98                 | 9 ± 0.07                   |

HESP, Hydroalcoholic extract of Stachys pilifera.

Effect of HESP on heat-induced hemolysis in human erythrocytes

The plant extract significantly inhibited heat-induced hemolysis in human erythrocytes. The HESP doses of 50, 100, 200, 400, and 800 µg/mL could inhibit hemolysis in a dose-dependent manner (P < 0.01 and P < 0.001). The inhibition of all four doses was greater compared to the hydrocortisone (200 µg/mL) inhibition, used as the standard drug (Table 1).

Effect of HESP on hypotonicity-induced hemolysis in human erythrocytes

Different doses of the HESP inhibited the hypotonicity-induced hemolysis in human red blood cells in a dose-independent manner. However, the inhibitory level of the hypotonicity-induced hemolysis was less compared to the heat-induced hemolysis. Also, doses of 100, 200, and 400 µg/mL of the extract had a significantly higher inhibitory effect (P < 0.05) compared with hydrocortisone (200 µg/mL) as a standard drug (Table 1).

Effect of HESP on IL-1β in carrageenan-treated paws

As shown in Fig. 4, 4 h after sub-plantar injection of carrageenan in the rat paw, a significant increase in IL-1β level was observed in the carrageenan-treated paw, compared to the normal paw. HESP (100 and 200 mg/kg) did not change the elevated level of IL-1β. Indomethacin (10 mg/kg) also did not affect the increased IL-1β level due to carrageenan injection.
Effect of HESP on TNF-α in carrageenan-treated paws

Sub-plantar injection of carrageenan significantly increased TNF-α concentration in the carrageenan-treated paw compared with saline-injected. As shown in Fig. 5, oral administration of the plant extract (100 and 200 mg/kg), 45 min before the sub-plantar injection of carrageenan, did not affect the increased level of TNF-α due to carrageenan injection. Indomethacin (10 mg/kg) also did not affect the TNF-α paw levels.

Effect of HESP on serum MDA level in carrageenan-treated paws

Serum MDA levels significantly increased 4 h after the sub-plantar injection of carrageenan (Fig. 6). HESP (100 and 200 mg/kg) reduced serum MDA levels significantly (P < 0.001) compared to the carrageenan group. Indomethacin (10 mg/kg) did not change the elevated MDA level.

Effect of HESP on the liver MDA levels in the formalin test

The liver MDA levels significantly raised after 7 days of treatment with formalin. HESP (100 and 200 mg/kg) significantly reduced the MDA level (P < 0.01), compared with the formalin group (Fig. 7). Indomethacin (10 mg/kg) did not affect the liver MDA levels.

Histopathological examinations:

As illustrated in Fig. 8, histological analysis of the formalin-treated paws revealed inflammation with extensive infiltration of leukocytes in the dermis and epidermis and accumulation of polymorph nuclear leukocytes around the capillaries. Oral administration of indomethacin (10 mg/kg) and HESP (100 and 200 mg/kg) significantly reduced the tissue damage induced by the sub-plantar injection of formalin.

Multiple topical TPA administrations induced severe inflammatory changes, such as epidermal hyperplasia, leukocyte infiltration in the dermis, and ear edema (Fig. 9). Significant improvements in the histological index of the inflammatory ear are observed with topical applications of the extract on the ear (5 mg/kg, Fig. 9c). Dexamethasone administration reduced the pathological alteration due to TPA (Fig. 9d).

Fig. 5. Effect of HESP on TNF-α concentration in rat paws. HESP (100 and 200 mg/kg), indomethacin (10 mg/kg), and normal saline were orally administered 45 min before sub-plantar injection of carrageenan. The data were expressed as mean ± SEM. ***P < 0.001 indicates significant differences compared to the saline group. HESP, Hydroalcoholic extract of Stachys pilifera; TNF-α, tumor necrosis factor alpha.

Fig. 6. Effect of HESP on serum MDA level in carrageenan test. HESP (100 and 200 mg/kg), indomethacin (10 mg/kg), and normal saline were orally administered 45 minutes before sub-plantar injection of carrageenan. The data were expressed as mean ± SEM. ***P < 0.001 indicates significant differences compared to the carrageenan group. HESP, Hydroalcoholic extract of Stachys pilifera; MDA, malondialdehyde.
Fig. 7. Effect of HESP on liver MDA level in the formalin test. HESP (100 and 200 mg/kg), indomethacin (10 mg/kg) normal saline were orally administered 45 minutes before sub-plantar injection of formalin every day for 7 consecutive days. The data were expressed as mean ± SEM. ***P < 0.001 indicates significant differences compared to the formalin group. HESP, Hydroalcoholic extract of *Stachys pilifera*; MDA, malondialdehyde.

Fig. 8. Histological examination of the formalin-treated paw. (A) Normal paw; (B) formalin-treated paw shows an inflammation with extensive infiltration of leukocytes in the dermis and epidermis; (C) indomethacin at 10 mg/kg; (D) HESP at 100 mg/kg; and (E) HESP at 200 mg/kg. Indomethacin and HESP significantly reduced the tissue damage. HESP, Hydroalcoholic extract of *Stachys pilifera*.

Fig. 9. Histological examination of ear tissue in repeated TPA test. (A) Normal ear; (B) multiple topical TPA administrations led to severe inflammatory changes, such as epidermal hyperplasia, leukocyte infiltration in the dermis, and ear edema; (C) HESP at 5 mg/ear; and (D) dexamethasone at 0.05 mg/ear. HESP and dexamethasone significantly reduced tissue damage. HESP, Hydroalcoholic extract of *Stachys pilifera*; TPA, 2-O-tetradecanoylphorbol-13-acetate.
DISCUSSION

Our data confirmed the anti-inflammatory effects of HESP in two subacute animal models of inflammation including formalin-induced arthritis in rats and multiple topical applications of TPA in mouse ears. Also, our data indicated that the inhibitory effect of this extract on serum and liver MDA levels, heat-induced hemolysis, and hypotonicity in human red blood cells.

The carrageenan-induced inflammatory model is a well-known model, widely used for the preliminary evaluation of the anti-inflammatory effects of various chemical compounds and medicinal plants (18). This type of inflammation is time-dependent biphasic acute inflammation. In the first phase (0 to 1 h after the sub-plantar injection of carrageenan) inflammatory agents such as histamine, serotonin, and bradykinin, and to some extent, also prostaglandins have crucial roles. While the second phase of inflammation, occurring 1 h after the injection of carrageenan, induces leukocyte infiltration, the production of free radicals derived from reactive oxygen species, the release of prostaglandins and proteases (26). Our previous study showed that the HESP inhibited inflammation induced by carrageenan in both phases (14).

Besides, carrageenan can induce some important pre-inflammatory cytokines such as IL-1β and TNF-α (27). The results of the present study confirmed that carrageenan increases the levels of IL-1β and TNF-α in inflamed paws, suggesting that both indicated cytokines are involved in the paw edema induced by carrageenan. These findings agreed with some previous findings (20,21). The plant extract could not decrease the tissue concentration of IL-1β and TNF-α due to carrageenan challenges. Indomethacin also did not have any effect on the levels of IL-1β and TNF-α that was consistent with the earlier findings (20).

Therefore, it seems that the anti-inflammatory effects of this extract were independent of the pathways of the synthesis and secretion of the pro-inflammatory cytokines, such as IL-1β and TNF-α.

Formalin injection into rat paws induces inflammation and pain. The formalin model of inflammation in the rat paw for consecutive days is a suitable model of subacute inflammation (28). This model is an appropriate experimental model for evaluating the antiproliferative and anti-arthritis activities of compounds, because of the similarity of this model to human arthritis (29). In subacute formalin inflammation, the rats were examined for 7 days. The plant extracts (100 and 200 mg/kg) and indomethacin (10 mg/kg) significantly reduced the progression of inflammation for seven consecutive days. Therefore, it could be suggested that the HESP is a potential therapeutic agent for treating subacute inflammation. Our data were confirmed by histopathological studies.

It has been reported that multiple applications of TPA on mouse ears generate edema, epidermal hyperplasia, and inflammatory cell infiltration. The inhibitors of cyclooxygenase and lipoxygenase, significantly inhibit acute inflammation, while the chronic inflammatory process is not suppressed by conventional cyclooxygenase inhibitors (30). Some studies have shown that TPA-induced skin cancer is closely related to inflammatory responses including the induction of pro-inflammatory cytokines and high expression of cyclooxygenase-2 and inducible nitric oxide synthase proteins (31). In the TPA-induced mouse ear inflammation, the expression of the indicated markers is regulated by the nuclear factor (NF)-κB (31). Also, other studies have shown that mitogen-activated protein kinase (MAPKs) contribute to the activation of NF-κB, in the TPA-induced skin inflammation in mice (33). As previously mentioned, plant extract has been effective in the acute model of inflammation by TPA (26). The present data, also indicated that the extract significantly inhibits the subacute inflammation resulted in the repeated application of TPA. In this line, one possibility is that the extract interferes with the MAPK pathway. Besides, the topical anti-inflammatory effects of the extract propose the presence of at least one lipophilic compound that crosses the skin barrier that exhibits topical antiphlogistic effects (34). Histological studies of TPA-treated ears revealed that the extract is an effective inhibitor of neutrophilic migration and pathological changes (26).
Other possible anti-inflammatory mechanisms of the extract including heat-induced or hypotonicity hemolysis in human erythrocytes and on MDA levels were studied. The plant extract (50-800 μg/mL) significantly inhibited heat or hypotonicity-induced hemolysis. The membrane of lysosomal and erythrocytes is morphologically similar, thus the RBC membrane is commonly used to examine the effects of new compounds on lysosomes. Then, inflammatory reactions trigger lyses of lysosomes and discharge of its contents (phospholipases), which further induce inflammatory conditions and result in several disorders. The membrane-stabilizing activity found with the extract indicates that lysosomal membrane stabilization can mediate part of its anti-inflammatory activities (35).

The lysosomal content of neutrophils contains proteases and bactericidal enzymes, which cause inflammation and tissue damage. The ingredients of the extract may exhibit this effect through pathways that promote the development of cell membranes and interactions with membrane proteins (36).

Reactive oxygen species play an important role in inflammation models induced by TPA and carrageenan (37). The antioxidant effects of S. pilifera have been reported in several earlier studies (8,9). The reaction of free radicals with unsaturated fatty acid chains of phospholipids leads to the breakdown of their double bonds, peroxidation, and cellular membrane degradation, and production of MDA, which is one of the final products during the peroxidation process of lipids. The results obtained from the measurement of serum MDA levels showed that the administration of carrageenan and formalin resulted in a significant increase in MDA levels in serum and rat liver tissue, which is consistent with previous studies. Lu et al. reported formalin or carrageenan sub-plantar injections to increase the MDA levels in the rat model. Also, Bilici et al. studied the protective effect of melatonin on carrageenan-induced inflammation. They also reported that carrageenan sub-plantar injection leads to increased tissue MDA levels (38,39). In this study, plant extracts significantly decreased the increased level of MDA in serum and liver in the carrageenan and formalin test. Therefore, S. pilifera may inhibit lipid peroxidation associated with anti-inflammatory processes due to its antioxidant effects. Our phytochemical screening data confirmed the presence of phenols and flavonoids in the plant. It has been reported that phenolic compounds and flavonoids inhibit the process of inflammation by regulating the production of pro-inflammatory molecules (40). Therefore, phenolic and flavonoids contents of the plant may participate in the anti-inflammatory activity observed with it.

**CONCLUSION**

In summary, the results of this study demonstrated the anti-inflammatory properties of S. pilifera in oral and topical administration in the subacute experimental models. Our findings also indicated that anti-inflammatory effects on the plant by inhibiting neutrophil infiltration, stabilizing the lysosomal membrane, and inhibiting the production or function of free radicals. Therefore, this study confirmed the beneficial potential of S. pilifera for treating topical and systemic inflammatory disorders.

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**Conflict of interest statement**

The authors declared no conflict of interest in this study.

**Authors' contribution**

H. Sadeghi and H. Sadeghi contributed to study concept and design, drafted the manuscript, and supervised the study; V. Zarezadeh, M. Mostafazadeh, and S.A. Abedi acquired the data; E. Panahi Kokhdan, J. Nikbakht, and H. Sadeghi analyzed and interpreted the data; and M. Molavi critically revised the manuscript for important intellectual content.
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