

Salvia Hypoleuca; A New Source of Compounds which are Useful for Treatment of Ocular Inflammation

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Summary

The aim of the present study was to determine the effects of *Salvia hypoleuca* extract on endotoxin-induced uveitis (EIU) in rats. In addition, the endotoxin-induced expression of the inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 proteins was determined in a mouse macrophage cell line (RAW 264.7) treated with *Salvia hypoleuca* extract in vitro, to clarify the anti-inflammatory properties. EIU was induced in male Wistar rats by a footpad injection of lipopolysaccharide (LPS). Immediately after the LPS injection, 1, 10, or 100 mg extract or 10 mg prednisolone was injected intravenously. After 24 hours, the aqueous humor was collected from both eyes, and the number of infiltrating cells, protein concentration, nitric oxide (NO), prostaglandin (PG)-E₂, and TNF- α levels in the aqueous humor were determined. RAW 264.7 cells treated with various concentrations of extract were incubated with 10 μ g/mL LPS for 24 hours. Levels of NO, PGE₂, and TNF- α were determined. The expression of iNOS and COX-2 proteins was analyzed by Western blot analysis. The number of inflammatory cells, the protein concentrations, and the levels of NO, PGE₂, and TNF- α in the aqueous humor in the groups treated with *Salvia hypoleuca* were significantly decreased in a dose-dependent manner. In addition, the anti-inflammatory effect of 100 mg *Salvia hypoleuca* was as strong as that of 10 mg prednisolone. The anti-inflammatory action of *Salvia hypoleuca* was stronger than that of either quercetin or anthocyanin administered alone. Extract also suppressed LPS-induced iNOS and COX-2 protein expressions in RAW 264.7 cells in vitro in a dose-dependent manner. The results suggest that *Salvia hypoleuca* has a dose-dependent anti-ocular inflammatory effect that is due to the direct blocking of the expression of the iNOS and COX-2 enzymes and leads to the suppression of the production of NO, PGE₂, and TNF- α .

Key words: Anti-ocular-inflammatory, *Salvia hypoleuca*, Endotoxin-Induced Uveitis

Introduction

The genus *Salvia*, one of the most important genera of Lamiaceae family, is widely used in flavouring and folk medicine all around the world (1). Fifty-eight species of this genus are documented in the Flora of Iran; 17 of them are endemic (1). The plants of the genus *Salvia*, which consist about 900 species (2) are generally known for their multiple pharmacological effects such as analgesic and anti-inflammatory (3), antioxidant (4), hepatoprotective, hypoglycemic activities (5), and antiischemia (6). In Iranian traditional medicine, *Salvia hypoleuca* is used for its pharmaceutical characteristics. Nowadays, medicinal plants receive attention to research centers because of their special importance in safety of communities. The

use of herbal medicine for the treatment of diseases and infections is as old as mankind. The curative properties of medicinal plants are due to the presence of various complex chemical substances of different composition which occur as secondary metabolites (7, 8). There have been no reports on the effects of *Salvia hypoleuca* on lipopolysaccharide (LPS)-induced inflammation in vivo or in vitro. Endotoxin-induced uveitis (EIU) is an animal model of acute anterior segment intraocular inflammation that is induced by an injection of LPS or lipoteichoic acid (9-11). In this model, LPS may directly activate the vascular endothelium, macrophages, and other cells. Cellular infiltration and protein extravasations in the anterior part of the eye reach a maximum 20 to 24 hours after LPS treatment. In the vitreous and retina, cellular infiltration reaches a maximum 48 hours after LPS treatment (12). Exposure to exogenous bacterial toxins, such as LPS, stimulates cellular inflammatory responses and releases factors such as nitric oxide (NO) (13, 14) and prostaglandin (PG)-E₂ (15-17) cytokines, including tumor necrosis factor TNF- α and eicosanoid mediators, which promote inflammatory responses (18). There are three types of nitric oxide synthase (NOS) isoforms in cells including; Endothelium NOS and neural NOS which are both constitutive NOS isoforms and act to maintain normal vasoactivity in an active state of vasodilation through a Ca⁺² dependent pathway and acts as a neurotransmitter in neuron signal transmission. NOS in macrophages and hepatocytes is an inducible (i) NOS isoform, and it acts in a Ca⁺² independent pathway. After exposure to endogenous and exogenous stimulators, iNOS is induced quantitatively in various cells, such as macrophages, smooth muscle cells, and hepatocytes, and triggers several disadvantageous cellular responses that can cause inflammation and stroke (19). The level of NO production induced by iNOS may reflect the level of inflammation, and therefore we might be able to evaluate the effect of an anti-inflammatory drug by measuring NO levels. Recently it has been reported that Ginkgo biloba extract prevents the inflammation of EIU by suppressing generation of NO (Ilieva, 2004). PGE₂, thromboxane B₂, and leukotriene B₄, are as major inflammatory mediators. At an inflammation the major COX product is PGE₂ which leads to local blood flow increases, edema, and pain sensitization. The common mechanism of action of nonsteroidal anti-inflammatory drugs is inhibition of cyclooxygenase (COX), and therefore prostaglandin production (20). As is now well appreciated, COX has two isoforms (21, 22) including; COX-1 which exists in places such as endothelium, stomach, and kidney, and COX-2 which is induced by endotoxins and proinflammatory cytokines in cells in vitro, and at inflammatory sites in vivo. In this study *Salvia hypoleuca* was subjected to anti-inflammatory screening on EIU in rats. The anti-inflammatory properties of *Salvia hypoleuca* was compared with that of prednisolone. In addition, to make clear the anti-inflammatory effects, we also investigated the expression of iNOS and COX-2 in a mouse macrophage cell line (RAW 264.7) treated with *Salvia hypoleuca* extract in vitro.

Materials and Methods

Plant materials

Salvia hypoleuca was collected from Guilan province in north of Iran, and then was identified by a botanist. Its leaves and fruits were dried under shade and powdered. The extract was prepared by the maceration method (80% ethanol in 300 g/lit for 48 hours), filtered with filter paper. After filtration ethanol was removed by rotary evaporator. The extract was stored in -4c° for more administration.

Animal treatment

Adult male albino rats of Wistar strain weighing 150 - 200 g used for the study were obtained from Razi Institute, (Karaj, Iran) and maintained according to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals, Razi Institute, Karaj, Iran. EIU was induced by a footpad injection of 200 µg LPS (100 µg each footpad) from *Salmonella typhimurium* (Sigma-Aldrich) that had been diluted in 0.1 mL phosphate-buffered saline (PBS, pH 7.4). Rats were injected intravenously with 1, 10, or 100 mg of *Salvia hypoleuca* extract or 10 mg prednisolone diluted in 0.1 mL PBS containing 0.1% dimethyl sulfoxide (DMSO; Sigma-Aldrich). Rats were killed, and the aqueous humor (15–20 µL/rat) was collected from both eyes by an anterior chamber puncture 24 hours after the LPS injection.

Cell Count and Protein Concentration

For cell counting, the aqueous humor sample was suspended in an equal amount of Turk stain solution, and the cells were counted with a hemocytometer under a light microscope. The number of cells per field was manually counted, and the number of cells per microliter was obtained by averaging the results of four fields from each sample. To determine the total protein concentration in the aqueous humor, A BCA protein assay reagent kit (Pierce, Rockford, IL) was used.

Cell Culture and LPS Stimulation

The mouse macrophage cell line (RAW 264.7) was obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in RPMI-1640 medium supplemented with 2 mM glutamine, antibiotics (100 U/mL penicillin and 100 U/mL streptomycin) and 10% heatinactivated fetal bovine serum (Invitrogen-Gibco, Grand Island, NY) and maintained at 37°C in an incubator containing 5% CO₂. The cells were seeded onto a 24-well plate (5×10^4 cells/well) for the experiments. The cells treated with 1, 10, or 100 µg/mL *Salvia hypoleuca* extract for 24 hours were induced with 10 µg/mL of LPS for 24 hours. *Salvia hypoleuca* extract was dissolved in 0.01% DMSO. For the control group, RAW cells were cultured with 0.01% DMSO alone.

Nitrite Concentration in the Aqueous Humor

NO was evaluated as its end product, nitrite, by using Griess reagent (Sigma-Aldrich). The culture supernatant (100 µL) was mixed with 100 µL of Griess reagent for 10 minutes, and the absorbance at 550 nm was measured in a microplate reader. The concentration of nitrite in the samples was determined from a sodium nitrite standard curve. The data represent the mean of eight determinations ± SD.

Levels of TNF- α , PGE₂, and Monocyte Chemoattractant Protein-1 in the Aqueous Humor

The aqueous humor was collected and accurately diluted 10-fold with PBS. ELISA kits (R&D Systems) were used to measure the levels of TNF- α , PGE₂, and monocyte chemoattractant protein (MCP)-1 in the aqueous humor.

Western Blot Analysis

Cells were washed with ice-cold PBS and then lysed in cold NP-40 lysis buffer (50 mM Tris-Cl [pH 7.6], 150 mM NaCl, 10% glycerol, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride, and 1 mg/mL each of leupeptin, aprotinin, and pepstatin) for 10 minutes at 4°C. Plates were then scraped, and crude lysates were cleared by centrifugation at 14,000g for 10 minutes at 4°C.

Aliquots of the cleared lysates were diluted with 2× SDS sample buffer, and SDS-polyacrylamide gel electrophoresis was performed. Protein expression was analyzed by Western blot analysis by the following standard procedures. The primary antibody (anti-NOS; Upstate Biotechnology) was developed with horseradish peroxidase– conjugated secondary antibody (Amersham Biosciences) and visualized by chemiluminescence (Amersham Biosciences).

Statistical Analysis

Data were expressed as mean ± standard deviation and were analyzed by analysis of variance (ANOVA). The Tukey-Kramer test was used to compare the two treatment groups. $P < 0.05$ was considered to be statistically significant.

Results

Effect of *Salvia hypoleuca* extract on EIU

In the LPS group, 24 hours after LPS treatment the number of inflammatory cells that infiltrated the aqueous humor was $23.6 \pm 8.7 \times 10^5$ cells/mL (mean ± SD, $n = 8$). In the groups treated with *Salvia hypoleuca* extract the number of inflammatory cells was significantly lower than that in the LPS group, and the reduction was dose-dependent (Fig. 1A). The effect of the 100-mg dose of *Salvia hypoleuca* extract on the number of cells in the aqueous humor was almost the same as that observed with the 10-mg prednisolone dose (Fig. 1A). There were no infiltrating cells in the aqueous humor from the control group (rats without LPS).

As it can be found from Fig. 1B, Fig. 1C, Fig. 1D, and Fig. 1E, the protein concentration and the levels of NO, TNF- α , and PGE2 in the *Salvia hypoleuca* extract treated groups were significantly lower than that observed in the LPS group respectively. The reduction in these parameters in the 100-mg *Salvia hypoleuca* extract group was almost the same as that in the prednisolone group (Figs. 1B–E). The MCP-1 level was 7.9 ± 2.6 pg/mL in the LPS group whereas there was not any MCP-1. The level of MCP-1 was significantly reduced by 100-mg *Salvia hypoleuca* extract and prednisolone in the aqueous humor (Fig. 1F).

Expression of the iNOS and COX-2 Proteins

For clarification of the inhibitory property of *Salvia hypoleuca* extract on LPS-induced NO and PGE2, the expression of the iNOS and COX-2 proteins was evaluated. Expression of the iNOS protein was high in LPS-stimulated RAW cells (Fig. 2A, lane 2). Clearly, it can be seen from figure 2A, lanes 3-5 that the expression of the iNOS protein significantly decreased in a dose-dependent manner within the concentration range from 1 to 100 μ g/mL *Salvia hypoleuca* extract. Although expression of COX-2 was detected in normal cells (Fig. 2B, lane 1), there was strong expression in LPS-stimulated cells (Fig. 2B, lane 2). Expression of the COX-2 protein decreased in a dose-dependent manner within the concentration range from 1 to 100 μ g/mL of *Salvia hypoleuca* extract (Fig. 2B, lanes 3–5).

FIGURE 1. Effect of *Salvia hypoleuca* extract on LPS-induced cell counts (A), protein concentration (B), NO production (C), TNF- α (D), PGE2 (E), and MCP-1 concentration (F) in aqueous humor. The aqueous humor was collected 24 hours after LPS treatment. Values are mean ± SEM ($n=10$). * $P < 0.05$ and ** $P < 0.01$ versus the LPS group. The dose of prednisolone (PSL) was 10 mg.

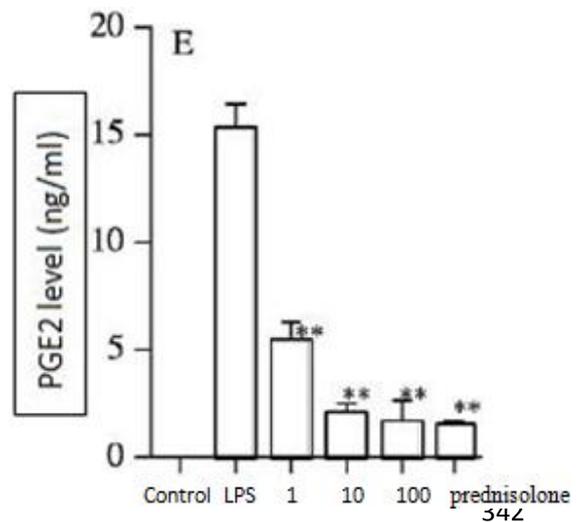
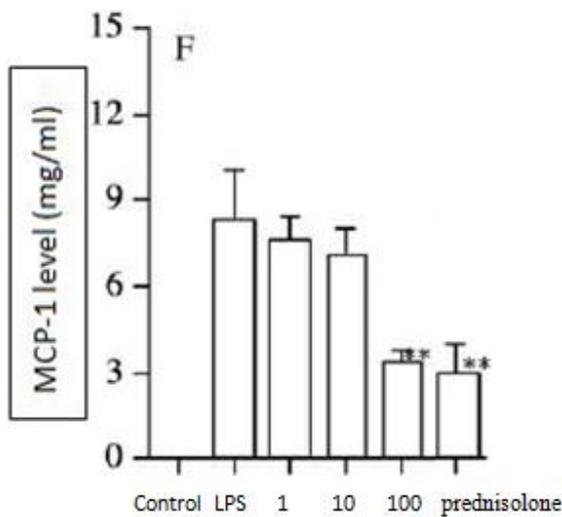
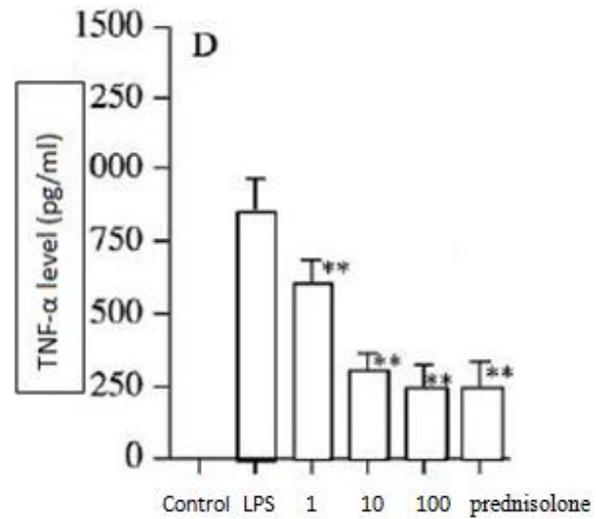
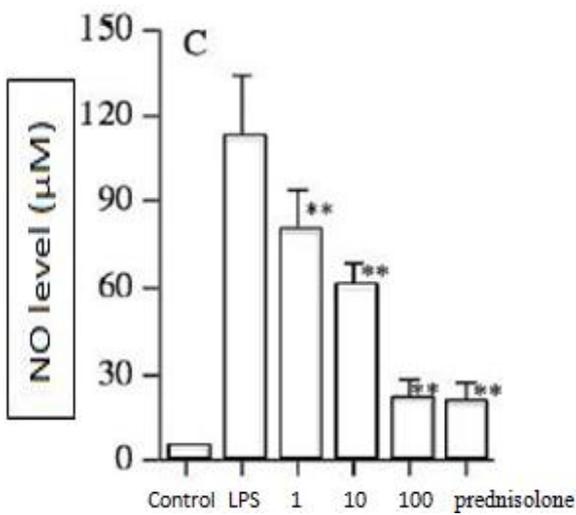
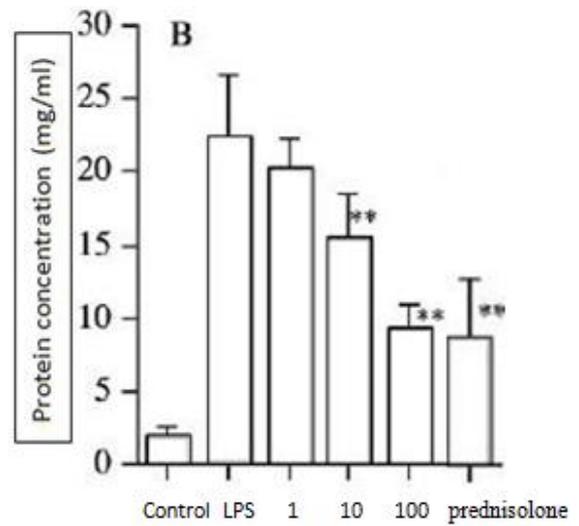
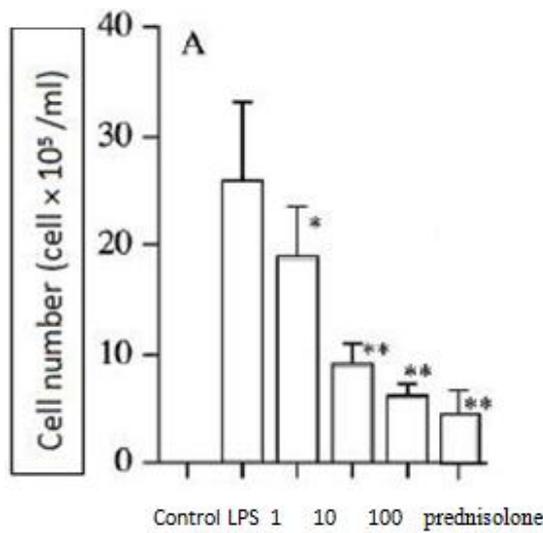
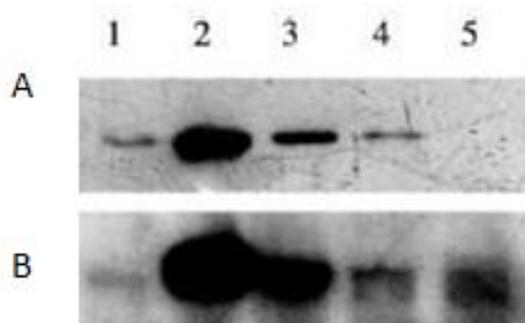


FIGURE 2. Western blot analysis of The effect of *Salvia hypoleuca* extract on expression of LPS-induced iNOS protein (A) and COX-2 protein (B) in RAW 264.7 macrophages. Lane 1: control; lane 2: LPS; lane 3: 1 $\mu\text{g}/\text{mL}$ of *Salvia hypoleuca* extract; lane 4: 10 $\mu\text{g}/\text{mL}$ of *Salvia hypoleuca* extract; lane 5: 100 $\mu\text{g}/\text{mL}$ of *Salvia hypoleuca* extract.



Discussion

The results of this study indicate that *Salvia hypoleuca* extract suppresses the development of EIU in a dose-dependent manner. Particularly, the anti-inflammatory effect of 100 mg *Salvia hypoleuca* extract was approximately as same as that observed with a 10-mg dose of prednisolone. Flavonoids and other phenolics have been suggested to play a preventive role in the development of cancer and heart disease (24). Antioxidant activity of some flavonoid compounds has been shown and given *Salvia hypoleuca* extract with high levels of polyphenol compounds may has Antioxidant activity. Oxidative stress has been known to be a major factor in triggering local inflammation and tissue damage during the inflammatory process, and *Salvia hypoleuca* extract's antioxidant properties have been proposed to underlie its beneficial effects on inflammation. It is known that NO production plays an important role in endotoxemia and inflammatory conditions (15). To elucidate the anti-inflammatory mechanism of *Salvia hypoleuca* extract, we focused our attention on *Salvia hypoleuca* extract's antioxidant activity and measured the concentration of NO in the aqueous humor in vivo. Our results showed that *Salvia hypoleuca* extract prevented NO production in the aqueous humor and the expression of the iNOS enzyme in a dose-dependent manner. These results are in agreement with those of our in vivo experiment. Large amounts of NO production induced by bacterial LPS or cytokines have been reported to play a central role in endotoxemia and inflammatory conditions (15). Therefore; we propose that *Salvia hypoleuca* extract, which inhibits NO production through the inhibition of iNOS enzyme expression, could have a beneficial therapeutic effect with regard to the treatment of inflammation. In EIU, LPS stimulates inflammatory cells to upregulate iNOS mRNA (25, 26). Increased NO levels have been detected in the aqueous humor in patients with Behc,et's disease (27). In this study, *Salvia hypoleuca* extract inhibited the development of EIU and suppressed LPS-induced iNOS expression in a dose-dependent manner. Therefore, the present results support their hypothesis. TNF- α produce principally by activated macrophages and monocytes, and the most important role of TNF- α is in nonspecific resistance to various

infectious agents (28, 29). The results show that the reduction in the TNF- α concentration has been occurred in a dose dependent manner by *Salvia hypoleuca* extract. According to previous studies, EIU in mice has been worsened by decreasing TNF- α and it is not directly involved in the pathogenesis of EIU and may protect against inflammatory processes in EIU (30, 31). In this study, LPS significantly increased TNF- α level in the aqueous humor, and the different effects of TNF- α on EIU may be due to differences in species (rat and mouse) or animal strain. PGE2 may have an important role in suppression of TNF- α by NO. According to previous studies, NO activates COX enzymes and thereby leads to a marked increase in PGE2 production (32, 33). The suppressive effect of PGE2 on TNF synthesis through elevated cAMP levels has been convincingly demonstrated (28, 33-35). Our results are in agreement with the previous studies, as we found that *Salvia hypoleuca* extract suppressed the levels of LPS-induced PGE2 and TNF- α in a dose dependent manner in vivo. In addition, our results indicated that the *Salvia hypoleuca* extract decreased the expression of the COX-2 enzyme in a dose-dependent manner. COX-2 is primarily responsible for increased PGE2 production during inflammation, and PGE2 is generally considered to be a proinflammatory agent. Results show that *Salvia hypoleuca* extract suppressed the development of EIU and decreased LPS-induced COX-2 expression in vitro in a dose-dependent manner. Therefore, our findings are in agreement with the results of previous studies (36-38) and suggested that blocking of COX-2 protein expression is one of the anti-inflammatory mechanisms of *Salvia hypoleuca* extract. In conclusion, this study indicates that *Salvia hypoleuca* extract has a dose-dependent anti-ocular-inflammatory effect on EIU. In particular, by increasing dose the effect of *Salvia hypoleuca* extract was remarkable. It appears *Salvia hypoleuca* extract performs its anti-inflammatory activity due to suppression of NO, PGE2, and TNF- α production through the direct inhibition of the expression of the iNOS and COX-2 enzymes. These findings suggest that *Salvia hypoleuca* extract may be useful for the treatment of ocular inflammation.

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