Original Research Article

Analgesic Effect and Immunomodulation Response on Pro-Inflammatory Cytokines Production by *Scrophularia megalantha* Extract

Abbas Azadmehr[1]**, Mohammad Sofiabadi**[1*] **and Reza Hajiaghaee**[2]

[1]*Cellular and Molecular Research Center, Qazvin University of Medical Sciences, Qazvin,* [2]*Department of Pharmacognosy and Pharmaceutics Department of Medicinal Plants Research Center, Institute of Medicinal Plants, ACECR, Karaj, Iran.*

*For correspondence: **Email:** mohasofi@yahoo.com; **Tel/Fax:** +982813336004*

Abstract

Purpose: *The remains unknown, To determine the analgesic and anti-inflammatory activities of Scrophularia megalantha in male rats in order to understand the scientific basis for its trado-medicinal uses, especially in inflammation.*
Methods: *The extract of Scrophularia megalantha was obtained with ethanol. In order to determine qualitatively the chemical components of the extract, thin layer chromatography (TLC) was used. The analgesic activity of the extract at various doses (25, 50, 100 and 200 mg/kg, i.p) was assessed using formalin test while pro-inflammatory cytokines were measured by enzyme-linked immunosorbent assay (ELISA), respectively. Diclofenac (5 mg/kg) was used as positive control.*
Results: *Phenolic compounds, flavonoids and phenyl propanoid were present in the extract. At doses of 100 and 200 mg/kg, the extract showed significant analgesic effects ($p < 0.05$, $p < 0.01$) in the first phases of formalin test, compared with the control. At 25, 100 and 200 mg/kg doses, the extract reduced significantly ($p < 0.05$, $p < 0.001$, $p < 0.001$) pain score in the chronic phases of the formalin test. In addition, at 50 - 200 µg/mL of the extractm both TNF-α and IL-6 proinflammatory cytokines were inhibited significantly ($p < 0.001$) on LPS-stimulated macrophages.*
Conclusion: *The extract of S. megalantha exerts analgesic and anti-inflammatory activities by inhibition of pro-inflammatory cytokines production. This lends support for the use of the plant as an analgesic in traditional medicine.*

Keywords: Scrophularia megalantha, Analgesic, Inflammation, Pro-inflammatory cytokines, Phenolics, Flavonoids

INTRODUCTION

Chronic pain is the most common symptom that brings a patient to the physician. Currently available analgesic drugs such as opiates and non-steroidal anti-inflammatory drug (NSAID) are not fully accepted due to their adverse effects. For example, opioids may induce physical dependence, tolerance and other side effects including respiratory depression, drowsiness, nausea, constipation and alterations of endocrine and autonomic nervous system activities. Also NSAIDs can aggravate peptic ulcers and cause hepatorenal complications. Consequently, there is renewed interest in new natural analgesics, especially of plant origin [1,2].

Inflammation plays role in the mechanism of pain. It can decrease pain threshold, increase nociceptive neuron firing rate and facilitate pain

transmission; these processes can augment pain [3,4]. On the other hand, the process of inflammation is necessary for healing of damaged tissues. In response to inflammation, a variety of innate immune cells such macrophages can be activated. However, uncontrolled inflammation induces tissue injury. Cytokine production is a primary step of the reaction of macrophages to inflammatory stimuli [5]. During inflammation, the over-production of inflammatory cytokines such as TNF-α, IL-6 and IL-1 by activated macrophages is a crucial event for triggering progressive stimulation of immune cells [6].

One of the plants said to have immuno-modulatory and anti-inflammatory effects is *Scrophularia megalantha Boiss* (Scrophularia-ceae) [7,8]. Several species of this genus have been used since ancient times as folk remedies for ailments such as inflammatory diseases, scabies, eczema, psoriasis, tumors, etc. In addition, we have previously demonstrated the inhibitory effect of *Scrophularia striata* extract on nitric oxide production using *in vitro* and *ex vivo* models [7]. Some species in this genus have shown anti-inflammatory activity [8]. Therefore, the objective of the present study was to investigate the effect of the ethanol extract of the aerial parts of *Scrophularia megalantha* on pro-inflammatory cytokines, including TNF-α and IL-6 denerated by LPS-stimulated macrophages, and formalin-induced inflammatory pain in male rats.

EXPERIMENTAL

Plant material and preparation of extract

The aerial parts of *Scrophularia megalantha* were collected from Kelardasht Region (Mazandaran Province) in the northern part of Iran, in May 2011 and air-dried at room temperature. The plant was identified by Mr. Ajani of the Department of Botany, Institute of Medicinal Plants (IMP) of Karaj, Iran. A voucher specimen (no. 1461) was deposited in the herbarium of the institute. The plant material was dried, powdered, and 100 g macerated with 80 % ethanol (900 ml) for 3 days with three changes of the solvent. The resulting extract was filtered and evaporated under vacuum to a dry powder (yield, 12.1 %). The plant extract was dissolved in dimethylsulfoxide (DMSO) to contain 0.1 %w/v of the extract and used at appropriate concentrations.

Phytochemical assay

In order to recognize chemical components of the extract, thin layer chromatography (TLC) was used. A variety of indicators including vanillin sulfuric acid; ferric chloride and natural product polyethylene glycol were used in this assay. The indictors were sprayed on prepared thin layers of fractions and observed at 260 and 280 nm wavelength under UV light.

Animals

Rats (230 – 250 g) were purchased from the Pasteur Institute of Iran (Tehran, Iran). All the animal experiments were approved by and performed according to the guidelines of the Ethical Committee of Cellular and Molecular Research Center (CMRC) of Qazvin University of Medical Sciences (approval ref no. 11.3.2011-CN157). The animals were maintained under standard laboratory conditions of temperature (23 ± 2 $^{\circ}$C) and light/dark cycle of 12 h/12 h, and received standard rat chow and water *ad libitum*.

Formalin test in rats

Analgesic activity was performed using formalin test as described by Dubuisson and Dennis [9]. Briefly, the rats were divided into 6 groups, each consisting of seven rats. The rats were moved to the test room 1 h before the experiment and acclimatized for 30 min in an acrylic observation chamber (30 cm in diameter and height) prior to intraperitoneal administration of the extract (25, 50,100 and 200 mg/kg) or diclofenac-Na (5 mg/kg) or normal saline (1 ml), Thirty minutes later, 50 μL of 2 % formalin was injected subcutaneously into the plantar surface of the right hind paw with a 30-gauge needle. Each rat was then immediately returned to the observation chamber, and pain behavior recorded. Pain behavior was scored (at 1-min interval for 60 min) as follows: 0, the injected paw was not favored; 1, the injected paw had little or no weight placed on it; 2, the injected paw was elevated and not in contact with any surface; and 3, the injected paw was licked or bit. The behavioral response of each rat during the early phase (1 – 7 min) and the late phase (15 – 60 min) was separately evaluated [9].

Isolation of peritoneal macrophages from rats

The rats were killed by cervical dislocation and peritoneal macrophages were harvested immediately by lavaging with ice cold sterile phosphate buffer saline (PBS). The cells were washed twice and plated in RPMI 1640 medium (Sigma Chemical Co) containing 10 % fetal bovine serum (Gibco), 100 U/ml penicillin and 100 μg/ ml streptomycin (Sigma Chemical Co), and incubated for 2 h at 37 °C in 5 % CO_2 humidified incubator. Non-adherent cells were

removed by gently washing with PBS and freshly prepared medium was added. Cell viability was checked by trypan blue exclusion technique. An aliquot of the cell suspension was mixed with an equal volume of 0.4 %w/v trypan blue in PBS and incubated for 10 min. The viability of the macrophages was > 98 %.

Measurement of pro-inflammatory cytokines

LPS (1 µg/ml) stimulated peritoneal macrophages were co-incubated with extract (10 – 200 µg/ml) or medium alone for 24 h. The supernatant was obtained by centrifugation at 2500 rpm for 20 min and assayed for TNF-α and IL-6 using ELISA kits (Bio-Source, Camarillo, CA, USA) according to the manufacturer's instructions.

Statistical analysis

Data are presented as mean ± standard deviation (SD). Statistical analyses were performed by one-way analysis of variance (ANOVA) to express difference among the groups followed by Bonferroni post hoc test. All analyses were performed using SPSS software, version 16. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Chemical components of the extract

Phytochemical assay by thin layer chromatography showed the presence of phenolic compounds, flavonoids and phenyl propanoids in *S. megalantha* extract.

S. megalantha extract reduced pain symptoms

Both acute (early) and chronic (late) phases of pain were observed in all groups after formalin injection (Fig 1).

In the acute phase, the extract decreased dose-dependently pain symptoms in comparison to control that was significant at 100 and 200 mg/kg dose ($p < 0.05$, $p < 0.01$) as shown in Fig 2A. The positive control, diclofenac-Na (5 mg/kg), significantly inhibited formalin-induced pain in the late phase ($p < 0.01$) (Fig 2B); however, there was no inhibition in the early phase (Fig 2A).

The extract significantly inhibited the late phase of formalin-induced pain, compared with control (Fig 2B). The inhibitory effect of extract (100 and 200 mg/kg) was stronger than that of the positive control, diclofenac-Na (5 mg/kg) ($p < 0.001$).
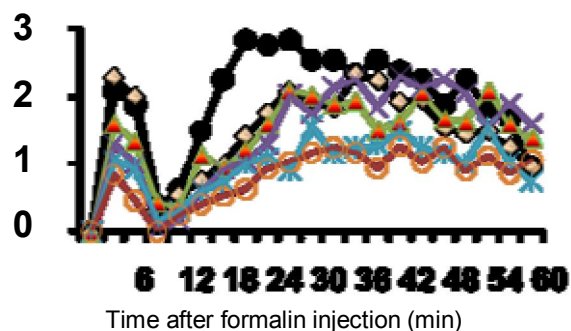

Fig 1: Effect of *S. megalantha* extract (25, 50, 100, 200 mg/kg, i.p.) and diclofenac (5 mg/kg, i.p.) on 2 % formalin-induced inflammatory pain in male rats. Data are presented as mean ± SD (n = 7) (● = control; ♦ = diclofenac; ▲ = Extract 25 mg)
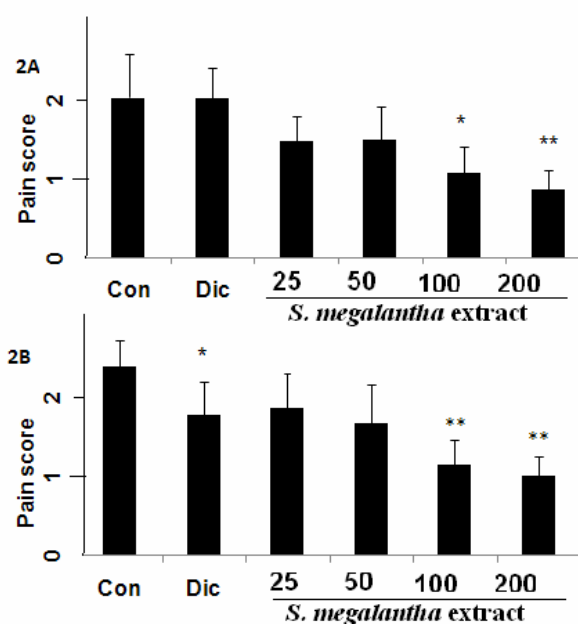

Fig 2: Effect of *S. megalantha* (25, 50, 100 and 200 mg/kg, i.p.) and diclofenac (5 mg/kg, i.p.) on 2 % formalin-induced inflammatory pain (early and late) phases in male rats. The extract reduced pain scores dose dependently in early phase 2A (0 – 5 min) and late phase 2B (15 – 40 min). Data are represented as mean ± SD (n = 7). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with the control group

S. megalantha extract reduced LPS-stimulated proinflammatory cytokines production in peritoneal macrophages

The effect of co-culture of LPS and extract for 24 h on the release of TNF-α and IL-6 pro-inflammatory cytokines into the medium is shown in Figs 3 and 4. Rat macrophages responded strongly to the addition of LPS by releasing TNF-α and IL-6 into the supernatant medium. Treatment of the cells with *S. megalantha* extract attenuated, in a concentration-dependent manner, the production of both pro-inflammatory cytokines by macrophages. The extract

significantly (*p* < 0.001) inhibited the production of both cytokines by LPS-activated macrophages in the concentration range of 50 - 200 µg/ml. The inhibition of TNF-α and IL-6 cytokines at the concentration of 200 µg/ml was approximately 65 and 25 % LPS only treated cells, respectively. Thus, inhibition of TNF-α was higher than of IL-6 after treatment of the macrophages with extract.
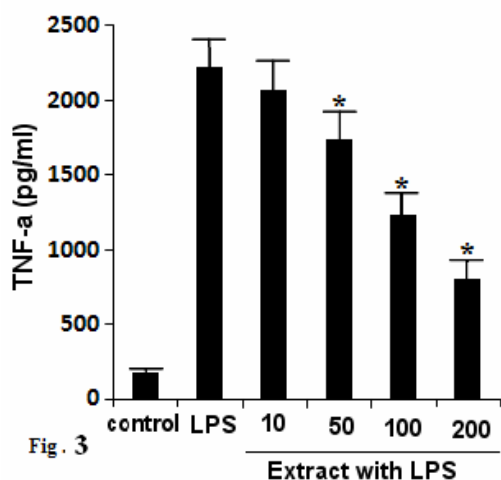

Fig 3: Effect of *S. megalantha* extract on LPS-stimulated TNF-α pro-inflammatory cytokine production in rat peritoneal macrophages. The extract significantly (*p* < 0.001) inhibited the production of TNF-α cytokine by LPS activated macrophages. Data are presented as mean ± SD.

DISCUSSION

S. megalantha is an important genus consisting of about 60 species in the Iran [10]. Most species belonging to *Scrophularia* genus have been used for anti-inflammatory therapy in folk medicine. A pervious study showed that some spices of *Scrophularia* have various flavonoids with anti-inflammatory properties; this study also indicates that *S. megalantha* extract contains flavonoids [11].

Phenylpropanoids play multiple important biologic roles, for example, anti-inflammatory action by reducing pro-inflammatory stimuli [12]. On the other hand, several reviews have reported the anti-inflammatory activity of phenols, attributing their properties not only to antioxidant capacity, but also to pro-inflammatory mediator modulation [13]. Our results show that phenolic compounds, flavonoids and phenyl propanoids are present in *S. megalantha* extract. Therefore, it is probable that the extract would exert analgesic and anti- inflammatory actions.
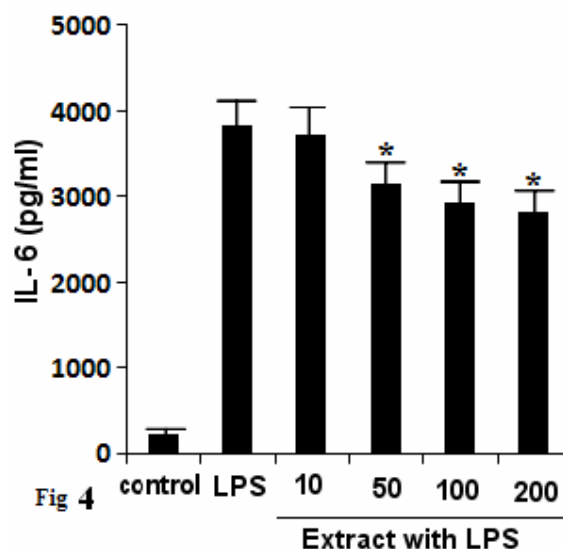

Fig 4: Effect of *S. megalantha* extract on LPS-stimulated IL-6 pro-inflammatory cytokine production in rat peritoneal macrophages.

The present results indicate that the ethanol extract of *S. megalantha* reduced symptoms of pain dose-dependently in both acute and chronic pain phases. This suggests that the extract exerts its analgesic action by both peripheral and central mechanisms. When formalin is injected into the footpad of an animal, it produces two phases of nociceptive behavior that are separated by a short period of quiescence in which there is no apparent pain. The first or acute phase typically occurs in the first 5 min while the second starts from 15 min and lasts about 40 - 60 min after injection. The early phase seems to be caused mainly by direct activation of both low-threshold mechanoreceptors and nociceptive primary afferent fibers due to peripheral stimulus. The late phase, also called the tonic phase, appears to be dependent on the combination of inflammatory reaction in the peripheral tissue and functional changes at some level of the central nervous system, spatially in the dorsal horn of the spinal cord, generally called central mechanisms [14].

In the present study, we observed more intense analgesic effect on the late (inflammatory) phase in the formalin test and the results show that the extract has potent analgesic effect. Therefore, it contains compounds that act peripherally and reduce inflammation [15]. In addition, our results indicate that the anti-nociceptive effect of the extract may be due to its anti-inflammatory effect. Pro-inflammatory cytokines, such as interleukin-6 (IL-6), is a cytokine initially indentified as T-cell derived factor regulating B-cell growth and differentiation. Furthermore, deregulation of IL-6 production has been implicated in a variety of

inflammatory diseases [16]. Pro-inflammatory cytokines such as IL-1 and tumor necrosis factor-α (TNF- α) markedly stimulate IL-6 production [17]. These mediators are able to recruit leukocytes, such as neutrophils, as reported for several experimental models [18,19]. In the present study, the levels of TNF-α and IL-6 were significantly decreased when treated with the extract. Thus, a putative anti-inflammatory mechanism could be associated with the degree of inhibition of pro-inflammatory mediators, such as TNF-α and IL-6. Our results indicate that the extract exhibited antinociceptive and anti-inflammatory activities. Although the anti-inflammatory mechanisms of *S.megalantha* are likely linked to the decrease in IL-6 and TNF-*α* pro-inflammatory cytokines, the effect of the extract on other inflammatory mediators is also essential.

CONCLUSION

Based on the findings of the present study, the ethanol extract of *S. megalantha* possesses analgesic and anti-inflammatory potentials. However, further studies are necessary to determine precisely the underlying mechanisms of these actions as well as to isolate the active compound(s) responsible for the activities.

ACKNOWLEDGEMENT

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