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Original article

Immunomodulatory and Anti-Inflammatory Effects of *Scrophularia megalantha* Ethanol Extract on an Experimental Model of Multiple Sclerosis

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Abstract

Background and objectives: *Scrophularia megalantha* is a native Iranian plant. In folk remedies, the species of the genus are used to treat stomach ulcers, goiter, eczema, cancer, psoriasis, and gall; however, there is not much research about *S. megalantha.* The current study aimed at evaluating the therapeutic effect of *Scrophularia megalantha*, a medicinal plant of Iran, on myelin oligodendrocyte glycoprotein 35-55 (MOG)-induced experimental autoimmune encephalomyelitis (EAE) as a model of multiple sclerosis (MS). **Methods:** The ethanol 80% extract of *S. megalantha* aerial parts was prepared by maceration method. The extract (100 mg/kg/day) was administered to C57BL/6 mice immunized with MOG (35-55) for 7 days, 3 weeks after EAE induction. The mice brain was removed and Hematoxylin-Eosin (H&E) was used to stain the sections. Moreover, spleen mononuclear cells from extract-treated or non-treated of EAE model mice were stimulated with MOG peptide and then culture supernatants were evaluated for IFN-ɣ, IL-17 and IL-10 cytokines using Enzyme-Linked Immuno Sorbent Assay (ELISA) kits. **Results:** Based on the obtained results, treatment with *Scrophularia megalantha* areal part extract significantly reduced inflammatory cells infiltration in the central nervous system (CNS) and also the disease severity in the experimental model of MS. Also, findings of the current study indicated that treatment with this medicinal plant in EAE mice model significantly decreased inflammatory cytokines including IFN-ɣ and IL-17 and vice versa significantly increased IL-10 as anti-inflammatory cytokine compared with non-treated of EAE model mice group. **Conclusion:** *Scrophularia megalantha* attenuated EAE by suppressing IFN-ɣ and IL-17 production and also increasing IL-10 cytokine. These findings suggested that this medicinal plant has the anti-inflammatory and immunomodulatory effects.

Keywords: anti-inflammatory; immunomodulatory**;** multiple sclerosis**;** *Scrophularia megalantha*

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Introduction

Multiple sclerosis (MS) is a chronic inflammatory disease with unknown causes; the demyeliniation in the CNS in this disease is related to the auto-immune response [1,2]. In the etiology of MS, in addition to genetic and environmental factors, immune responses have the main role [3]. It has been found that innate and acquired immune systems play important roles in inflammation and tissue damage, but TCD4 cells are involved in the early stages of inflammation [4,5]. The auto reactive T cells, by disrupting the blood-brain barrier (BBB), attack oligodendrocytes and myelin around the axon in the CNS [4,6]. Myelin destruction of neurons is due to the activation and penetration of mononuclear cells, pro-inflammatory cytokines secretion, chemokines and oxidative stress through excessive production of active oxygen species and nitrogen species such as hydrogen peroxide, peroxide and peroxynitrite [7,8]. On the other hand, the inflammatory cytokines produced by Th1 play a critical role in MS pathology [9]. Experimental autoimmune encephalomyelitis (EAE) is an experimental autoimmune disease developed by auto-reactive T cell response [9]. EAE model is the most popular animal model used to study MS [1,9]. In the EAE model, T cells consider myelin of oligodendrocytes as foreign agents. There is evidence that Th17 and Th1 are associated with immunopathology of demyelination in the EAE [8,10,11].

Th1 and Th17 cells penetrate in the CNS before causing the early signs of EAE, which may trigger inflammation in the CNS by activating the microglia cells [10]. In this regard, activated microglia has a major role in the process of inflammation of the neural tissue by releasing various toxic factors such as matrix metalloproteinase (MMP), TNF-α, IL-1β and IL-6 pro-inflammatory cytokines [12-14]. On the other hand, Th2 and T regulator cells as well as the associated cytokines, IL-5 and IL-10, can control the symptoms of the disease [15]. Drugs made for treatment of MS are expensive that have side effects and toxicity, so finding new drugs with less complications and better therapeutic effects are important [16]. *Scrophularia megalantha* Rech. f. is a native Iranian herb that grows in Kelardasht in north of Iran. Various species of this genus are used to treat stomach ulcers, goiter, eczema, cancer, psoriasis, and gall [17]. There are species of *Scrophularia* known to be rich in iridoid glycoside, aucubin and catapol. These substances in this medicinal plant and others herbs have choleretic, liver protective, anti-cancer, anti-inflammatory and antimicrobial activities [18,19]. Quercetine, isorhamnetin-3-O-rutinoside and nepitrin, flavonoid compounds, have been identified in this herb that have antioxidant properties [20]. Previous in vitro studies have indicated the inhibitory effect of Scrophulariaceae species in the production of nitric oxide and pro-inflammatory cytokines such as TNF- α, PGE2, IL-1β by macrophages [21]. However, there is not much research about *Scrophularia megalantha*. Therefore, the present study aimed at investigating the anti-inflammatory and immunomodulatory effects of *Scrophularia megalantha* on an EAE model.

Material and Methods

Ethical considerations

The Ethical Committee of Qazvin University of Medical Sciences (IR.QUMS.REC.1394.266, 2015) and also Institute of medicinal plant, Karaj, Iran approved animal experiments and handling procedures.

Prepration of *Scrophularia megalantha* extract

Aerial parts of *Scrophularia megalantha* was collected from Kelardasht area of Mazandaran province, northern Iran, in May 2011. The collected plants were identified by Mr. Ajani, department of botany, Institute of Medicinal Plants (IMP), Karaj, Iran. A voucher specimen (No. 1461) was deposited at the Herbarium of the institute. Aerial parts of *S. megalantha* were washed and dried at room temperature and manually crushed into powder (100 g). They were macerated in ethanol 80% for 3 days. The extract was filtered and vacuum evaporated and the dried powder was collected [22]. The extract of *S. megalantha* areal part (ES) was dissolved in dimethyl sulfoxide (DMSO) with non-toxic 1% v/v concentration before use.

Animals

In this study, nineteen female C57BL/6 mice weighing 18-20g (seven-to nine week-old), were provided by the laboratory of Animal Center of Pasteur institute of Iran. The mice were housed under optimal hygienic conditions and also the standard mouse chow and water. Mice were

randomly classified into three groups including eight mice in extract treated of EAE model, eight mice in non-treated of EAE model group and three mice in normal control group.

Induction of EAE

We induced EAE as an animal model for MS study, according to the manufacturer's instructions, Hooke Kits (Hooke laboratories, USA). These kits consist of two components, each of which was provided in pre-filled syringes of (MOG35-55) antigen in CFA (complete freund's adjuvand) and PTX (Pertussis toxin) emulsion in PBS. Briefly, subcutaneous injection of 0.1 mL antigen emulsion was performed on the upper back and lower back of mice in day zero. Within 2 h of emulsion injection, 0.1 mL PTX was intraperitoneally (i.p.) injected to each mouse. Twenty-four h after injection of this emulsion, the second intraperitoneal injection of PTX was performed. The plant extract (100 mg/kg) seven days before induction of disease (day zero) and twenty-one days after induction of disease was administered orally to treated mice. The non-treated group received DMSO as vehicle.

Evaluation of EAE experimental symptoms

Clinical symptoms were evaluated after treatment with the extract. The mice were observed for clinical scores based on the following symptoms: 0= lack of disease (normal); 1=impairment of tail motion; 2=paralyzed of tail; 3= disruption of movement; 4=paralyzed of one leg; 5=paralyzed of both leg;s 6=full paralyzed of hands and feet; 7=death [23,24].

Histopathology

In order for histopathologic evaluation, the mice were sacrificed following anesthesia, and the brains of mice were separated and fixed with formaldehyde 10%. Then, the paraffin-embedded segments were cut into 4-7-µm sections and Hematoxylin-Eosin (H&E) was used to stain the segments. Inflammatory infiltrated stained cells were observed under 40 x magnifications for each mouse.

Cytokine concentration assay by ELISA

Spleen mononuclear cells (2×10^6 cell/well) from extract treated and non-treated mice were separated and stimulated with MOG 35-55 peptide for 72 h. Then, the concentration of IFN-ɣ, IL-17 as inflammatory and IL-10 as anti-inflammatory cytokines were measured in cell culture supernatant by ELISA kits (IFN-ɣ ;cat no:558258, IL-10; cat no: 555252 BD, OptEIA™ and also IL-17; cat no: BMS6001 eBiosciences) method regarding to instruction of manufactures'.

Statistical analysis

Since data were unpaired, the non-parametric test (Mann-Whitney U) was used for statistical analysis; also ANOVA for parametric test was used. The results were expressed as mean ± SD. The p value <0.05 was considered as significance level.

Results and Discussion

EAE was induced in all mice immunized with MOG33-55. There was a significant difference between the treated group which were administrated 100 mg/kg/day *scrophularia megalantha* extract orally, and the non-treated group in terms of experimental parameters and severity of the disease (figure 1). The mean of disease severity in the non-treated group was higher than the mice which were treated with the extract. During the 28 days of observation, this medicinal plant significantly improved the clinical symptoms and suppressed disease progression in the extract treated group. Our findings indicated that the extract could control EAE progression and severity of clinical signs. We examined whether there was a relationship between the manifestations of EAE and the histopathology of CNS in the non-treated and treated groups. Histological analysis in extract treated group was compared with non-treated group as shown in figure 2. Our findings in the mice treated with ES showed less inflammation in CNS compared to the non-treated group. These results indicated that histopathology of CNS in mice treated with ES was associated with clinical signs in mice. The mice spleen mononuclear cells were isolated and cultured for 72 h in the presence of MOG35-55. In the extract treated group, IFN-ɣ was significantly ($p<0.05$) less than the non-treated group of EAE model mice (figure 3). In addition, the amount of IL-17 secreted from Th17 cells was significantly ($p<0.05$) lower in the extract treated group compared to the non-treated group. In contrast, IL-10 cytokine in the extract treated group was much more than the non-treated group. EAE is an autoimmune disease of CNS that is caused by auto-reactive $TCD4^+$ lymphocyte specific for MOG and also EAE is an appropriate experimental model for MS disease [25].

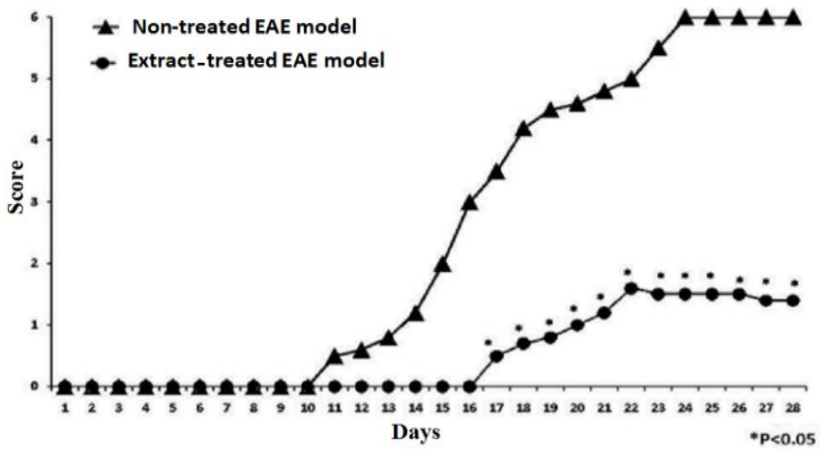

Figure 1. Evaluation of experimental score in EAE model. The results indicated that *Scrophularia megalantha* could control EAE progression and experimental severity. All data were expressed as mean ± SD. P values <0.05 were compared with non-treated group of EAE model.

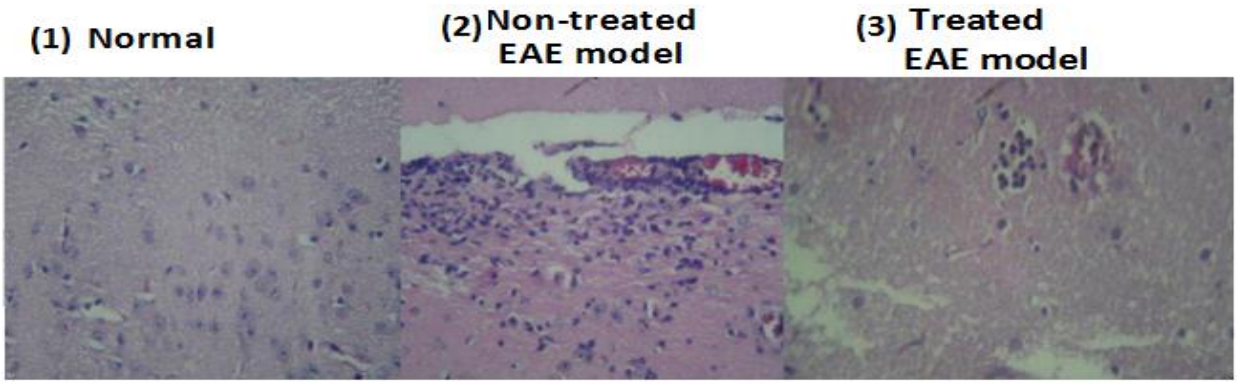

Figure 2. Histopathology of the normal brain group (1), Non-treated group of EAE model (2) and *Scrophularia megalantha* extract treated group of EAE model (3). Infiltrations of inflammatory cells in the mice EAE group treated with *Scrophularia megalantha* were compared with non-treated group of EAE model and normal groups that stained with H & E.

It has been shown that Th1 cells and the associated inflammatory cytokines play main roles in the pathogenesis of MS [26]. On the other hand, an increase in the production of interferon gamma, a pro-inflammatory cytokine, is associated with inducing this disease [27].

The protective properties of *Scrophularia megalantha* are attributed to its immunomodulatory and anti-inflammatory. As it was shown in the results, *S. megalantha* suppressed the production of inflammatory cytokines IL-17 and IFN-γ in cell cultures supernatant. Moreover, we have previously indicated that this medicinal plant has in vitro cytotoxic effects in cell culture and immune response activities in an in vivo model [28].

It has been made clear that Th1 cytokines are increased and vice versa Th2 cytokines are reduced in MS. On the other hand, Th2 cytokines are involved in the survival of neurons and Th1 cytokines are key factors in the demyelination of neurons. Th1 response and cellular immune activators, such as IFN-γ, secreted from Th1, increase the process of demyelination, apoptosis of oligodendrocytes, and loss of axon [9]. Moreover, several studies demonstrated that the production of Th2 cytokine such as IL-10 can be effective in improving EAE [29-34]. IFN-β and glatiramer acetate are medications prescribed for the treatment of MS that have anti-inflammatory and immunomodulatory effects [35,36]. The results of the measurement of cytokines in this

study showed that Th1 and Th2 responses were balanced by *S. megalantha*. So this medicinal plant has immunomodulatory and anti-inflammatory effects in the control of MS model. In addition, a pervious study showed that some spices of *Scrophularia* have various flavonoids with anti-inflammatory properties [37]. Several reviews have reported the anti-inflammatory activity of phenolic compounds, attributing their pro-inflammatory mediator modulation [38]. Our previous study showed that phenolic compounds, flavonoids and phenyl propanoids were present in *S. megalantha* extract [22].

In the present study, the inflammatory cells infiltration and clinical manifestations in the treatment group were significantly lower than the non-treated group.

These findings indicated that histopathology of the central nervous system in mice treated with ES was associated with clinical signs in mice. Therefore, this medicinal plant could control clinical signs of EAE model disease.

The Th17 cells produce a variety of cytokines such as IL-17, IL-6, IL-21, IL-22, IL-23, IL-26, GM-CSF and TNF-α. IL-17 is a specific cytokine in these cells. In addition to direct pro-inflammatory effects, IL-17 induces the production of other soluble intermediates, including IL-6, IL-1, TNF-α, GM-CSF and MMP in the cell [39]. In this study, the ELISA test showed that the IL-17 inflammatory cytokine decreased, so this plant may play a main role by inhibiting the production of IL-17 and reducing its damaging effects by Th17 cells in controlling of MS. However, further research is needed. On the other hand, T regulator cells play a key role in preventing autoimmune disorders; so, reducing the number or function of these cells can be effective in the development of autoimmune diseases [40]. It has been shown that T regulator produces IL-10 as an anti-inflammatory and immune-inhibitor cytokine that plays the role of suppressing IL-17 function [41]. We believe that IL-10 produced by T regulator cells plays the role of IL-17 suppression in mice treated with ES; however, further research for measuring of the T regulator number cells in the spleen tissue is needed to prove this hypothesis. Briefly, our findings showed that the inflammatory cytokines from Th17 and Th1, including IL-17 and IFN-γ, decreased, and IL-10 as anti-inflammatory cytokine secreted by T suppressor cell increased. The results of this study indicated that *S. megalantha* can be effective against prolonged and severe neurological complications of MS with anti-inflammatory and immunomodulatory effects. However, more researches are suggested in this regard.

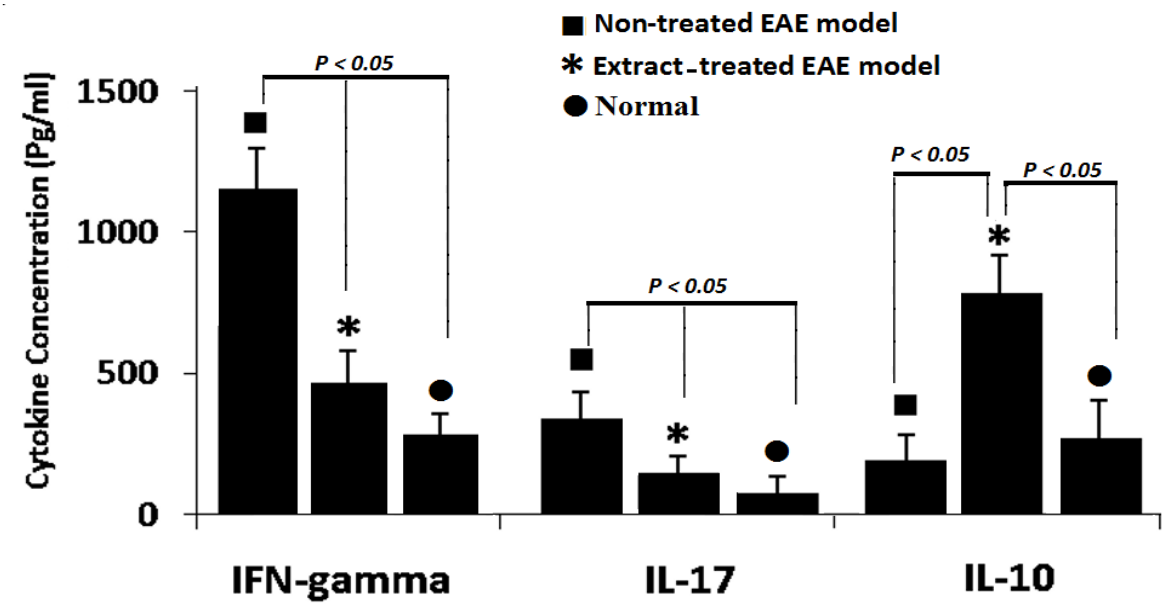

Figure 3. Evaluation of cytokines production by spleenocytes after treatment with the extract. The values are mean of triplicates, and the error bars represent SD. P values <0.05 were compared with non-treated group of EAE model.

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Author contributions

Abbas Azadmehr and Mahdi Goudarzvand designed, supervised, wrote and revised the manuscript and performed the experiments; Payam Saadat and Hadi Ebrahimi supervised and contributed in the writing of the manuscript; Reza Hajiaghaee supervised, prepared the plant extract and contributed to the revision of the manuscript; Niloufar Sadat Miri, Somayeh Fallahnezhad, Reza Norian, Abolfazl Rahmani, Masoud Baee contributed in the preparation and revision of the manuscript, statistical analysis and performing some parts of the experiments.

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

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Abbreviations

MOG: myelin oligodendrocyte glycoprotein 35-55; EAE: experimental autoimmune encephalomyelitis; MS: multiple sclerosis; H&E: hematoxylin-eosin; ELISA: enzyme-linked immuno sorbent assay; CNS: central nervous system; BBB: blood-brain barrier; MMP: matrix metalloproteinase; DMSO: dimethyl sulfoxide; CFA: complete freund's adjuvand; PTX: pertussis toxin; PBS: phosphate buffered saline