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Original article

Splenocyte proliferation, NK cell activation and cytokines production by extract of *Scrophularia variegata*; an *in vitro* study on mice spleen cells

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Abstract

Background and objectives: *Scrophularia variegata* M. Beib. (Scrophulariaceae) is a medicinal plant, used for various inflammatory diseases in Iranian Traditional Medicine. In the present study, we evaluated the immune modulation and antioxidant effects of the hydroalcoholic extract of *S. variegata*. **Methods:** The splenocytes were harvested from the spleen of Balb/c mice and were cultured. The splenocyte proliferation, NK cell activity, cytokines production and antioxidant effects were evaluated by MTT assay, enzyme- linked immunosorbent assay (ELISA) and DPPH assay, respectively. **Results:** The *S. variegata* extract significantly increased splenocyte proliferation. The results indicated that the extract increased NK cell cytotoxicity of Yac-1 tumor cells and at the concentration of 50-200 µg/mL significantly increased IFN-γ and IL-2 cytokines, although the level of IL-4 cytokine was significantly reduced. The antioxidant activity was observed in the extract with IC_{50} 302.34±0.11 µg/mL.**Conclusion:** The increasing in the splenocyte proliferation, anti-tumor NK cell cytotoxicity and cytokine secretion were indicated as potent immunomodulatory effects. These results suggest that *S. variegata* could be considered in the treatment of immunopathological disorders such as allergy and cancer; however, future studies are necessary.

Keywords: cytokines, immune modulation, NK cell, *Scrophularia variegata*, splenocyte

Introduction

In a healthy immune system, there is a balance between T helper 1 (Th1) and T helper 2 (Th2) responses. The immune responses balance is critical for immunoregulation and their imbalance causes various immune mediated disorders such as allergy, autoimmune diseases, cancers and infections. The Th1/Th2 balance is controlled not only by Th1 and Th2 cell cytokines, but also by various regulatory factors including regulatory T cells, sexual factor, chemokines, transcription factors and signal transduction pathway [1-3]. Therefore, the creation of Th1/Th2 balance in immune mediated diseases is an important goal of research by applying imunomodulator drugs

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such as medicinal plants.
The genus *Scrophularia* with about 300 species, is one of the most important genera belonging to the Scrophulariaceae family. Some species of this family have been used since ancient times as folk remedies for many medical conditions (scrofula, scabies, wounds, psoriasis, eczema, tumors, inflammations, etc.) [4].
Our previous studies on several species of *Scrophularia* demonstrated different effects, including immunomodulatory activity, anti-cancer activity by induction of apoptosis on leukemia cell lines, inhibitory effect on matrix metalloproteinases, nitric oxide and pro-inflammatory cytokines production and anti-asthmatic and neuroprotective effects [5-9]. In the present study, we have evaluated the immune modulation effects of *S. variegata* extract with *in vitro* splenocyte culture from spleen of Balb/c mice. To the best of our knowledge, this study is the first evaluation of *S. variegata* extract on the lymphocyte proliferation, NK cell activity and cytokines production.

Experimental

Plant material and preparation of the extract
The aerial parts of *S. variagata* were collected from Taleqan region (Alborz province, Iran) in May 2012 and verified by Dr. Ajani, Institute of Medicinal Plants (IMP), ACECR, Karaj, Iran. A voucher specimen was placed at the Herbarium of the institute (No. 1463). The aerial parts of the plant was dried, powdered (100 g) and macerated with 90% ethanol solution for 3 days with three according refreshes of the solution. The extract was filtered and dried under vacuum. The dried extract was stored at –20 ºC**.** Before testing, the extract was dissolved in dimethylsulfoxide (DMSO) (% 0.1 v/v) and used in appropriate concentrations.

Antioxidant and total flavonoid assay
The antioxidant capacity of the plant extract was estimated by DPPH (2,2-diphenylpicrylhydrazyl) test. One thousand microliters of various concentrations of the extract was added to 4 mL of 0.004% methanol solution of DPPH. After 60 min incubation period at room temperature, the absorbance was read against a blank at 517 nm. Inhibition percentage of free radicals by DPPH (I %) was calculated in the following way:

$I\ \% = [(A_{blank} - A_{sample}) / A_{blank}] \times 100$
A_{blank} =Absorbance of the control reaction (containing all reagents except the test compound).
A_{sample} =Absorbance of the test compound.

Extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotted from inhibition percentage against the extract concentration. IC_{50} values were compared to IC_{50}% value of a standard antioxidant agent (ascorbic acid, AA).
The total flavonoid content was evaluated by aluminum chloride colorimetric assay as described previously [5,10,11].

Cell culture and splenocytes preparation
The splenocytes were harvested from spleen of Balb/c mice and cultured in RPMI 1640 medium (Sigma, USA) with 10% heat-inactivated fetal calf serum (Gibco, USA), 2 mM L-glutamine, 100 µg/mL streptomycin and 100 U/mL penicillin (Robin Teb Gostar, Iran). In summary, for measuring splenocytes proliferation, the spleens were aseptically taken out of the Balb/c mice and single cell suspension was provided by grinding the spleen and later, they were separated by a density gradient centrifugation on a Ficoll-Hypaque solution (Sigma, USA). Cell survival was measured using the trypan blue dye exclusion technique with the results showing >98% of viable cells.

In vitro assay of splenocyte proliferation
For measuring the splenocyte proliferation, MTT (3-(4,5-dimethylthiazoyl)-2,5diphenyltetrazolium bromide) (Sigma, USA) assay was used. In summary, 10^5 cells/well were seeded in flat-bottomed micro-culture plates in the presence or absence of various concentrations (10- 200 µg/mL) of the extract (in triplicate) with or without Concanavalin A (Con A) (10 µg /mL) and incubated at 37 ºC in a 5% humidified CO_2

incubator for 48 h (Con A is a mitogen that stimulates lymphocyte proliferation for *in vitro* studies). Ten µL of MTT (5 mg/mL) was added to each well afterwards and incubation was carried for 4 h at 37 ºC. The supernatants were aspirated carefully, and 150 µL of DMSO was added to each well. The plates were shaken for an extra 10 min, and the absorbance values were read at 570 nm with an ELISA reader. Then, the stimulation index (SI) was calculated as follows:
SI= absorbance in the test group/ absorbance in the negative control.

Cytokines assay by ELISA
The concentration of each cytokine was measured by commercial Enzyme Linked ImmunoSorbent Assay (ELISA) kits (Biolegend, USA). In summary, the splenocytes at 10^5 cells/well along with Con A (10 µg/mL) were seeded in flat-bottomed micro-culture plates in the presence or absence of various concentrations (10- 200 µg/mL) of the extract and incubated at 37 °C in a 5% humidified CO_2 incubator for 48 h. Afterwards, the amounts of cytokines were measured according to the manufactures instruction.

Cytotoxicity assay of NK cells
For NK cell activity evaluation, the splenocytes as the effector and Yac-1 as the target cells (effector (E) at 1×10^6 cell/well was added to 2×10^4 target (T) cells/well to give E/T ratio 50/1) were cultured in 96-well plate in the presence or absence of various concentrations (10- 200 µg/mL) of the extract and incubated at 37 ºC in a 5% humidified CO_2 incubator for 24 h. Afterwards, tumor killing activity of NK cell was evaluated by the MTT assay. NK cell activity was computed according to the following equation:

(%) NK cell activity= (OD target control – (OD sample – OD effector control))/ OD target control × 100.

Statistical analysis
Statistical analyses were performed by one-way analysis of variance (ANOVA) followed by post–hoc Bonferroni's test. All analyses were performed using SPSS software16. The results were presented as mean±SD. *p* value of less than 0.05 was considered as significant.

Results and Discussion
There is evidence that antioxidants may be useful in preventing more than one hundred disorders (for example immunologic disorders) in humans and interest is growing in applying natural antioxidants from herbs and medicinal plants [12]. DPPH assay is an easy, rapid and sensitive way to survey the antioxidant activity of a specific compound or plant extracts [13]. It has been reported that flavonoids show antioxidant activity and their effects on human health are considerable [14]. The total flavonoid contents of the dry extract were used for analysis of plant. These were expressed as milligrams of rutin equivalents per 1 g dry extract (55.84±3.27); besides, the extract showed antioxidant activity in DPPH assay (IC_{50}302.34±0.11µg/mL compared to ascorbic acid as the positive control; IC_{50}35.2µg/mL).
The results of our preliminary experiment on splenocytes indicated that no significant toxicity was observed at higher doses (up to 200 µg/mL). Therefore, the cells were incubated with extract (0-200 µg/mL concentrations). The splenocytes proliferation analysis showed a direct dose-response relationship with *S. variagata* extract. As shown in figure 1, the extract at the 50-200 µg/mL with or without Con A significantly (p<0.05) induced splenocyte proliferation compared to the negative (only cells) and positive (Con A treated) controls. The stimulation index results indicated that the extract at 200 µg/mL increased lymphocyte proliferation approximately 3- fold versus the negative control. In addition, our findings showed that the extract at 200 µg/mLwith Con A increased lymphocyte proliferation index approximately 2- fold versus the positive control.
In the present study, IFN-γ, IL-2, IL-4 and IL-10 concentrations were measured in the culture supernatant of Con A by enzyme- linked immunosorbent assay (ELISA).
The significant increases in cytokine production

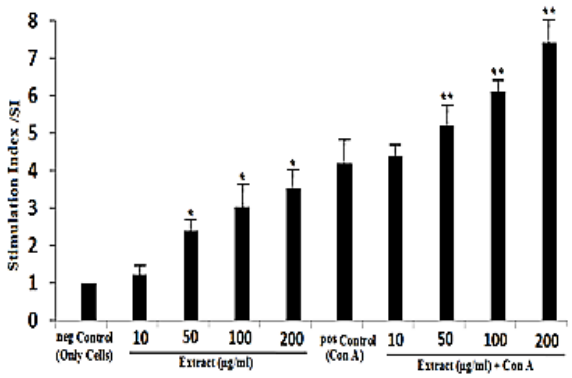

Figure 1. Effect of *S. variegata* extract on splenocyte proliferation; (*significant compared with negative control, ** significant compared with positive control). The results shown are representative of three independent experiments.

was shown in mouse splenocytes treated with Con A, compared with control. These results showed that Th1 cytokines including IFN-γ and IL-2 significantly increased in a dose dependent manner (figure 2A and 2B).

As shown in figure 2, our results indicated that the extract at the 200 µg/mL increased both IFN-γ and IL-2 cytokines approximately 1.5- fold versus the negative control group. Also, our findings showed that the extract significantly reduced IL-4 cytokine (figure 2C) compared to the control. Moreover, the results exhibited that *S. variegata* extract had no significant effect on the IL-10 cytokine production (Figure 2D). The splenocyte cytotoxicity was examined against NK-sensitive tumor cells (Yac-1). As shown in figure 3, the NK cell cytotoxicity was significantly increased after exposure to the extract. The results indicated that the extract significantly increased the NK cell cytotoxic activity approximately 4- fold versus the control group which indicated that the extract can modulate the innate immune response. In this study, we investigated the immune modulation activity of *S. variagata* extract as a medicinal plant used in traditional medicine.

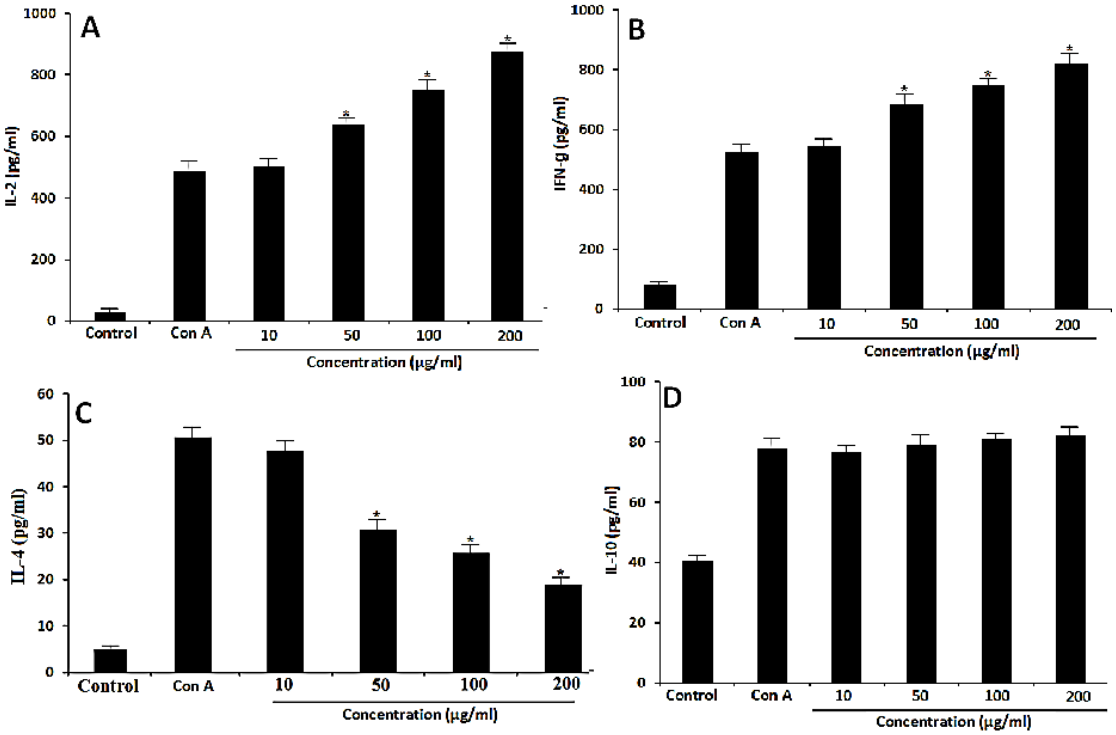

Figure 2. Effect of *S. variegata* extract on the cytokines production; * $p<0.05$ compared to control

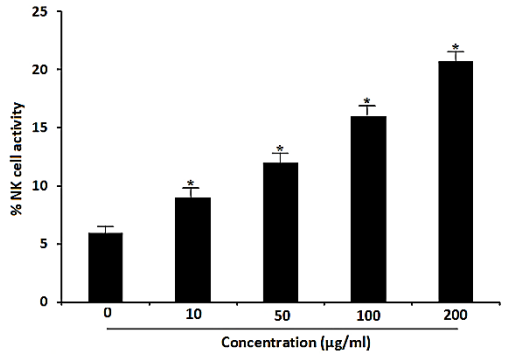

Figure 3. Effect of *Scrophularia variegata* extract on the NK cell activity; * $p<0.05$ compared to control

The splenic lymphocytes play important roles in the cellular and humoral immune responses against the antigens in the blood. Several studies indicated the immunomodulatory and antitumor activity of some medicinal plants [15,16].The immunomodulatory effect of *S. variagata* extract was evaluated by proliferating the splenocytes. Our results indicated that the extract up to 200 µg/ mL did not show any significant toxicity in the splenocytes for 48 h. In addition, the extract of *S. variagata* significantly stimulated the splenocyte growth and exhibited co-mitogenic activities in combination with Con A at 10-200 µg/ mL. Also, the extract increased IL-2 cytokine. This increase may be due to the enhancement in the production of IL-2 lymphocyte growth factor [17]. In addition, the immunomodulatory and anti-inflammatory effects of some species of *Scrophularia* on the lymphocyte have been shown by other investigators [7,8,16]. On the other hand, modulation of Th1 and Th2 balance in the body has appeared as a major determinant for the therapeutic activity of plant extracts. Th1 cells produce the IFN-γ and IL-2 cytokines as anti-cancers and for infections disorders whereas Th2 cells produce IL-4 for humoral response and allergic conditions [18]. Our results indicated that the extract increased both IFN-γ and IL-2 cytokines. The results in this study showing a significant augment in the production of IL-2 in cultures treated with the extract, demonstrated the capacity of *S. variagata* to increase activation of the T cells and release of IL-2 cytokine. Moreover, our findings showed that the extract significantly reduced IL-4 cytokine compared to the control group. The present study showed that the extract could modulate immune response balance to Th1 activity. NK cell activity was evaluated to determine the immune modulation effect of the extract on the innate immunity. The results indicated that the extract significantly increased the NK cell cytotoxic activity. The NK cell cytotoxicity against NK-sensitive tumor cell (Yac-1) significantly increased after exposure to extract, so the extract could increase the innate immunity response against tumor cell.

Phytochemical assays have shown the presence of the main components including phenyl propanoids, phenolic compounds and flavonoids in the *S. variagata* extract. It has been shown that phenolic compounds have an antioxidant activity, anti-inflammatory and anti-tumor affect [19,20].

The results of this study suggest that the extract of *S. variagata* may modulate Th1 pathway polarization and regulate some Th1/Th2 imbalance responses such as allergy, infections and cancers. Nevertheless, study of the T cell signaling activity and the expression of T-bet and GATA-3, requires further investigation.

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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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