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Full Length Research Paper

Evaluation of *in vivo* immune response activity and *in vitro* anti-cancer effect by *Scrophularia megalantha*

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Many studies have showed that plant extracts possess immunomodulatory and anti-cancer effects. In this study, these effects of *Scrophularia megalantha* extract, a native plant in Iran, were investigated *in vitro* and *in vivo.* Microculture tetrazolium test (MTT assay) was employed to evaluate the effects of *S. megalantha* on human Jurkat (lymphoblast-like) cell line cytotoxicity. Furthermore, delayed type hypersensitivity (DTH) and hemagglutination tests in mice employed to evaluate the effects of *S. megalantha* on cellular and humoral responses, respectively. *In vitro* exposures of the Jurkat cells with various concentrations of *S. megalantha* extract (0.05, 0.1 and 0.2 mg/mL) significantly suppressed their growth in a dose-dependent manner. The maximum tumor cell growth inhibition was observed with 0.2 mg/mL of the extract. Moreover, the production of specific antibody to sheep red blood cell (SRBC) antigen in immunized mice significantly increased by different concentrations of *S. megalantha* extract. In conclusion, our results indicated that *S. megalantha* extract could be an immunomodulator plant and a good candidate for further investigations in order to develop a natural compound as an immunomodulator and anticancer agent.

Key words: *Scrophularia megalantha*, extract, tumor growth, immune response.

INTRODUCTION

Tumor is one of the most common causing deaths worldwide. Chemotherapy and surgery are standard methods for treatment of these diseases, although not been fully effective. Most of the anti-tumor drugs currently used in chemotherapy are toxic to normal cells and cause toxicity for immune cells. Therefore, the identification of new anti-cancer drug with low side effects on immune system has become an essential goal in many studies of immunopharmacology (Haishun et al., 2009). One of plants proposed to have immunomodulatory and anti-cancer effect was *Scrophularia megalantha* Boiss (Scrophulariaceae), collected from Kelardasht region (Mazandaran province) in North of Iran. Several species of this genus have been used since ancient times as folk remedies to treat ailments such as scrophulas, scabies, tumors, eczema, psoriasis, etc. Some species in this genus have shown anti-inflammatory activity (Azadmehr et al., 2009; Diaz et al., 2004; Bas et al., 2007a). However, no report on the effect of this species has been published in the literature. There have been no previous studies of the immunomodulatory activities and anti-cancer effects of *S. megalantha* on Jurkat (lymphoblast-like) cell line. In the present study, we investigated the *in vitro* anti-cancer effect and *in vivo* immunomodulatory activities of *S. megalantha* ethanolic extract.

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MATRERIALS AND METHODS

Plant collection

The aerial parts of *S. megalantha* Boiss were collected from the north part of Iran, in the Kelardasht region (Mazandaran province) and were air dried at room temperature. The plant was identified by Mr. Ajani from the Department of Botany, Institute of Medicinal Plants (IMP) of Karaj, Iran. A voucher specimen (herbarium No. 1461) was deposited in the herbarium of the above mentioned center.

Preparation of *S. megalantha* extract

Aerial parts of the plant were dried, powdered (100 g) and macerated with an 80% ethanol solution for 3 days with three changes of the solution. The resulting extract was filtered and evaporated under vacuum into a dried powder extract (12.1g, 12.1%). The plant extract was dissolved in dimethylsulfoxide (DMSO) with 0.1% v/v concentration that was not toxic and used at appropriate concentrations (0.001, 0.01, 0.05, 0.1 and 0.2 mg/ml).

Mice and cell line

Balb/c mice (6 to 8 weeks old) 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 Institute of Medicinal Plants. The human Jurkat (lymphoblast-like) cell line was prepared from the National Cell Bank of Iran, Pasteur Institute of Iran (Tehran, Iran) and maintained by culturing in RPMI 1640 medium (GIBCO BRL, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (GIBCO), 2 mM L-glutamine, 100 µg/ml streptomycin and 100 U/ml penicillin (all prepared from Sigma Co.) in a humidified incubator at 37°C and 5% CO_2.

MTT colorimetric assay

To determine the toxicity of *S. megalantha* extract against Jurkat cancer cell line, a colorimetric assay using 3-(4, 5-dimethylthiazoyl)-2, 5-diphenyltetrazolium bromide (MTT) was used. This assay measures the cellular capacity to reduce MTT to the blue formazan products by various mitochondrial dehydrogenase enzymes. Briefly, cells were added onto flat-bottomed micro-culture plates in the presence or absence of various concentrations of the extract (in triplicate) and incubated at 37^{o}C in a 5% humidified CO_2 incubator for 72 h. Then, 10 µl of MTT (5 mg/ml, Sigma) was added to each well and incubation was continued for a further 4 h at 37^{o}C. In each well, 100 µl/well of solubilization solution, contained isopropanol and 10% SDS in 0.01 M HCl were then added. After complete solubilization of dye, the optical density (OD) of the samples was read at 570 nm on an ELISA micro-plate reader. The mean optical density (OD) ± SD for each group of the replicates calculated. Percent growth inhibition of cells exposed to treatments was calculated as follows: % Inhibition = 100 - (Test OD/Non-treated OD) × 100).

Humoral antibody synthesis

Animals (Five groups of five mice) were immunized intraperitoneally (ip) with 5x10^{9} SRBC on days 0 and +7. In three groups, different doses of extract (1, 50 and 100 mg/kg of *S. megalantha* extract) administrated on days -2, -1, 1 and 2 immunization. The mice in the fourth group were injected with levamisol as positive control on the same day (2 mg/kg, ip). The fifth group was considered as non-treated control and injected only with equal amount of the vehicle. Blood samples were obtained from each mouse on day +7 for evaluating primary response and on day +14 for secondary response. Antibody titer was determined by hemagglutination test. 25 ∝l of 0.1% SRBC suspension was added to 25 ∝l of two-fold diluted serum samples in V-shape micro-titration plates. After 1 h of incubation, the last dilution of serum samples which caused hemagglutination was considered as antibody titer. To compare the results the mean Log2 of the titers was then calculated.

Delayed type hypersensitivity response

Sheep red blood cell (SRBC) was used as antigen for delayed type hypersensitivity reaction. SRBC collected in Alsever's solution, were washed three times in large volumes of pyrogen free 0.9% normal saline and standardized to 5×10^{9} cells/ml for injection. Mice were divided into four groups, each group containing five mice. Different concentrations of the *S. megalantha* extract (1, 50 and 100 mg/kg) immunized intraperitoneally in three groups at days -2, -1, 0, 1 and 2. The vehicle was injected at the same days in group four as the control. Mice were immunized subcutaneously by injecting (10^{8} SRBC/100 µl) on day 0. The mice were then challenged by injection of SRBC suspension in right hind foot pad at day 7. The thickness of the right hind foot pad was measured using vernier caliper after 24 h.

Statistical analysis

The results are presented as the means ± SD of at least three separate experiments. Statistical analyses were performed by one-way analysis of variance (ANOVA) to express the difference among the groups. All analyses was performed using SPSS software16. Data considered statistically significant at $P < 0.05$.

RESULTS

Scrophularia megalantha inhibited tumor cell growth.

The *S. megalantha* extract exhibited growth inhibitory effect on Jurkat cell line under experimental conditions in 72 h treatment. *In vitro* exposures of Jurkat cells with various concentrations of *S. megalantha* extract (0.05, 0.1 and 0.2 mg/mL) significantly suppressed Jurkat cancer cell growth in a dose-dependent manner ($P<0.001$). The maximum inhibition of Jurkat cells due to exposure to *S. megalantha* was found at 0.2 mg/mL of the extract (% 38 inhibitions, Figure 1).

Effect of *S. megalantha* on antibody response

The effect of the *S. megalantha* extract on specific anti-body synthesis is showed in Figure 2. The mean antibody titer for 50 mg/kg of the extract was 9.6±0.4 versus 8.1±0.4 in non-treated mice at primary response and 10.8 ± 0.8 versus 8.4 ± 0.5 at secondary response ($p<0.001$). Moreover, the mean antibody titer for 100 mg/kg of the extract was 10.3±0.5 versus 8.1±0.4 in non- treated mice at primary response and 11.7 ± 0.6 versus 8.4 ± 0.5 at

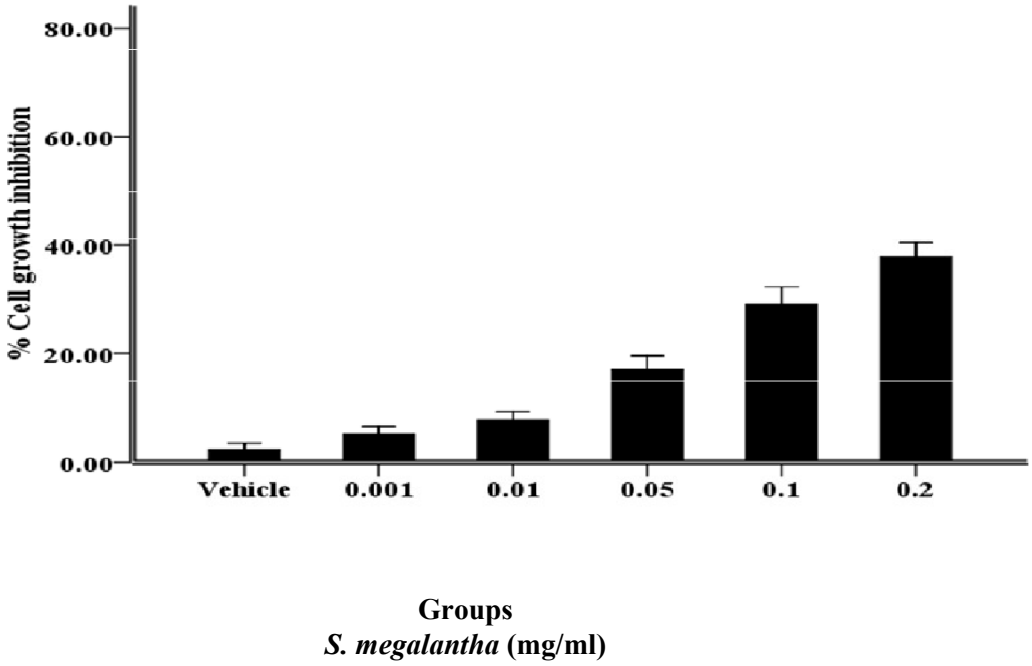

Figure 1. Effect of *S. megalantha* extract on Jurkat cell growth inhibition.

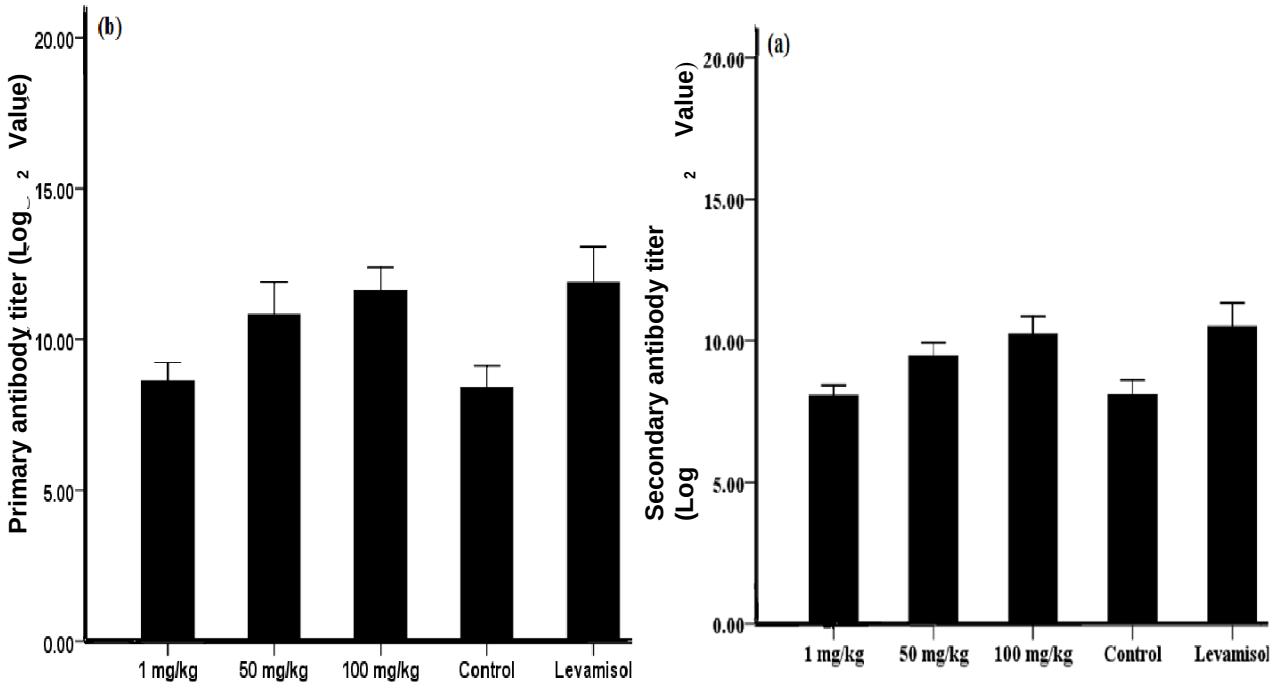

Figure 2. Effect of different doses of *S. megalantha* extract on antibody synthesis in mice (a): Primary antibody titer and (b) Secondary antibody titer.

at secondary response ($p<0.001$). The stimulatory effect of the extract on specific antibody production at these concentrations was comparable to the effect of levamisol (2 mg/kg) as a positive control at both primary (10.5±0.6) and secondary (11.8±0.9) response.

Effect of *S. megalantha* on delayed hypersensitivity response

Mice immunized with SRBC as antigen for delayed hypersensitivity reaction.

The mean footpad thickness of all mice groups treated with 1, 50 and 100 mg/kg of the *S. megalantha* extract at 24 h after immunization of extract-treated mice with SRBC was measured. The mean footpad thickness of the mice groups treated with *S. megalantha* extract had not significantly different compared to non-treated mice.

DISCUSSION

In the present study, the immunomodulatory effects of *S. megalantha* extract on immune response and tumor cell growth inhibition were investigated. *S. megalantha* is a traditional herb in Iran and has been used to treat various inflammatory diseases such as rheumatics and chronic inflammatory disorders. In previous studies on *Scrophularia* species, some immunomodulatory effects have been reported. Some species in this genus have shown anti-inflammatory activity (Azadmehr et al., 2009; Schinella et al., 2002; Bas et al., 2007b). We have previously demonstrated the inhibitory effect of *S. striata* extract on matrix metalloproteinases (MMPs) in Wehi-164 cell line (Hajiaghaee et al., 2007). In a previous study, flavonoids, cinamic acid and phenylpropanoid were isolated from aerial parts of *S. striata* Nepitrin, flavonoid glycoside, and Acteoside1, phenylpropanoid glycoside, were identified from 80% methanolic fraction (Monsef-Esfahani et al., 2010). In this study, the ethanolic extract of *S. megalantha* was studied for its probable anti-tumor activity against Jurkat cell line which is a kind of human leukemic cell line and immunomodulatory effects in vivo. According to our results which seen *in vivo*, *S. megalantha* extract has potential immunomodulatory effect for specific humoral response to SRBC. This extract had not significant effect on delayed hyper-sensitivity reaction in mice. Finding of the present study showed stimulatory effect of *S. megalantha* on humoral immunity in mice. However, no anti-cancer effect of this species has ever been reported. In the past few years, a number of Iranian herbal medicines with potent anti-cancer activity were reported, such as *Dionysia termeana, Linum persicum and Euphorbia cheiradenia* (Amirghofran et al., 2006a, b; 2007). *S. megalantha* is a promising anti-tumor herb whose mechanism of action is mostly unclear. Inhibition of cancer growth has been a continuous effort in tumor treatment. Suppression in cell growth and induction of cell death are two major means to inhibit cancer growth (Huang et al., 2003). In this study, we showed that *S. megalantha* extract could cause significant growth inhibition of Jurkat cell line in a dose-dependent manner. The extract of *S. megalantha* inhibited tumor cell growth *in vitro* and showed immunomodulatory effects *in vivo*. In conclusion, the enhancement of antibody synthesis and Jurkat growth inhibition indicated that extract contains bioreactive components that stimulate immune response and have anti-tumor effect. Moreover, our results indicated that *S. megalantha* extract could be an immunomodulator plant and a good candidate for further investigations in order to develop a natural compound as an immunomodulator and anti-cancer agent. However, further studies is needed.

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