ORIGINAL ARTICLE
Iran J Allergy Asthma Immunol
December 2011; 10(4): 281-288

Immunomodulatory Effects of *Astragalus gypsicolus* Hydroalcoholic Extract in Ovalbumin-Induced Allergic Mice Model

Mehri Ghafourian Boroujerdnia[1], Mohammad Ebrahim Azemi[2], Ali Asghar Hemmati[3], Amin Taghian[4], and Abbas Azadmehr[5]

[1] *Department of Immunology, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran*
[2] *Medicinal Plant Research Center, Department of Pharmacognosy, School of Pharmacy, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran*
[3] *Department of Parmacology, School of Pharmacy, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran*
[4] *School of Pharmacy, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran*
[5] *Immunology Division, School of Medicine, Qazvin University of Medical Sciences, Qazvin, Iran*

Received: 20 October 2010; Received in revised form: 12 May 2011; Accepted: 22 July 2011

ABSTRACT

Several studies have demonstrated that herbal extracts possess various biological effects including anti-inflammatory and anti-cancer activities. The present study was aimed to investigate the protective effects of the *Astragalus gypsicolus* (AG) hydroalcoholic extract in early allergic sensitized mice induced by ovalbumin.

Phytochemical assay was used to recognize the main active constituents in the AG hydroalcoholic extract. Mice were immunized with subcutaneous injection of ovalbumin and aluminum hydroxide. Efficiency of sensitization was assessed by serum IgE levels and eosinophil count. After sensitization, two doses of extract (250 mg/kg and 500 mg/kg) were injected intrapritoneally.

On day 14, mice were challenged with intrapritoneal injection of ovalbumin. IL-4 and IFNγ levels in broncoalveolar lavage fluid, which had been collected on day 15, were assessed by Enzyme-Linked Immunosorbent Assay (ELISA) kit.

Our results indicate two main active constituents including flavonoids and terpenoids are present in the AG hydroalcoholic extract. Intrapritoneal injection of the AG hydroalcoholic extract was able to decrease IL-4 and increase IFNγ. It seems the AG hydroalcoholic extract has the potential to modulate the balance of Th1/Th2 cytokines in allergy.

Keywords: *Astragalus gypsicolus*; Extract; Allergy; IL-4; IFNγ

INTRODUCTION

The imbalance of Th1/Th2 status immune response in human beings may deteriorate infection, autoimmune disorders[1] and allergic diseases.[2] The prevalence of allergic airway disease such as rhinitis and asthma has significantly increased over the recent decades. Cumulative evidence shows that airway allergic inflammation are attributed to T-helper (Th)

Corresponding Author: Mohammad Ebrahim Azemi; MD; Pharmacognosy Department, School of Pharmacy, Ahwaz Jundishapur University of Medical Sciences, Ahvaz, Iran. Tel/ Fax: (+98 611) 3738 381, Email: M.E.Azemi@ajums.ac.ir

type 2 cells response as well as other inflammatory factors, including mast cells, eosinophils, B cells, cytokines and chemokines.[3] They are known to produce IL-4 and IL-13 contributing to IgE production by B cells, mucus hyper secretion, airway hyper responsiveness (AHR) and to participate in the initiation of Th2 inflammatory responses. IL-5 is vital for growth, differentiation, recruitment and survival of eosinophils.[4] Therefore, several attempts are being made to reduce the inappropriate Th2 response to reduce allergic airway diseases. Therapeutic concepts include Th2 cytokine inhibitors, neutralizing antibodies directed against IgE, histamine and leukotriene blockers, as well as other targets.[5,6]

Medicinal plants have been demonstrated to be a source of a numbers of useful ingredients such as anti-inflammatory and anti-cancer compounds. The genus *Astragalus* is very large group of more than 2,000 species distributed worldwide, and is commonly known as Gavan in Iran. In the past few years, a number of Iranian herbal medicines with potent anti-inflammatory, immunomodulatory and anti-tumor activity were reported, such as Scrophularia striata, Haussknechtia Elymatica, Dionysia termeana, Linum persicum and Euphorbia cheiradenia.[7-12] About 800 species of genus *Astragalus* are distributed in Iran. However, no report on the effect of this species has been published in literature. *Astragalus* root is one of the oldest and most frequently used crude drugs for oriental medicine in Korea, China, Japan and other Asian countries, and is well known to strengthen the host defense system.[13,14] It has also effects on circulation and immune system, and enhances the cell metabolism in vitro.[15] In the present study, we investigated the effects of the *Astragalus gypsicolus* (AG) hydroalcoholic extract, a plant native to Iran, on Th1/Th2 cytokines in ovalbumin-induced murine allergic model.

MATERIALS AND METHODS

Plant Collection

AG herb was collected in May 2008 from North of Masjed Soliman city located in Khuzestan province of Iran and authenticated by herbalist. A voucher specimen for plant was deposited at the Herbarium of the Pharmacognosy Department, Jundishapur Medical University of Sciences, Ahvaz, Iran (voucher No. J4852).

The AGMM was dried at room temperature for 72 hours on sunshade, and then weighed and stored in cool-dry place until extraction. In this study the whole body of plant was used.

Extract Preparation

The dried plant was powdered by a grinder. Powdered plant (200g) was macerated in ethanol 70% for 72hr in laboratory temperature (25-30°C). The extract was filtered using a wattman filter paper No.10. The filtered extract was then evaporated under vacuum below 45°C in a vacuum drier to give a final yield of 14.98g (7.49% w/w).[16]

Phytochemical Assay

In order to identify chemical components of AG, thin layer chromatocheraphy (TLC) was used. A variety of indicators including dragendorff, and wagner for detection of alkaloids; vanillin sulfuric acid and vanillin phosphoric acid for trepenoids; ferric chloride for phenol components; natural product-polyethylenglycol (NP/PEG) for flavonoids; and kef and blood agar tests for saponins were used in this assay.[17]

The indictors were sprayed on prepared thin layers of the plant which were then observed at 280 and 260nm wavelengths under UV light and the results recorded finally.

Animals

Six- to 8-week-old male NMARI mice were purchased from the Animal Research and Care Center of Ahvaz Jondishapur University of Medical Sciences (AJUMS). Animals were housed in colony cages (8 mice per cage) in our laboratory conditions which maintained at an ambient temperature of 23±3ºC with a relative humidity of 30-70 % and a light/dark cycle of 12h during the experiment and for at least one week prior to sensitization period (for acclimatization purpose).

All mice had access to standard laboratory rodent chow and water ad libitum. All procedures involving animals were conducted in accordance with the Guidelines for Laboratory Animal Experiments in AJUMS Animal Research and Care Center.

Table 1. Description of experimental groups

	Experimental groups	Substances injected	Route of injection
1	control group	normal saline	Ip
2	negative control group	normal saline + low dose extract (250mg/kg)	Ip
3	negative control group	normal saline + high dose extract (500mg/kg)	Ip
4	positive control group	OVA + AL (OH)3+ normal saline	Sc
5	treatment group (sensitized)	low dose extract (250mg/kg)	Ip
6	treatment group (sensitized)	high dose extract (500mg/kg)	Ip

Ovalbumin (OVA) Sensitization and Allergic Challenge

Active sensitization was performed by two subcutaneous (Sc) injections of 100 and 200 µg of ovalbumin (Sigma, USA) absorbed in 1mg of aluminum hydroxide (Merk, USA) as adjuvant in 0.1 ml of pyrogen-free saline on days 1 and 7. Efficiency of sensitization was assessed by measurement of blood total IgE levels and eosinophil count on day 8. This sensitization procedure induced high levels of total IgE in serum of mice[18]. On day 14, mice were challenged with intraperitoneal (Ip) injection of 10 µg of ovalbumin in 0.2 ml of saline. Eosinophili count was repeated on day 15. Control groups injected (Sc) either 0.2ml of pyrogen-free saline or 1 mg aluminum hydroxide in 0.1ml of pyrogen-free saline.

Drug Administration

In this study mice were separated in 6 experimental groups. The control group (1) injected normal saline (vehicle). Negative control groups (2 and 3) injected normal saline plus low dose extract (250mg/kg) or high dose extract (500mg/kg) respectively. Positive control group (4) was immunized by subcutaneous injection of a emulsion containing 100µg of ovalbumin and 1mg aluminum hydroxide in 0.2ml of saline on days 1 and 7. The treatment groups (5 and 6) were sensitized by ovalbumin and then treated with the low dose extract (250mg/kg) or high dose extract (500mg/kg) on days 8 to 14 respectively. Then groups 5 and 6 were injected 10µg ovalbumin in 0.2 ml of saline (ip) on day 14th. Finally lung lavaging was collected on day 15. Groups are summarized in the table 1.

Measurement of Total Serum IgE Levels

Blood samples were collected from the tail vein of the mice on 8 day. Serum samples were separated from the blood and stored at -20˚ C until analyzed. Levels of total mouse IgE were determined by using an enzyme immunoassay kit (KOMA BIOTECH; Catalog no: K0231082; S: Koria), as described by the manufacturer. The minimum and maximum detection levels of IgE were 3.9 and 250ng/ml, respectively.

Eosinophil Count

Blood samples were obtained from the tail vein of the mice on days 8 and 15. Smears the from heprinized blood prepared on slide and eosinophil number was determined by specialist who was blinded to the groups of the study in Shafa Hospital laboratory, a teaching hospital, affiliated to Ahvaz university of Medical sciences

Measurement of IFN-γ and IL-4 Cytokines in Bronchoalveolar Lavage (BAL) Fluid

For preparation of bronchoalveolar lavage (BAF) fluid the thorax cavity of each mouse was opened after sheering the omohyiod and stylohyoid muscles, Thus a needle or a fine polyethylene tube was fixed in trachea (for prevention of lavage reflux) and 1 ml of normal saline at 37°C was injected to the fixed tube via insulin syrange and then it was aspirated. This operation was repeated until 2 ml of BAL fluid was taken. The process was performed for all the mice in the six groups.

The level of IL-4 and IFNγ in BAL fluid were determined by enzyme-linked immunosorbent assay (ELISA) kits (KOMA BIOTECH; Catalog no: K0231082; for IL-4 and S: Koria) according to the manufacturer's protocol. Cytokine concentrations were determined with a standard curve derived from known amounts of the relevant cytokine reading absorbance at 450 nm on a spectrophotometer (TECAN). The minimum detection levels of IL-4 and IFNγ kits were 32 and 16 pg/ml, respectively.

Table 2. Phytochemical results of hydroalcoholic extract of *Astragalus gypsicolus*

Categories of compounds	Tests, reagent used	Results
Alkaloids	Mayer's test	+
	Dragendorff reagent	+
	Wagner's reagent	+
Terpenoids and phenylpropanoids	Vanillin sulfuric acid reagent	+
Terpenoids, lignins and cucurbitacins	Vanillin phosphoric acid	+
Phenolic compounds	Ferric chloride	+
Flavonoides	Natural product reagent	+
Saponins	Kef and blood agar tests	+

Table 3. Serum concentration of IgE in different mice groups

Experimental groups	Type of treatment	Mean±SEM (ng/ml)
Group 1	Normal saline	19.17±5.53
Group 2	AL (OH)3	20.43±9.1
Group 3	OVA (100ug/mice) + AL(OH)3	77.65±18.3
Group 4	OVA (200ug/mice) + AL (OH)3	85.5±19.6

Statistical Analysis

The results were analyzed with one-way variance (ANOVA) test to assess mean difference and TUKEY test to assess meaningful difference between various groups. Data were presented as mean values ± S.D. and $P < 0.05$ was considered statistically significant.

RESULTS

Chemical Components of AG Extract

Phytochemical assay by thin layer chromatography showed main components of genus *Astragalus* including trepenoids, flavonoids and saponins are present in the plant. The results also showed the plant has alkaloids and phenol components (Table 2).

Ovalbumin-induced Allergic Mouse Model

The IgE level increased significantly in the groups receiving 100 and 200µg of ovalbumin compared to control group receiving normal saline (Table 3, Figure 1) (p<0.001). There was not significant difference in serum IgE level between group 3 and 4 (p=0.62). So, the two doses had the same effects. IgE level did not show significant increase in group 2 compared to group 1. Eosinophil count was negative in prepared smears from all groups on day 8. However, high eosinophil count was seen in the group receiving 100µg of ovalbumin on day 15 (5.30±2.80). In other groups, no eosinophil was detected in the blood smears on day 15.

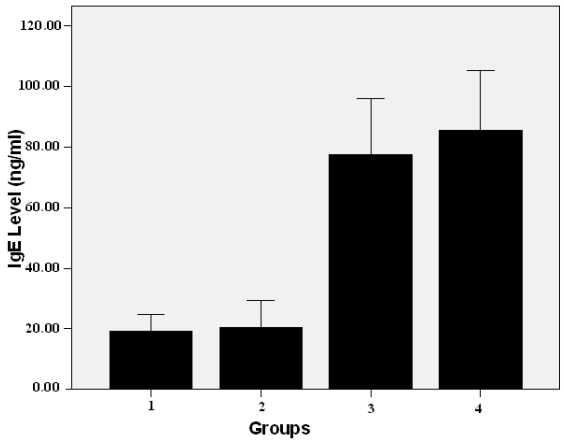

Figure 1. Serum concentration of IgE in mice.
The IgE level increased significantly in both groups receiving 100 and 200µg of ovalbumin in comparison with control group (*P<0.001). There was not significant enhancement of IgE concentration in the group receiving AL(OH)3 compared to control group. (Mean±SEM)
1- Control group (normal saline)
2- Group receiving AL (OH)3
3- Group receiving OVA with dose 100ug/mice+AL (OH)3
4- Group receiving OVA with dose 200ug/mice+AL (OH)3

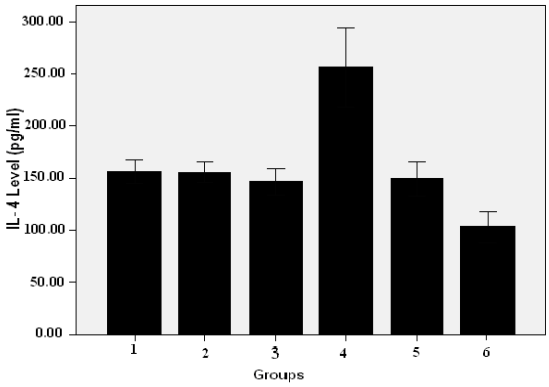

Figure 2. IL-4 levels in bronchoaloveolar lavage (BAL) fluid of different groups.

IL-4 in BAL fluid was significantly higher in positive control group compared to other groups (* p<0.001). IL-4 level in healthy mice groups receiving both low and high doses of extract was similar to control group. IL-4 level in sensitized mice groups receiving both low and high doses of the extract significantly diminished compared to positive group (*p*<0.001).

1-Control group (normal saline)

2- Mice receiving low level extract

3- Mice receiving high level extract

4- Positive control (OVA + AL (OH)3 + saline)

5- Sensitized mice group receiving low level extract

6- Sensitized mice group receiving high level extract

Cytokine Assay

Maximum concentration of IL-4 in BAL fluid was found in control positive group and minimum concentration in sensitized mice group receiving high dose of extract (Figure 2). IL-4 level did not significantly decrease in normal mice groups receiving high and low doses of the extract in comparison with control group (p=1.00 and p=0.96, respectively). Moreover, no significant difference was found between two healthy mice groups receiving high and low doses of the extract (p=0.98). However ,production of IL-4 level decreased significantly in sensitized mice groups receiving high and low doses of the extract in comparison with positive control group (p<0.001) in which it was more significant in the group with high dose to low dose. (p=0.005). Maximum concentration of IFNγ in BAL fluid was found in healthy mice receiving high dose of the extract and minimum concentration in positive control group on day 15. IFNγ level increased significantly in mice receiving high and low doses of plant extract in comparison with control group (p<0.001 and p<0.001, respectively). Similarly these two doses affected on sensitized mice groups compared to positive control group (p<0.001). It was noticed that the increased level of IFNγ in sensitized mice groups were less than normal groups (Figure 3).Extract with 500mg/kg induced more significant enhancement of IFNγ concentration in both normal and sensitization mice groups than extract with 250mg/kg (p<0.001). Thus, dose 500mg/kg was more effective.

DISCUSSION

Asthma is a chronic inflammatory disorder characterized by reversible airway obstruction, bronchial hyperresponsiveness and airway inflammation.

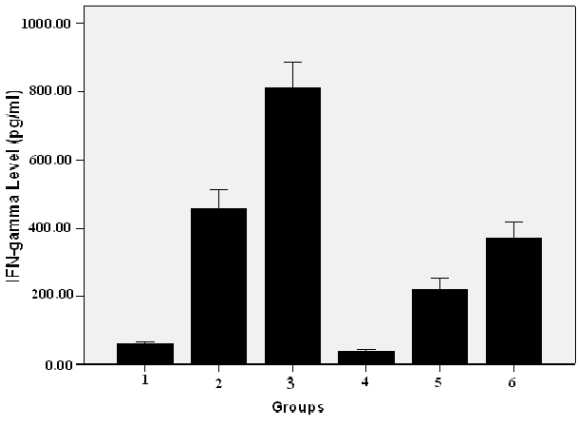

Figure 3. IFNγ level in bronchoaloveolar lavage (BAL) fluid in different groups.

IFNγ significantly increased in healthy mice groups receiving both low and high doses of the extract compared to control group (*p<0.001). This enhancement was also seen in sensitized mice groups receiving both low and high doses of the extract compared to positive control group (*p<0.001). Note, increased level of IFNγ in sensitized mice groups were less than healthy mice groups. The extract with 500mg/kg dose was more effective than 250mg/kg in sensitized mice groups.

1-Control group (normal saline)

2- Mice receiving low level extract

3- Mice receiving high level extract

4- Positive control (OVA + AL (OH)3+ saline)

5- Sensitized mice group receiving low level extract

6- Sensitized mice group receiving high level extract

Although potent anti-inflammatory drugs, such as glucocorticoids, are available to treat asthma, these drugs produce unwanted side effects and exhibit limited efficacy in treatment. In the present study, we investigated the effects of the AG hydroalcoholic extract, a native plant in Iran, on Th1/Th2 cytokines in ovalbumin-induced murine allergic model in comparison with control group. Our results indicated main active components including flavonoids, saponins and terpenoids are present in the AG hydroalcoholic extract. These chemical components detected in different species of *Astragalus* with various effects. Triterpene saponins extracted from roots of *Astragalus* species in Turkish folk medicine showed a prominent IL-2 inducing activity by in vitro study.[19] Different contents from three herbs of *Astragalus*, including total extract, flavonoids extract, saponins extract, polysaccharides extract, and amino acids extract, showed different effects in which polysaccharides were the major constituents of the three herbs with different quantities.[20] A positive involvement of the TLR4 molecule in *Astragalus* polysaccharides mediated macrophage activation also was demonstrated[21]. Improvement of CD4/CD8 ratio in chickens following four Chinese herbal polysaccharides administered at vaccination has been reported[22]. Although phytochemical assay was not able to reveal polysaccharide fraction in our experiment, it is supposed the extract possessed this fraction because of immunomodulation effects. IL-4 cytokine predominates in allergic patients. In present study significant IgE levels in sensitized mice was detected in comparison to control groups. Increase of IgE concentration can occur following IL-4, IL-5 and IL-13 production. IL-5 is a potent eosinophil activating cytokine which enhances the ability of eosinophils to release granule contents similar to mastocytes and basophiles. Eosinophilia observed on day 15, was at a significant numbers in the study. IL-4 produced by Th2 cells may enhance expression of adhesion molecules for eosinophils to recruit and infiltrate.[23] Reversing Th2 dominant is thought to be a promising strategy in treatment of asthma and allergy. Intrapritoneal injection of the AG hydroalcoholic extract was able to decrease IL-4 and increase IFNγ cytokine levels in the present study. IFNγ secreted dominantly by Th1 and NK cells, promotes further Th1 differentiation and inhibits the proliferation of Th2 cells.[23] IL-4 and IL-10, as the natural antagonists, suppress the function and production of IFNγ. The production of Th2 cytokines and promoting effects of Th1 cytokines revealed that AG is a promising candidate for treating Th2-biased diseases including allergy. In agreement with present study, using *Astragalus membranaceus* on a mouse model of chronic asthma it was found enhanced IFNγ and suppressed elevation of IL-5, IL-13 in BAL fluid.[24] *Astragalus membranaceus* administration significantly decreased inflammatory infiltration and mucus secretion in the lung tissues of the allergic mice.[24] Astragali-Cordyceps Mixtura, a traditional Chinese herbal medicine, greatly improves the symptoms of asthma airway remodeling by inhibiting the expression of TGF-β and upregulating the amount of Smad7.[25] Parallel with these studies, in a case - control study *Astragalus membranaceus* showed increase in expression of T-bet mRNA and Th1 cytokines in patients with asthma.[26] Recently efficacy and safety of *Astragalus membranaceus* in the treatment of patients with seasonal allergic rhinitis have been demonstrated.[27]

Inhibitory effects of astragaloside IV, a new extract of *Astragalus membranaceus*, on ovalbumin-induced chronic experimental asthma have also been reported. The effects were significant reduction of eosinophilic airway inflammation, airway hyperresponsiveness, and decrease IL-4 and IL-13 levels in BAL fluid and total IgE levels in serum.[28] In the present study, AG increased level of Th1 cytokine (IFNγ) and reduced Th2 cytokines (IL-4) in BAL fluid of sensitized mice in two different concentrations. The IFNγ/IL-4 ratios which express Th1/Th2 ratio was greater in mice treated with 500mg/kg dose of the extract. Although it has been shown that *Astragalus* species have a very low toxicity[29], it is recommended the use AG in dose 250mg/kg and even less for achieving a balance of Th1/Th2 to normal situation. Two different concentrations of the extract increased IFNγ in both healthy and sensitized mice groups and decreased IL-4 only in the sensitized mice groups. It was not as expected a decrease of IL-4 in normal mice group when IFNγ increased. Two reasons can be suggested: sanitization in mice may cause activation the immune system of pathways that increase IFNγ and decrease IL-4, and other doses of the extract may induce this effect in normal mice group which requires more investigations. In conclusion, it seems the AG hydroalcoholic extract has the potential effect to modulate the balance of Th1/Th2 cytokines in allergy.

ACKNOWLEDGEMENT

We would like to express our gratitude to all the people who contributed to this work specially Sadigheh Yosef-Nanaei who authenticated AG herb. This project of study is part of a pharmacy thesis of Mr Amin Taghian and financially supported by Ahvaz Jondishapur University of Medical Sciences (No U-86142).

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