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Effects of *Viola tricolor* Flower Hydroethanolic Extract on Lung Inflammation in a Mouse Model of Chronic Asthma

Elham Harati[1,2], Maryam Bahrami[2], Alireza Razavi[3], Mohammad Kamalinejad[4], Maryam Mohammadian[5], Tayebeh Rastegar[6], and Hamid Reza Sadeghipour[2]

[1] Iranian Center of Neurological Research, Neuroscience Institute, Imam Khomeini Hospital, Tehran University of Medical Sciences, Tehran, Iran
[2] Department of Physiology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran
[3] Department of Pathobiology, Division of Immunology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran
[4] Department of Pharmacognosy, School of Pharmacy, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[5] Department of Physiology, Faculty of Medicine, Kermanshah University of Medical Sciences, Kermanshah, Iran
[6] Department of Anatomy, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran

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ABSTRACT

Asthma is a chronic inflammatory disease of the lungs driven by T cell activation. *Viola tricolor* L. as a traditional medical herb could suppress activated T lymphocytes and has been used empirically for asthma remedy. In the present study, we investigated the anti-inflammatory effect of *Viola tricolor* and its underlying mechanism on asthma characteristics induced by ovalbumin (OVA) in mice.

BALB/c mice were randomly divided into six groups: normal control, Ovalbumin (OVA) control, OVA mice treated with *Viola tricolor* (50, 100 and 200 mg/kg) and dexamethasone (3 mg/kg). All mice except normal controls were sensitized and challenged with OVA. Asthmatic mice were treated orally in the last 7 days of OVA challenge. The total and differential leukocyte counts, Interleukin (IL)-4 and interferon (IFN)-γ levels in bronchoalveolar lavage fluid (BALF) were determined. H&E staining for lung inflammation was performed.

Viola tricolor treatment at 200 mg/kg significantly decreased IL-4 level but did not considerably affect the IFN-γ level. Therefore, it effectively reduced asthma characteristics including infiltration of leukocytes particularly eosinophil and peribronchial inflammation as compared to dexamethasone. However, *Viola tricolor* at 100 mg/kg had the most prominent inhibitory effect on the IL-4 level and also markedly increased IFN-γ level. As result, it prevented further reduction of inflammatory parameters in this group compared to the *Viola tricolor*-treated group at 200 mg/kg.

Corresponding Author: Hamid Reza Sadeghipour, PhD; Department of Physiology, Faculty of Medicine, Tehran University of Medical Sciences, Porsina Ave, Tehran, Iran. Tel/Fax: (+98 21) 6641 9484, E-mail: sadeghipour@tums.ac.ir

Co-Corresponding Author: Elham Harati, PhD; Iranian Center of Neurological Research, Neuroscience Institute, Imam Khomeini Hospital, Tehran University of Medical Sciences, Tehran, Iran. Tel/Fax: (+98 21) 6119 2297, E-mail: eharati@alumnus.tums.ac.ir

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Our study demonstrated that *Viola tricolor* has anti-inflammatory effects via inhibition of T-helper type 2 (Th2) cytokine production and validated its empirical usage in traditional medicine.

Keywords: Asthma; Eosinophil; Inflammation; Ovalbumin; Viola

INTRODUCTION

Allergic asthma is a chronic T-cell-mediated inflammatory lung disease characterized by accumulation of inflammatory cells particularly eosinophil in the bronchial mucosa and airway inflammation.[1] In susceptible persons, this inflammation causes the recurrent manifestation of wheezing, breathe shortness and cough.[2] Many pathologic features of asthma are induced by Th2 cells which produce cytokines such as IL-4. In addition, Il-4 induces proliferation of Th2 cells, raises the serum immunoglobulin E and stimulates airway eosinophil infiltration. In turn, IgE and eosinophil inducing the release of inflammatory mediators. These secreted mediators lead to airway inflammation, increased vascular permeability and recruitment of inflammatory cell such as eosinophils.[3-7] Eosinophils are the most abundant inflammatory cell recruited into the airways of asthmatic patients and contribute to the many of the pathological processes associated with asthma.[1,8] Compared with Th2 cells, the role of the Th1 cytokine such as IFN-γ in asthma pathogenesis is ambiguous. It is believed that Th1 by inhibiting Th2 responses, has a protective effect in asthma[9] but there is contradictory evidence that shows the level of IFN-γ correlated with asthma severity and inflammatory response.[10,11] Therefore, it is unclear if a shift in balance of Th2 cytokines to Th1 cytokines production may improve asthma symptoms or worsen this situation.[12]

The most common anti-inflammatory drugs used in asthma are glucocorticoids. Nonetheless, prolonged intake of these medications is usually associated with numerous side effects. Because of these complications and the chronic nature of asthma, many patients traditionally tend to use medicinal herbs due to the presence of effective ingredients and fewer side effects. Therefore, it is necessary to explore new strategies with natural dietary products.[13,14] There is increasing scientific evidence demonstrating that medicinal plants have the potential for treating asthma.[15-19] *Viola tricolor* L., belonging to the Violaceae family, is one of the medicinal plants widely spread throughout the world.[20] The major constituents of the plant are flavonoids particularly rutin, and other compounds, like anthocyanins, coumarins, tannins, saponins, phenolic acids and cyclotides.[20,21] *Viola tricolor* has a long history in folk medicine in treating bronchitis and asthma. It has remarkable pharmacological activities like anti-inflammatory, antioxidant, antitussive, expectorant properties.[22,23] In addition, this herb has immunosuppressive activity and can block proliferation of activated lymphocytes.[23] Therefore, this study was designed to investigate the protective effects and immunological mechanism of *Viola tricolor* herb on airway inflammation in the mouse model of OVA-induced allergic asthma. Also, assess the controversial role of IFN-γ in the asthma management.

MATERIALS AND METHODS

Preparation of *Viola tricolor* Hydroethanolic Extract

The *Viola tricolor* L. was collected from the garden in Tehran (its seed imported from the Netherlands). The flowers were dried in shade at room temperature for 7 days. The dried flowers were powdered with the help of grinder. Then, the powdered material (100 gr) was macerated with 800 mL of 96% ethyl alcohol (Kimia alcohol Zanjan Co., Iran) and water (1:1) and kept in the laboratory for 72 hours. After filtration, it was concentrated by the rotatory evaporator (Heidolph, Germany) and dried extract was obtained. Concentrated *Viola tricolor* hydroethanolic extract kept in 2-8 ºC and was freshly dissolved in normal saline before used.

Mice

Sixty male BALB/c mice (6–8 weeks old) (18±2 g), were purchased from Pasteur Institute of Iran and kept in the animal house one week prior to the study for acclimatization. Mice maintained in a stainless steel cage at 23±2°C with 12 h light/dark cycle and water and food available *ad libitum*. This study was carried

out in strict accordance with the guidelines for the care and use of laboratory animals of the Tehran University of Medical Sciences. Study procedures were approved by the Animal Ethics Committee of Tehran University of Medical Sciences (Number: IR.TUMS.REC.1395.2465).

Experimental Protocol

BALB/c mice were randomly divided into six groups: normal control, OVA (asthma) control, OVA mice treated with *Viola tricolor* (50 mg/kg, 100 mg/kg and 200 mg/kg) and dexamethasone (3 mg/kg). For asthma modeling, mice were sensitized by i.p. injection of OVA (20 µg) (grade V, Sigma, USA) and aluminum hydroxide (2 mg) (Sigma, USA) on days 0 and 14. Next, the mice were challenged with 1% OVA aerosol in a closed plexiglass chamber (dimensions 40×40×70 cm) connected to a nebulizer (Omron CX3, Japan, particle size 3–5 µm and output of 5 l/min) for 30 min. The challenge was carried out from day 21 of protocol for 8 weeks, 3 times in a week.[24,25] The normal control animals were received i.p. injections of 2 mg aluminum hydroxide gel and further were challenged with normal saline. Mice were treated orally (via gavage) with *Viola tricolor* at different doses (50 mg/kg, 100 mg/kg or 200 mg/kg) in the last 7 days of OVA challenge. Dexamethasone (3 mg/kg) (Iran hormone Co., Iran), used as reference drug.[26] Normal group and model group were received only saline. Animals were sacrificed 24 h after the last challenge (thus on day 75) to investigate the anti-asthmatic effects of *Viola tricolor*. The schedule for asthma modeling and treatment is shown in Figure 1.

Bronchoalveolar Lavage Fluid (BALF) Collection

At the end of the experimental period (day 75), the animals were anesthetized with ketamine (80 mg/kg) and xylazine (8 mg/kg). The trachea was cannulated and lungs were lavaged with 0.4 mL sterile saline for three times. The BALF sample was centrifuged at 1500 rpm for 10 minutes. The supernatant was stored at -70 °C until analysis of cytokines (IL-4 and IFN-γ) levels. The cell pellet was resuspended in 500 µL normal saline and total WBC count was determined following staining in Turk's solution using a Neubauer counting chamber. To perform the differential cell counts, 0.1 mL of the cell suspension was smeared on a slide and stained with Wright-Giemsa solutions. According to staining and morphological criteria, at least 200 cells were counted under a light microscope (400x magnification) and the percentage of each cell type was calculated.

Measurement of Cytokine Levels in BALF

The mouse enzyme-linked immunosorbent assay (ELISA) kits (IBL, Germany) were used according to the manufacturer's brochures for the quantitative detection of IL-4 and IFN-γ in BALF. Sensitivities of the ELISA kits were 2 pg/mL for IL-4 and 5.3 pg/mL for IFN-γ.

Histopathological Analysis

The lungs were fixed in formalin and embedded in paraffin blocks. Tissue sections were prepared at 4 µm thicknesses and stained with hematoxylin-eosin (H&E) solution to determine inflammation. The peribronchial inflammation score in five randomly distributed airway sections was analyzed for each animal and their average scores were calculated. The grading system of peribronchial inflammation was: 0, no cells; 1, a few cells; 2, a ring of cells 1 cell layer deep; 3, a ring of cells 2–4 cells deep; 4, a ring of cells 4–6 cells deep; and 5, a ring of cells >6 cells deep.[27]

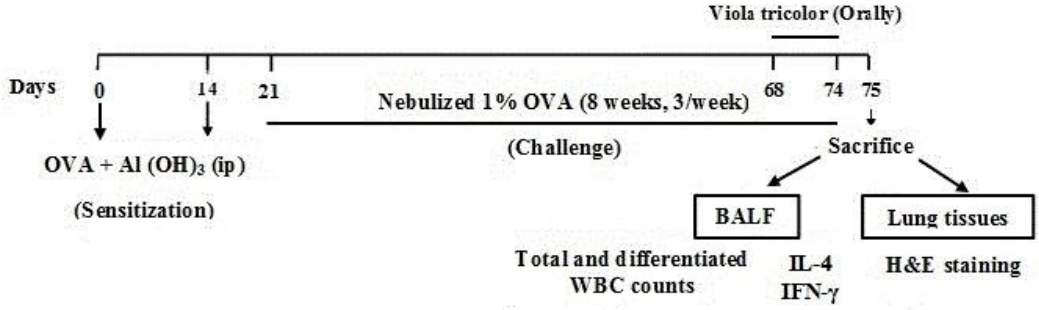

Figure 1. Timeline for mouse model of chronic asthma and treatment with *Viola tricolor*

Statistical Analysis

The results are expressed as the mean ± standard error of mean (SEM) for each group. One-way ANOVA with Tukey's post hoc test was performed using GraphPad Prism version 5.00 for Windows, (GraphPad Software, San Diego, California, USA). Differences were considered to be statistically significant at $p<0.05$.

RESULTS

Effect of *Viola tricolor* on the Infiltration of Inflammatory Cells in BALF

As shown in Figure 2, OVA challenge significantly increased total WBC counts ($p<0.01$) and the percentage of eosinophils ($p<0.001$) in BALF of asthmatic mice compared with normal control group (Figure 2A and B). In contrast, lymphocyte percentage in OVA control animals was reduced compared to normal control animals (non-significant) (Figure 2C). However, treatment with *Viola tricolor* at all three doses ($p<0.05$ or $p<0.01$) as well as dexamethasone ($p<0.05$) markedly reduced total leukocyte counts compared to non-treated asthmatic animals. The total WBC count in Viola tricolor-treated group at 100 mg/kg was little higher than the 50 mg/kg (Figure 2A). Interestingly, treatment with *Viola tricolor* at 200 mg/kg had the most potent inhibitory effect on eosinophil infiltration and significantly suppressed the percentage of eosinophils ($p<0.001$) in BALF compared with the asthmatic group. Also, animals treated with standard drug dexamethasone significantly decreased the percentage of eosinophils ($p<0.01$). Nevertheless, *Viola tricolor* at 50 and 100 mg/kg had no significant differences in eosinophil percentage compared with the asthmatic group (Figure 2B). In addition, *Viola tricolor* at 50 and 100 mg/kg decreased lymphocyte percentage even more than OVA control animals (Figure 2C). *Viola tricolor* at 200 mg/kg increased monocyte percentage as compared to asthmatic animals ($p<0.01$) and normal control (non-significant) (Figure 2D).

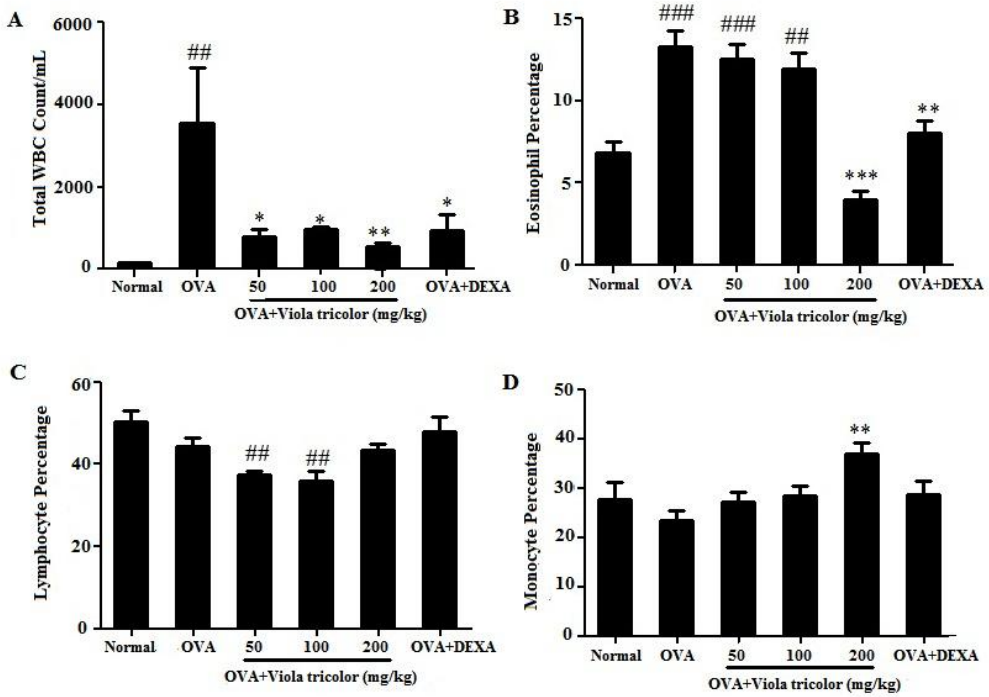

Figure 2. Effect of *Viola tricolor* extract on the infiltration of inflammatory cells in BALF of asthmatic mice. (A) Total WBC count; (B) the percentage of eosinophils; (C) lymphocytes and (D) monocytes in NC, normal control animals (n=7), OVA (asthma) control animals (n=9) and asthmatic animals treated with *Viola tricolor* (50, 100 and 200 mg/kg) or dexamethasone (DEXA) (3 mg/kg) (n=9). Data are expressed as means±SEM. $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ vs. normal control and $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ vs. OVA control.

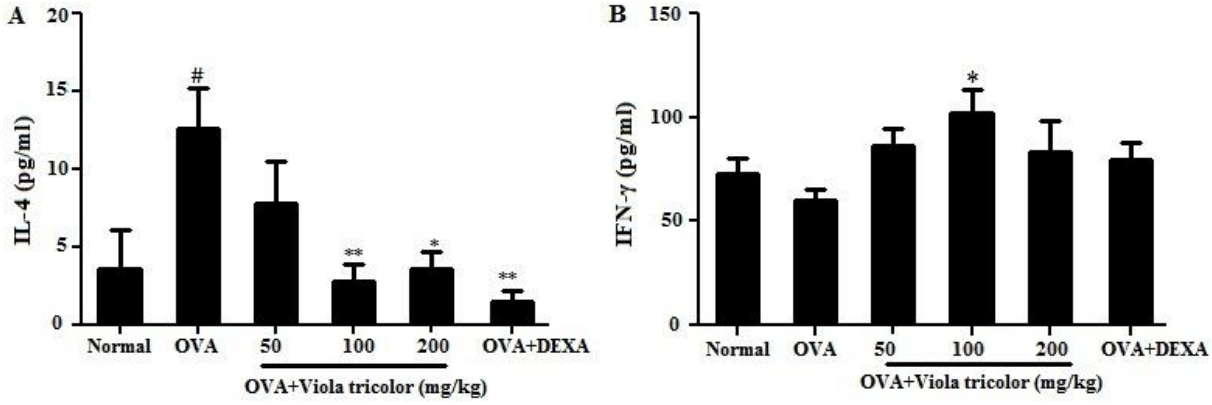

Figure 3. Effect of *Viola tricolor* extract on cytokines production in BALF of asthmatic mice. (A) IL-4; (B) IFN-γ in normal control animals (n=7), OVA (asthma) control animals (n=9) and asthmatic animals treated with *Viola tricolor* (50, 100 and 200 mg/kg) or dexamethasone (DEXA) (3 mg/kg) (n=9). Data are expressed as means±SEM. $^{\#}p<0.05$, $^{\#\#}p<0.01$ vs. normal control and $^{*}p<0.05$, $^{**}p<0.01$ vs. OVA control.

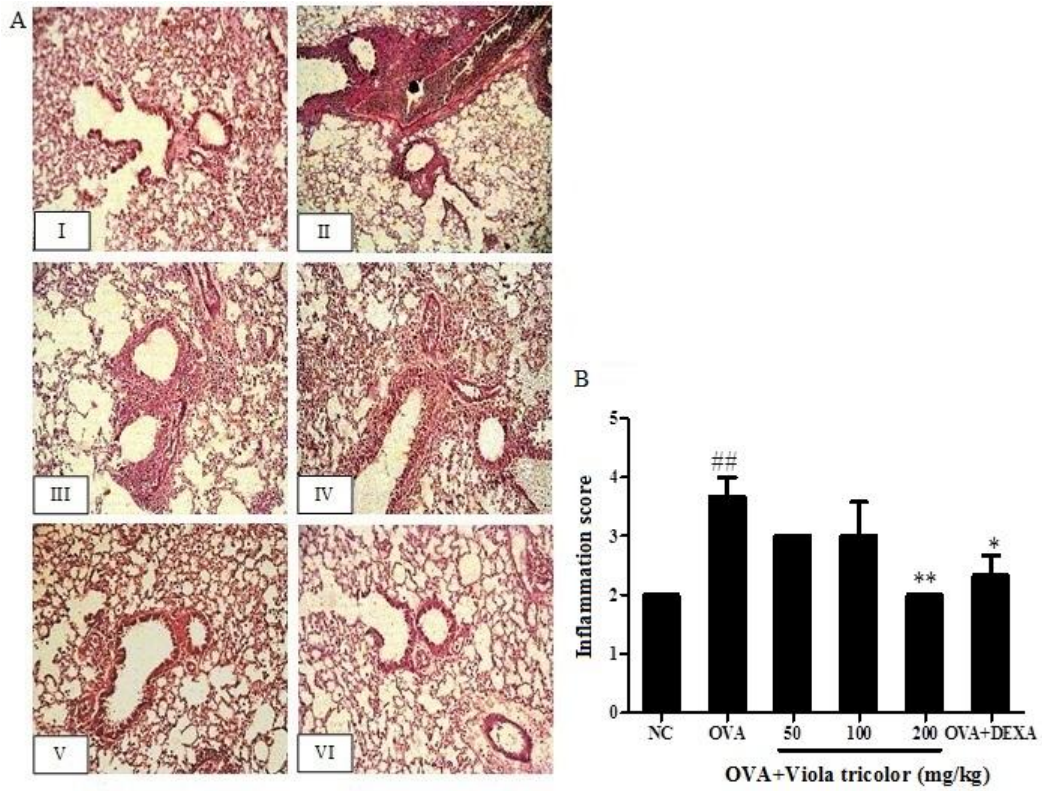

Figure 4. Effect of *Viola tricolor* extract on peribronchial inflammation of asthmatic mice. (A) Lung sections were stained with H&E solution (10×10 magnification), (B) Scoring of peribronchial inflammation in lung sections: I, normal control animals; II, OVA (asthma) control animals and III, IV, V and VI asthmatic animals treated with *Viola tricolor* (50, 100 and 200 mg/kg) or dexamethasone (DEXA) (3 mg/kg) respectively. Data are expressed as means ±SEM. $^{\#\#}p<0.01$ vs. normal control and $^{*}p<0.05$, $^{**}p<0.01$ vs. OVA control.

Effect of *Viola tricolor* on the Cytokines Production in BALF

To determine whether *Viola tricolor* influenced Th2 and Th1 cytokines production into BALF, we measured the levels of IL-4 and IFN-γ in BALF. The level of IL-4 significantly increased ($p<0.05$) but IFN-γ level slightly decreased (although not statistically significant) in BALF of asthma group compared to those in the normal control group. Treatments with *Viola tricolor* at 100 mg/kg ($p<0.01$) and 200 mg/kg ($p<0.05$) as well as treatment with dexamethasone ($p<0.01$) markedly decreased IL-4 level versus asthmatic animals (Figure 3A). In the IFN-γ (Figure 3B), administration of *Viola tricolor* only at a dose of 100 mg/kg significantly increased the IFN-γ production into BALF more than the other treatment groups ($p<0.05$).

Effect of *Viola tricolor* Extract on Peribronchial Inflammation

Peribronchial inflammation score was significantly elevated in asthmatic compared to normal control group ($p<0.01$). *Viola tricolor* at all doses reduced peribronchial inflammation; however, significant differences were observed for 200 mg/kg and dexamethasone only ($p<0.01$ and $p<0.05$ respectively) (Figure 4A and B).

DISCUSSION

Asthma is one of the most common chronic diseases globally and its prevalence is increasing during recent decades.[28] Although conventional therapy such as inhaled corticosteroid and long-acting β2 agonist have been used to control asthma symptoms, herbal therapy is still in use around the world.[13] *Viola tricolor* is traditionally used in folk medicine to treat respiratory disorders.[23] To our knowledge, this study for the first time indicated that *Viola tricolor* was able to reduce asthma-related inflammation induced by OVA in mice and validated its empirical usage in traditional medicine.

A mouse model is a valuable tool for evaluating new therapies for asthma. Although the mouse model does not exactly reproduce human asthma, in this study we used the chronic mouse model of asthma which is more similar to asthma in humans.[29] It is well known that OVA sensitization induces allergic asthma by Th2 predominant responses but there are controversies about Th1 responses.[30] In our study, the Th2-related cytokine IL-4 was significantly elevated in asthma group (Figures 3A) but the Th1-related cytokine IFN- γ was not affected by OVA sensitization (Figure 3B). One possible reason could be the presence genetically dominant Th2 immune responses in BALB/c mice.[31-33] Moreover, Th2-produced IL-4 induces asthma features including recruitment of inflammatory cells into the airways and bronchial inflammation[3,5,30,34] In our experiment, OVA mice showed higher levels of Th2-related cytokine IL-4, total WBC number and eosinophil percentages and obvious peribronchial inflammation compared to normal non-sensitized mice which confirmed an effective asthma modeling. However, lymphocyte percentage in OVA control animals was reduced compared to normal control animals (Figure 2C). This reduction in lymphocyte percentage is perhaps due to increased percentage of eosinophil in asthmatic animals. Although lymphocyte percentage was reduced, we speculated an increase in infiltration rate and absolute number of lymphocyte due to marked rise in total WBC count. The percentage of lymphocytes was multiplied by the white blood cell count to give the absolute lymphocyte number. In fact, decreased percentage of the lymphocyte using similar method of animal sensitization was reported in previous studies.[35,36]

Since Th2 cytokines produced by T lymphocytes play an important role in asthma pathogenesis,[3] the therapeutic agent suppressing the proliferation of T cells could be beneficial for resolution of asthma. *Viola tricolor* is one of the medical herbs which has immunosuppressive properties and attenuates proliferation of activated lymphocytes.[23] We observed a reduction in lymphocyte percentage in *Viola tricolor* at 50 and 100 mg/kg even more than OVA control animals. This effect is indicative of the immunosuppressant action of Viola tricolor on activated lymphocyte as it can block proliferation of these cells. Surprisingly, we did not observe this effect for 200 mg/kg. The possible explanation for this can be the masking effect of potent reduction of eosinophil percentage.

In our study, *Viola tricolor* treatment of asthmatic mice reduced IL-4 production and increased IFN-γ level. This suggests that Viola tricolor's immunosuppressive effect was particularly on Th2 cells. Moreover, *Viola tricolor* shifted T helper polarity towards Th1 and as result we observed an increased in

IFN-γ level. Furthermore, treatment at 100 mg/kg had prominent inhibitory effect on IL-4 production (Figure 3A), but also increased IFN-γ level profoundly as compared to 200 mg/kg (Figure 3B). Even though the dose of 100 mg/kg greatly reduced IL-4 production, the profound rise in proinflammatory IFN-γ prevented further reduction of inflammatory markers. At this dose, total WBC number (Figure 2A), eosinophil percentage (Figure 2B) and inflammation score (Figure 4A and B) were less improved and were higher than 200 mg/kg. We observed a little higher total WBC count in Viola tricolor-treated group at 100 mg/kg compared to the 50 mg/kg. Since IFN-γ is a proinflammatory cytokine, it seems that significant increase in IFN-γ level in 100 mg/kg may have prevented further reduction in WBC number in this group when compared to 50 mg/kg. Our findings are in accordance with a previous study which demonstrated that some effects of Th2 cytokines in the lungs are potentiated by IFN-γ such as inflammation. Therefore, increased IFN-γ level can inhibit certain therapeutic effects of reduced Th2 cytokines in asthma.[10-12] In contrast to our findings, previous researches have suggested that IFN-γ induces therapeutic effects in asthma.[37,38]

We interestingly found that 200 mg/kg extract-treated animals showed the greatest reduction of leukocyte infiltration particularly eosinophil (Figure 2B) as well as peribronchial inflammation (Figure 4) compared to other doses of *Viola tricolor* as well as dexamethasone treated group. These effects may have resulted from a decrease in the level of Th2-related cytokine IL-4, which mediates eosinophilic inflammation in the asthmatic lungs.[1] This finding is in agreement with the result of a study which indicated that *Viola tricolor* gel has anti-inflammatory effects in a model of sunburn in rats.[39] We observed an increase in monocyte percentage in 200 mg/kg *Viola tricolor*-treated animals compared to normal control animals (non-significant) and OVA control animals ($P<0.01$) (Figure 2D). This rise in monocyte percentage in 200 mg/kg does not indicate the side effect of this herb as it happened due to significant reduction in eosinophil percentage. Since total WBC reduced in 200 mg/kg, the monocyte absolute number and its infiltration rate also decreased.

Overall, in spite of the finding that the most inhibition of Th2 cytokine production was at 100 mg/kg *Viola tricolor*, the most beneficial effects of *Viola tricolor* as asthma remedy were observed at the dose of 200 mg/kg. It seems that optimal inhibition of IL-4 production and normalization of IFN-γ level by the appropriate dose of *Viola tricolor* (200 mg/kg) reduced asthma hallmarks and led to the best therapeutic effects. Despite excessive inhibition of IL-4 production, 100 mg/kg *Viola tricolor* was less effective in asthma remedy as it induced a considerable increase of IFN-γ level. However, this result suggests that Th1-related cytokine IFN-γ could prevent most beneficial effects mediated by reduced Th2 cytokine in asthma therapy.

The current study did not perform flow cytometric analysis for the quantitative leukocyte count in BAL samples. Moreover, remodeling indices like collagen deposition and mucus production were not evaluated. Therefore, there is a need for future studies to evaluate effects of Viola tricolor on the airway remodeling as well as the clinical investigations are warranted.

In conclusion, the current study demonstrated that *Viola tricolor* could effectively suppress infiltration of leukocytes particularly eosinophil and peribronchial inflammation. Our findings support the traditional application of *Viola tricolor* as a therapeutic drug for patients with allergic asthma.

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