

The effect of *Viola tricolor* L. flower hydro-alcoholic extract on anxiety-like behavior in a mouse model of chronic asthma

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Abstract

Background: Since anxiety may aggravate asthma outcomes and current anti-anxiety drugs may cause respiratory depression, the development of new anxiolytic therapies for asthmatic patients is critically needed. *Viola tricolor* L. has been used empirically for asthma remedy, but its anxiolytic effect has not been evaluated yet. Here, we investigated the effect of *Viola tricolor* L. hydro-alcoholic extract on anxiety-like behavior in ovalbumin (OVA) sensitized mice.

Methods: BALB/c mice were randomly divided into six groups: normal control, OVA (asthma) control, OVA + *Viola tricolor* (50, 100 and 200 mg/kg) and OVA + dexamethasone (3 mg/kg). Allergic asthma was induced in mice by sensitization and challenge with ovalbumin. Asthmatic mice were treated orally in the last 7 days of the OVA challenge. One hour after the last administration of therapeutic regimen, the anxiolytic activity was evaluated by elevated plus maze. Next day, the body weight of the animals and OVA-specific immunoglobulin (Ig) E levels in serum were measured.

Results: *Viola tricolor* at all three doses as well as dexamethasone significantly suppressed OVA-induced IgE production, although IgE level in dexamethasone-treated group remained significantly higher than the normal control group. *Viola tricolor* treatment particularly at 200 mg/kg increased open arm activity and improved body weight in asthmatic mice. However, treatment with dexamethasone in asthmatic animals did not induce significant changes in open arm activity and body weight.

Conclusions: Unlike corticosteroid therapy which did not improve anxiety, *Viola tricolor* can be a good remedy for treating asthma associated anxiety.

Key words: Anxiety, Asthma, Immunoglobulin E, *Viola tricolor* L

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Introduction

In the recent years, asthma has emerged as a major public health problem and affects about 300 million people worldwide (1). Allergic asthma is a chronic inflammatory respiratory disease driven by Th2 lymphocytes activation which leads to increased synthesis and release of IgE from B-cell following allergen exposure. IgE is a critical mediator of early and late phase of allergic reaction and plays an important role in the pathophysiology of allergic asthma (2-4). Although the cellular mechanisms underlying allergic asthma are well understood, little is known about its effects on brain function and behavior. Moreover, recent evidence has reported that allergic responses could modulate brain functioning in the areas that are involved in behavior, and these allergic responses are implicated in the development of psychiatric diseases, such as asthma associated anxiety (5, 6). Clinical studies have reported a high prevalence of anxiety in asthma patients (7, 8). The comorbidity of anxiety disorders is responsible for poor prognosis of asthma and impaired quality of life. Therefore, managing anxiety is a critical aspect of asthma treatment (9).

Some classes of currently used anxiolytic drugs (e.g. benzodiazepines) may increase the possibility of asthma exacerbation and induce adverse effects such as addiction and respiratory depression. Therefore, anxiety-reducing medications may not be ideal for managing comorbid anxiety in patients with asthma (10, 11). Although, steroid therapies are effective in controlling asthma, they have anxiogenic effects and may induce insomnia, and neuropsychiatric disorders (12, 13). Therefore, a drug bearing anti-inflammatory and anxiolytic potentials may impart significant benefit for managing comorbid anxiety in asthma patients.

There is increasing scientific evidence demonstrating that traditional medicine has the potential for treating asthma and comorbid conditions, such as asthma associated anxiety (14). *Viola tricolor*, belonging to the *Violaceae* family, is a traditional medicinal plant widely distributed throughout Europe, Asia, America and Australia (15). It contains flavonoids particularly rutin, and other compounds, like anthocyanins, coumarins, tannins, saponins, phenolic acids and cyclotides (15, 16). *Viola tricolor* has a long history in folk medicine for treating bronchitis and asthma. It has remarkable pharmacological properties like anti-inflammatory, antioxidant, antitussive, expectorant effects (17, 18). In addition, this herb has immunosuppressive activity and can block proliferation of activated lymphocytes (18). Keeping in view the traditional use of *Viola tricolor* against asthma, the current study was designed to evaluate the anxiolytic effect of *Viola tricolor* L. hydro alcoholic extract on asthma associated anxiety-like behavior in ovalbumin (OVA) sensitized mice.

Materials and Methods

Plant extraction

The *Viola tricolor* L. was purchased from the garden in Tehran. Flowers of the plant were dried at room temperature. 100 grams of dried flowers were ground, added to 800 ml of 96% ethyl alcohol (Kimia alcohol Zanjan Co., Iran) and water (1:1) and kept for 3 days. The suspension was filtered through filter paper and concentrated using rotary evaporator vacuum system (Heidolph, Germany) until the solvent was evaporated. The resultant product was kept in 2-8 °C and freshly dissolved in normal saline before used.

Animals

Sixty adult male BALB/c mice (6–8 weeks old) (18 ± 2 g) were purchased from Pasteur Institute of Iran. The animals were acclimatized to the laboratory conditions one week prior to the study. They were maintained at 23 ± 1 °C in a 12 hour light-dark cycle with free access to rodent food and water. 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).

Establishment of a murine model of chronic asthma and treatment regimen

Sensitization and challenge were performed as described by Mohammadian M (2016), with some modifications (19). Mice were randomly divided into six groups as follows ($n=10$): normal group, OVA (asthma) group, OVA + *Viola tricolor* treated groups (with 50 mg/kg, 100 mg/kg, and 200 mg/kg), and OVA + dexamethasone (DEXA) group.

For asthma modeling, the mice were sensitized with intraperitoneal injection of 20 µg chicken OVA (grade V; Sigma, USA) and 2 mg aluminum hydroxide (Sigma, USA) on days 0 and 14. Then mice were challenged with a 1 % OVA solution in normal saline, using an ultrasonic nebulizer (Omron CX3, Japan) for 30 minutes daily. The challenge was carried out from day 21 of protocol for 8 weeks, 3 times in a week (19). The normal group received intraperitoneal injections of 2 mg aluminum hydroxide gel and were challenged with normal saline alone. Mice were orally treated with *Viola tricolor* at different doses (50 mg/kg, 100 mg/kg, and 200 mg/kg) from day 68 to 74 (the last 7 days of OVA challenge). Dexamethasone (3 mg/kg) (Iran hormone Co., Iran), used as the reference drug for the positive control (20), was orally administered from day 68 to 74. Normal group and OVA group received only saline. Animals were sacrificed 24 hours after the last challenge (thus on day 75) to investigate the therapeutic effects of *Viola tricolor*. A schematic diagram of the treatment schedule is shown in Figure 1.

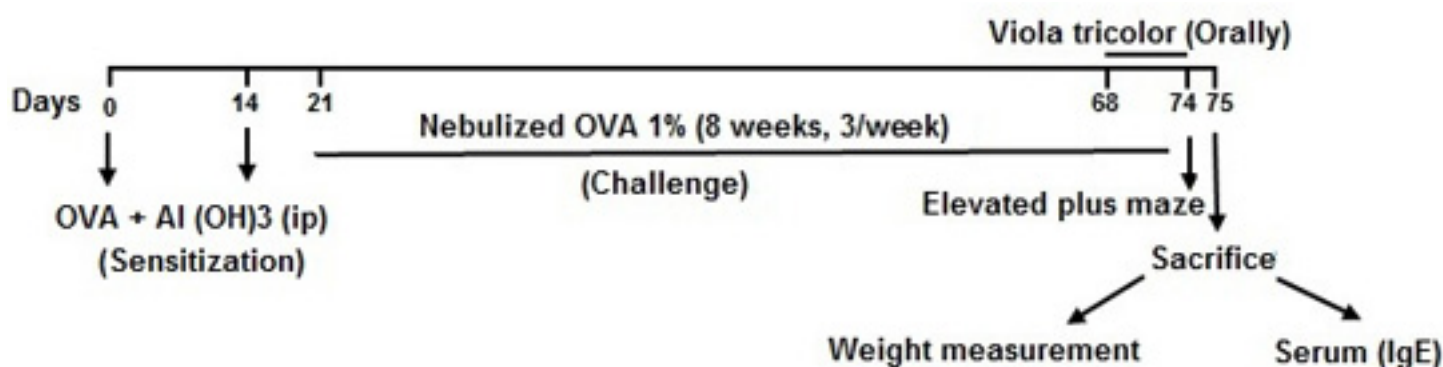


Figure 1: Time schedule of the chronic asthma mouse model and treatment with *Viola tricolor*.

Elevated plus maze test

The elevated plus maze test was performed to measure anxiolytic properties of pharmacological agents as described previously by Lister (21). The EPM apparatus consisted of two open arms (35 cm × 5 cm) and two closed arms (35 cm × 5 cm × 15 cm) that extended from a central platform (5 cm × 5 cm) which was elevated to a height of 50 cm above the floor.

One hour after last administration of extract, on the day of the last challenge (day 74), the mouse was placed in the center of the maze facing toward an open arm. The number of entries and the time spent in closed and open arms were recorded over 5 minutes. The criterion of an entry was the presence of all four paws inside an arm. The maze was cleaned with 96% ethanol and dried after each trial to prevent a bias based on olfactory cues.

The percentage of open arm entries ($100 \times \text{open}/\text{total}$ entries) and open arm time ($100 \times \text{open}/\text{open} + \text{closed}$ arm time) was calculated for each animal. An increase in open arm activity (duration and/or entries) indicates anti-anxiety behavior. The total number of entries (numbers of entries into open and closed arms) was determined as an index of locomotor activity.

Measurement of body weight

At the end of the experiment, the body weight of the animals was measured before euthanasia, using the digital electronic balance (Vibra, SJ 620 model, Japan), 620 g capacity and sensitivity of 0.01 g.

Measurement of OVA-specific IgE level in serum

Blood was obtained from the heart; sera were collected by centrifugation (3000 rpm, 10min) and stored at -70°C . Then, the serum level of OVA-specific IgE was measured by a sandwich enzyme-linked immunosorbent assay (ELISA) kit using commercially available reagents, according to the manufacturer's instructions (BioLegend, Cat. No. 439807, USA).

Statistical analyses

Data are represented as mean \pm standard error of mean (SEM). Statistical analysis was performed using GraphPad Prism Software (the version of 5.0). Differences between experimental groups were first analyzed by ANOVA. When a statistical significance was detected, post hoc Tukey test was used to determine statistical significance between

multiple testing groups. P-values less than 0.05 were considered significant.

Results

Viola tricolor increased open arm activity in the elevated plus-maze

As it can be seen in Figure 2, animals from the OVA group in the elevated plus maze test showed a slight reduction in the percentage of both time spent in the open arms (OAT %) and the number of entries to open arms (OAE %) when compared with animals in the normal group, but the differences did not reach statistical significance. Animals treated with *Viola tricolor* (50, 100, or 200 mg/kg) showed an increase in both percentage of time spent in the open arms and the percentage of open arm entries when compared with the OVA group and this increase was significant only in the 200 mg/kg *Viola tricolor*-treated group. Administration of the extracts at all three doses had no effect on locomotor activity (Figure 2C). However, dexamethasone at 3 mg/kg had no significant effects on any of the parameters that were measured on the EPM (Figure 2).

Viola tricolor extract reduced the level of OVA specific-IgE in the serum

Figure 3 shows that the serum level of OVA-specific IgE significantly increased in the asthmatic group compared with normal control group ($P < 0.001$). However, treatment with *Viola tricolor* at all three doses as well as dexamethasone significantly suppressed OVA-induced IgE synthesis ($P < 0.001$ and $P < 0.01$) but the level of OVA-specific IgE in the DEXA group did not reach the normal value and was significantly greater than the normal control group.

Viola tricolor extract improved body weight of the asthmatic animals

The body weight of the animals at the end of the experiment is presented in Table 1. The weight of animals in OVA-group was significantly lower than the animals in the normal control group ($p < 0.05$). However, the weight of the mice treated with *Viola tricolor* at all three doses was significantly higher than the OVA-group ($P < 0.005$).

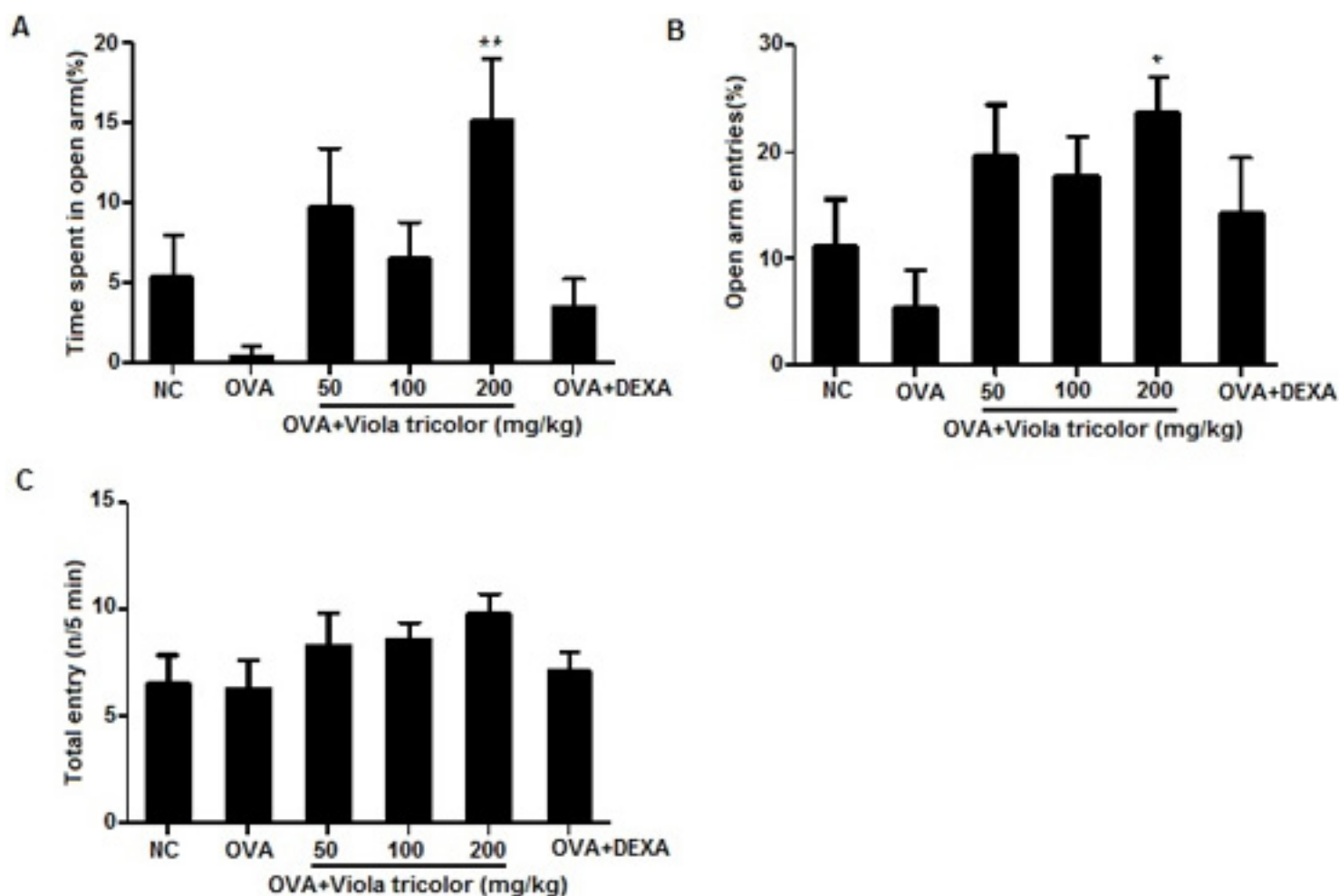


Figure 2: The effect of *Viola tricolor* extract on (A) the percentage of time spent in the open arms; (B) the percentage of open arm entries; (C) the numbers of total entries of the elevated plus-maze during a 5 minute test. In NC, Normal control animals, OVA (asthma) animals and asthmatic animals treated with *Viola tricolor* (50, 100 and 200 mg/kg) or dexamethasone (DEXA) (3mg/kg). Data are expressed as means \pm SEM (n=9/group). *P<0.05, **P<0.01 vs. OVA group.

Treatment with dexamethasone in asthmatic animals did not induce significant changes in body weight. Furthermore, dexamethasone-treated mice had lower body weight compared to normal control and extract-treated groups.

Table 1: Effect of *Viola tricolor* extract on weight of asthmatic animals

Groups	Weight (gr)	P-value
Normal control	23.37 \pm 0.77	
OVA (Asthma)	20.70 \pm 0.83	P = 0.05 #
OVA + <i>Viola tricolor</i> 50 mg/kg	24.63 \pm 0.45	P = 0.003 *
OVA + <i>Viola tricolor</i> 100 mg/kg	25.03 \pm 0.99	P = 0.000 *
OVA + <i>Viola tricolor</i> 200 mg/kg	25.04 \pm 0.29	P = 0.001 *
OVA + Dexamethasone (3mg/kg)	19.77 \pm 0.96	P = 0.015 #

Data are expressed as means \pm SEM (n=9/group); # Significant difference from normal control group; * Significant difference from OVA group. OVA = Ovalbumin

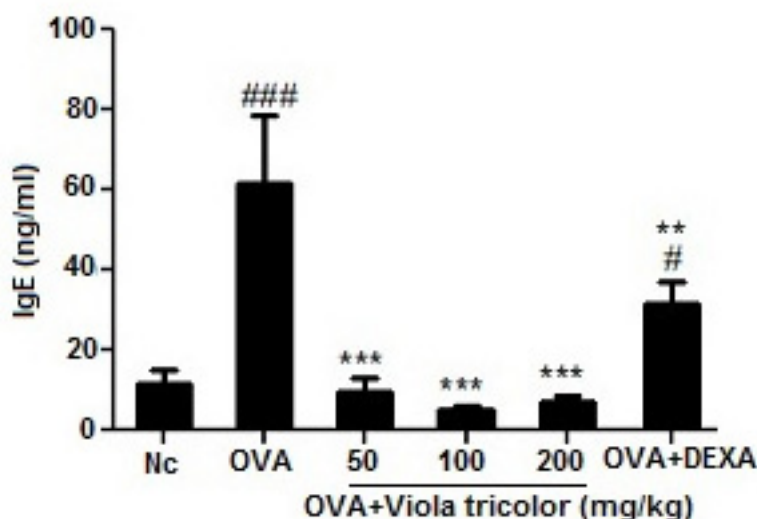


Figure 3: Effect of Viola tricolor extract on the level of OVA specific-IgE in the serum. In NC, normal control animals, OVA (asthma) animals and asthmatic animals treated with Viola tricolor (50, 100 and 200 mg/kg) or dexamethasone (DEXA) (3mg/kg). Data are expressed as means \pm SEM (n=9/group). #P<0.05, ###P<0.001 vs. normal control, **P<0.01, ***P<0.001 vs. OVA group.

Discussion

The prescription of anxiolytic drugs in asthmatic patients is frequent and these medications tend to have adverse effects on the respiratory system (11). Therefore, there remains a crucial need for the development of new anxiolytic therapies for patients with asthma. Viola tricolor is a medicinal herb that has been used for centuries in the traditional treatment of asthma (18). To the best of our knowledge, this is the first study that investigated the effects of Viola tricolor on asthma-associated anxiety in the chronic asthma mouse model.

Elevated serum allergen-specific IgE is the hallmark of allergic asthma and is correlated with the disease severity (3, 22, 23). In our study, the OVA-sensitized animals showed higher levels of serum OVA-specific IgE versus normal control animals (Figure 3), confirming that the sensitization was effective. Although dexamethasone significantly inhibited OVA-induced IgE synthesis, IgE levels did not return to the normal levels and remained significantly higher than the normal control group (Figure 3). Consistent with our findings, some evidence suggests that dexamethasone may have little or even no effect on serum IgE levels (24, 25). Interestingly, treatment with Viola tricolor at all three doses reduced IgE titer to the normal level, suggesting that the extract effectively reduced sensitization process in mice (Figure 3).

Numerous studies demonstrated that psychiatric disorders, mainly anxiety and depression are more prevalent in asthmatic patients than the general population (7, 8, 26). Similar to the human studies (8, 26), results of this study demonstrate that asthmatic animals rated higher in anxiety tests as compared to the normal group (Figure 2). Basso et al. found serum IgE levels to be positively correlated with anxiety (5). Therefore, increased anxiety in asthmatic

animals (Figure 2) may be at least partially attributed to asthma-induced rise in IgE levels (Figure 3).

There are a lot of natural herbs that can reduce stress and anxiety without causing many side effects as currently used anti-anxiety medications do (27, 28). Our study demonstrates that treatment with Viola tricolor, especially at the dose of 200 mg/kg, reduced behavioral markers of anxiety in OVA-challenged mice (Figure 2). This effect of extract may be partially related to the suppressive effect of Viola tricolor on IgE production. Jyonouchi showed that controlling allergy symptoms with the use of a humanized anti-IgE antibody unequivocally improves neuropsychiatric symptoms (27). Viola tricolor and dexamethasone both resulted in reduced IgE levels. However, anxiety levels were markedly reduced with Viola tricolor treatment. These findings suggest the presence of another prominent mechanism beside IgE that would have led to the anxiolytic effect of Viola tricolor. We speculated that the anxiety-reducing effect of Viola tricolor may be associated with the stimulation of GABA receptor by rutin, the main component of Viola tricolor. A previous study suggested that rutin induces anxiolytic-like effects through modulation of GABAA receptors in the basolateral amygdala (29, 30).

In this study, dexamethasone as a conventional anti-asthma drug had no effect on anxiety (Figure 2) and body weight (Table 1). On the other hand, Viola tricolor, a traditional remedy for asthma reduced anxiety (Figure 2) and improved body weight of the asthmatic animals (Table 1). These results suggest that Viola tricolor induced improvement in body weight may be attributed to its anxiolytic effects and not to its anti-asthmatic effects. In accordance with our findings, a recent study indicates that rutin treatment has anti-anxiety effects and ameliorates various impairments related to physical fatigue including body weight (31).

Furthermore, previous studies indicate that psychotropic drugs increase appetite and result in weight gain over a long-term period of consumption (32, 33). A study shows that GABAergic neurons within the hypothalamus positively regulate feeding behavior and body weight (34). Since rutin, the major component of *Viola tricolor*, has GABA agonistic property (29, 30), *Viola tricolor*'s effect on body weight may have been exerted through stimulation of GABA receptors.

Conclusion

The results of the present study showed that the hydro alcoholic extract of *Viola tricolor* reduces anxiety-like behavior in OVA-sensitized mice. These results suggest unlike corticosteroid therapy which cannot improve anxiety, *Viola tricolor* appears to be beneficial for managing asthma-associated anxiety. However, future studies are warranted to evaluate the role of GABA receptors in mediating anxiolytic effects of *Viola tricolor*.

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