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Anti-Inflammatory Effects of *Viola odorata* Aqueous Extract in an Ovalbumin-Induced Murine Model of Allergic Asthma

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ABSTRACT

Asthma represents a persistent inflammatory condition affecting the airways. Viola odorata has long been utilized in traditional medicine for managing various inflammatory disorders. This research sought to examine the potential anti-inflammatory actions of the aqueous extract derived from V. odorata in an experimental mouse model of asthma. A total of forty-eight female Balb/c mice were assigned to six groups, with eight mice per group. The normal control group was given distilled water, whereas asthma was induced in the other groups via ovalbumin sensitization. Following this, one asthmatic group was treated with dexamethasone, while three others received the extract at doses of 100, 200, and 400 mg/kg daily for seven days. Afterward, eosinophil numbers and concentrations of interleukins 4, 5, and 13 were quantified in bronchoalveolar lavage fluid (BALF). Lung sections were examined for pathological modifications. Treatment led to a marked decline in both eosinophil numbers and interleukin 4, 5, and 13 concentrations within BALF. Mice receiving the extract showed diminished goblet cell hyperplasia, lymphoid hyperplasia, as well as reduced inflammation around bronchi and vessels. Across all assessed indicators, the outcomes from V. odorata aqueous extract mirrored those achieved with dexamethasone. This investigation marks the initial documentation of anti-inflammatory benefits from V. odorata aqueous extract in an allergic asthma mouse model. Consequently, the findings position V. odorata as a candidate for further development as a therapeutic option targeting asthma-related inflammation.

Keywords: Asthma, Eosinophilia, Inflammation, Interleukins, Ovalbumi

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Introduction

Asthma constitutes a varied chronic inflammatory disorder of the respiratory tract, manifesting in multiple clinical forms [1]. Typical features include wheezing, sensations of chest constriction, and/or coughing, accompanied by excess mucus secretion and swelling of the mucosa, collectively resulting in reduced airway diameter. In certain individuals with asthma, the reversal of airflow restriction can remain incomplete [2, 3]. As a significant global health concern, asthma impacts over 330,000,000 people, with projections indicating a 20% rise in cases within the coming two decades [4, 5].

The condition generally appears in allergic or non-allergic variants. The allergic variant is driven by T helper 2 (Th2) cellular activity, leading to secretion of type 2 cytokines such as interleukins IL-4, IL-5, IL-9, and IL-13 [6]. Such cytokines facilitate extensive eosinophil buildup in airway tissues, heightened mucus generation, and IgE production by B cells responsive to specific allergens [7]. Environmental triggers, including workplace exposures, commonly provoke this variant [8]. In contrast, non-allergic asthma often develops later in life and predominates in women and those with obesity [9]. Further classifications involve Th2-high patterns (eosinophilic, tied to raised eosinophil counts in blood or elevated FeNO levels) versus Th2-low patterns (non-eosinophilic, at times neutrophilic or linked to metabolic factors) [10]. The eosinophilic subtype, coordinated by

cytokines like IL-4, IL-5, and IL-13 associated with Th2 pathways, tends to be more intense than its non-eosinophilic counterpart. Neutrophilic variants are typically sparked by Th17 cell involvement [1, 8]. The prevailing asthma pattern relies on Th2-driven mechanisms, termed Th2-high asthma [3]. Allergen inhalation allows binding to IgE, prompting mast cells to liberate substances including leukotrienes (LTs), histamine, and various interleukins, thereby causing airway constriction [1]. Moreover, IL-5 supports the development, ripening, and lung-directed movement of eosinophils, which then secrete factors amplifying mast cell release of histamine and LTs. IL-13 drives mucin production from epithelial cells, promoting structural changes, scarring, and tissue overgrowth in pulmonary areas [1, 11]. Despite the utility of primary options like corticosteroids and antihistamines in asthma control, a subset of patients shows inadequate response alongside frequent exacerbations [12, 13]. Extended application of these agents often yields substantial unwanted effects. Hence, the pursuit of alternative asthma therapies is vital. Increasing efforts are directed toward discovering superior medicinal approaches for asthma sufferers. Multiple earlier works have highlighted the antioxidant, anti-inflammatory, and antimicrobial potentials of plant-based treatments.

Belonging to the Violaceae family, Viola odorata L. offers a range of therapeutic applications and is found in regions spanning Asia, North Africa, and Europe [14]. In traditional practices, it has been applied to alleviate cough, fever, colds, headaches, sleep disturbances, seizures, bowel issues, heart palpitations, breathing difficulties, urinary problems, and certain dermatological conditions [15-18]. Compounds in the aqueous extract of V. odorata, including salicylic acid, flavonoids, anthocyanins, and saponins, contribute to its anti-inflammatory and antimicrobial characteristics [5, 19, 20]. This work was undertaken to explore how Viola odorata aqueous extract influences inflammatory processes and structural alterations in the airways using a Balb/c mouse model of allergic asthma provoked by ovalbumin.

Materials and Methods

Ethical considerations

All procedures involving mice complied with the National Institute of Health (NIH) standards for laboratory animal care and use. The study protocol was granted approval by the ethics committee of Shahid Beheshti University of Medical Sciences, Tehran, Iran, under the reference code IR.SBMU.AEC.1402.109.

Chemicals

Ovalbumin (OVA) was sourced from Sigma-Aldrich Company, USA. Aluminum hydroxide originated from the Razi Vaccine and Serum Research Institute, Iran. Phosphate-buffered saline was formulated in-house using the relevant salts. Dexamethasone was obtained from Aboryhan Company, Iran. Ready-to-use enzyme-linked immunosorbent assay (ELISA) kits for cytokine detection were acquired from Abcam Company, USA.

Plant material and extraction

The above-ground portions of Viola odorata were bought from a traditional herb market in Tehran, Iran, and verified at the Herbarium of the Traditional Medicine and Materia Medica Research Center, Shahid Beheshti University of Medical Sciences (specimen code: HMS-581). To prepare the aqueous extract, powdered plant material was boiled in distilled water at a 1:10 ratio for 10 min. The resultant solution was evaporated to dryness at 70 °C. Quantification of total phenolic compounds in the extract was performed via the Folin-Ciocalteu assay [21].

Animals

A cohort of forty-eight female Balb/c mice, ranging in age from 6 to 8 weeks and in weight from 15 to 20 g, was supplied by the Pasteur Institute, Tehran, Iran. Prior to the start of sensitization, the mice were acclimatized for one week in a facility maintained at 22 ± 2 °C, with a 12 h light/dark rhythm, humidity levels of 55 ± 10%, and an environment devoid of allergens and pathogens. During the entire experiment, the animals had unlimited access to standard chow and drinking water.

Experimental groups

Animals were allocated randomly into six groups, each containing eight mice. The first group functioned as the negative control and was administered distilled water via intraperitoneal injection. The second group, serving as

the positive control, underwent ovalbumin (OVA) sensitization alone. The third group received OVA sensitization followed by oral dexamethasone treatment. The fourth, fifth, and sixth groups were subjected to OVA sensitization and subsequently given Viola odorata aqueous extract via oral gavage at daily doses of 100, 200, and 400 mg/kg, respectively.

Induction of allergic asthma

Asthma development in the model required a 30-day period. On days 1 and 14, mice in groups 2 through 6 were given intraperitoneal injections of 20 µg OVA combined with 20 µL aluminum hydroxide in 100 µL phosphate-buffered saline (PBS, pH 7.4). Additional sensitization involved 30-min exposures to nebulized 5 mL of 1% OVA in sterile saline, delivered via an ultrasonic nebulizer (NB-500, Rossmax, Switzerland), on days 24, 26, 28, and 30. On those same days, the negative control group inhaled aerosolized 0.9% saline intranasally. Oral treatments commenced from day 23 through day 30: groups 4–6 received the respective extract doses (100, 200, 400 mg/kg/day), and group 3 was given 5 mg/kg dexamethasone. Euthanasia occurred on day 31, after which each group was split evenly—four mice for bronchoalveolar lavage fluid (BALF) retrieval and four for lung tissue collection and pathology evaluation [22].

Cytokine measurement and eosinophil counting

Interleukin 4, 5, and 13 concentrations in BALF specimens were measured employing dedicated ELISA kits, following the supplied manufacturer instructions precisely. Eosinophils in BALF were enumerated across all samples and presented as a proportion of the overall leukocyte population.

Histological evaluation

On day 31, lung samples were collected from four animals per group as outlined. Tissues were preserved in 10% neutral buffered formalin for 14 days before paraffin embedding. Thin sections were prepared with a microtome and subjected to staining using hematoxylin and eosin (H&E) as well as periodic acid–Schiff (PAS) techniques. Mounted slides were reviewed under microscopy by a qualified pathologist.

Statistical analysis

Data are displayed as mean ± SD. Intergroup comparisons were carried out via one-way analysis of variance (ANOVA), supplemented by Tukey's post hoc analysis. Distribution normality was assessed with the Kolmogorov-Smirnov test. Statistical processing was performed using GraphPad PRISM software, version 7. Differences were deemed significant at $p < 0.05$.

Results and Discussion

The phenolic content of the dried extract was quantified as 4.7% in pyrogallol equivalents.

Figure 1 displays the influence of the extract on eosinophil percentages in BALF across the studied mice. Findings confirmed that OVA challenge in the positive control substantially boosted eosinophil infiltration into BALF relative to healthy controls ($p<0.0001$). Dexamethasone administration in asthmatic animals caused a profound drop in eosinophil numbers compared to the OVA group ($p<0.0001$). The extract at 100 mg/kg failed to produce a meaningful reduction in eosinophils versus the positive control. Conversely, higher doses of 200 mg/kg ($p = 0.026$) and 400 mg/kg elicited clear reductions in eosinophil proportions within BALF against the OVA-challenged group ($p<0.0001$), (F-value= 31.45).

Figures 2a–2c illustrate how treatment with V. odorata aqueous extract affected levels of key pro-inflammatory cytokines (IL-4, IL-5, and IL-13) in BALF from mice with OVA-induced asthma. The positive control showed markedly higher IL-4 in BALF than the negative control ($p<0.0001$). Dexamethasone effectively curbed this rise relative to the positive control ($p<0.001$). The 400 mg/kg extract dose notably lowered IL-4 concentrations ($p=0.021$) **(Figure 2a)** ($F = 19.58$). **Figure 2b** indicates a strong increase in IL-5 within the OVA group compared to healthy animals ($p<0.0001$), which was countered by both dexamethasone and the 400 mg/kg extract ($p<0.0001$, $p = 0.007$) (F-value = 39.74). As depicted in **Figure 2c**, OVA sensitization raised IL-13 levels versus the healthy group ($p<0.001$), but dexamethasone and the 400 mg/kg extract significantly suppressed this elevation relative to the positive control ($p<0.0001$) ($F = 60.45$).

Lung pathology across treatment groups is presented in **Figure 3**. The healthy control **(Figure 3a)** exhibited normal tissue with absence of peribronchial/perivascular inflammation and goblet cell overgrowth. In contrast, the OVA positive control revealed prominent lymphoid hyperplasia and mononuclear cell invasion into alveolar regions **(Figure 3b).** Dexamethasone-treated sections **(Figure 3c)** demonstrated clear mitigation of inflammatory infiltration and lymphoid tissue expansion. Of particular note, the 400 mg/kg extract dose visibly alleviated goblet cell overgrowth, mucus overproduction, and general tissue inflammation when compared to OVA-exposed animals **(Figure 3d).**

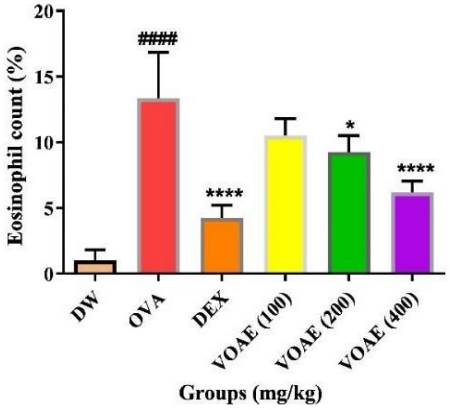
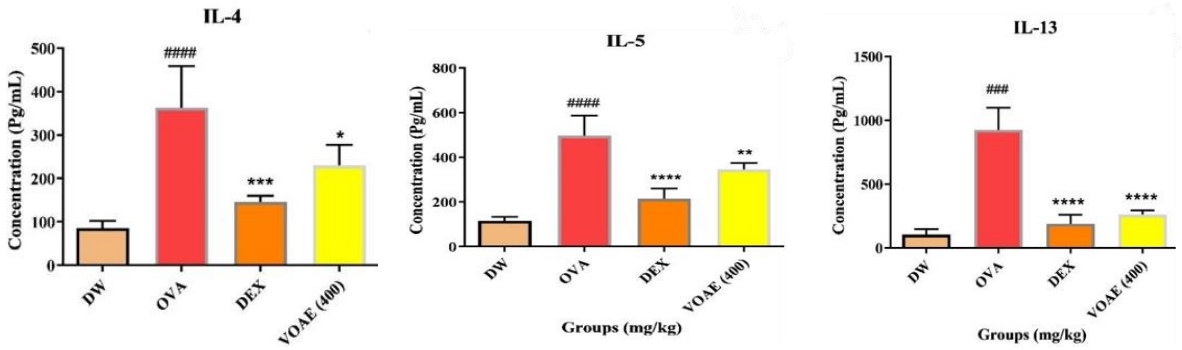
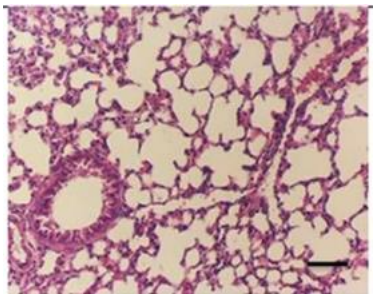


Figure 1. Influence of Viola odorata aqueous extract (VOAE) on eosinophil percentages in BALF of mice sensitized with ovalbumin (OVA). The negative control received distilled water (DW), while the positive control was given OVA. Asthmatic animals induced by OVA were administered dexamethasone (Dex) or various VOAE doses. Results are mean ± SD. $p<0.05$ and $p<0.0001$ versus the ovalbumin group; ####$p<0.0001$ versus the DW (healthy control) group.

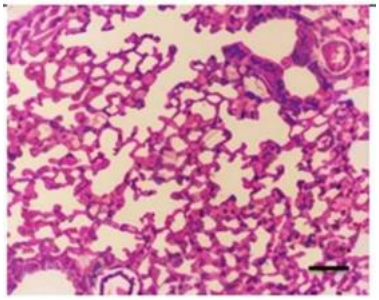


a) b) c)

Figure 2. Impact of Viola odorata aqueous extract (VOAE) administration on BALF concentrations of IL-4 (a), IL-5 (b), and IL-13 (c) across study groups. Negative and positive controls were treated with distilled water (DW) or ovalbumin (OVA). OVA-challenged asthmatic mice received dexamethasone (Dex) or differing VOAE doses. Values shown as mean ± SD. $p<0.05$, $p<0.01$, $p<0.001$, and $p<0.0001$ compared to OVA group; ####$p<0.0001$ and ###$p<0.001$ compared to healthy control.



a1



a2

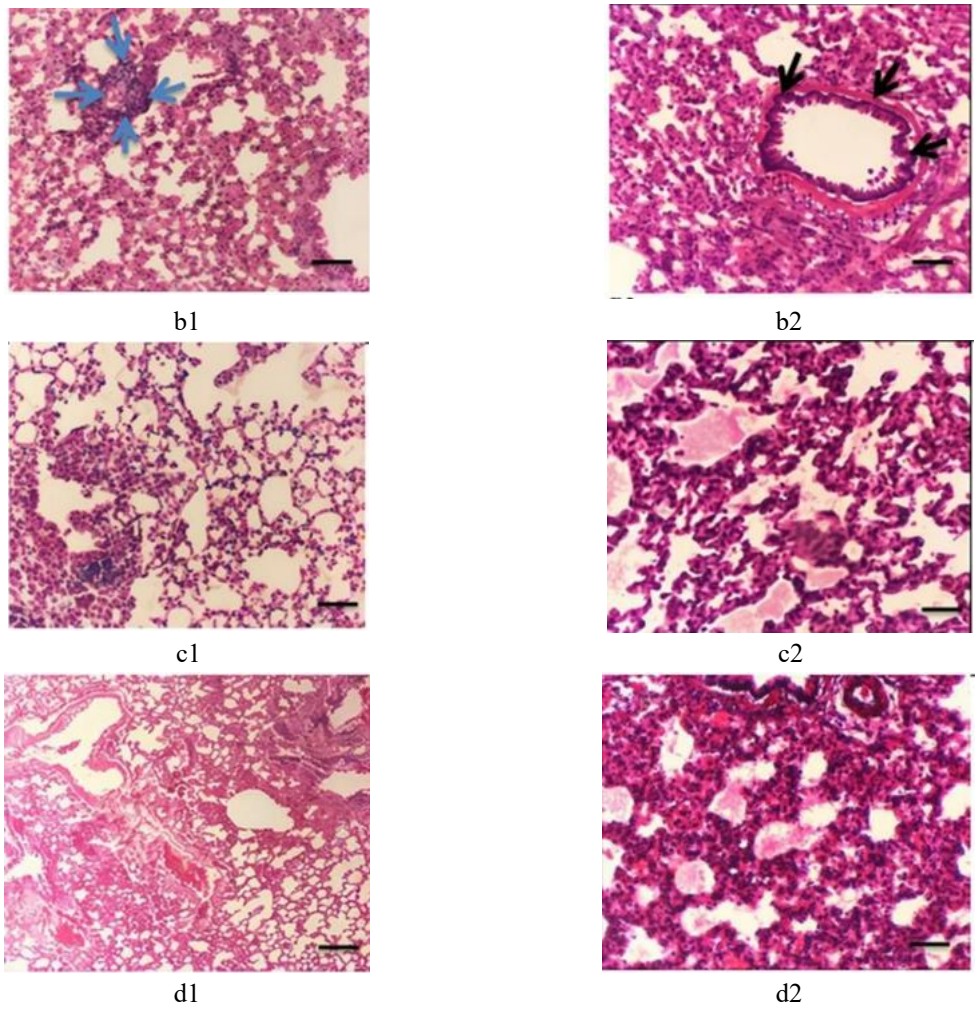


Figure 3. Microscopic examination of lung specimens from experimental groups stained with hematoxylin-eosin (H&E) and periodic acid-Schiff (PAS). a1 and a2: Normal tissue from negative control given distilled water; b1 and b2: Positive asthmatic control sensitized with ovalbumin; c1 and c2: Asthmatic group treated with dexamethasone; d1 and d2: Samples from asthmatic mice given VOAE at 400 mg/kg. Blue arrows highlight inflammatory cells within alveoli; black arrows mark goblet cell hyperplasia; scale bar indicates 100 µm (a, b, c, and d).

Previous studies have indicated that certain plant-based remedies can exert anti-inflammatory actions on mediators including tumor necrosis factor-α (TNF-α), IL-4, cyclooxygenase-2 (COX-2), and prostaglandin E2 (PGE2) [23]. Various parts of *V. odorata* have been widely employed in managing numerous disorders [5]. Within Iranian traditional medicine (ITM), *V. odorata* has been applied to alleviate fever, intense thirst, uremic itching, cough, pneumonia, and pleurisy [24]. The plant harbors compounds capable of improving respiratory performance, attenuating lung inflammation, and lessening edema in mucous membranes [25].

In the current work, the anti-inflammatory capacity of *V. odorata* aqueous extract was evaluated in the respiratory tracts of mice exhibiting allergic asthma.

The underlying pathways contributing to the anti-asthmatic properties of this herb were also explored. Allergic asthma was established through ovalbumin administration. Findings revealed that ovalbumin provoked typical asthmatic features in mice, primarily through elevating eosinophil numbers in BALF. Administration of *Viola odorata* aqueous extract notably impeded this rise in eosinophils within BALF. Dexamethasone, serving as a reference anti-asthma agent, similarly lowered eosinophil counts relative to ovalbumin-exposed animals.

Allergic asthma is characterized by heightened Th2-high markers, including sputum eosinophils [26]. Moreover, levels of IL-4, IL-5, IL-13, along with IL-1β, IL-6, and TNF-α, are markedly elevated in BALF and pulmonary tissues of affected individuals [27]. This represents a Type 2 inflammatory pattern that promotes eosinophilia, increased IL-4, and raised total serum IgE in the airways. Both IL-5 and IL-13 play key roles in driving

eosinophilic inflammation within the airways [28]. Severe asthma manifests as a multifaceted and varied condition, with pronounced eosinophil recruitment serving as a prominent feature. Eosinophils interact with Th2 cells and type 2 innate lymphoid cells, which subsequently release Th2 cytokines such as IL-4, IL-5, and IL-13 [29, 30].

In this research, ovalbumin exposure in Balb/c mice led to elevated eosinophil numbers and associated asthmatic manifestations, yet the extract curtailed this cellular expansion, suggesting protective benefits against eosinophilic forms of asthma. The aqueous extract of *V. odorata* was shown to diminish production of IL-4, IL-5, and IL-13. These outcomes aligned closely with those observed using dexamethasone as the benchmark therapy for asthma. Data from the present study further demonstrated that ovalbumin exposure in the asthmatic cohort substantially raised IL-4, IL-5, and IL-13 concentrations in BALF. Both dexamethasone and *V. odorata* aqueous extract effectively lowered the protein concentrations of these cytokines in BALF. Notably, the extract's impact on these interleukins paralleled that of dexamethasone.

Administration of *V. odorata* aqueous extract to the animals prevented goblet cell overgrowth, excessive mucus production, and inflammatory processes in pulmonary tissues compared to the ovalbumin-sensitized cohort. Additionally, histopathological analysis indicated that the extract successfully limited the migration of inflammatory cells into perivascular and peribronchial regions in the airways of mice with ovalbumin-induced asthma. In a study by Beibei Zhang *et al*., the prospective anti-asthmatic activity of *Ephedrae herba* was assessed in ovalbumin-treated rats, revealing that its benefits likely stemmed from blocking inflammatory cell entry into the airways [31]. In a formalin-induced lung injury model in rats, the anti-inflammatory attributes of *V. odorata* were confirmed, as prophylactic treatment mitigated tissue damage and pathological alterations in bronchiole epithelium, supporting its utility for pulmonary inflammatory conditions [15]. Another investigation using a wound healing model found that polysaccharide fractions from *V. odorata* and *Malva pusilla* displayed anti-inflammatory effects by inhibiting various inflammation phases and altering capillary permeability [32]. Research conducted by Lee *et al*. explored the anti-inflammatory and anti-asthmatic potential of *Viola mandshurica* W. Becker in a murine model of airway inflammation, demonstrating substantial reductions in total serum IgE, cytokines including IL-3 and IL-4, airway hyperreactivity, eosinophilia, and mucus overproduction [33]. Work by Ali Zainab Aziz *et al*. verified the occurrence of umbelliferone in the ethyl acetate fraction of Iraqi *V. odorata* aerial parts and confirmed its anti-inflammatory action [16, 34]. Additionally, findings from Karimfar *et al*. revealed that *V. odorata* extract markedly suppressed nuclear factor kappa B (NF-κB) expression across penumbra, core, and subcortical regions. Given that NF-κB governs multiple genes linked to pro-inflammatory responses, innate and adaptive immunity, apoptosis, cell survival, and proliferation, the observed anti-inflammatory effects of *V. odorata* aqueous extract here may arise from downregulation of NF-κB [35].

IL-5 serves as the main cytokine driving the maturation, recruitment, and activation of eosinophils [36]. This cytokine promotes eosinophil degranulation while extending their lifespan [37]. IL-13, secreted by CD8+ memory T cells, type 2 innate lymphoid cells (ILC2), and basophils, represents a key cytokine capable of initiating asthma pathogenesis. Blocking IL-13 eliminates house dust mite (HDM)-induced airway hyperresponsiveness, airway inflammation, and mucus hypersecretion [38]. Impaired immune reactions to viral pathogens may occur in type 2 inflammatory states, where interferon production shows an inverse relationship with rising airway eosinophilia, IL-4 concentrations, and total serum IgE [28]. The beneficial impacts of

V. odorata aqueous extract against ovalbumin-induced allergic asthma observed here may stem from its ability to inhibit expression of Th2-related interleukins and eosinophil expansion. This points to a potential pathway underlying the anti-inflammatory and anti-asthmatic actions of V. odorata in this mouse model of allergic asthma. A key advantage of this study lies in its comprehensive examination of the primary inflammatory pathways central to allergic asthma. Subsequent research should explore additional pathways contributing to the onset and advancement of allergic asthma, including T-helper 1 inflammatory mechanisms.

Conclusion

This research demonstrated the anti-inflammatory and anti-asthmatic properties of V. odorata aqueous extract in a mouse model of ovalbumin-induced asthma. Based on the findings, V. odorata appears to achieve these benefits by regulating eosinophil expansion, type 2 inflammation, excessive mucus production, and histopathological alterations arising from inflammatory cell infiltration into the lungs and airways. Moreover, the current data confirm that the influence of V. odorata on asthmatic features was similar to that of dexamethasone. Overall, these

results indicate that V. odorata holds promise as a therapeutic candidate for managing hyper-responsiveness in pulmonary tissues and airways amid the inflammatory processes characteristic of asthma.

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Conflict of Interest: None

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Ethics Statement: None

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