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Research Article

Evaluation of Anti-Inflammatory and Immunomodulatory Potential of a Traditional Herbal Drug in Experimental Model of Bronchial Asthma

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Abstract

The present study experimentally evaluated the effects of the standardized extract of an Indian traditional medicinal plant, *Adiantum venustum,* in ovalbumin (OVA) induced model of bronchial asthma in rats. Wistar rats were sensitized with OVA adsorbed on aluminium hydroxide on day 0 followed by OVA challenge on day 14 to experimentally induce bronchial asthma. Different doses of standardized extract of *Adiantum venustum* were administered daily for 14 days and effects on airway hyprresponsiveness to spasmogen and airway inflammation were assessed. Enhanced pause (*p-enh*), a marker of airway hyperresponsiveness, was measured in response to inhaled methacholine using whole body plethysmography, following which rats were anesthetized and blood and bronchoalveolar lavage fluid (BALF) were collected and analyzed for OVA specific IgE, pro-inflammatory cytokine (TNF-α) and the Th-2 cytokine (IL-4). The results showed that in OVA immunized and challenged rats, methacholine exposurre induced enhancements in airway hyper-responsiveness as measured by elevated *p-enh* values. Assay of blood and BAL fluid showed elevated OVA specific IgE and the pro-inflammatory cytokines thus validating the experimental model (Disease control). Administration of standardized extract of *Adiantum venustum* resulted in attenuation of *p-enh* values, as well as levels of OVA specific IgE, TNF-α and IL-4 as compared to that in the vehicle treated Disease control group, in a dose-related manner. The results with *Adiantum venustum* were comparable with that of prednisolone (10 mg/kg) on all parameters tested The results showed that *Adiantum venustum* reduced both (a) airway hyper-responsiveness to the bronchoconstrictor; and (b) the biomarkers of airway inflammation and immunity, and validates the observed therapeutic benefits of this Indian traditional medicinal plant for bronchial asthma.

Keywords: *Bronchial Asthma; Ovalbumin; Methachloine; Airway Hyperresponsiveness; Prednisolone*

Abbreviations

AHR: Airway Hyperresponsiveness; ANOVA: Analysis of Variance; BALF: Bronchoalveolar Lavage Fluid;ELISA: Enzyme Linked Immunosorbent Assay; IgE: Immunoglobulin E; IL: Interleukin; OVA: Ovalbumin; P-enh: Enhanced Pause; TNF-α: Tumour Necrosis Factor Alpha.

Introduction

Bronchial asthma is a chronic airway disease which is characterized by airway inflammation, bronchial hypersensitivity and airway obstruction [1,2]. Asthma is mediated through T-helper cells type-2 (Th2) immune response. On exposure to various allergic as well as

non-allergic factors, a series of immune-responses are initiated leading to airway inflammation. Furthermore, increased levels of Th2 cells trigger release of immunoglobulin IgE and cytokines through mast cell activation. IgE activation increases release of inflammatory mediators such as leukotrienes, histamine and cytokines such as IL-4, IL-13 etc. that result in eosinophilic inflammation. These mediators result in characteristic symptoms of asthma such as edema, bronchospasm and mucus hypersecretion [3,4]. Further, these cytokines mediate various inflammatory responses leading to chronic airway inflammation and bronchial hyperreactivity (late-phase asthmatic response).

Pharmacotherapy of bronchial asthma consists of anti-inflammatory agents (e.g., corticosteroids) and bronchodilators (e.g., β-2-agonists). These drugs are generally accompanied with side effects and many cases are refractory to conventional pharmacotherapy. Thus, there is a need to explore newer drugs/adjuncts for better management of bronchial asthma. The drug development from synthetic molecules is an expensive and time-consuming proposition which may also have higher possibility of failure.On the other hand herbal drugs have not been given the desired recognition in the management of asthma, due to lack of data. Thus, the naturally available alternatives used in traditional medicine are being explored.

Adiantum venustum is one of the plants used in traditional folklore medicine for management of bronchial asthma. *Adiantum venustum* (Black Hansraj) is native to Himalayas and traditionally used for the treatment of cold, headache, hydrophobia and chest inflammation. Studies have reported analgesic and anti-inflammatory properties at 50 mg/kg as well as 100 mg/kg [5]. Plant fronds are used as expectorant, tonic, diuretic, astringent and for the treatment of scorpion sting [6]. The rhizome extract can be used to treat diabetes, liver problems as well as diuretic [7]. It has been reported that ethanolic extract of leaves and stem of the plant contains terpenoids, phytosterols, flavanoid and saponins which are useful in regulating cancer activities [8]. Studies have reported effective antimicrobial, anti-viral, antibacterial, anticancer and antioxidant activity of *Adiantum venustum* [9, 10, 11]. Therefore, the plant was selected for assessing its anti-asthmatic potentials in ovalbumin induced model of asthma in rats by utilizing modern scientific methodology to validate the traditionally described effects. The study was conducted to evaluate the efficacy of *Adiantum venustum* against hyperresponsiveness to spasmogen and airway inflammation in experimental model of bronchial asthma.

Materials and Methods

Animals: Wistar rats of either sex (180-220 g) were used for the study (n=6/group). They were housed in standard laboratory conditions (25 ± 2°C, 12 hours light and dark cycle).Care of the animals was done as per the guidelines of Committee for the purpose of control and supervision of experiments on animals (CPCSEA), New Delhi. The study protocol was approved by Institutional Animal Ethics Committee (IAEC).

Drugs and Chemicals: The standardized drug *Adiantum venustum* was procured from National Innovation Foundation (NIF), Ahmedabad, India.Ovalbumin, Methacholine, Prednisolone, were procured from Sigma Aldrich-USA. All other routine chemicals were of analytical grade andprocured from Sisco Research Laboratories (SRL, New Delhi). Cytokine assay kits Ovalbumin specific IgE, TNF–α and IL-4 were procured from Weldon Biotech, New Delhi.

OVA-induced model of airway inflammation/bronchial asthma

As described by Kwasnieski., *et al.,* [12] rats were sensitized with intraperitoneal administration of Ovalbumin (10 mg/rat) adsorbed to 10 µg of aluminium hydroxide on day 0. On 14th day the animals were challenged with Ovalbumin (1 mg per rat) in 0.5 ml of isotonic saline.

Experimental protocol

Rats were divided into following 6 groups (n=6/group):

- Normal control: rats were sensitized with ovalbumin (10 mg per rat, i.p) adsorbed on aluminium hydroxide (10 µg) and treated orally with distilled water for 14 days (vehicle) without exposure to challenge dose of OVA.
- Disease control: rats were sensitized and treated orally with distilled water for 14 days and challenged with ovalbumin as mentioned below.
- Positive Control: rats were sensitized and treated orally with prednisolone (10 mg/kg) from day 1 to 14, followed by sensitization with ovalbumin.
- (4-6) AV1, AV2 and AV3: rats were sensitized and treated orally with *Adiantum venustum* at the dose of 15.5 mg/kg, 31 mg/kg, or 62 mg/kg from day 1 to 14, followed by challenge with ovalbumin. The dose of *Adiantum venustum* was calculated from human dose used in Traditional system of medicine.

After 24 hour of ovalbumin sensitization, the animals were anaesthetized with ketamine (24 mg/kg, i.p.) and blood was collected by cardiac puncture. Blood was centrifuged at 3000 rpm for 10 minutes at 4ºC and the serum was separated and stored at -80ºC for biomarker analysis. After blood collection, BAL fluid was collected by lavaging the airways through a tracheal cannula with 0.9% sodium chloride solution and centrifuged at 1500 rpm for 10 minutes at 4ºC and supernatant was recovered and stored at -80ºC for assay of various biomarkers [13].

Measurement of bronchial responsiveness and airway resistance to methacholine

After 24 hours of Ovalbumin sensitization, airway hyper-responsiveness was assessed in response to inhaled methacholine using whole body plethysmography and expressed as *enhanced pause* (P-enh) [14]. Briefly, rats were placed in a whole-body chamber and basal readings were obtained and averaged for a 3 min period. Subsequently, increasing doses of methacholine (2.5-20 mg/mL), were aerosolized for 3 min, and readings were taken and averaged for 3 min after each nebulization [15].

Cytokine assay

Blood and BAL fluid samples were analysed for OVA specific IgE, TNF-α and IL-4 using commercially available enzyme linked immunosorbent assay (ELISA) kits. TNF-α and IL-4 levels were measured using sandwich ELISA method following the instruction of the manufacturer. The absorbance of the colored product was read at a wavelength of 450 nm using ELISA plate reader and values are expressed in pg/ml.OVA specific IgE was measured using double antibody sandwich ELISA method. Briefly, the microtiter plate was pre-coated with an antibody specific to OVA specific IgE to which samples were added and after incubation, secondary OVA specific IgE antibody labeled with biotin was added. Streptavidin-HRP conjugate was added in order to form antibody-antigen-antibody immune complex and incubated. Chromogenic solutions were added to develop colour and the reaction was stopped by adding stop solution. The absorbance of colored microtiter plate was measured using software based microtiter plate reader at 450 nm and values are expressed in ng/ml.

Statistical analysis

All data are expressed as Mean ± S.E.M and analyzed by one way ANOVA followed by Dunnett's test. A p value < 0.05 was used as a level of significance in all statistical tests.

Results

Effect of *Adiantum venustum* on bronchial hyperresponsiveness

P-enh, a marker of airway-responsiveness was measured in response to increasing doses of methacholine (2.5 mg/ml, 10 mg/ml and 20 mg/ml) using whole body plethysmography. Immunization followed by OVA challenge in the Disease control group resulted in increased degree of responsiveness on exposure to different doses of methacholine. Treatment with *Adiantum venustum* (15.5 mg/kg, 31 mg/kg and 62 mg/kg) showed significant reduction in *P-enh* values as compared with Disease control group ($p < 0.05$). Amongst different treatment groups, maximum reduction in *P-enh* values was observed at highest dose level of *Adiantum venustum* (i.e. 62 mg/kg). Prednisolone treated group also showed significant reduction in *P-enh* values as compared to Disease control group ($p < 0.05$). These results are summarized in figure 1.

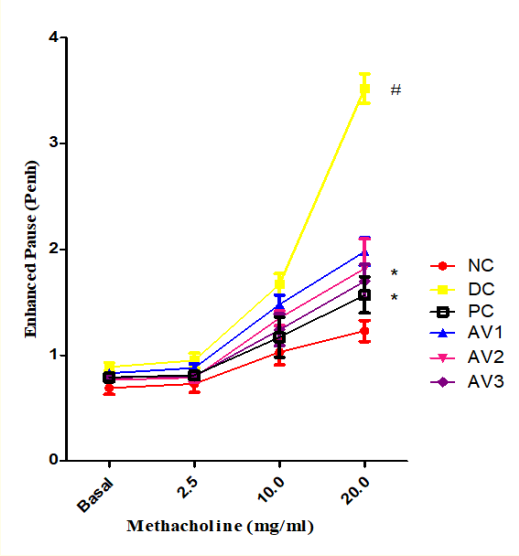

Figure 1: *Effects of Adiantum venustum on enhanced pause (p-enh) as measured by whole body plethysmography. Normal control (NC): OVA sensitized; Disease control (DC): OVA sensitized+challenged rats treated with vehicle; Positive Control (PC): OVA sensitized and challenged rats treated with Prednisolone (10 mg/kg); AV1, AV2 and AV3: OVA sensitized and challenged rats treated with Adiantum venustum at doses of 15.5 mg/kg, 31 mg/kg and 62 mg/kg extract orally. Data are expressed as Mean ± SEM. #p < 0.05 vs Normal control (NC) group;*p < 0.05 versus Disease control (DC) group.*

Effect of *Adiantum venustum* on OVA specific IgE levels in blood and BALF

Immunization (day 0) + challenge (day 14) with OVA resulted in significant increased levels of IgEin Disease control group as compared tothat seen in normal control rats ($p < 0.05$). The aqueous extract of *Adiantum venustum* (15.5 mg/kg, 31 mg/kg and 62 mg/kg) was administered daily for 14 days and its effects were assessed on OVA specific IgE levels in blood and BAL fluid samples. Treatment with *Adiantum venustum* at dose of 31 mg/kg and 62 mg/kg induced significant attenuation in OVA specific IgE levels in both blood (by approximately 43% and 52% with respective doses) and BAL fluid (approximately 19% and 26%)as compared to that of Disease control group ($p < 0.05$). On the other hand, pretreatment with *Adiantum venustum* (15.5 mg/kg) did not influence the OVA specific IgE levels in both blood (19%) and BAL fluid (10%) by any appreciable extent ($p > 0.05$). Pre-treatment with prednisolone also induced significant attenuations in OVA-specific IgE levels by 55% in blood and by 22% in BALF as compared to that of Disease control group ($p < 0.05$). These results are summarized in figure 2.

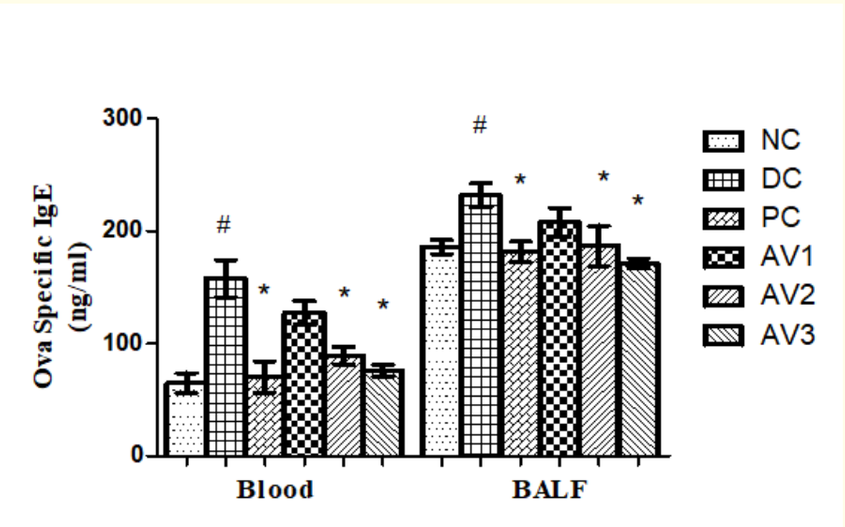

Figure 2: *Effects of Adiantum venustum extract on OVA specific IgE levels in blood and BAL fluid of OVA sensitized+challenged rats. Normal control (NC): OVA sensitized and treated with vehicle; Disease control (DC): OVA sensitized and challenged rats treated with vehicle; Positive Control (PC): OVA sensitized and challenged rats treated with Prednisolone (10 mg/kg); AV1, AV2 and AV3: OVA sensitized and challenged rats treated with Adiantum venustum at doses of 15.5 mg/kg, 31 mg/kg and 62 mg/kg extract orally. Data are expressed as Mean ± SEM. #p < 0.05 versus Normal control group;*p < 0.05 versus Disease control group.*

Effect of *Adiantum venustum* on TNF-α in blood and BALF

Assay of TNF-α showed significant increase in TNF-α level in Disease control group i.e. OVA sensitized and challenged rats as compared to that seen in the normal control group ($p < 0.05$). Pretreatment with *Adiantum venustum* resulted in attenuation in TNF-α level at all doses but significant attenuations were observed at 31 mg/kg and 62 mg/kg dose in blood and at 62 mg/kg in BALF ($p < 0.05$). *Adiantum venustum* administration at the three doses showed suppression in TNF-α level in blood by 16%, 33% and 43%, respectively, while in BAL fluid, TNF-α level were suppressed by 14%, 18% and 23% respectively. Pre-treatment with prednisolone (10 mg/kg) showed significant attenuations of TNF-α levels by 42% in blood and by 27% in BAL fluid as compared to Disease control group ($p < 0.05$).These results are summarized in figure 3.

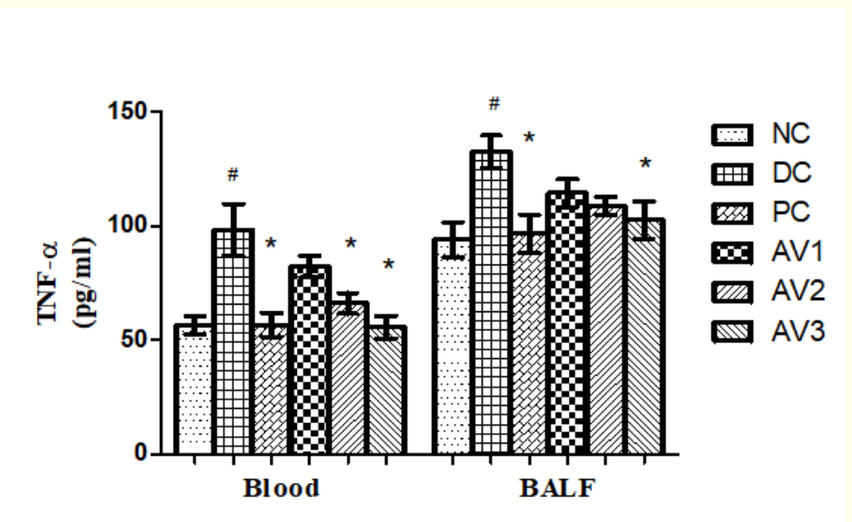

Figure 3: *Effects of Adiantum venustum on TNF-α level in blood and BAL fluid of OVA sensitized and challenged rats. Normal control (NC): OVA sensitized and treated with vehicle; Disease control (DC): OVA sensitized and challenged rats treated with vehicle; Positive Control (PC): OVA sensitized and challenged rats treated with Prednisolone (10 mg/kg); AV1, AV2 and AV3: OVA sensitized and challenged rats treated with Adiantum venustum at doses of 15.5 mg/kg, 31 mg/kg and 62 mg/kg extract orally. Data are expressed as Mean ± SEM. #p < 0.05 versus Normal control group;*p < 0.05 versus Disease control group.*

Effect of *Adiantum venustum* on IL-4 levels in Blood and BAL fluid

OVA immunized + challenged rats (Disease controls) showed significant elevations in IL-4 levels as compared to that of normal control rats($p < 0.05$) in both blood and BAL fluid. Interestingly, *Adiantum venustum* treated animals showed significant attenuations in IL-4 levels as compared to that of Disease control group ($p < 0.05$) in both blood and BAL fluid.Administration of *Adiantumvenustum*at the dose of 15.5 mg/kg, 31 mg/kg and 62 mg/kg, showed suppression in IL-4 levels by 64%, 74% and 88% in blood, while in BAL fluid, IL-4 levels were suppressed by 42%, 47% and 60%, respectively at the three dose levels. Pre-treatment with Prednisolone (10 mg/kg) showed significant attenuation in IL-4 levels by 79% in blood and by 57% in BAL fluid as compared to Disease control groups ($p < 0.05$). These results are summarized in table 1.

Experimental Groups	IL-4 levels (pg/ml)	
	Blood	BAL fluid
Normal Control	117.6 ± 27.05	192.2 ± 9.70
Disease Control	307.7 ± 77.22$^{\#}$	294.3 ± 25.47$^{\#}$
Positive Control	65.16 ± 27.82*	125.2 ± 18.27*
Adiantum venustum 15.5 mg/kg	109.8 ± 27.58*	172.0 ± 39.5*
Adiantum venustum 31 mg/kg	73.77 ± 9.14*	155.7 ± 24.05*
Adiantum venustum 62 mg/kg	31.62 ± 9.25*	117.9 ± 18.25*

Table 1*: Effects of Adiantum venustum on IL-4 levels in blood and BAL fluid of OVA sensitized and challenged rats.*

Data are expressed as Mean ± SEM. $^{\#}p < 0.05$ versus Normal control group;$^{*}p < 0.05$ versus Disease control group. Normal control: OVA sensitized and treated with vehicle; Disease control: OVA sensitized and challenged rats treated with vehicle; Positive Control: OVA sensitized and challenged rats treated with Prednisolone (10 mg/kg).

Discussion

Bronchial asthma is a respiratory disease characterized by airway obstruction, airway inflammation as well as airway hyperresponsiveness mediated through complex cellular and humoral events. Exposure to various allergens resulted in a cascade of phenomena resulting in a Th-2 type mediated immune response which releases various inflammatory mediators such as cytokines from mast cells, inflammatory cells (eosinophils and neutrophils), and chemokines which trigger and maintain the disease pathology. Mast cells upon activation through IgE mediated pathways play an important role in allergic diseases [16,17].

Pharmacotherapy is crucial for bronchial asthma and mainly requires long term treatment with controllers (corticosteroids) and relievers (beta agonists).However, these are associated with various adverse effects and refractoriness to responses [18,19]; which in compromise compliance and adherence to therapy. In response to search for viable alternatives or safer options, focus has been shifted towards traditional systems of medicine and especially medicinal plants and their derived products. Although, use of these medicinal plants are well documented in traditional systems of Indian medicine but there is need for scientific validation so as to establish them as pharmacoeconomically viable alternatives or complimentary therapy. *Adiantum venustum* (Hansraj) is used for the treatment in cold, headache, hydrophobia and inflammation of chest in traditional folklore medicine. Studies have reported analgesic and anti-inflammatory properties at 50 mg/kg as well as 100 mg/kg in rats [5]. Therefore, the present study evaluated the efficacy of *Adiantum venustum* against hyper-reactivity to spasmogen and airway inflammation in experimental model of bronchial asthma. Inhibition of inflammatory response and symptomatic relief remains one of the main mechanisms for drugs in asthma. Immunization of rats followed by antigen challenge

were performed to experimentally induce bronchial hyperresponsiveness/airflow restriction to simulate bronchial asthma and induce release of cytokines eg. TNF-α, IL-4, IL-5, IL-13 as well as initiate an inflammatory response, marked by mobilization of mediators such as IgE.

In the experimental model of bronchial hyper-responsiveness in OVA immunized rats, effect of *Adiantum venustum* was assessed on methacholine challenged rats using Whole Body Plethysmography. Enhanced Pause (*P-enh*) is positively correlated with airway resistance [20, 21] and airway responsiveness in several asthma studies [14,22]. In our study, enhanced pause (*P-enh*), a marker of airway resistance and airway hyperresponsiveness was measured through whole body plethysmography in response to methacholine at different doses of 0 (basal), 2.5 mg/ml, 10 mg/ml and 20 mg/ml. Higher *P-enh* values are indicative of higher airway resistance and airway hyper-responsiveness. Increased airway resistance was observed in the Disease control group of rats which may be attributed due to bronchospasm as well as bronchoconstriction following antigenic challenge as evidenced by high *P-enh* values (with maximum at highest dose level, 20 mg/ml of the methacholine). Treatment with *Adiantum venustum* extract showed significant reductions in *P-enh* values following methacoline challenge (20 mg/ml) which may be an indicator of reduced airway resistance and hyper-responsiveness. Prednisolone treated group also showed significant lowering of *P-enh* values after methacholine and these effects were comparable with those of the *Adiantum venustum* extract. These results reflected reversal of bronchial hyper-responsiveness by *Adiantum venustum* extract.

IgE is a reaginic antibody which plays vital role in airway inflammation and other allied allergic reactions. Higher levels of IgE are reported in adults and children with asthma and are associated with greater asthma severity, airway hyperresponsiveness and lower baseline lung function [23,24]. Our results showed elevated levels of OVA specific IgE following OVA immunization + challenge in experimental rats. However, pretreatment with *Adiantum venustum* (15.5 mg/kg, 31 mg/kg and 62 mg/kg) reduced the levels of IgE in both blood and BAL fluid as compared to OVA-induced sensitized and challenged group (Disease controls). A clear dose dependent effect was observed confirming the pharmacological relevance of the herbal agent in this experimental asthma model in rats. These effects of *Adiantum venustum* were comparable with that seen after prednisolone treatment – indicating an anti-inflammatory role for this herbal agent.

TNF-α is a pro-inflammatory cytokine and primarily produced by macrophages but also from other cells like T lymphocytes, mast cells and epithelial cells. TNF-α production is also under the regulatory influence of IL-1, GM-CSF and IFN-γ. Higher levels of TNF-α have been observed in induced sputum and lung biopsies of subjects with severe asthma [25,26]. Moreover, studies have shown that inhalation of TNF-α in normal individuals and those with asthma cause an influx of neutrophils into the airways and also increase airway hyper-responsiveness [27,28]. In our study, OVA sensitization+challenge treatment resulted in increased TNF-α levels in both blood and BAL fluid compared to normal control rats which resulted in increased airway hyperresponsiveness. However, treatment with *Adiantum venustum* (15.5 mg/kg, 31 mg/kg and 62 mg/kg) reduced TNF-α level in both blood and BAL fluid, thus reaffirming a significant anti-inflammatory response of *Adiantum venustum.*

IL-4 is a Th-2 cell specific cytokine and actively participates in allergic airway inflammation and in the pathophysiology of bronchial asthma by stimulating mucus producing cells, fibroblasts, inducing expression of vascular cell adhesion molecule-1 (VCAM-1) on endothelial cells, VCAM-1 in turn enhances adhesiveness of the endothelium for T-cells, eosinophils, basophils and monocytes, which are also key players in allergic reactions [29, 30]. IL-4 differentiates naïve T-helper cells to Th-2 cells and suppresses Th-1 immune response, which upon activation results in mast cell activation and releases various mediators. In this study, OVA immunization + challenge of rats resulted in increased IL-4 level in both blood and BAL fluid compared to normal control rats which is characteristic of increased inflammation in experimental animals. However, pretreatment with herbal agents, *Adiantum venustum* (15.5 mg/kg, 31 mg/kg and 62 mg/kg) reduced the levels of these cytokines in blood and BAL fluid as compared to OVA-induced sensitized and challenged group (Disease control).

Taken together, pretreatment with *Adiantum venustum* attenuated bronchial hyperresponsiveness (as evidenced by lowered *p-enh*), and lowered levels of IgE, TNF-α and IL-4 in both blood and BAL fluid, thus indicating that the herbal extract may reverse the biomarkers of airflow restriction, and airway inflammation, thus validating the reported beneficial effects of this herbal agent in bronchial asthma.

Conclusion

The present study demonstrated that OVA-induced model of bronchial asthma was marked by increased levels of IgE, TNF-α and IL-4 in both blood and BAL fluid and elevated enhanced pause (during whole body plethysmography). Adiantum venustum (I5.5 mg/kg, 31 mg/kg and 62 mg/kg) reduced the airway bronchial hyperresponsiveness to spasmogens in the OVA sensitized and challenged rats which was accompanied with reduction in the cellular markers of inflammation and immunity. These findings validated ethno-traditional claims related to anti-asthmatic properties of *Adiantum venustum* and delineated its cellular and molecular mechanism of action using modern methodology/technology. Such translational studies using reverse pharmacology also helps in providing new molecular leads in the field of drug discovery, development and rational therapy of various complex diseases.

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