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Research Article

Cellular and molecular mechanisms involved in the protective effects of *Aerva Lanata* Linn. on airways remodeling in experimental model of bronchial asthma in rats

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ABSTRACT

Bronchial asthma is a chronic inflammatory respiratory disease characterized by bronchoconstriction and hyperresponsiveness of the bronchial airways. The treatment for bronchial asthma is mainly aimed at relieving symptoms by bronchodilators and controlling asthmatic attacks with anti-inflammatory agents. These therapeutic strategies are associated with several adverse drug effects and high cost that may negatively affect the compliance to therapy. Chronic asthma also results in structural remodeling of the airways which enhances the incidence of refractory asthma and increases morbidity/mortality. It is therefore important to explore adjuncts from alternative forms of therapy to compliment/supplement the conventional treatment. The study assessed the effects of *Aerva Lanata* Linn., a medicinal plant used in the traditional systems of medicine, on airway remodeling in an experimental model of asthma in rats. Wistar rats were immunized on day 0 with ovalbumin and Al(OH)₃ and challenged with aerosolized ovalbumin from day 15 to 22. The rats were treated orally with various doses of standardized aqueous extract of whole plant of *Aerva Lanata* Linn. or prednisolone (10 mg/kg) for 22 days. After 24 hrs of last challenge, blood, bronchoalveolar lavage (BAL) fluid and lung tissue were collected and assayed for markers of structural remodeling viz, (a) cytokine levels (TGF- β and IL-13); (b) hydroxyproline content, and (c) histopathology. The results showed that *Aerva Lanata* Linn. markedly reduced the levels of TGF- β , IL-13 in both blood and BAL fluid and hydroxyproline content in lung homogenates. Histopathological examination of lung tissue confirmed the modulatory effects of *Aerva Lanata* Linn. on airway remodeling as evidenced by reductions in goblet cell hyperplasia, inflammatory cells infiltration and sub epithelial fibrosis, as compared to that in the experimental (disease control) group. The results showed that *Aerva Lanata* Linn. Attenuated the histopathological changes and biochemical markers of airway remodeling by altering the levels of IL-13 and TGF β , and the study suggested that it could be beneficial in cases of chronic bronchial asthma.

Keywords: *Aerva Lanata* Linn; Airway inflammation; Airway remodeling; Bronchial asthma

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INTRODUCTION

Asthma is indeed a chronic inflammatory airway disease that can lead to tissue injury. Over time, this can cause abnormal structural changes in the airways, which is known as airway remodeling. These changes can further contribute to the symptoms and complications associated with asthma¹. These changes involve tracheal wall thickness, epithelial alterations², sub epithelial fibrosis³, inflammatory cell infiltration, goblet cell hyperplasia⁴, hypertrophy⁵, increased vascularity⁶ and hyperplasia of tracheal smooth muscle. The airway smooth muscle cells do

indeed play a crucial role in increasing inflammation and remodeling in asthma. Asthma can be stimulated by various inflammatory mediators such as Th2 cytokines such as IL-13, IL-5, IL-9 etc., transforming growth factor- β (TGF- β), and extracellular matrix (ECM) proteins⁷. According to the Global Network asthma study, India has an estimated prevalence of approximately 34.3 million individuals with asthma. This account for about 13.09% of the worldwide burden of asthma⁸. The current treatment of asthma includes bronchodilators and inhaled and systemic corticosteroids, but the high cost of inhalers, incidence of side effects and refractoriness to these drugs is a major problem⁹⁻¹⁰. There has

been a constant search for novel therapeutic strategies with potential to reduce morbidity and mortality in bronchial asthma¹¹. In recent times, herbal drugs have regained immense importance all over the world and extensive research is being carried out to scientifically validate the traditionally proven drugs. There is a national initiative to strengthen and promote technological innovations as well as traditional knowledge using new methodology and tools. The literature was surveyed for herbal drugs used in asthma and *Aerva lanta* Linn. was selected on the basis of its use in the traditional system of medicine. *Aerva lanta* Linn. is a type of herbaceous plant that can be found growing erect or prostrate along roadsides. It is characterized by its white axillary bunches of small woolly flowers. This plant is abundant in the warmer regions of India, particularly on the plains, and can be found in various locations up to an altitude of 3000 m¹². Few synonyms mentioned for this plant are: Aadanapaki, Shatakabhedha, Valliyaka, Tripatra, Krsnavalli, Prayanika¹³. *Aervalanata* is indeed endowed with various chemical compounds including steroids, flavonoids, alkaloids, polysaccharides, and saponins. The plant includes alkaloids such as ervine, methylervine, ervoside, aervine, ervolanine, Hentriacontane, β -sitosterol and its D-glucoside, α -amyrin and aervolanine; β -sitosteryl palmitate, α -amyrin and β -sitosterol and tannin.

It is traditionally used as an antimicrobial, antitussive, antioxidant, anti-asthmatic, anti-diabetic, nephroprotective, immunomodulatory, hepatoprotective, etc. and has anti-inflammatory effects in bronchitis¹⁴⁻¹⁶. In a preliminary study, during screening to find novel candidates as anti-inflammatory agents, the ethanolic extract of *Aerva lanata* Linn showed inhibitory activity against mast cell degranulation in experimental model of inflammation in mice¹². Ethanolic extract of *Aerva lanata* Linn. also exhibited dose dependent reduction in histamine induced goat tracheal chain contraction. The purpose of the present study was to investigate the potential modulatory effects of *Aerva lanata* Linn. on airway remodeling in an experimental model of asthma in rats. This study was conducted in light of the traditional claims and reported anti-asthmatic activity associated with *Aerva lanata* Linn. Airway remodeling refers to the structural changes that occur in the airways of asthma patients, which can contribute to the chronicity and severity of the condition.

Materials and Methods

Experimental animals

Inbred Wistar rats (200-250g, both male and female) were used. Animals were kept in separate cages under standard laboratory conditions and had free access to water and food (as per the CPCSEA guidelines). The study protocol was approved by the Institutional Animal Ethics Committee (VPCI/IAEC/2019/22) of the Vallabhbhai Patel Chest Institute (VPCI), University of Delhi, Delhi.

Drugs and chemicals

The following drugs and chemicals were used: Ovalbumin, Methacholine, Prednisolone, Hydroxyproline, Chloramine-T, aluminium hydroxide, etc. (all from Sigma-Aldrich), and other routine chemicals. Cytokine assay kits of IL-13 and

TGF- β . The standardized aqueous extract of *Aerva Lanata* Linn was procured from Kshipra Biotech Pvt. Ltd., Nagpur.

Experimental groups and treatment

Rats were divided into six groups (n=5 per group) viz. Normal control, Disease control (experimental group), Prednisolone (10 mg/kg, positive control) group; and three doses (25, 50 and 100 mg/kg) of *Aerva lanta* Linn. All groups, except normal controls were OVA sensitized and challenged as mentioned below. Rats of normal control and disease control groups received vehicle (distilled water), whereas all drug treated groups received either prednisolone or *Aerva lanta* Linn. from day 1 to day 21, respectively.

Immunization and challenge

The animals were sensitized and challenged with ovalbumin as per the procedure of Rai et al.¹⁷. Briefly, rats were sensitized by intraperitoneal injection of ovalbumin (50 mg per rat) emulsified with 100 mg of aluminium hydroxide in 1 ml of normal saline on day 1 of the experiment. Thereafter, rats were daily exposed to aerosolized ovalbumin (2% in vehicle) from day 15 to 21 for 30 minutes/day. The challenge was carried out in a Plexiglas box (38cm x 30cm x 22cm) by using ultrasonic nebulizer (Aeroneb Lab Nebulizer System, Ireland). After 24 hours of last challenge, all animals were anesthetized. Through cardiac puncture Blood was collected and centrifuged at 3000 rpm for 10 minutes at 4°C and the serum was separated and stored at -80°C for assay of IL-13 and TGF- β .

Blood and BAL fluid collection

After 24 h of the last challenge, the animals were sacrificed and the normal saline was slowly injected into the lung by tracheal cannulation. The thorax was given a gentle massage and saline was slowly withdrawn, the samples (BAL fluid) were collected and maintained at -4°C and centrifuged at 1500 rpm for 10 minutes and the supernatant was collected for biochemical analysis. The supernatant of BAL fluid was separated and stored at -80°C for assay of IL-13 and TGF- β .

Histopathological studies

In the experimental model of airway remodeling, after 24 hour of last challenge of OVA in rats, the blood and BAL fluid were collected as mentioned above and lungs were removed. Right lung was kept for assay of hydroxyproline (as mentioned below) and Left lung was fixed in formalin solution (10%) and processed. The lungs sections were stained with H&E (hematoxylin-eosin) and histopathological examination were carried out and reported by a pathologist, who was blinded to the various treatment groups.

Assay for Hydroxyproline

Hydroxyproline, a marker of collagen content, is formed by hydroxylation of proline by prolylhydroxylase following protein degradation. The right lung of each rat was used for determining hydroxyproline content, a marker of the total collagen content that indicates lung fibrosis. It was evaluated by determining hydroxyproline content according to the standard procedure of Stegemann and Stalderet¹⁸. Briefly, lung tissues were weighed and homogenized in normal saline and then digested in 6N HCl for 8 hour at 130°C. Each sample was neutralized and then freshly prepared chloramine-T

reagent was added. The samples were left at room temperature for 20 minutes and then dimethylaminobenzaldehyde-perchloric acid reagent was added to each sample. The samples were incubated at 60°C for 15 minutes and then cooled under tap water. Finally, the absorbance of each sample was read at 550 nm on a UV-Vis Spectrophotometer (Spectronic 200, Thermo scientific), and the results were expressed in pg/ml.

Assay for TGF- β levels

Blood and BAL fluid samples were assayed for TGF- β levels using commercially available ELISA kits, and as per the manufacturer's instructions. Briefly, the microtiter plate was pre-coated with an antibody specific to rat TGF- β . Blood and BAL fluid samples were then added to the appropriate wells of the microtitre plate with biotinylated anti-rat TGF- β and incubated for 2 hours at room temperature. After incubation, microtitre plate was washed with washing solution. Then, biotin-conjugate was added and again incubated for 1 hours. Again after incubation, microtitre plate was washed with washing solution. Then, Streptavidin-HRP was added and again incubated for 30 minutes. Again after incubation, microtitre plate was washed with washing solution. TMB substrate was added to produce a colored reaction product and again incubated for 30 minutes. The absorbance was read at a wavelength of 450 nm using an ELISA microplate reader (Spectra Max190, Molecular Devices) and the results were expressed in pg/ml.

Assay for IL-13 levels

Blood and BAL fluid samples were assayed for IL-13 levels using commercially available ELISA kits and as per the manufacturer's instructions. Briefly, the microtiter plate was pre-coated with an antibody specific to rat IL-13. Blood and

BAL fluid samples were then added to the appropriate wells of the microtitre plate with biotinylated anti-rat IL-13 and incubated for 1 hour at room temperature. After incubation, microtitre plate was washed five times and add chromogen solution A and B then incubate for 10 minute at 37°C. The enzyme-substrate reaction was quickly stopped by adding stop solution. The absorbance was read at a wavelength of 450 nm using ELISA microplate reader (Spectra Max190, Molecular Devices), and the results were expressed in pg/ml.

Statistical analysis

All values were expressed as Mean \pm SEM and analyzed by using one-way ANOVA followed by post hoc Tukey's multiple comparison test. GraphPad Prism software (version 5) was used for statistical analysis. A p value of at least 0.05 was considered as the level of significance in all statistical tests.

RESULTS

1. Histopathological Studies:

The histopathological study of lung tissue section from the disease control group rats (ovalbumin sensitized/challenged) showed marked structural changes, viz. (a) increased number of inflammatory cells infiltrations in airways, (b) increased goblet cell hyperplasia and (c) sub epithelial fibrosis, as compared to that observed in normal controls. Rats treated with *Aerva lanata* Linn. showed dose dependent reductions in above histopathological changes that were observed in disease control group of rats. The reduction of histopathological changes was comparable to the positive control, prednisolone (10 mg/kg) treated group. These results are shown in Figure 1.

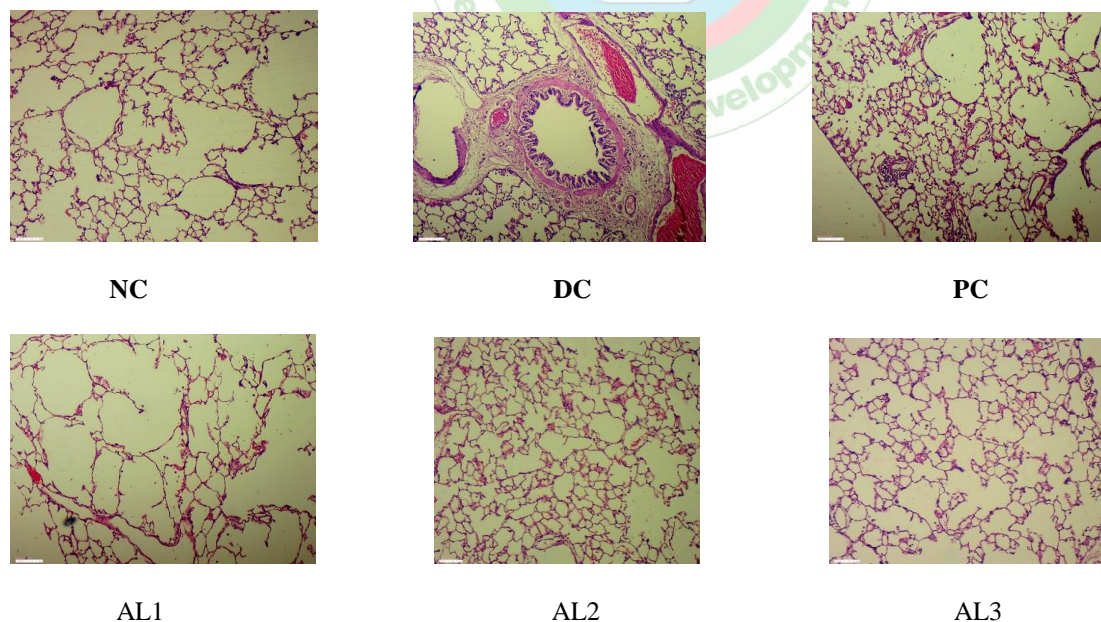


Figure 1: Effect of *AervaLanata* Linn. (AL) on histopathological changes in lungs tissue of ovalbumin sensitized rats

NC: normal control i.e. ovalbumin sensitized rats treated with vehicle; DC: normal control i.e. Ova sensitized and challenged rats treated with vehicle; PC: positive control i.e. ovalbumin sensitized rats and challenged treated with prednisolone (10mg/kg); AL1, AL2, and AL3: i.e. ovalbumin sensitized rats and challenged treated with *Aerva lanata* Linn. 25mg/kg, 50mg/kg and 100mg/kg, respectively. Stained with hematoxyline & eosin (H&E), 10x magnification.

2. Effect of *Aerva lanata* Linn on the level of Hydroxyproline levels lung tissue of OVA sensitized rats

Hydroxyproline, a marker of collagen content, is formed by hydroxylation of proline by prolyl hydroxylase following protein degradation. Changes in the hydroxyproline content of lung homogenate can be indicative of alterations in collagen levels, which are often associated with tissue remodeling processes, including fibrosis. Level of hydroxyproline in lungs homogenate were high in disease control group as compared that of normal control group. The level were 115.8 ± 20.54 $\mu\text{g/g}$ lung tissue and 243.3 ± 13.43 $\mu\text{g/g}$ lung tissue, in normal (NC) and disease control (DC) rats, respectively, i.e. the level were increased by 110% in the latter group – suggestive of airway remodeling. Results showed that the treatment with *Aerva lanata* Linn. reduced the hydroxyproline content in lung tissue homogenate by all three doses of in a dose related manner (26%, 36% and 43% reduction by 25mg/kg, 50mg/kg, 100mg/kg doses of *Aerva lanata*, respectively, as compared to DC group). The reduction was statistically significant at the 100mg/kg dose level and this was comparable to that of prednisolone treated group that showed reduction by 55% as compared to DC group. The results are shown in Fig. 2

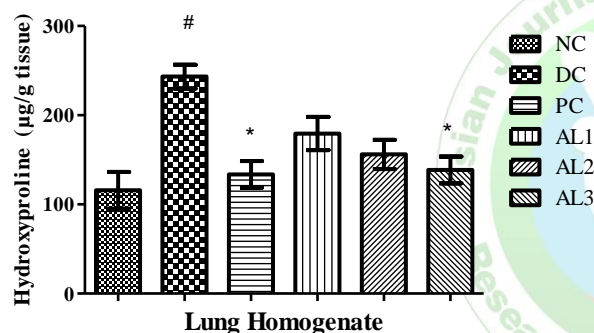


Figure 2: Effect of *Aerva Lanata* Linn. on the level of Hydroxyproline in ovalbumin sensitized and challenged rats in lung homogenate.

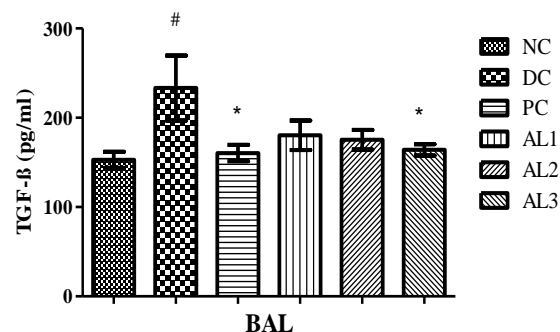
NC: normal control i.e. OVA sensitized rats treated with Vehicle; DC: disease control i.e. Ovalbumin sensitized and challenged rats treated with vehicle; PC: positive control i.e. ovalbumin sensitized and challenged rats treated with Prednisolone (10mg/kg); AL1, AL2, and AL3: ovalbumin sensitized and challenged rats treated with *Aerva lanata* Linn. 25mg/kg, 50mg/kg and 100mg/kg, respectively. All data are expressed as Mean \pm SEM, n=5 per group. *p<0.05 vs disease control, #p<0.05 vs normal group.

3. Effect of *Aerva lanata* Linn. on the levels of TGF- β in Blood and BAL fluid of OVA sensitized and challenged rats

Transforming growth factor-beta (TGF- β) is a critical cytokine that plays an important role in tissue remodeling in the asthmatic lung, particularly in the context of fibrosis. Analysis of data showed that ovalbumin sensitization and challenge (disease controls) resulted in significant increased level of TGF- β as compared to normal group. The levels were increased by 36% in blood and by 75% in BAL in disease control group as compared to that in normal control group. The effect of *Aerva lanata* was assessed on TGF- β in BAL fluid and blood of ovalbumin sensitized and challenged rats. Results showed that administration of all three doses of

Aerva lanata (25mg/kg, 50mg/kg, 100mg/kg), reduced the TGF- β level by 19%, 21% and 28% (p<0.05) in blood and 23%, 25% and 30% (p<0.05) in BAL fluid respectively, as compared to disease control group. The results with the high dose (100 mg/kg) of *Aerva lanata* Linn were comparable with that of prednisolone treated group. The results are shown in Figure 3.

(A)



(B)

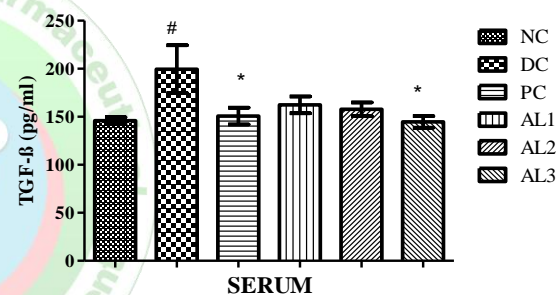


Figure 3: Effect of *Aerva lanata* Linn. on the level of TGF β of ovalbumin sensitized and challenged rats in A) BAL fluid and B) serum.

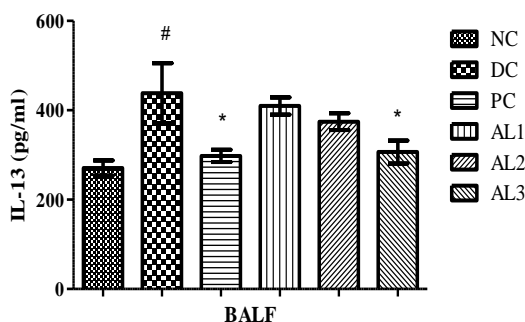
NC: normal control i.e. ovalbumin sensitized rats treated with Vehicle; DC: disease control ovalbumin sensitized and challenged rats treated with vehicle. PC: positive control i.e. ovalbumin sensitized and challenged rats treated with Prednisolone (10mg/kg). AL1, AL2, and AL3: ovalbumin sensitized and challenged rats treated with *Aerva lanata* Linn. 25mg/kg, 50mg/kg and 100mg/kg, respectively. All data are expressed as Mean \pm SEM, n=5 per group. *p<0.05 vs Normal control; #p<0.05 vs disease control.

4. Effect of *Aerva lanata* Linn. on the level of IL-13 in Blood and BAL fluid of OVA sensitized and challenged rats

IL-13 is a key cytokine from Th2 cells mediating airway inflammation and remodeling in allergic asthma. Analysis of results showed that OVA sensitization followed by challenge significantly increased level of IL-13 i.e. the level was increased by 54% in blood and 61% in BAL fluids as compared to that in normal group (only sensitized). Treatment with *Aerva lanata* Linn. (25mg/kg, 50mg/kg, 100mg/kg) and prednisolone for 21 days reduced the level of IL-13 as compared to that in disease control group. There were reduction in the level of IL-3 by *Aerva lanata* (25, 50 and

100 mg/kg doses reduced it by 21%, 27% and 30% in blood and 7%, 15% and 27% in BAL fluid, respectively) as compared to disease control group rats. The reductions were statistically significant by the 100 mg/kg dose in both BAL and blood. Prednisolone (10mg/kg) treatment (positive control) also decreased the level of IL-13 by 34% in both blood and by 32% in BAL fluid ($p < 0.05$). These results are shown in Figure 4.

(A)



(B)

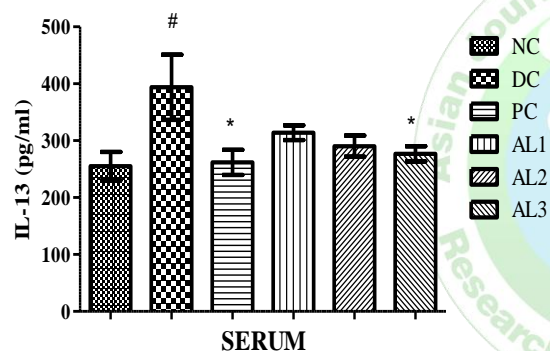


Figure 4: Effect of *Aerva lanata* Linn. on the level of IL-13 in ovalbumin sensitized and challenged rats in Serum and (A) BAL fluid and (B) Serum.

NC: normal control, i.e. OVA sensitized rats treated with Vehicle; DC: disease control i.e. ovalbumin sensitized and challenged rats treated with vehicle; PC: positive control, i.e. ovalbumin sensitized and challenged rats treated with Prednisolone (10mg/kg); AL1, AL2, and AL3: ovalbumin sensitized and challenged rats treated with *Aervalanata* Linn. 25mg/kg, 50mg/kg and 100m/kg respectively. All data are expressed as Mean \pm SEM, $n=5$ per group. # $p < 0.05$ vs Normal control; * $p < 0.05$ vs disease control.

DISCUSSION

Bronchial asthma is a chronic inflammatory airway disease characterized by recurring episodes of wheezing, breathlessness, chest tightness, and coughing. Antigen sensitization and its prolonged exposure plays a critical role in asthma pathophysiology leading to airway inflammation, hyperresponsiveness, reversible airflow obstruction, and eventually airway remodeling. Respiratory inflammation is a complex process involving interactions between various immunological mediators produced by different types of inflammatory cells such as eosinophils, basophils,

neutrophils, dendritic cells and lymphocytes. These cells play crucial role in the immune response to pathogens, allergens, and other irritants in the respiratory system¹⁹.

Chronic abnormal inflammatory response of airways result in airway remodeling i.e. structural changes in the airways viz. thickening of the airway walls, increased smooth muscle mass, and alterations in the extracellular matrix²⁰. The histopathologic highlights of airway remodeling are inflammatory cell infiltration, bronchial smooth muscle cell hypertrophy, and epithelial detachment, thickening of the basement membrane or sub epithelial fibrosis and hyperplasia along with increased mucus production. The increase in the number and size (morphology) of goblet cells and submucosal glands lead to excessive production of mucus that further contributes to airway obstruction and airway hyperresponsiveness. In the current study, the histopathological examination of lung tissue following immunization and challenge to ovalbumin revealed increased number of inflammatory cells infiltration in airways, goblet cell hyperplasia and sub epithelial fibrosis, i.e. clearly indicative of airway remodeling. In rats pretreated with *Aerva lanata* Linn. such changes, specifically goblet cell hyperplasia and sub epithelial fibrosis were markedly reduced, thus suggesting the protective effect of the herbal drug against airway remodeling.

One of the most important features of airway remodeling in respiratory disease is fibrosis caused by the deposition of collagen which is also a probable marker for disease severity in chronic asthmatic patients²¹. Collagen is important proteins which has the major amino acids proline and hydroxyproline, and measurement of hydroxyproline content of lung is widely accepted as a reliable marker of collagen production during lung fibrosis. The results of current study showed that ovalbumin challenge resulted in enhanced level of Hydroxyproline in lung homogenates of disease control rats. Pretreatment with *Aerva lanata* Linn. (100 mg/kg) for 21 days significantly reduced the levels of hydroxyproline as compared to that in disease control rats, thus emphasizing the reduction of airway remodeling and lung fibrosis following administration of *Aerva lanata* Linn. The results confirmed the histopathological observations and anti-remodeling effect of the *Aerva lanata* Linn. in the experimental model of asthma. Further studies were conducted to delineate the possible cellular and molecular mechanisms involved in such effect.

Transforming Growth Factor-beta (TGF- β) and Interleukin-13 (IL-13) are two key mediators implicated in airway remodeling. These mediators are often associated with Th2-type immune responses. TGF- β is a crucial cytokines involved in tissue remodeling in asthmatic lung²². It is a potent pro-fibrotic cytokine that promotes proliferation of goblet cells, mucus secretion, and sub-epithelial fibrosis by

stimulating fibroblast and myofibroblast cells that secrete interstitial collagen and increase the apoptosis of airway epithelial cells²³. In the current study, ovalbumin immunization and challenge resulted in significant increase in the TGF- β levels in both blood and BAL fluid in disease control rats. Similar increased expression of TGF- β has been reported earlier also that correlated with the severity of asthma and airway remodeling²⁴⁻²⁵. Further, administration of TGF- β in mice has been shown to enhance collagen deposition, subepithelial fibrosis, and airway hyper-responsiveness²⁶. Daily administration of *Aerva lanata* Linn. (100 mg/kg,) for 21 days significantly reduced TGF- β levels in both blood and BAL fluid in ovalbumin-immunized and challenged rats thus suggesting its beneficial effect in airway remodeling that may be mediated through TGF- β .

IL-13 plays a crucial role in orchestrating various aspects of airway remodeling in sub-epithelial fibrosis, goblet cell hyperplasia and infiltration of inflammatory cells. A Th-2 cytokine, IL-13, is considered a key mediator of allergic asthma and airway remodeling²⁷⁻²⁸. Moreover, it stimulates various cells that are involved in bronchial asthma, such as epithelial cells eosinophils, mast cells, B-cell, airway smooth muscle cells, and fibroblasts²⁹. In the present study, the IL-13 levels were found to be significantly increased in disease control rats which were reduced by treatment with different doses of *Aerva lanata* Linn (25, 50 and 100 mg/kg,) in both blood and BAL fluid of ovalbumin immunized and challenged rats, however it reached the level of significance ($p < 0.05$) at the dose of 100 mg/kg only. During asthmatic attacks the levels of IL-13 are reported to be increased in humans³⁰. Yang et al.³¹ also showed that the administration of anti-IL-13 monoclonal antibody effectively reduced airway remodeling, airway hyper responsiveness as well as inflammatory cells infiltration. IL-13 is also an important fibrogenic factor that is associated with the sub epithelial fibrosis mediated through the activation of TGF- β ³⁶. The results corroborate the earlier findings that *Aerva lanata* Linn. has anti-remodeling effect in the airways of ovalbumin immunized and challenged rats by altering the levels IL-13, which is responsible for regulating inflammation, mucus secretion, and airway hyperresponsiveness.

CONCLUSION

In conclusion, the study showed that *Aerva Lanata* Linn. has anti-remodeling potentials as confirmed by histopathological and biochemical (hydroxyproline levels) studies. The effect may possibly be mediated by reducing the levels of TGF- β and IL-13 which play an important role in mediating allergic asthma, inflammation and airway remodeling. These results indicate that *Aerva Lanata* Linn could provide a lead molecule with anti-remodeling potential and this could contribute to its therapeutic benefit in bronchial asthma.

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Conflict of Interest: Authors have no personal or financial conflicts of interest in relation to the publication of this manuscript.

Author's Contribution: The study was conceptualized by HS and KG. The methodology, investigation, data analysis, writing, quality draft preparation, editing and revision were conducted by HS and KG. Supervision, critical revision and approval for the final manuscript by KG and AR. All authors read and approved the final manuscript.

Ethical Statement: The study was conducted after ethical approval from the Animal Ethical Committee (IAEC), V P Chest Institute, and University of Delhi-110007.

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