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Original Research Article

Anti-Inflammatory and Immunomodulatory Effects of *Withania Somnifera* In. an Experimental Model of Bronchial Asthma in Normal and Stressed Rats

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Abstract

Introduction: Airway inflammation and hyperresponsiveness are distinctive features of bronchial asthma and chronic inflammation may lead to structural changes known as airway remodelling. Complex and interacting mechanisms regulate the pathophysiology of asthma and confounding factors like emotional stress have been implicated. *Withania somnifera* (WS) is a potent anti-stress and immunomodulator in view of which it could be considered as possible therapeutic agent for asthma.

Objective: The present study examined the anti-inflammatory and immunomodulatory effects of s WS root extract on markers of airway inflammation and immunity in normal as well as stressed rats in experimentally induced bronchial asthma.

Methods: Wistar rats (200-225 g) were immunized with ovalbumin (OVA) + aluminium hydroxide on day 1 and challenged with OVA on day 14. The rats were divided into stressed (RS x 15 days) and non-stressed groups and were administered WS extract (200 and 400 mg/kg). Following this, the animals were sacrificed, and Broncho alveolar lavage fluid (BALF) and blood were collected for assay of immune and inflammatory markers of the airways.

Results: WS extract significantly decreased OVA-induced elevations in IgE, IL-4, TNF-α and IL-13 levels in both blood and BALF in RS exposed and non-RS rats with the higher dose of WS. These WS effects were comparable to those seen after dexamethasone treatment. Further, the OVA-induced responses in RS-exposed rats and their modulation by WS were of greater magnitude as compared to their non-RS groups.

Conclusion: Our study findings indicate that WS extract effectively attenuated markers of immunity and airway inflammation in stressed and non-stressed rats, in the OVA model of asthma and suggests that anti-inflammatory, immunomodulatory and anti-stress effects could contribute to these responses.

Keywords: *Withania somnifera*, Bronchial Asthma, Stress, Airway Inflammation, Immunomodulation

Introduction

Bronchial asthma is a complex syndrome in which airway inflammation, bronchial hyperresponsiveness and airflow restriction are characteristic features. Further extraneous factors like exposure to allergens and irritants, exercise, infections, etc. could trigger the onset of the disease [1]. Airway inflammation is a distinctive feature of bronchial asthma, and chronic or persistent inflammation may lead to structural changes known as airway remodelling [2]. Several cellular and humoral components are involved in the pathophysiology of asthma and a plethora of cells like eosinophils, neutrophils, cytokines and other immune markers) have been implicated. Emotional and environmental stressors are known to disrupt the physiological milieu and result in a variety of disease conditions including those of the respiratory tract [3]. The effects of such stressors are also known to impact the immune system and inflammatory processes and targeted stress pathways could be an important therapeutic strategy for treating stress-related disease states. Drug therapy of bronchial asthma primarily comprises of bronchodilators, anti-inflammatory agents and others [4]. Chronic use of such agents can result in adverse reactions which could affect compliance issues and costs. Hence there is an unmet need for devising therapeutic strategies to effectively deal with the multicomponent syndrome of asthma. Medicinal plants are documented for being safe and effective in the Indian traditional system of medicine and have contributed to many landmark drugs in contemporary medicines [5]. Integration of traditional and modern medicinal concepts are an emerging area of investigation to promote the use of phototherapeutic agents as alternatives/adjuncts in chronic disorders. *Withania somnifera* (Ashwagandha, Dunal, winter cherry) is a Rasayana herb primarily used as an adaptogen and rejuvenator in traditional medicine. Immunomodulatory and anti-inflammatory effects of this medicinal plant are also well documented [6]. However, the role of WS as a modulator of inflammation and immunity in the respiratory tract is not clearly defined. In view of this, we evaluated the effects of standardised *Withania somnifera* extract on inflammatory and immune biomarkers in normal as well as stressed rats by using an experimental model of bronchial asthma.

Materials & Methods

Animals

Wistar rats (150-200 gm) of either sex were used. Rats were housed at a constant temperature of 22±2°C and a 12h light:12 h dark cycle. The animals (n=6 per group) had free access to food and water throughout the experiments. Animal care was maintained under standard laboratory conditions following CPCSEA guidelines. The study protocol was approved by the Institutional Animal Ethics Committee (IAEC), Jamia Hamdard, and New Delhi.

Drugs

The standardized root extract of *Withania somnifera* (WS) was a generous gift of

Natural Remedies Pvt. Ltd., (Bengaluru, India). The aqueous extract was prepared from the dried mature root of *Withania somnifera* (L) Dunal (Family Solanaceae) by using water solvent. Physical and chemical parameters were tested and certified to have passed the prescribed standards. HPLC method was used to investigate the presence of the bioactive phytochemical markers in the root extract as well as to analyse their content. The total withanolide content was 2% in the portion of root dry extract which complied with the specification (≥ 1.5 % total withanolides).

The peaks at the retention time of reference compounds; withanolide A and withanoside IV in the HPLC chromatogram were found at a similar retention time in sample extract. Ovalbumin (OVA, Grade III) was procured from Sigma Aldrich Chemical Co., USA.

The specific rat Enzyme-linked immunosorbent assay (ELISA) kits for TNF-α, IL-4, and IL-6 (Diaclone SAS, France), HDAC2 and specific IgE(BT Lab, China)were purchased from Amplicon Biotech, India. Dexamethasone sodium was procured from Abbott Laboratories, India was purchased from the local pharmacy. All the buffers were freshly prepared, and all routine drugs/chemicals needed for the various assays were of high analytical grade and obtained from SRL Labs, New Delhi, India.

Immunization

The animals were sensitized and challenged with OVA as per the method of Kwasniewski *et al*. [7]. Briefly, on day 1, all rats, except normal controls, were immunized with OVA (10 mg per rat i.p.) emulsified with and 1 mg of aluminum hydroxide in 0.5 ml of isotonic saline was added as an adjuvant. Fourteen days after sensitization, except for groups I and II, all rats were received i.p. injection of 1mg OVA in 0.5 ml of isotonic saline as the challenged dose.

Experimental groups received oral administration of WS (200 and 400 mg/kg) or dexamethasone (1mg/kg i.p) which were given 30 minutes prior to OVA exposure from day 1 to day 14. Following this, on day 15, rats received ketamine (50 mg/kg i.p) and xylazine (10mg/kg i.p.). Blood was collected by cardiac puncture from anesthetized animals and centrifuged (3500×10 minutes). In addition, 0.5 ml of blood was collected in heparinized tubes for eosinophil count. For the collection of BALF, a slit was made in the neck, the trachea exposed, and a polyethylene cannula was inserted. The trachea and lungs were perfused with normal saline (0.5 ml × 3 times) and the cardiac massage was done to increase the output of Broncho alveolar lavage fluid (BALF). The collected BALF was then centrifuged (1500 rpm × 10 minutes), and the supernatant of serum and BALF supernatant were stored at -80 ºC for biochemical analysis [8,9].

Stress procedure

For stress (RS), animals were restrained in Plexiglas restrainers for 1 hour at room temperature. This is a well-documented research procedure to induce psychological stress in rodents with all the typical features found in stress response [10].

In this method, the rats are immobilized with minimum scope of movement without causing pain to the animal. Following RS, the animals were anesthetized with ketamine at 50mg/kg. Blood was collected by cardiac puncture, serum was stored at -80℃ for various biochemical assays. Similarly, for BALF, the trachea was cannulated, and 0.9% sodium chloride solution was injected for lung lavage as per the procedure described earlier.

Immunological assay

The collected blood and BALF samples were assayed for cytokines by ELISA method using a commercially available assay kit. Solid phase sandwich ELISA was performed

by following the manufacturer's instructions for the various assays.

Statistical analysis

All data were expressed as Mean ± SE and analyzed using one-way ANOVA followed by Tukey`s post-hoc test for intergroup comparisons, using Graph Pad prism software (version 8.3.0). A p-value of less than 0.05 was fixed for significance testing in all statistical tests.

Results

IgE

WS extract treatment effects were evaluated on IgE levels in OVA immunised rats, in both blood and BALF. Analysis of data showed that the IgE levels following different treatments were significantly different across F (7, 40 = 214.4); p< 0.001, for BALF; and F (7, 40=182.6); p< 0.001. Pretreatment with WS (200 and 400 mg/kg)x 14 days markedly decreased IgE levels, in a dose-related manner, in both BALF and blood, when compared to the disease control group; p< 0.05, in each case. Further, the attenuating effects of WS 400mg/kg on IgE levels were comparable to that of the comparator drug Dexamethasone 1mg/kg i.p.

In stressed rats similar elevation in IgE levels was observed in both blood and BALF samples when compared with controls;(p<0.01 in both cases). Pretreatment with WS extract doses (x 14 days) markedly lowers IgE levels in both body fluids, when compared to the OVA group (Disease control, DC) and RS; (p<0.01 in each case). Interestingly the WS induced reduction in IgE levels in BALF and blood were more marked in the stressed group of rats as compared to their normal counterparts. These results are shown in Fig 1.

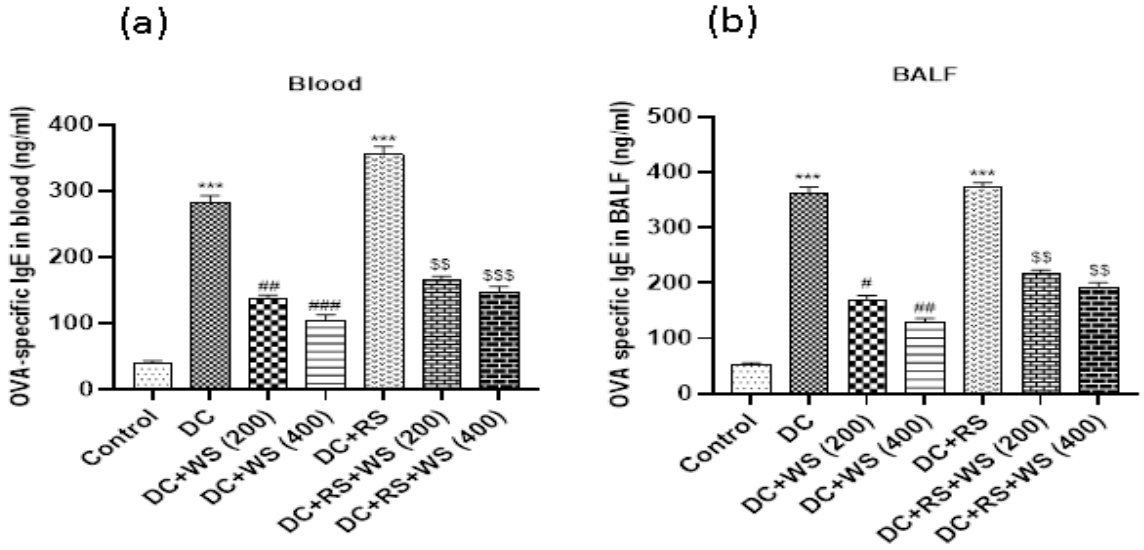

Figure 1: Effects of *Withania somnifera* (WS) on IgE levels in (a) blood and (b) BALF in OVA induced model of bronchial asthma in normal and stressed rats.

Control- OVA sensitized only; DC-Disease Control (OVA sensitized + challenged); RS-Restraint stress. ***p< 0.001 (compared to controls); # p < 0.05; ## p < 0.01; ### p < 0.001 (compared to DC group); $$ p < 0.01; $$$ p < 0.001 (compared to DC+RS).

IL-4

The effects of WS extract were evaluated on IgE levels in OVA immunised rats, in both blood and BALF. Data analysis showed that the IL-4 concentrations were significantly different across

all treatment groups F (7, 40 = 86.34); p< 0.001, for BALF; and F (7, 40= 253.6); p< 0.001, for blood. Pretreatment with WS (200 and 400 mg/kg) x 14 days markedly decreased IL-4 levels, in a dose-related manner, in both BALF and blood, when compared to the disease control group; p< 0.05, in each case. Further, the attenuating effects of WS 400mg/kg on IL-4 levels were comparable to that of the comparator drug Dexamethasone 1mg/kg i.p.

In stressed rats similar elevation in IL-4 levels were observed in both blood and BALF samples in the OVA group as compared to the respective values of the control group;(p< 0.01, both cases). Pretreatment with WS extract doses (x 14 days) markedly lowers IL-4 levels in both body fluids, as compared to DC and RS group; (p<0.01, both cases). These results are summarized in Fig 2.

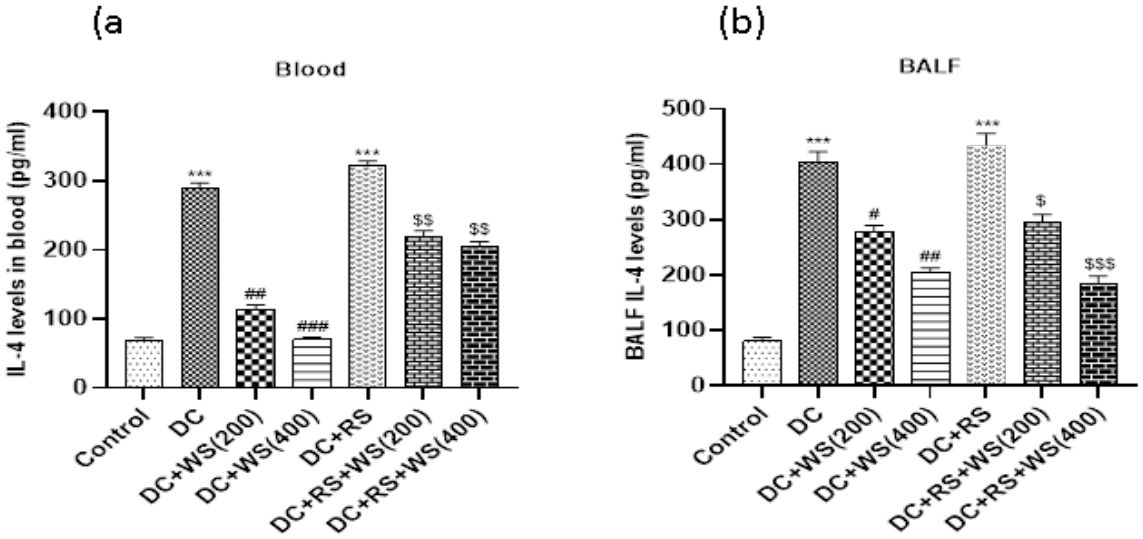

Figure 2: Effects of *Withania somnifera* (WS) on IL-4 in (a) blood and (b) BALF in OVA induced model of bronchial asthma in normal and stressed rats.

Control- OVA sensitized only; DC-Disease Control (OVA sensitized + challenged); RS-Restraint stress. ***p< 0.001 (compared to controls); # p < 0.05; ## p < 0.01; ### p < 0.001 (compared to DC group); $ p < 0.05; $$ p < 0.01; $$$ p < 0.001 (compared to DC+RS).

TNF-α

The effects of WS extract were evaluated on TNF-α levels in OVA immunised rats. Data analysis showed TNF-α levels were significantly different across the various treatment groups F (7, 40 = 22.09); p< 0.001, for BALF; and F (7, 40=20.72); p< 0.001 for blood. Pretreatment with WS (200 and 400 mg/kg) x 14 days markedly decreased TNF-α levels, in both blood and BALF when compared to DC rats; (p< 0.05, both cases). Further, the effects of the higher dose of WS 400mg/kg on TNF-α levels were comparable with the comparator drug Dexamethasone (1mg/kg i.p).

In stressed rats, similar elevation in TNF-αlevels was observed in both blood and BALF samples when compared with controls (p< 0.01, both cases). Pretreatment with WS extract doses (x 14 days) markedly lowers TNF-α levels in both body fluids, as compared to DC and RS group; p<0.01 in each case. These results are shown in Fig 3.

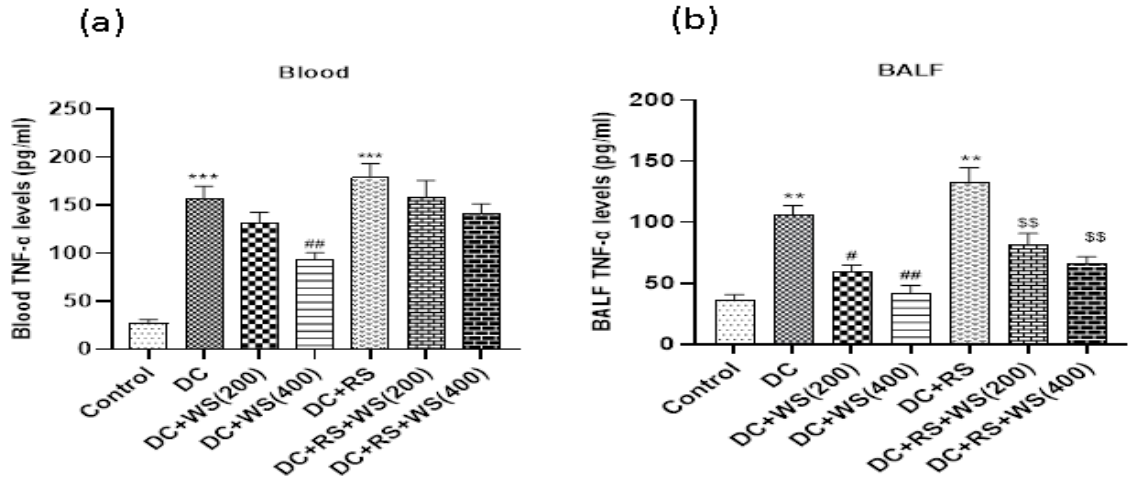

Figure 3: Effects of *Withania somnifera* (WS) on TNF-α levels in (a) blood and (b) BALF in OVA induced model of bronchial asthma in normal and stressed rats.

Control- OVA sensitized only; DC-Disease Control (OVA sensitized + challenged); RS-Restraint stress. ***p< 0.001 (compared to controls); # p< 0.05; ## p < 0.01 (compared to DC group); $$ p < 0.01 (compared to DC+RS).

IL-13

The effects of WS extract were evaluated on IL-13 levels in OVA immunised rats, in both blood and BALF. Data analysis showed that IL-13 levels were significantly different across all treatment groups F (7, 40 =53.06); p< 0.001, for BALF; and F (7, 40=33.29); p< 0.001 for blood. Pretreatment with WS (200 and 400 mg/kg) x 14 days markedly decreased IL-13 levels, in both BALF and blood, when compared with the DC group; (p< 0.05, in each case). Further, the attenuating effects of WS 400mg/kg on IL-13 levels were comparable to that of the comparator drug Dexamethasone 1mg/kg i.p.

In stressed rats, similar elevation in IL-13 levels was observed in both blood and BALF samples, as compared to the control group (p< 0.01, both cases). WS Pretreatment (×14 days) markedly lowers IL-13 levels in both body fluids, as compared to DC and RS exposed rats; (p<0.01). These results are shown in Fig 4.

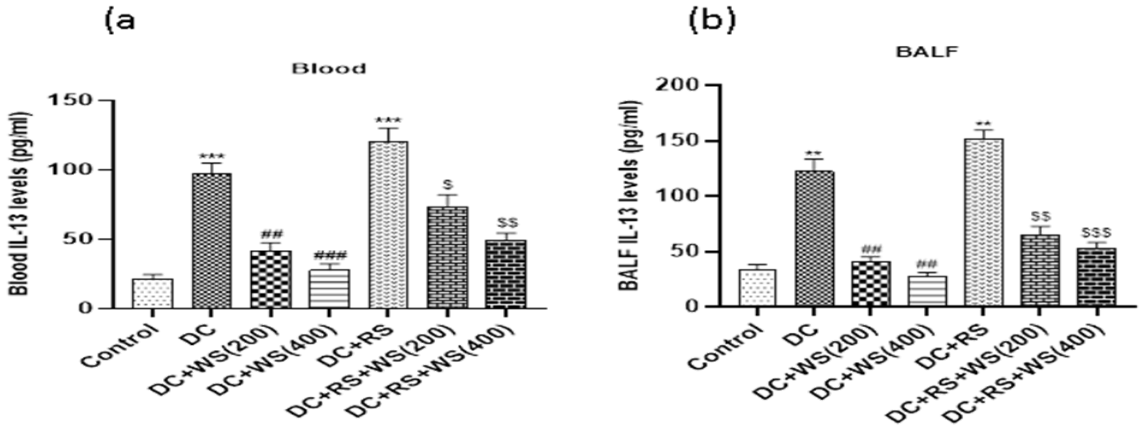

Figure 4: Effects of *Withania somnifera* (WS) on IL-13 levels in (a) blood and (b) BALF in OVA induced model of bronchial asthma in normal and stressed rats.

Control- OVA sensitized only; DC-Disease Control (OVA sensitized + challenged); RS-Restraint stress. ** $p < 0.01$; ***$p< 0.001$ (compared to controls); ## $p < 0.01$; ### $p < 0.001$ (compared to DC group); $ $p < 0.05$; $$ $p < 0.01$; $$$ $p < 0.001$ (compared to DC+RS).

Discussion

The pathophysiology of bronchial asthma is driven by complexly interacting cellular and molecular processes and thus the therapeutic intervention should ideally be multi-targeted. Though airway inflammation and immune modulation are crucial, other factors such as physiological/psychological stressors can be compounding factors for the disease process and which in turn could impact the treatment outcome [11].

Stress is known to influence inflammation and immune response of the respiratory tract and it is likely that stress induced modulation of airway inflammation and immune regulation could influence the disease progress [12]. Long-term conventional drug therapy (bronchodilators and corticosteroids) for asthma [13] is usually associated with safety and compliance issues and hence there is a need for an integrated approach for disease management to reduce morbidity and mortality.

Medicinal plants have been well documented for their therapeutic benefits in respiratory diseases including asthma by virtue of their holistic approach [5]. In this study, interactions between traditional and contemporary medicinal concepts were evaluated for rationalizing drug therapy for bronchial asthma. This study evaluated the effects of WS in OVA induced (immunized + challenged) model of bronchial asthma in normal and stressed rats.

In our study, the results showed that immunological markers were markedly higher in the DC group (immunized + challenged) as compared with the control group (only immunized and not challenged) –thus confirming the development of the asthma model. OVA sensitized and challenged rats showed higher levels of IgE levels than the control group suggesting the development of an allergic asthma model and an agreement with earlier studies [14].

This could also result from an imbalance between Th1 andTh2 cells- a predominant Th2 phenotype resulted in allergic airway inflammation [15]. In this study, in addition to elevated IgE levels, increases in IL-4 (Th2 cytokine) levels were also seen. It is well documented that Th2 cytokine induces infiltration of eosinophils and causes the production of IgE by mast cells [16]. Increased production of IgE in turn stimulates the secretion of other inflammatory mediators which facilitates bronchial hyperreactivity, mucus hypersecretion and airway inflammation- all clinical hallmarks of asthma [14-17]. The fact that *Withania somnifera* markedly attenuated both IL-4 and IgE levels in blood and BALF strongly suggests its ameliorating effect on airway inflammation. Further, WS extract lowered IgE and IL-4 levels in both non-stressed and stressed rats with the effect being greater in the stressed group. The anti-stress property of WS is well documented [18] and it is possible that WS could have modulated the stress-immune axis to produce this beneficial effect.

Both TNF-alpha and IL-1 β play are important for the inflammatory process viz cell activation and infiltration in allergic asthma [19]. TNF-αenhances the release of cytokine, eosinophils/neutrophils recruitment, increased expression of cell adhesion molecules and myofibroblasts activation [20]. Our study showed that OVA immunized + challenged rats had higher levels of cytokine in BALF and blood when compared with controls, and treatment with WS decreased the levels of these proinflammatory cytokines in a graded fashion and the higher dose

(400mg/kg) were more prominent- thus emphasizing the pharmacological basis of this response.

Further, WS pre-treatment reduced TNF- α levels in our experimental model of asthma in normal (no stress) and stressed rats with the effects being more marked in stressed rats. Our data are in agreement with another study which showed downregulation of TNF-α mRNA after an active glycowithanolide (Withaferin A) in the lungs of OVA immunized mice [6].

IL-13, is another Th2 cytokine which is known for its pro-inflammatory role in allergic disorders including asthma. IL-13 is of special significance because it can also produce structural changes in the airways viz airway smooth muscle proliferation, hyperplasia of goblet cells and subepithelial fibrosis– which are characteristics of chronic intractable asthma [21]. In the present study, IL-13 expression was increased in DC (OVA immunized) as compared to the control rats. Further, WS pre-treatment attenuated such elevations in IL-13 in blood and BAL fluid, in a dose-related manner. As seen earlier, WS attenuated IL-13 levels in both stressed and non-stressed rats with the response in the stress group being greater. This suggested that, in addition to anti-inflammatory and immunomodulatory effects, the anti-stress effect of WS could also have contributed to the beneficial effects in asthma pathophysiology in our experiments.

Taken together, the result of the present study indicates that WS attenuated the biomarkers of airway inflammation and immunity in the OVA immunized model of allergic asthma suggesting that airway inflammation attenuating and immune regulatory effects of the medicinal agent could have contributed to this effect.

Further, WS protected against OVA-induced immunological and biochemical changes in both normal and stressed rats –indicating the anti-stress effects of the plant extract could have complimented the anti-inflammatory and immunomodulatory effects. This study is of translational significance as the multidimensional effects of WS in asthma seen in our preclinical studies have the potential of being taken forward to randomized clinical trials for asthma patients.

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Author contribution

Maaz Naqvi participated in the conduction of the experiments, collection of data and data analysis. Sana Rehman contributed to the study design, data analysis and interpretation, and drafting and finalizing of the manuscript. Nafaa Hasan Ali contributed to data curation and analysis. Kavita Gulati participated in the study design and manuscript review. Arunabha Ray participated in the study design, and data interpretation and the final draft of the manuscript was accepted by all authors.

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