VIWITHAN: A STANDARDIZED ASHWAGANDHA EXTRACT AMELIORATES OVALBUMIN-INDUCED AIRWAY-INFLAMMATION AND OXIDATIVE STRESS IN MOUSE MODEL

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ABSTRACT

Objective: *Withania somnifera*, commonly known as Ashwagandha, Indian ginseng, has been used in Ayurvedic and indigenous medicinal preparations for various disease conditions since long time. In the present study, we investigated the protective effects of Viwithan, a standardized proprietary extract from Ashwagandha roots, against airway-inflammation and oxidative stress modulation in an ovalbumin (OVA)-induced murine model of inflammation.

Methods: Allergic asthma was initiated in BALB/c mice by sensitizing with OVA on days 1 and 14, followed by intranasal challenge with OVA on days 27, 28, and 29. Mice were administered Viwithan (200 and 400 mg/kg) by oral gavage before challenge. Then, mice were evaluated for the presence of airway inflammation, production of allergen-specific cytokine response, lung pathology, and oxidative stress modulation.

Results: The results showed that treatment with Viwithan attenuated OVA-induced lung inflammation in mice. Viwithan significantly attenuated inflammatory cell infiltration into the bronchoalveolar lavage fluid and markedly reduced the levels of pro-inflammatory cytokines, interleukin-10, and transforming growth factor-β1 in lung tissues. Viwithan treatment considerably reduced the lung weight in OVA-sensitized mice. Viwithan markedly attenuated the OVA-induced generation of reactive oxygen species in lung tissues.

Conclusion: Together, these results suggested that Viwithan alleviates OVA-induced airway-inflammation and oxidative stress, highlighting the potential of standardized Ashwagandha extract as a useful therapeutic agent for pulmonary fibrosis management.

Keywords: Ashwagandha, Lung inflammation, Oxidative stress, Withaferin A, *Withania somnifera*.

INTRODUCTION

Asthma is a complex and chronic inflammatory disorder of the lung tissue characterized by the infiltration of inflammatory cells, airway inflammation, bronchial hyper-responsiveness, and mucus hypersecretion [1,2]. The incidence and prevalence of asthma is rapidly increasing around the world and it has become a significant cause of morbidity and mortality in developed countries [3]. A few patients suffering from airway inflammation develop honeycomb lung and suffer mortality due to the irreversible loss of pulmonary function [4]. Airway inflammation results in the upregulation of a large variety of reactive oxygen species (ROS) in the lungs, thereby causing oxidative damage to the airways. Oxidative stress can initiate and propagate airway inflammation, highlighting the importance of pro-oxidants in inflammatory airway disorders [5]. Eosinophils are well known for their contribution to inflammation in airway disorders through the release of ROS and specific granules [6]. Recent research has been focusing on exploration of various drugs for the treatment of airway inflammation; however, they are not particularly effective and more often accompanied by adverse side effects during long-term treatment [7].

Medicinal plants provide an extensive resource in the search for effective medical treatments with a low risk of adverse effects. Recently, herbal preparations have drawn greater attention for treating respiratory diseases, including airway inflammation, asthma, cystic fibrosis, and chronic bronchitis [8,9]. *Withania somnifera* (*Solanaceae*) commonly known as Ashwagandha, Indian ginseng, is an important medicinal plant used long since in indigenous system of medicine including Ayurveda. Ashwagandha provides benefits against many human illnesses such as epilepsy, depression, arthritis, diabetes, and palliative effects such as analgesic, rejuvenating, regenerating, and growth-promoting effects [10,11]. Phytosteroids such as withaferin A and withanoloids are the main biologically active compounds and most of the therapeutic properties of Ashwagandha are attributed to them. These compounds are reported to have anti-inflammatory, immunomodulatory and anti-tumor properties [12].

Previous studies conducted in animal models showed that withaferin A could attenuate brain injury by suppressing inflammation [13]. In addition, withaferin A has been reported as an effective treatment to reduce the progression of fibrosis [14]. Therefore, it is hypothesized that withaferin A may also be a potential candidate for the treatment of airway inflammation. Hence, the present investigation is aimed to assess the therapeutic efficacy of Viwithan, a proprietary extract of Ashwagandha roots containing 5% withaferin A in ovalbumin (OVA)-induced airway-inflammation model.

METHODS

Preparation of plant extract

Viwithan™, a proprietary extract of Ashwagandha roots containing 5% withaferin A was obtained from the Department of Quality control and Assurance, Vidya Herbs Private Limited, Bengaluru.

Animals

Thirty healthy male BALB/c mice weighed 25–35 g were purchased from commercial animal breeder, Biogen Private Limited, Bengaluru, India. Mice were kept for acclimatization, 7 days before dosing. Animals were housed in groups of five per standard cage, on 12 h light/dark cycle; and air temperature was maintained at 22±2°C. Experiments were carried out in accordance with guidelines and approved by Institutional Animal Ethical Committee of Vidya Herbs Private Limited, Bengaluru.

The mice were randomly divided into five groups (n=6): (i) Group I: Normal control group; (ii) Group II: Treated with OVA; (iii) Group III: Treated with OVA and dexamethasone (2 mg/kg); (iv) Group IV: Treated with OVA and Viwithan 200 mg/kg; and (v) Group IV: Treated with OVA and Viwithan 400 mg/kg.

Experimental protocol

The airway-inflammation was induced by OVA (Sigma-Aldrich) using the method described previously [15]. Briefly, all the mice except normal control group were sensitized by intraperitoneal (i.p.) injection of 50 μg of OVA with 1 mg of aluminum hydroxide (Sigma-Aldrich) on day 1st and day 14th. The OVA-challenged mice were exposed to intranasal challenge with 2% OVA solution from days 27th to 29th after the second sensitization. Group IV and Group V of asthma mice were orally administrated once daily with 200–400 mg/kg of Viwithan, respectively, and Group III was treated with dexamethasone 2 mg/kg daily, from days 15 to 26. Normal control group was only sensitized and challenged with phosphate buffered saline (PBS). Mice were sacrificed to determine the pathophysiological features of asthma 24 h after the last challenge.

Cytokine analysis

The levels of interleukin-10 (IL-10) and transforming growth factor-β1 (TGF-β1) in the lung tissue homogenates were estimated using commercially available enzyme-linked immunosorbent assay (ELISA) kit (USCN Life science Inc., Wuhan, China) according to the manufacturer's protocol.

Histological analysis

The lung tissue samples were embedded in paraffin and stained with hematoxylin and eosin to examine the histological changes. Photomicrographs were taken using a light microscope.

Inflammatory cell infiltration in the bronchoalveolar lavage fluid (BALF)

Following treatment with OVA and Viwithan, the mice in each group were sacrificed and their BALF was collected by washing lungs 3 times with chilled PBS using a tracheal cannula. The BALF samples were then centrifuged at ×1000 g for 10 min at 4°C and the cell pellet was re-suspended in PBS. The total BALF cell number was determined using a hemocytometer. The cells were then cytospin onto microscope slides and stained with Wright-Giemsa (50% in PBS) using a cytospin for total 6 min at room temperature for cell classification. The percentages of BALF neutrophils, macrophages, and lymphocytes were obtained by counting the leukocytes under light microscopy at ×400.

Estimation of reduced glutathione (GSH) content

GSH level was determined by the method of Ellman (1959) with slight modification [16]. Briefly, 100 μl of lung homogenate was mixed with 100 μl of 10 mM Ellman's reagent. The volume was made up to 1 ml using 0.2 M Tris buffer with 1 mM ethylenediaminetetraacetic acid (EDTA), pH 8.2 and the yellow color developed was read at 412 nm. The concentration of GSH in the sample was determined by calculating from the linear equation or the regression curve generated from GSH standard.

Superoxide dismutase (SOD) activity

For total SOD assay, 3.0 ml reaction mixture contained 50 mM sodium carbonate (pH 10.2), 96 μM nitroblue tetrazolium (NBT), 0.1 mM EDTA, 20 mM hydroxylamine hydrochloride, 0.6% (v/v) Triton X-100, and 50 μl lung homogenate. The absorbance was recorded at 560 nm for 5 min. One unit of SOD activity was defined as the amount of enzyme required that caused 50% of NBT reduction at 25°C [17].

Lipid peroxidation assay

Lipid peroxidation in the lung homogenate was measured by estimating the formation of thiobarbituric acid reactive substances (TBARS). Malondialdehyde (MDA) is an end product of lipid peroxidation, which reacts with thiobarbituric acid to form pink chromogen TBARS [18]. The reaction mixture contained 0.1 ml of lung homogenate, 0.9 ml of 10% trichloroacetic acid, and 1.0 ml of 0.8% thiobarbituric acid in 20% acetic acid. Solution was incubated at 95°C in water bath for 60 min. After incubation, solution was cooled, and absorbance was measured at 532 nm. Results were expressed as μmol MDA/mg protein.

RESULTS

Viwithan administration ameliorates OVA-induced lung injury by suppressing pulmonary fibrosis in mice

There was no significant difference in body weight observed in all the treatment groups following the OVA-challenge compared with the control group. The lung weight of the OVA-challenged mice slightly increased compared to that in the control group. In contrast, treatment with Viwithan (200 mg/kg) slightly decreased the lung weight. Our results suggest that Viwithan inhibited the OVA-induced lung weight gain in airway tissues (Table 1).

Viwithan reduced the number of inflammatory cells in BALF

BALF was collected 24 h after the last OVA-challenge; the effect of Viwithan on OVA-induced total cells and leukocytes (i.e., macrophages, lymphocytes, basophils, and eosinophils) was evaluated in BALF. OVA administration markedly increased the total cell count and the number of differential leukocytes in BALF, which were significantly suppressed by Viwithan compared with that of the control, suggesting that the inflammation leading to fibrosis was alleviated by Viwithan (Fig. 1).

Effects of Viwithan on the level of pro-inflammatory cytokines in the lung tissues of OVA-challenged mice

The levels of pro-inflammatory cytokines in response to OVA-sensitization were measured in lung tissue homogenates by ELISA. The OVA-challenged mice showed a marked elevation of IL-10 and TGF-β1 secretion compared with that for the PBS-treated controls. Treatment with Viwithan and dexamethasone suppressed the production of IL-10 and TGF-β1 compared with those in the OVA group though it was not significant (Fig. 2). However, Viwithan at the concentration of 400 mg/kg showed a slight increase in the TGF-β1 levels than the OVA group.

Viwithan ameliorated pathological changes of lung tissues in airway-inflammation model

In the present study, we investigated the effects of Viwithan on the morphological changes of lung tissues in asthma mice. A clear bronchitis was observed in OVA-induced mice which were characterized by epithelial and goblet cell hyperplasia, extensive fibrosis, mucous secretion, and alveolar hyperplasia with inflammation (Fig. 3B). However, these pathological changes were hardly observed in the
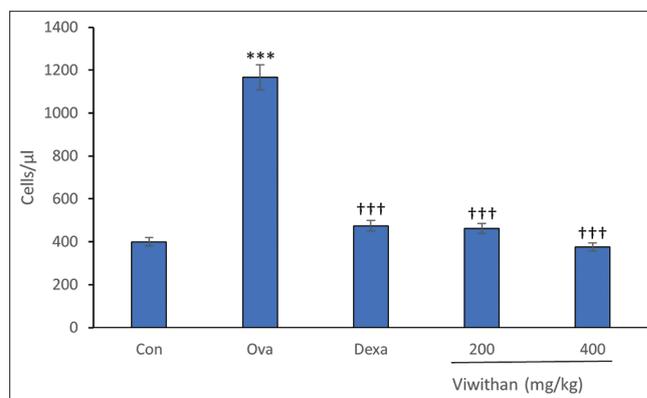
Fig. 1: Effect of Viwithan on inflammatory cells response in airway-inflammation model. Values are expressed as mean±SD, n=6. * Significant difference when compared with control group (p<0.05). † Significant difference when compared with ovalbumin group (p<0.05)

Table 1: Effect of Viwithan on body weight and lung weight in airway-inflammation model

Groups	Body weight (g)					Lung weight (mg)	Relative Lung weight (mg)
	0	7	14	21	28		
Control	30.98	31.38	32.37	32.15	32.55	275.38	847.27
OVA	31.50	31.56	33.33	33.83	34.35	309.60	918.50
OVA + Dexamethasone	33.42	33.67	35.45	33.78	33.64	287.38	861.33
OVA + Viwithan (200 mg/kg)	33.66	35.18	36.02	35.85	36.31	326.43	891.50
OVA + Viwithan (400 mg/kg)	33.19	33.87	35.06	34.45	34.19	343.13	1057.37

OVA: ovalbumin
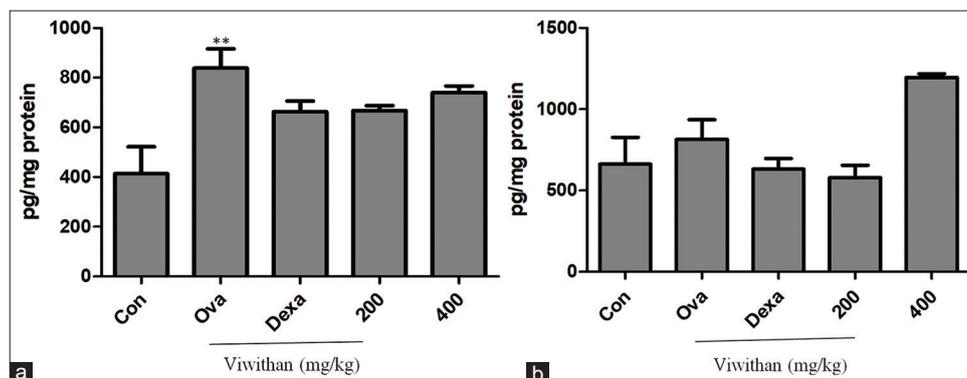
Fig. 2: Effect of Viwithan on pro-inflammatory cytokine levels in the lung tissues. (a) Interleukin-10 and (b) transforming growth factor-β1. Values are expressed as mean±SD, n=6. *Significant difference when compared with control group (p<0.05)
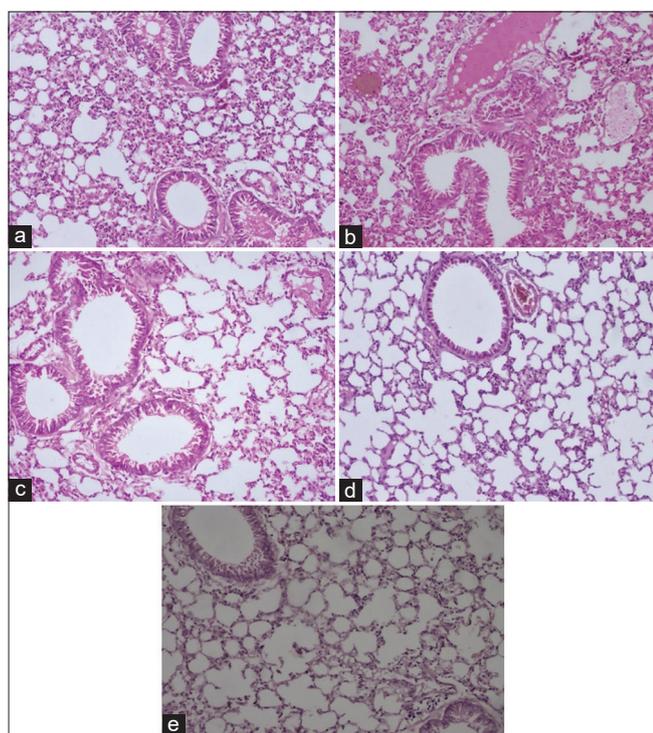
Fig. 3: Effect of Viwithan on ovalbumin (OVA)-induced histopathological alterations in the lung tissues. (a) Control; (b) OVA; (c) OVA+ Dexamethasone; (d) OVA + Viwithan (200 mg/ kg); and (e) OVA + Viwithan (400 mg/kg)

control group (Fig. 3A). Treatment with dexamethasone (Fig. 3C) and Viwithan (Fig. 3D and E) reversed OVA-induced allergic inflammation response around the bronchial trees. Treated mice showed reduced leukocyte inflammatory infiltration evidenced by near normal appearance of alveoli and mild bronchiole epithelial hyperplasia.

Effect of Viwithan on oxidative stress induced by OVA in lung tissues

In this study, we examined the inhibitory effect of Viwithan on ROS production in lung tissues from OVA-challenged mice. GSH level was significantly increased in the treated groups compared to OVA-induced group (Fig. 4A). The concentration of MDA in the OVA-induced group was significantly higher (p<0.05) than the control group and all treatment groups significantly (p<0.05) reduced the MDA levels in the lung tissues (Fig. 4B). SOD activity was also significantly increased in the Viwithan treated group compared to OVA-induced group (Fig. 4C).

DISCUSSION

Ashwagandha (*W. somnifera*) root powder is being used in Ayurvedic system of medicine and remedies that have been in practice in India for 1000 years. Toxicological studies have found that, Ashwagandha, in its reasonable dose, is a non-toxic, safe, and edible herb [19]. Administration of hydroalcoholic extract of Ashwagandha containing 4.5% withaferin A showed no evidence of toxic effects or mortality up to the concentration of 2000 mg/kg/day for 28 days in Wistar rats [20]. Standardized extract of Ashwagandha and withaferin A has been found to be promising for their anticancer activity and immunomodulatory effects in animal models [21,22]. In the present study, using a mouse model of OVA-induced airway inflammation, we investigated the protective effects of Viwithan, a standardized extract of Ashwagandha containing 5% withaferin A against allergic airway-inflammation. Results demonstrated that the treatment with Viwithan (200 mg/kg) protected against OVA-induced airway-inflammation by regulating inflammatory cytokines, reducing lung pathological alterations and oxidative stress.

Inflammation of the airway epithelium has typically been considered as the first defensive barrier, which minimizes allergen access to the body [23]. OVA has been demonstrated to induce airway inflammation in animal models for experiments [24]. The airway-inflammation induced in the present study model was characterized by excessive airway eosinophils and other inflammatory cell infiltration, goblet cell hyperplasia with excessive airway mucus, and elevated cytokines.
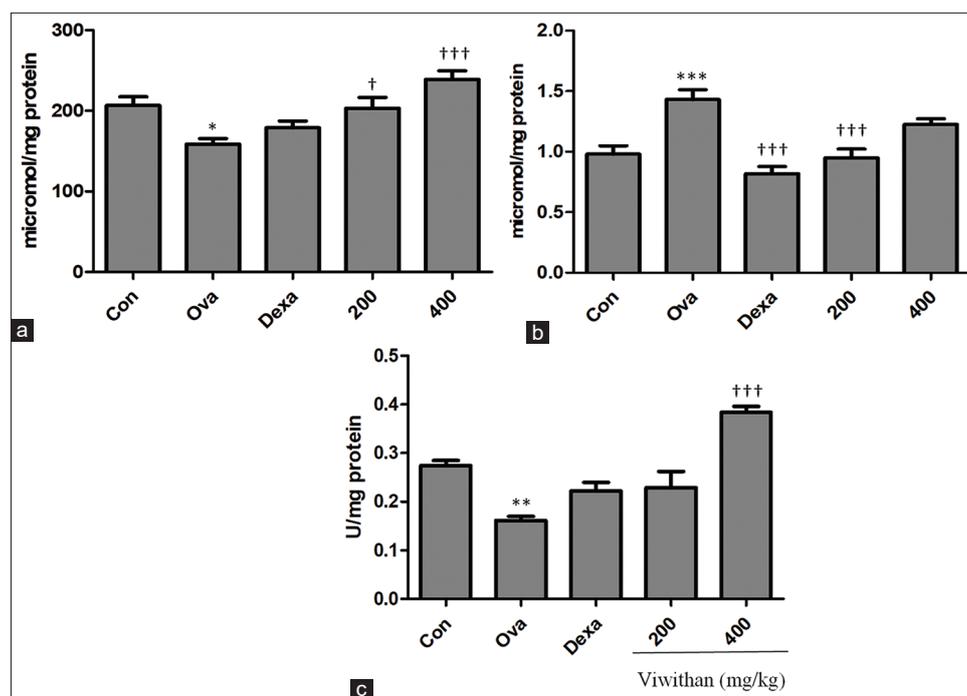
Fig. 4: Effect of Viwithan on oxidative stress induced in the lung tissues in airway inflammation model. (a) Superoxide dismutase; (b) malondialdehyde; and (c) glutathione. Values are expressed as mean±SD, n=6. * Significant difference when compared with control group (p<0.05). †Significant difference when compared with ovalbumin group (p<0.05)

Furthermore, inflammatory immune cells, including eosinophils, neutrophils, monocytes, and epithelial cells, generate high levels of ROS to damage lung tissues [25,26]. Hence, asthma progression may be ameliorated by regulating the activity of cytokines and reducing the infiltration of inflammatory immune cells. Viwithan treatment evidently lowered cytokines and inflammatory cells especially eosinophils in BALF and lung homogenates.

Previous studies have indicated that pro-inflammatory cytokines, including IL-10 and TGF-β1, are important in the pulmonary inflammatory response [27,28]. TGF-β1 is associated with pulmonary fibrosis, during which it initiates fibroblast differentiation into myofibroblasts, and subsequently causes excessive collagen accumulation and extracellular matrix deposition [29]. TGF-β1 also known to promote epithelial mesenchymal transition (EMT) during fibrotic diseases. EMT induced by TGF-β1 is a key issue in the pathogenesis of tissue or organ fibrosis; it has been reported that the inhibition of TGF-β1 may reverse EMT in hepatic fibrogenesis [30]. Therefore, suppression of TGF-β1 is potentially beneficial for the treatment of airway inflammation and pulmonary fibrosis. In the present study, TGF-β1 level has been increased in OVA-induced animals compared to normal control and treatment with Viwithan significantly reduced the TGF-β1 level at the concentration of 200 mg/kg. Similarly, the increased levels of IL-10 in OVA-induced mice were significantly decreased after treatment with Viwithan. These results were in agreement with the previous reports where some plant extracts alleviated airway inflammation by regulating the production of inflammatory cytokines [31,32]. Our results also showed that Viwithan significantly attenuated the infiltration of eosinophils and other inflammatory cells.

Oxidative stress is closely associated with allergic and immunological disorders, such as asthma, atopic dermatitis, and allergic rhinitis [33]. Studies have shown that increased ROS production in allergic respiratory diseases contributes, in part, to tissue damage at sites of inflammation [34]. MDA is the product of lipid peroxidation and a classic indicator of oxidative stress and SOD is one of the major enzymes responsible for the inactivation of superoxide and hydrogen

peroxide [35]. GSH, reduced GSH, is a key antioxidant responsible for primary defense system for protection from oxidative damage [36]. In the present study, Viwithan treatment significantly reduced oxidative stress induced by OVA-sensitization as indicated by decrease in MDA levels and increase in SOD activity and GSH levels. The results suggested that Viwithan has an antioxidant activity and is responsible for the decrease in the production of ROS, which finally may inhibit the tissue damage caused due to oxidative stress. Previous reports also support the potential antioxidant activity of Ashwagandha in various disease conditions [37].

CONCLUSION

Collectively, we demonstrated that treatment with Viwithan has a significant anti-inflammatory effect in allergic asthma and may prove to be an effective therapeutic reagent for airway-inflammation by suppressing pro-inflammatory cytokines, oxidative stress, and inflammatory cell infiltration. The present study warrants further evaluation at the molecular level for the treatment of airway inflammation.

AUTHORS' CONTRIBUTIONS

All authors have contributed equally.

CONFLICTS OF INTEREST

No conflicts of interest disclosed.

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