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# Pharmacological evaluation of cellular and molecular mechanism of Anti-Inflammatory and Immunomodulatory effects of traditional herbal drug extract of *Hedychiumspicatum*in Experimental Models of Bronchial Asthma

**Kavita Gulati[1], Hemlata Sharma[1], Suresh Thokchom[1], Pankaj Verma[1], Arunabha Ray[2]**

[1]Departments of Pharmacology, Vallabhbhai Patel Chest Institute (VPCI), University of Delhi.

[2]Hamdard Institute of Medical Sciences and Research (HIMSR), Jamia Hamdard, New Delhi.

## A B S T R A C T

The study assessed the effects of standardized extracts of *Hedychiumspicatum* on airway inflammation and immunomodulatory parameters in experimental model of bronchial asthma in rats. Wistar rats were immunized with ovalbumin (OVA) adsorbed on to aluminium hydroxide (i.p.) and challenged with aerosolized ovalbumin from day 15 to 22. Standardized extracts of *Hedychiumspicatum* were administered orally for 22 days in different treatment groups. After 24h of last challenge, rats were anesthetized and blood and bronchoalveolar lavage fluid (BALF) were collected, centrifuged and analyzed for proinflammatory cytokines TNF-α, NF-κB, Th2 type cytokines IL-4 and cell count.The results showed that eosinophil cells levels were elevated in the immunized rats which were reduced in the treatment group *Hedychiumspicatum* extract as compared to control. These results were comparable with the effects of the standard drug prednisolone. The results showed that extract of *Hedychiumspicatum* has anti-inflammatory and immunomodulatory effects. Thus, the results suggest that the beneficial effects of *Hedychiumspicatum* in bronchial asthma could be due to balancing influence on prooxidant-antioxidant status and reducing the airway inflammation.

**Keywords:** Bronchial asthma; *Hedychiumspicatum*; Airway inflammation; Immunomodulation

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*Address for Correspondence:
Shubhangi Manikpuriya, Late Bhagirathi Yashwantrao Pathrikar College of Pharmacy, Maharashtra, India

## INTRODUCTION

Asthma is indeed a chronic respiratory condition characterized by inflammation and narrowing of the airways, which can lead to various symptoms including wheezing, shortness of breath, coughing, and chest tightness. These symptoms can vary in severity and frequency from person to person and can be triggered by various factors such as allergens, irritants, exercise, respiratory infections, and stress[1]. Asthma can certainly become life-threatening during severe asthma attacks if not managed promptly and effectively. Projections about future prevalence are subject to change based on various factors, including changes in environmental conditions, healthcare access, and advancements in treatment and prevention strategies. Therefore, while it's possible that the number of people affected by asthma may continue to increase, predictions about the 100 million of affected individuals in 2025 may vary.According to WHO, 455000 deaths globally were reported in 2017 due to this chronic condition[2]. Some important factors contributing to the increasing incidence of asthma, including exposure to allergens like pollen, indoor dust, and environmental pollutants. These allergens can trigger inflammation and exacerbate asthma symptoms in susceptible individuals. While glucocorticosteroids are indeed considered one of the mainstays of asthma treatment due to their anti-inflammatory properties, long-term use can lead to various adverse effects, including osteoporosis, cataracts,

weight gain, and immune suppression, among others. Therefore, finding alternative or complementary therapies to manage asthma is crucial for minimizing reliance on glucocorticosteroids and reducing the risk of adverse effects[3].

Pharmacology has played an important role in the development of new drugs from naturally occurring chemicals/or substances using a scientific and logical approach. The early allopathic medicines for asthma were derived from plants such as, Justiciaprocumbens, Daturastramonium, Atropa belladonna, Euphorbia pilulifera, Rauvolfiaserpentine, and Digitalis purpurea,. The gold standard treatment for asthma is inhalers of beta 2 agonist and corticosteroids. In the last 40 years of research, leukotriene antagonists have been introduced in anti-asthmatic drugs, which are less effective than existing treatments and corticosteroids, whereas highly effective therapies are not acceptable because of carrying risks of side effects. Therefore, a new approach to the further improvement of treatment for asthma is a challenge. Urticadioica (nettle), Ammivisnaga (khell), and Medicago sativa (alfalfa), are the common traditional medicines in Saudi Arabia. Theseplants have been used in folk medicine to treat and prevent asthma and they have antihistaminic, anti-allergic, anti-inflammatory, immunomodulatory, and smooth muscle relaxant properties. However, the clinical uses of these drugs in asthma remain unclear. Therefore, we established the protective effect of these drugs on animal models of allergy and asthma in the present study[4].

*Hedychiumspicatum*, commonly known as spiked ginger lily or Kapur Kachri, indeed belongs to the family Zingiberaceae. It's native to the Himalayan region and is known for its aromatic rhizomes, which is used as traditional medicine. The plant typically reaches a height of 1-2 meters, featuring green leaves and large, showy flowers in shades of orange with whitish accents. Its hardiness and resilience make it a popular choice for gardens in temperate regions[5].Hedychiumspicatum has a wide distribution across various regions, including India, Bhutan, Nepal, Japan, Thailand, Pakistan, and China[6]. Its rhizomes have been traditionally utilized for treating a variety of ailments such as tuberculosis, asthma, liver complaints, inflammatory conditions, and for blood purification purposes[7]. Additionally, the rhizomes are incorporated into formulations for syrups, tablets, tonics, and dietary supplements, showcasing its versatility in traditional medicine systems across these regions[8].Recent study shown that *Hedychiumspicatum* have Anti-Inflammatory and Immunomodulatory potentials in cellular and molecular mechanism experimental model of bronchial asthma.

## MATERIALS AND METHODS

### Experiment Animals

Wistar rats of either sex, weighing 180-220 g were used for the study. They were housed in standard laboratory cages and kept in environmentally controlled room (25 ± 2°C, 12 hours light and dark cycle). Animals were acclimatized for one week before treatment. They were fed with standard laboratory food pellets and water ad libitum. The study protocols were approved by Institutional Animal Ethics Committee (VPCI/IAEC/2020/26), following the guidelines of CPCSEA (Committee for the purpose of control and supervision of experiments on animals), which complies with international guidelines of Indian National Science Academy (INSA), New Delhi.

### Drugs and Chemicals

The standardized aqueous rhizome extract of *Hedychiumspicatum (H. spicatum*) was procured from Shivayu Ayurved Limited (Nagpur). The aqueous extract was prepared by dissolving in distilled water and administered orally as per the calculated dosage schedule. Ovalbumin, Methacholine and Prednisolone were procured from Sigma Aldrich-USA. All other routine chemicals were procured from SRL, New Delhi. Cytokine assay kits such as Ovalbumin specific TNF-α, NF-κB, and IL-4 were procured from Weldon Biotech, New Delhi.

### Experimental Protocol

Animals were randomly divided into the following six groups (n = 6 for each): the normal control group, OVA-control group, OVA + *H. spicatum* (100, 200 and 400 mg/kg) treatment groups, and OVA + Prednisolone 5mg/kg) group. The animals were actively sensitized by intra-peritoneal injection (1.0 ml) of allergen suspension containing ovalbumin 40 mg and aluminum hydroxide 2 mg on day 1. Fifteen days after immunization, rats were challenged by exposure to a 1% OVA in phosphate-buffered saline (PBS) aerosol once daily during 20 min per day for 8 consecutive days i.e., from day 15 to day 22by the method of Raiet al[9]. Control group rats were exposed to nebulized sterile saline using the same method by using an ultrasonic nebulizer, the Aerosol flow rate was 3 ml/min. Exposure to aerosol was done in a closed chamber, dimensions 40×32×32 cm24 hours after challenging with the antigen.

### Blood and BAL Fluid Collection

After 24 hour of OVA challenge, animals were anesthetized 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 blood was separated and stored at -80ºC. After blood collection, BAL fluid was collected by lavaging the airways through a tracheal cannula with normal saline given in a volume of 1ml for three times. The collection of BAL was approx. 1.2 to 1.8 ml. The sample was centrifuged at 1500 rpm for 10 minutes at 4ºC and supernatant recovered and stored at -80ºC for biochemical assay. The precipitated pellets in BAL fluid were resuspended in 100 µl of normal saline. Neutrophil and Eosinophil counts in blood and BAL fluid samples were carried out using Neubauer's chamber after Leishman staining.

### Measurement of bronchial responsiveness to spasmogen

In the experimental model of airway remodeling, after 24 hour of last challenge of OVA, airway responsiveness was assessed in response to inhaled methacholine using whole body plethysmography and expressed as enhanced pause (Penh). Penh is an index of bronchial hyperresponsiveness and airflow limitation in experimental animals. Briefly, rats were placed in animal chamber (main chamber) of the whole body plethysmography and basal Penh readings were recorded with the help of transducer connected to the amplifier and averaged for a 3 minutes period. Subsequently, increasing doses of methacholine (2.5–20 mg/ml), were aerosolized for 3 minutes, and readings were taken and averaged for 3 minutes after each

nebulization. Between two doses of methacholine, 10 minutes relaxation period was provided.

### Assay for the levels of Cell Count

Eosinophil & neutrophil in blood and BAL fluid were counted using Neubauer's chamber.

### Assay for levels of OVA-specific IgE

Assay for OVA-specific IgE OVA-specific IgE (OVAsIgE) or allergan specific IgE is used to assess the role of allergen in aggravating IgE levels in experimental animals of bronchial asthma. OVA-specific IgE (OVAsIgE) in serum and BAL fluid were analysed by using commercially available ELISA test kits as per manufacturer's instructions. Briefly, the microtiter plate was pre-coated with an antibody specific to OVA-specific IgE (OVAsIgE). Standard, Test samples and HRP-labeled conjugate specific for OVA-specific IgE (OVAsIgE) were simultaneously incubated for specified periods. Then, Chomogen A and B were added to produce a coloured reaction. The absorbance was read at a wavelength of 450 nm using ELISA plate reader and results were expressed as ng/ml.

### Assay for levels of TNF-α

Assay for TNF-α in Blood and BAL fluid samples was analyzed by using commercially available enzyme linked immunosorbent assay (ELISA) kits as per manufacturer's instructions. TNF-α level was measured using sandwich ELISA method. Antigen and biotin-conjugated poly clonal antibody preparation specific for TNF-α was added to microtiter plate pre-coated with polyclonal antibody specific to TNF-α and incubated for specified periods. Then, streptavidin horse-radish peroxidase and TMB substrate were added to produce a colored reaction product. The-enzyme-substrate reaction was stopped by adding sulphuric acid. The absorbance of the colored product was read at a wavelength of 450 nm using ELISA plate reader and values were expressed in pg/ml.

### Assay for the levels of IL-4

Blood and BAL fluid samples were assayed for IL-4 levels using commercially available ELISA kits. Briefly, the microtiter plate was pre-coated with an antibody specific to rat IL-4. Blood and BAL fluid samples were then added to the appropriate wells of the microtitre plate with biotinylated anti-rat IL-4 and incubated for 4 hours at room temperature. After incubation, microtitre plate was washed three times with washing solution. Then, Streptavidin- HRP was added and again incubated for 30 min. Again after incubation, microtitre plate was washed with washing solution. TMB substrate was added to produce a colored reaction product and again incubated for 15 minutes. After the incubation H2SO4 stop solution was added. The absorbance was read at a wavelength of 450 nm using ELISA microplate reader and the results were expressed in pg/ml.

### Assay for the levels of NF-kB

Blood and BAL fluid samples were assayed for NF-kB levels using commercially available ELISA kits. Briefly, the microtiter plate was pre-coated with an antibody specific to rat NF-kB. Blood and BAL fluid samples were added to the appropriate wells of the microtitre plate with biotinylated anti-rat NF-kB and incubated for 30 min at 37$^{o}$ C temperature. After incubation, microtitre plate was washed with washing solution. Then, Streptavidin-HRP conjugate was added and again incubated for 30 minutes. After incubation, microtitre plate was washed with washing solution and then Chromogen solution A and B were added and again incubate for 15 minutes. Then Stop Solution was added. The absorbance was read at a wavelength of 450 nm using ELISA microplate reader and the results were expressed in ng/ml.

### Assay for the levels of HDAC

Blood and BAL fluid samples were assayed for HDAC levels using commercially available ELISA kits. Briefly, the microtiter plate was pre-coated with an antibody specific to rat HDAC. Blood and BAL fluid samples were added to the appropriate wells of the microtitre plate with biotinylated anti-rat HDAC and incubated for 30 min at 37$^{o}$ C temperature. After incubation, microtitre plate was washed with washing solution. Then, Streptavidin-HRP conjugate was added and again incubated for 30 minutes. After incubation, microtitre plate was washed with washing solution and then Chromogen solution A and B were added and again incubate for 15 minutes. Then StopSolution was added. The absorbance was read at a wavelength of 450 nm using ELISA microplate reader and the results were expressed in ng/ml.

### Statistical analysis

Statistical analysis was performed using appropriate parameteric and non-parametric tests. A p value of $\leq 0.5$ was taken as level of significance in all the statistical tests perform

## RESULTS-

**Effects of standardized aqueous rhizome extract of *Hedychiumspicatum* on bronchial hyperresponsiveness to spasmogen in OVA sensitized rats**

Bronchial hyperressponsiveness is one of the characteristics features of bronchial asthma. The effect of methacholine inhalation on OVA induced airway hyperresposiveness was evaluated in -vivo 24 hour after last ovalbumin challenge by using whole body plethysmography and represented as enhanced pause (Penh). Penh is an index of bronchial hyperresposiveness and airflow limitation in experimental animals when using whole body plethysmography. Enhanced pause (P-enh) is a dimensionless value which is calculated by multiplying the ratio of peak expiratory height to peak inspiratory height with pause. Animals were placed in the nebulization chamber, where animals were exposed to different doses of inhaled methacholine. Animals were then placed in the second chamber where the difference in the breathing pressure between the animal chamber and the reference chamber was recorded using a transducer connected to the amplifier. Changes in the air volume leading to changes in pressure resulted in the changes in box pressure (reference chamber). Results showed that in experimental control group the responsiveness to methacholine (5-20 mg/ml) was significantly increased as compared to that in normal control group. This increase in Penh value was reversed after the treatment with 100, 200 and 400mg/kg of Hedychiumspicatum and results were comparable with prednisolone. The reduction in value of Penh was maximum with 400 mg/kg of Hedychiumspicatum. These results are summarized in Figure 1.

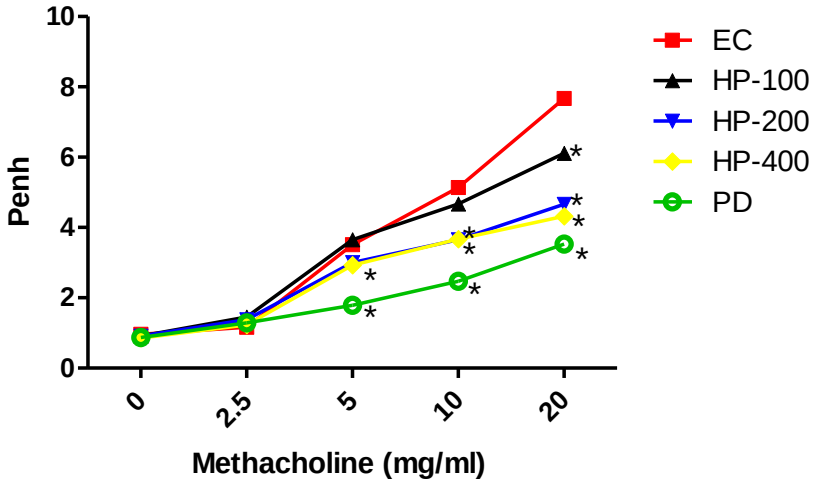


**Figure 1:** Effects of standardized aqueous rhizome extract of Hedychiumspicatum on enhanced pause (Penh) values to methacholine (Mch) in OVA sensitized rats *p<0.05 compared to EC. EC- Experiment Control (sensitized and challenged with OVA), HS - Hedychiumspicatum at dose 100 mg/kg, 200 mg/kg and 400 mg/kg respectively, PD- Prednisolone (10 mg/kg).

**Effects of standardized aqueous rhizome extract of *Hedychiumspicatum* on eosinophils and neutrophils counts in blood and BAL fluid**

Analysis of the data showed that there were significant differences in eosinophils levels across all the groups [F (5, 24) = 48.85, P<0.05 in blood; and F (5, 24) = 97.92, P<0.05 in BAL fluid, one way ANOVA]. Exposure to OVA for 22 days in experimental control group resulted in significant (p<0.05) increase in eosinophil counts as compared to normal control (NC) group, thus validating the experimental model of bronchial asthma. Result showed that pre-treatment with standardized aqueous rhizome extract of *Hedychiumspicatum* (100, 200 and 400 mg/kg, p.o. for 22 days) significantly (p<0.05) reduced the number of eosinophil in blood when compared to experimental control (EC) group of rats. Eosinophil counts in BAL were reduced significantly (p<0.01) after treatment with *Hedychiumspicatum*. Prednisolone used as positive control also reduced eosinophil numbers to a significant level (p<0.01) in blood and BAL fluid. These results are summarized in Figure 2.

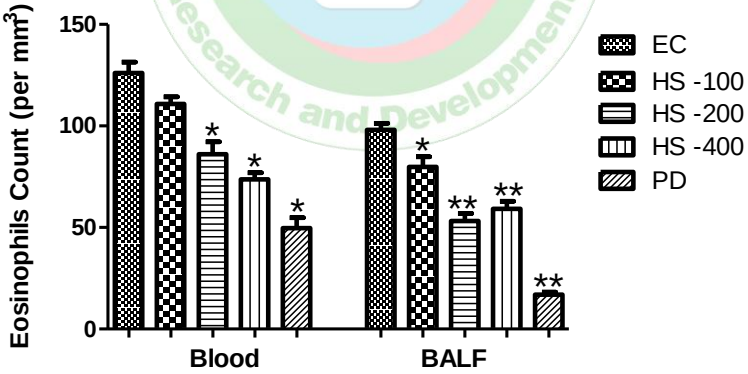


**Figure 2:** Effects of standardized aqueous rhizome extract of Hedychiumspicatum on eosinophils count in blood and BAL fluid in experimental model of bronchial hypereactivity *p<0.05, **P < 0.01 compared to EC. EC- Experiment Control (sensitized and challenged with OVA), HS - Hedychiumspicatum at dose 100 mg/kg, 200 mg/kg and 400 mg/kg respectively, PD- Prednisolone (10 mg/kg)

Analysis of the data showed that there were significant differences in neutrophils levels across all the groups [F (5, 24) =88.23, P<0.05 in blood; and F (5, 24) = 120.60, P<0.05 in BAL fluid, one way ANOVA]. Exposure to OVA for 22 days in experimental control group resulted in significant (p<0.05) increased in neutrophils counts as compared to normal control (NC) group. Result showed that pre-treatment with *Hedychiumspicatum* (100, 200 and 400 mg/kg, p.o. for 22 days) significantly (p<0.05) reduced the number of neutrophils in both blood and BAL fluid when compared to experimental control (EC) group of rats. The dose of 200 mg/kg of *Hedychiumspicatum* was associated with greater reduction in neutrophils counts (p<0.05) in both blood and BAL fluid. Prednisolone used as positive control also reduced neutrophil numbers significantly (p<0.05) in blood and BAL fluid. These results are summarized in Figure 3.

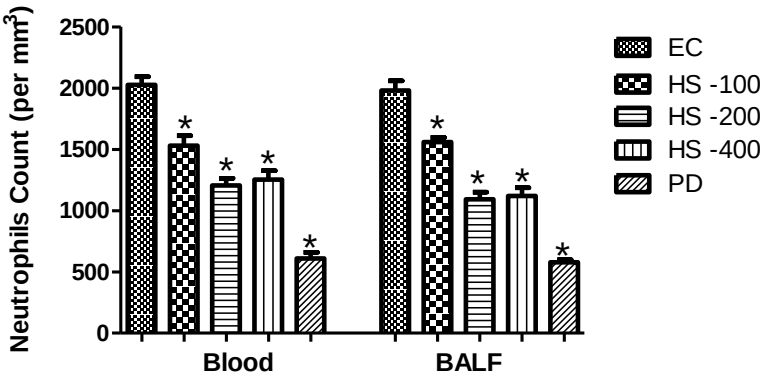


**Figure 3**: Effects of standardized aqueous rhizome extract *of Hedychiumspicatum* on neutrophils count in blood and BAL fluid in experimental model of bronchial hypereactivity *p<0.05 compared to EC. EC- Experiment Control (sensitized and challenged with OVA), HS -*Hedychiumspicatum* at dose 100 mg/kg, 200 mg/kg and 400 mg/kg respectively, PD- Prednisolone (10 mg/kg)

**Effects of standardized aqueous rhizome extract of *Hedychiumspicatum* on ovalbumin specific IgE (OVA sIgE) levels in blood and BAL fluid**

Analysis of the *Hedychiumspicatum* data revealed that there were significant differences in OVA sIgE levels across all the groups [F (5, 24) = 11.28, P<0.05 for OVA sIgE in blood; and F (5, 24) = 106.90, P<0.05 for OVA sIgE in BAL fluid, one way ANOVA]. Rats challenged with OVA aerosols from 15$^{ht}$ day to 22$^{nd}$ day for 20 minutes in experimental control (EC) rats showed significant (p<0.05) increase in IgE levels in comparison to normal control (NC) rats. Treatment with *Hedychiumspicatum* (100, 200 and 400 mg/kg) for 22 days, attenuated the effect of OVA sIgE in both blood and BAL fluid as compared to experimental control group with significant (p<0.05) reduction in the dose group 200 and 400 mg/kg in blood and (p<0.05) reduction in 100 mg/kg and (p<0.01) at the doses of 200 and 400 mg/kg in BAL fluid. The results are more prominent in BAL fluid as compared to blood sample. The OVA sIgE levels were reduced by 21%, 44%, 43% in blood and 21%, 51%, 55% in BAL fluid respectively with 100, 200 and 400mg.kg. Prednisolone also reduced OVA sIgE levels by 39% and 32% in blood and BAL fluid and the results were comparable with that of *Hedychiumspicatum* treated groups. These results are summarized in Figure 4.

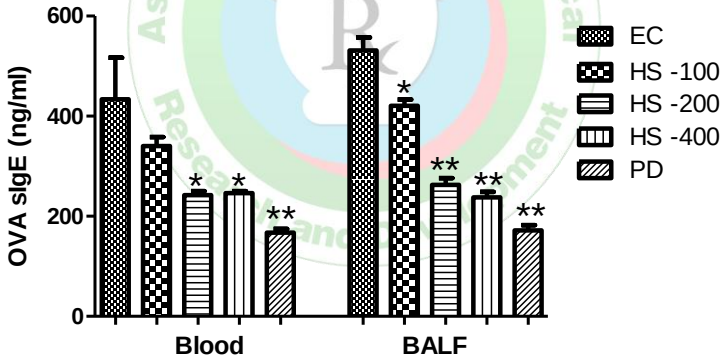


**Figure 4:** Effects of standardized aqueous rhizome extract of Hedychiumspicatum on ovalbumin specific IgE (OVA sIgE) in blood and BAL fluid in experimental model of bronchial hypereactivity *p<0.05, **P < 0.01 compared to EC. EC- Experiment Control (sensitized and challenged with OVA), HS - Hedychiumspicatum at dose 100 mg/kg, 200 mg/kg and 400 mg/kg respectively, PD- Prednisolone (10 mg/kg)

**Effects of standardized aqueous rhizome extract of *Hedychiumspicatum* on TNF-α levels in blood and BAL fluid**

In the present study, TNF-α levels were measured in BAL fluid and serum using ELISA kits. The overall analysis of data using ANOVA showed that there was a significant difference in TNF-α levels across various treatment groups (Ovalbumin, *Hedychiumspicatum* and prednisolone) [F (5,35) = 9.94, for BAL fluid; and F (5,35) = 11.76, for serum; p<0.05 for both BAL fluid and serum]. Sensitization of rats followed by challenge treatment with OVA resulted in a significant increase in TNF-α levels in both BAL fluid and serum as compared to that of normal control rats (non-challenged rats) which is an indicator of increased inflammation in the disease control group. The TNF-α levels were 61.08 ± 5.03 pg/ml in BAL fluid and 31.67 ± 1.99 pg/ml in the serum of normal control rats which were found to be significantly increased to 106.10 ± 5.67 pg/ml in BAL fluid and 75.34 ± 5.70 pg/ml in serum in response to OVA sensitization and challenge treatment in disease control rats. Administration of 100 mg/kg, 200 mg/kg, and 400 mg/kg doses of *Hedychiumspicatum* for 22 days resulted in a dose-dependent reduction of TNF-α levels by 21.25%, 28.26% (p<0.05) and 32.56% (p<0.05) in BAL fluid and 28.19%, 44.71% (p<0.05) and 51.36% (p<0.05) in serum respectively, as compared to TNF-α levels in disease control rats. There was significant reduction of TNF-α levels by 37.86% (p<0.05) in BAL fluid and 51.22% (p<0.05) in serum by the treatment with prednisolone (10 mg/kg) respectively, as compared to that of disease control rats (p<0.05). The results are summarized in Figure 5.

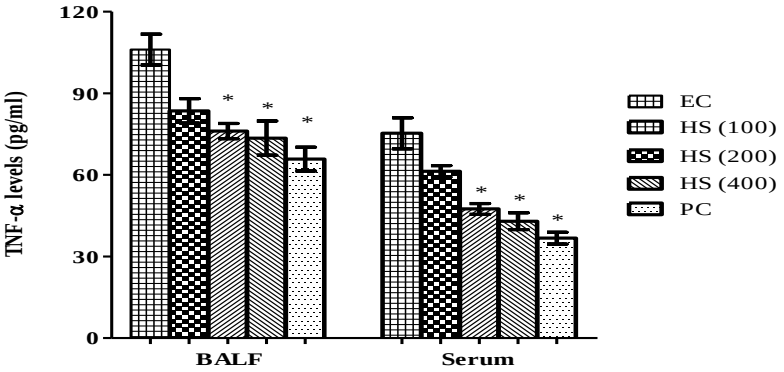


**Figure 5.** Effect of *Hedychiumspicatum* on TNF-α levels in BAL fluid and serum of OVA-induced airway inflammation in rats. *p<0.05, **p<0.01 versus EC group. EC- Experiment Control (sensitized and challenged with OVA), HS - Hedychiumspicatum at dose 100 mg/kg, 200 mg/kg and 400 mg/kg respectively, PC- Prednisolone (10 mg/kg)

**Effects of standardized aqueous rhizome extract of *Hedychiumspicatum* on IL-4 levels in blood and BAL fluid**

IL-4 levels were measured in BAL fluid and serum using ELISA kits and values are expressed in pg/ml. The overall analysis of data using ANOVA showed that IL-4 levels were significantly different across various treatment groups [F (5, 35) = 11.41, for BAL fluid; and F (5,35) = 8.53, for serum; p<0.05 for both BAL fluid and serum]. Assay of IL-4 showed significantly higher levels of IL-4 in BAL fluid and serum of the OVA-sensitized and challenged disease group as compared to that of the normal control group (OVA-sensitized group). The IL-4 levels were 193.70 ± 9.65pg/ml in BAL fluid and 141.90 ± 12.64 pg/ml in serum of disease control rats which were significantly higher as compared to 73.9 ± 8.99 pg/ml in BAL fluid and 47.60 ± 7.05 pg/ml in serum of normal control rats. Intergroup analysis showed that there was suppression of IL-4 levels by 18.62%, 34.30% (p<0.05) and 42.94% (p<0.01) in BAL fluid and 33.69%, 47.54% (p<0.05) and 57.17% (p<0.01) in serum by treatment with *Hedychiumspicatum* at 100 mg/kg, 200 mg/kg or 400 mg/kg doses respectively as compared to that of disease control group. Treatment with prednisolone (10 mg/kg) resulted in a significant reduction of IL-4 levels by 54.63% (p<0.01) in BAL fluid and 65.21% (p<0.01) in serum as compared to that of disease control rats. These results are summarized in Figure 6.

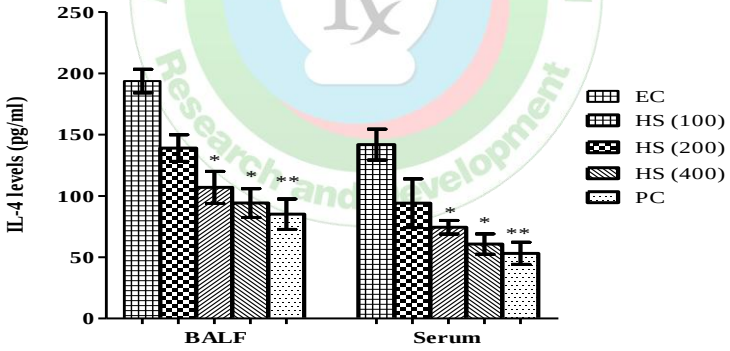


**Figure 6:** Effect of Hedychiumspicatum on IL-4 levels in BAL fluid and serum of OVA-induced airway inflammation in rats. *p<0.05, **p<0.01 versus EC group. EC- Experiment Control (sensitized and challenged with OVA), HS - Hedychiumspicatum at dose 100 mg/kg, 200 mg/kg and 400 mg/kg respectively, PC- Prednisolone (10 mg/kg)

**Effects of standardized aqueous rhizome extract of *Hedychiumspicatum* on NF-κBlevels in blood and BAL fluid**

NF-κB levels were measured in BAL fluid and serum using ELISA and values are expressed in pg/ml.The overall analysis of data using ANOVA showed that there was a significant difference in NF-κB levels across various treatment groups [F (5,35) = 5.48, for BAL fluid; and F (5,35) = 4.51, for serum; p<0.05 for both BAL fluid and serum]. Assay of NF-κB showed increased NF-κB levels in BAL fluid and serum of OVA-sensitized and challenged rats as compared to that of normal control rats (p<0.05). The NF-κB levels were 273.9 ± 18.99pg/ml in BAL fluid and 77.60 ± 13.05 pg/ml in serum in normal control rats as compared to the NF-κB levels which were increased to 468.4 ± 20.22 pg/ml in BAL fluid and 244.0 ± 19.34 pg/ml in serum in disease control rats. Administration of 100 mg/kg, 200 mg/kg, and 400 mg/kg doses of *Hedychiumspicatum*for 22 days suppressed the NF-κB levels by 15.35%, 25.11%, and 30.66% (p<0.05) in BAL fluid and by 27.66%, 46.43% and 58.28% (p<0.05) in serum respectively, as compared to NF-κB level of disease control group. Pre-treatment with prednisolone (10 mg/kg) showed a significant reduction of NF-κB levels by 35.85% (p<0.05) in BAL fluid and by 63.58% (p<0.05) in serum as compared to that of disease control rats. These results are summarized in Figure 7.

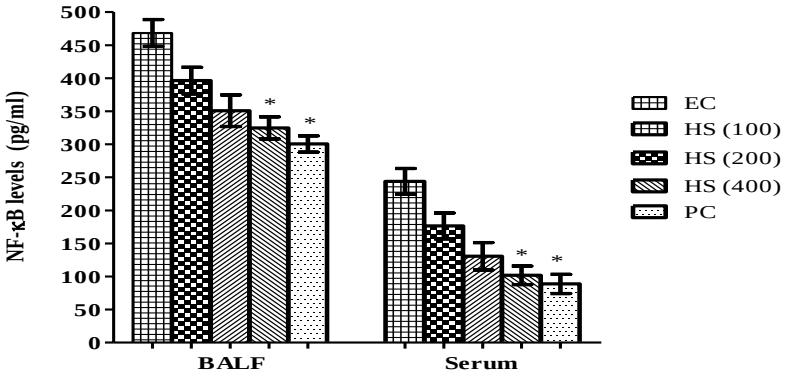


**Figure 7:** Effect of *Hedychiumspicatum* on NF-κB levels in BAL fluid and serum of OVA-induced airway inflammation in rats. *p<0.05, versus EC group. EC- Experiment Control (sensitized and challenged with OVA), HS - Hedychiumspicatum at dose 100 mg/kg, 200 mg/kg and 400 mg/kg respectively, PC- Prednisolone (10 mg/kg)

**Effects of standardized aqueous rhizome extract of *Hedychiumspicatum* on HDAC levels in blood and BAL fluid**

HDAC levels were measured in BAL fluid and serum using ELISA and values are expressed in ng/ml. Overall analysis of data using ANOVA showed significant difference in HDAC levels across various treatment groups [F (5,35) = 8.75, for BAL fluid; and F (5,35) = 5.32, for serum; p<0.05 for both BAL fluid and serum]. Sensitization of rats followed by challenge with OVA showed significantly increased HDAC levels in both BAL fluid and serum as compared to that of normal control rats (only sensitized rats). The HDAC levels were 134.08 ± 7.01 pg/ml in BAL fluid and 108.24 ± 9.48 pg/ml in serum in normal control rats which were found to be decreased to 61.37 ± 3.15 pg/ml in BAL fluid and 44.50 ± 5.90 pg/ml in serum in disease control rats. The results showed that administration of 100 mg/kg, 200 mg/kg, and 400 mg/kg doses of *Hedychiumspicatum* showed increase in HDAC levels by 26.02%, 50.68% and 65.18% (p<0.05) in BAL fluid and by 35.46%, 61.12% and 70.34% (p<0.05) in serum respectively as compared to HDAC level of disease control rats. The results showed that administration of prednisolone (10 mg/kg) showed a significant increment of HDAC levels by 79.40% (p<0.01) and 106.36% (p<0.01) in BAL fluid and serum as compared to that of disease control rats. These results are summarized in Figure 8.

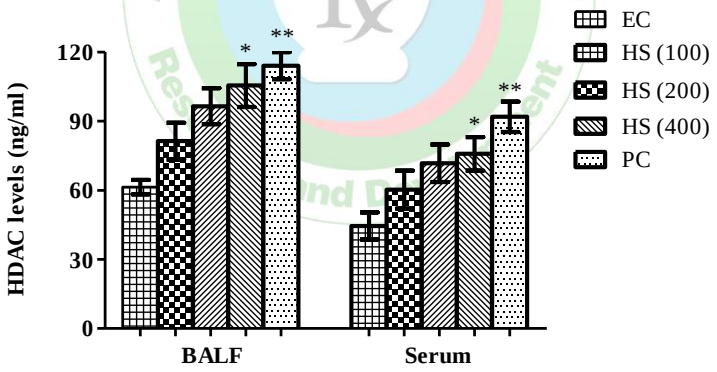


**Figure 8:** Effect of *Hedychiumspicatum* on HDAC levels in BAL fluid and serum of OVA-induced airway inflammation in rats. *p<0.05, **p<0.01 versus EC group. EC- Experiment Control (sensitized and challenged with OVA), HS - Hedychiumspicatum at dose 100 mg/kg, 200 mg/kg and 400 mg/kg respectively, PC- Prednisolone (10 mg/kg)

## Discussion

Bronchial asthma is a disease of the airways mainly characterized by airway obstruction, airway inflammation as well as airway hyperresponsiveness mediated through cellular and humoral events[11]. Exposure to various allergens caused a cascade of events which resulted in a Th-2 type mediated immune response which releases various inflammatory mediators such as cytokines, mast cells, inflammatory cells (eosinophils and neutrophils), chemokines which triggers symptoms into an individual. Pharmacotherapy of bronchial asthma mainly requires long term treatment with controllers (corticosteroids) and relievers (beta agonists) but these are associated with various side effects and refractoriness responses[12-13], which in turn raised question/growing concern towards issues such as reduced efficacy and safety towards use of these drugs. In response to the search for viable alternatives or safer options, the focus has been shifted towards a traditional system of medicine that involves medicinal plants and their derived products[14]. Although the use of these medicinal plants is well documented in the traditional system of Indian medicine, there is a need for scientific validation so as to replicate their potential therapeutic values into lead compounds and drugs. Therefore, the effects of *Hedychiumspicatum* were evaluated using standardized experimental models of bronchial asthma. This study was performed to evaluate various anti-inflammatory and immunomodulatory markers to determine the underlying

molecular and cellular mechanisms involved in their therapeutic effects.

In our investigation, we assessed Enhanced Pause (P-enh), a recognized marker of airway hyper responsiveness (AHR), in response to aerosolized methacholine (at concentrations of 0, 2.5 mg/ml, 5 mg/ml, 10 mg/ml, and 20 mg/ml) utilizing whole-body plethysmography, with measurements averaged over a 3-minute period. Enhanced pause has been established as positively correlated with both AHR and airway resistance in previous studies[15]. Previous research on OVA-sensitized and challenged mice has indicated elevated P-enh values, which are in turn positively associated with airway neutrophilia and epithelial injury[16]. In this study, OVA-sensitized and challenged rats exhibited a notable increase in enhanced pause in response to methacholine compared to normal control rats. Treatment with Hedychiumspicatum for 22 days led to a significant reduction in P-enh levels compared to those observed in disease control rats. These findings were consistent with those of the positive control, prednisolone.

Asthma is characterized by a deregulated immune response to environmental triggers, antigen interaction s, leading to chronic inflammation of the airway including eosinophils, neutrophils, lymphocytes, basophils and macrophages, followed by initiation and release of various proinflammatory mediators and cytokines,. Understanding these inflammatory mechanisms is crucial for developing effective treatments that target specific aspects of the disease process[17-18]. Th2 cells are pivotal in initiating and propagating the inflammatory cascade in asthma through the release of cytokines such as IL-4, IgE, IL-5, and IL-13, which contributes to airway hyper responsiveness and remodeling. These are well known mediators, and a potent modulator of immune and inflammatory response resulting in influx of neutrophils and eosinophils as well as bronchial hyperreactivity. Inhibition of inflammatory responses mediated through inflammatory cells and their cytokines, is a basic therapeutic aim in treatment of asthma[19-20].Present study showed significant suppression of IgE responses by administration of Hedychiumspicatum in BAL fluid samples as compared to control. Inflammation and infiltration of the airways with eosinophils and neutrophils is the characteristic feature of asthma and in most of the phenotype is accompanied with increased levels of these cells in tissue, blood and bronchoalevolar lavage (BAL) fluid in general and has direct correlation with disease severity[21]. The result of our experiments showed that treatment with Hedychiumspicatumreduced the number of eosinophil and neutrophil cells in BAL fluid of ovalbumin immunized and challenged rats. Prednisolone treatment, which acted as positive control group, also decreased the number of eosinophils and neutrophils cells in separate group of rats indicating the comparability of our data.

TNF-α is a proinflammatory Th-2 cytokine that is responsible for promoting inflammation in bronchial asthma. TNF-α has been known to act as chemo attractant for neutrophils and eosinophils, also involved in the activation of T-cells and increases epithelial expression of adhesion molecules[22-23].In the present study, the effect of different doses of *Hedychiumspicatum* was evaluated on TNF-α levels in serum and BAL fluid of the ovalbumin-induced airway inflammation model in rats In our findings, OVA-sensitized and challenged rats showed significantly increased levels of TNF-α in serum and BAL fluid as compared to that of normal control groups which is an indicator of increased inflammation and thus, increased asthma symptoms in disease control rats. These results are corroborated with other findings which reported increased TNF-α levels in serum and BAL fluid following OVA-sensitization and challenge treatment in experimental rats[24-25].Therefore, reduction of TNF-α levels may be contributing to the observed beneficial effect of this folklore herbal drug considered as an ideal treatment strategy for the treatment and management of bronchial asthma. Treatment with different doses *Hedychiumspicatum* showed attenuation of TNF-α levels in both BAL fluid and serum in a dose-related manner with maximum effect observed with the highest dose in an experimental model of bronchial asthma which also reflects the anti-inflammatory effects of the herbal drug.

The present study evaluated the effects of *Hedychiumspicatum* in ameliorating IL-4 levels in serum and BAL fluid of OVA-sensitized and challenged rats. In bronchial asthma, levels of Th2 cytokines such as IL-4, IL5 were increased as compared to Th1 cytokine i.e., IFN-ϒ and this imbalance plays a significant role in the pathogenesis of bronchial asthma[26-27]. IL-4 is a Th-2 specific cytokine that helps in the differentiation of Th-2 cells from naive T-cells which results in the formation of more Th-2 cells and over-production of various cytokines such as IL-4, which are implicated in the development and progression of asthmatic symptoms in experimental rats. In our study, antigenic (OVA) sensitization and challenge treatment resulted in significantly increased levels of IL-4 in serum and BAL fluid as compared to that of normal control rats which is an indicator of increased production of Th-2 mediated cytokines and inflammation in disease control rats. Treatment with different doses of *Hedychiumspicatum* for 22 days showed significant reductions in IL-4 levels in serum and BAL fluid as compared to that of disease control rats. IL-4 is also responsible for the production of IgE from activated B-lymphocytes. The binding of IgE on FcεRI receptors has been reported to stimulate mast cell activation which results in the release of various cytokines as well as inflammatory mediators such as histamine, prostaglandins, leukotrienes, bradykinin, etc.[28-29]. Reduction of IL-4 in serum and BAL fluid by treatment with different doses of *Hedychiumspicatum* for 22 days may also be responsible for decreased IgE production and thus, decreased release of inflammatory mediators in experimental rats.

Thenuclear factor-kappa light chain enhancer of activated B cells (NF-κB) is a transcription factor that is responsible for the expression of genes involved in the proliferation of cells, apoptosis, stress as well as innate immune responses[30].Respiratory diseases such as bronchial asthma are characterized by airway inflammation, obstruction, and hyperresponsiveness which are attributed to the increased expression of various inflammatory genes that are regulated by pro-inflammatory transcription factors, NF-κB. In the present study, NF-κB levels were found to be increased in serum and BAL fluid of OVA-sensitized and challenged rats as compared to that of normal control rats which is an indicator of an increase in transcription gene factor and thus increased production of proinflammatory cytokines such as TNF- α, etc. Administration of herbal drug, *Hedychiumspicatum* attenuated NF- κB levels and provided evidence for decreased gene expression and thus reduced degree of airway inflammation.

The results are in line with the earlier reports that have shown that exposure to allergens leads to increased release of transcription gene factor which in turn resulted in the production of pro-inflammatory cytokines (TNF-α) and other inflammatory cells and aggravates asthma.

Nuclear factor-κB (NF-κB) is a transcription factor that is crucial role during processes, inflammation and results in activation of histone acetyltransferase (HAT) responsible for acetylation of histones. Histone acetylation plays an important role in regulating gene expression in inflammatory lung diseases, whereas HDAC2 suppresses these inflammatory genes through the recruitment of corepressor proteins to switch off gene transcription. Hence, HDAC2 could be a useful target for the development of new anti-inflammatory agents, particularly in diseases where there is active corticosteroid resistance such as bronchial asthma[31]. Patients with severe asthma and asthmatics who smoke had lower HDAC2 levels compared to healthy individuals[32]. A study reported that exposure to cigarette smoke reduced the expression of HDAC2 in blood and lung tissue in mice[33]. Furthermore, the downregulation of HDAC2 levels has been notably associated with corticosteroid resistance in patients with severe asthma[34]. Similarly, the results showed that the levels of HDAC were increased in both BAL fluid and serum in ovalbumin-sensitized and challenged rats after *H. spicatum* treatment which was comparable to the prednisolone-treated group. It can be suggested that *H. spicatum* may reverse the histone acetylation by recruiting histone deacetylase and thereby switch off the inflammatory genes, and this may explain the observed beneficial effects of *H.spicatum* in the treatment and management of bronchial asthma.

## CONCLUSION

Taken together, the present study demonstrates the attenuating effects of *H.spicatum* extract on airway inflammation in an experimental model of allergic bronchial asthma. The statement suggests that the plant extract has shown influence in a way that indicates it has anti-inflammatory, immunomodulatory, and antioxidant properties. Since airway inflammation is central to the pathophysiology of asthma, and *H.spicatum* suppressed inflammatory cell infiltration and downregulated several inflammatory and pro inflammatory cytokines, it could have good potential as a disease modifying agent and/or therapeutic adjunct in bronchial asthma. Interactions of traditional and modern medicinal concepts are a rapidly emerging concept and the present results are thus of translational value. Such studies may form the basis for a new multi targeted pharmacotherapeutic strategy in bronchial asthma. Further experimental and clinical studies to discover the potential effects of *H.spicatum*and its active phytoconstituents for the treatment of asthma and associated airway remodeling are highly recommended.

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