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Protective Effect of *Biophytum sensitivum* Against Ovalbumin Induced Lung Inflammation in an Animal Model of Asthma

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ABSTRACT

The present study was conducted to evaluate the effect of methanolic extract of *B.sensitivum* on mast cell Degranulation induced by Egg albumin, the antioxidant activity, differential WBC count in BAL fluid and histopathology of lungs in ovalbumin induced lung inflammation in an animal model of asthma. The methanolic extract of aerial part of *Biophytum sensitivum* (MBS) was prepared. Animals were divided into 6 groups (Normal control, Disease control, Standard group (Dexamethasone-4mg/kg) and Treatment groups (MBS-100 mg/kg, 200 mg/kg, and 400 mg/kg) in Ovalbumin induced model. Ovalbumin was given by i.p on days 7 and 14, by aerosol on days 28 to 30. Treatment groups and the Standard group were treated orally for last 10 days with MBS (100 mg/kg, 200 mg/kg and 400 mg/kg) and Dexamethasone (4mg/kg) respectively. In Egg albumin induced mast cell degranulation model, mast cell derived from mesentery divided into 6 groups including MBS at doses 50 µg/gm, 100 µg/gm, 200 µg/gm. Percentage protection of mast cells was calculated. Antioxidant activity was done for lung homogenate. Results were analyzed by using one way ANOVA. Methanolic extract of *Biophytum sensitivum* (400mg/kg) significantly ($p<0.05$) inhibited the lung inflammatory cells like Total WBC, Eosinophils, Neutrophils which show the inhibition of lung inflammation in asthma model. MBS showed significant protection in mast cell degranulation induced by Egg albumin. Animal treated with MBS showed the higher level of Catalase, SOD, GSH and lower level of Nitrite than those observed in disease group. As compare to disease group, MBS treated group of animals showed protective effect in lung histopathology. It can be concluded that Methanolic extract of Aerial part of *Biophytum sensitivum* has protective effect on lung inflammation in experimental animals.

Keywords: Lung inflammation, Asthma, Ovalbumin, *Biophytum sensitivum*

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INTRODUCTION

Bronchial asthma is characterized by hyperresponsiveness of tracheobronchial smooth muscle to a variety of stimuli, resulting in narrowing of air tubes, often accompanied by increased secretion, mucosal edema and mucus plugging. Symptoms include dyspnoea, wheezing, cough and may be limitation of activity. [1] Asthma is characterized by: 1) Inflammation of airways, 2) Bronchial hyper-reactivity, 3) Reversible airways obstruction.[2] Asthma can be classified as an extrinsic or intrinsic based on whether symptoms are precipitated by allergens (extrinsic) or intrinsic. Furthermore, there is chemical induced asthma, exercise induced asthma, and occupational asthma.[3]

In terms of the extent and duration of disability, asthma is the 14th most severe disorder in the world. The asthma burden is most common for children aged 10-14 and elderly aged 75-79. The prevalence of asthma is found to be an approximately 4.5 per cent in the world. Across the world there are about 334 million patients with asthma affecting all age groups. The prevalence of asthma has increased over time and an additional 100 million people worldwide to develop asthma by the year 2025. 10 per cent of the asthma which is prevalent in the world is happened in India. Three different estimate models (INSEARCH, GINA and WHO survey) refers that the prevalence of asthma in India varies between 2.05 to 3.5 per cent (17-30 million patients) as per latest analysis. The expected cost of asthma treatment per year for the year 2015 has been calculated to be about Rs.139.45 billion. An estimated 15 million disability adjusted years (DALYs) are lost due to asthma. The treatment of asthma costs about Rs.139.45 billion per year up to 2015. An estimated 15 million disability adjusted years (DALYs) are lost due to asthma.[3, 4] For asthma causes are not known. In earlier, Investigators refers some genetic and environmental factors which interact to cause asthma. These factors are[3] an inherited tendency to develop allergies, called atopy (AT-o-pe), asthmatic patients, Childhood respiratory infection, Tobacco smoke (active and passive), Some Drugs like- Beta-blockers and NSAIDs (except-Paracetamol and Nimesulide are safe), Psychological or other unaccustomed stress, exercise (in which the stimulus may be cold air and/or drying of the airways), Airway inflammation is considered to be the primary pathologic event in asthma. An interaction of many cells types is involved in the airway inflammation and many within the airways results in the pathophysiological features of asthma. Bronchial inflammation and airflow limitation result in recurrent episodes of cough, wheeze, and shortness of breath. Bronchial inflammation and airflow limitation result in recurrent episodes of cough, wheeze, and shortness of breath. These interactive events occur by which processes and lead to clinical asthma are still not known. These include Cells responsible for Inflammation like

Mast cells, Lymphocytes, Neutrophils, Eosinophils, Dendritic cells, Macrophages, Resident cells of the airway and epithelial cells. Furthermore, it also includes Inflammatory Mediators like Cytokines, Chemokines, Cysteinyl leukotrienes, Nitric oxide and Immunoglobuln E.[6] Asthma is one type of airway inflammatory disorder. So the main goal for the asthma treatment is to inhibit inflammation by anti inflammatory drugs and also to decreases the inflammatory mediators. There are two broad groups for treatment of asthma, 1) control group and 2) reliever group. Recently available asthmatic treatment includes bronchodilators, mast cells stabilizers, corticosteroids (systemic and inhaled), leukotrienes modulators, monoclonal IgE antibodies, leukotrines modulators. All shows effective action in asthma.[1]

Patients want complementary and alternative medicine for asthma because of very severe side effect like tachycardia, muscle tremor and convulsions observed in allopathic medication which is used for asthma. In Ayurveda and other Indian literature many herbal plant description are mention for their different use. India has about 45,000 plant species and among them most of possesses medicinal properties. For the treatment of bronchial asthma and allergic disorders, there is number of drugs from indigenous plant sources.[7] Now a day's many herbal plants widely used for the treatment of asthma *Boerhhavia diffusa, Datura metel, Phyllanodif lora, Tribulus terrestris, Ginkgo biloba, Adhatoda vasica, Glycyrrhiza glabra* and many more.[8, 9] *B.sensitivum* is traditionally used plant in asthma. [8] Anti inflammatory, anti ulcer, anti diabetic and many other activities are also reported for *B. sensitivum.*[10] But there is no evidence to prove its anti asthmatic activity. Therefore this study evaluate the effect of *B.semsitivum* on Ovalbumin induces lung inflammation in an animal model of asthma.

MATERIALS AND METHOD

Plant collection and Authentification

Aerial part of *B.sensitivum* plant was collected from Paniya, Amreli, Gujarat. Authentication of plant was done at NISCAIR (National Institute of Science Communication and Information Resources), New Delhi.

Plant extraction

Aerial parts of *Biophytum sensitivum* was washed thoroughly with water and shade dried at room temperature. After drying chopped into pieces and then powdered material was extracted with methanol in a soxhlet apparatus.

Preliminary Phytochemical screening of MBS[11]

Preliminary phytochemical screening was performed in the methanolic extract of *Biophytum sensitivum* for the presence of alkaloids, glycosides, tannins, flavanoids, saponins, steroids,

carbohydrates and amino acid.

LC-MS analysis of Methanolic extract of aerial part of *Biophytum sensitivum*

An efficient liquid chromatography– electrospray ionization–tandem mass spectrometry (LC–ESI–MS/MS) method was developed for identification and simultaneous determination of Bioflavonoid in Aerial part of *B.sensitivum*.

Specification of LC-MS method: (1) Column: Gemini C18,(250mm x 4.6mm, 5µm), (2) Mobile Phase: 5mM Ammonium Acetate in water: Methanol,(30:70) (3) Flow rate : 1.00mL/min (4) Column oven temperature : 40±0.3˚C (5) Auto sampler temperature: 15±3˚C (6) Volume of injection: 20µl (7) Detector: PDA & Mass detector (8) Detection wavelength: 254 nm (9) Run time: 20.0 minutes (10) Ion Source: Electro spray ionization (11) Polarity: Positive & Negative.

Animals Housing and Approval:

Experiment will be conduct according to the CPCSEA guideline and the study was approved by the institutional animal Ethics Committee. (Protocol no. BKMGPC/ IAEC19/RP25/2017). Mice (20-25 Gms) will be used and maintained under standardized condition and provide pelleted diet and purified drinking water. Throughout the experiments, animals will be process according to the suggested ethical guideline for the care of laboratory animals.

In vivo Ovalbumin induced Asthma model [12]

Animals were divided into following groups:

Group 1	Normal Control	Normal saline for day 1,14 & 28 to 30
Group 2	Asthma-model control	20 ug OVA+ 2 mg $Al(OH)_3$ i.p on day 1 and 14, 28 to 30 day inhalation.
Group 3	Asthma treated with standard	On day 28 to 30 days-Dexamethasone (4 mg/kg) (i.p)
Group 4	Asthma treated with MBS (100 mg/kg)	On day 28 to 30 MBS (100 mg/kg) (P.O)
Group 5	Asthma treated with MBS (200 mg/kg)	On day 28 to 30 MBS (200 mg/kg) (P.O)
Group 6	Asthma treated with MBS (400 mg/kg)	On day 28 to 30 MBS (400 mg/kg) (P.O)

Where, MBS- methanolic extract of *B.sensitivum*, OVA-Ovalbumin

Evaluation parameters

Bronchoalveolar lavage (BAL) fluid collection and cell counting

After 48 h of the last challenges, mice will be sacrificed and a tracheotomy will be performed. After cold PBS will be instilled into the lungs, bronchoalveolar lavage fluid (BALF) will be obtained by aspiration two times via tracheal cannulation. BALF will be centrifuged & stored. Cell counting will be done with the help of hemocytometer.

Histopathological studies

The tissue samples from lung for histology will be fixed overnight in 10% neutral buffer formalin and will be processed, sectioned (4 µm thick) and stained with haematoxylin and eosin. Each sample will be observed and evaluated in laboratory.

Anti-oxidant Parameters

Catalase Level (CAT) [13]

Sample will be added to a cuvette containing 2 mL of phosphate buffer (pH 7.0) and 1 mL of 30 mM H_2O_2. Catalase activity will be measured at 240 nm for 1 min using spectrophotometer. The molar extinction coefficient of H_2O_2, 43.6 M cm1 will be used to determine the catalase activity. One unit of activity is equal to 1 mmol of H_2O_2 degraded per minute and is expressed as units per milligram of protein.

Reduced glutathione (GSH) estimation [13]

The tissue homogenate in 0.1 M phosphate buffer pH 7.4 will be taken and added with equal volume of 20% trichloroacetic acid (TCA) containing 1 mM EDTA to precipitate the tissue proteins. The mixture will be centrifugated for 10 min at 2000 rpm. To the supernatant (200 lL) than added 1.8 mL of the Ellman's reagent (5,50-dithiobis-2- nitrobenzoic acid (0.1 mM) prepared in 0.3 M phosphate buffer with 1% of sodium citrate solution). After completion of the total reaction, solutions are measured at 412 nm against blank.

Superoxide dismutase (SOD) method [14]

Although superoxide anion is a weak oxidant, it ultimately produces powerful and dangerous hydroxyl radicals as well as singlet oxygem, both of which contribute to oxidative stress. Supernant (0.1ml) of sample will be mixed with 0.1 ml EDTA, 0.5 ml of carbonate buffer (pH 9.7) and 1 ml of epinephrine. The optical density of formed adrenochrome will read at 480nm for 3 min at an interval of 30 sec. the enzyme activity will be expressed in terms of U/mg protein. One unit of enzyme activity is defined as the concentration required for the inhibition of the chrogen production by 50% in one min under the defined assay condition.

Nitrate estimation [15]

Accumulated nitrite (NO) in the homogenate will be spectrophotometrically determined based on the Griess reaction. The samples (100 µl) will be incubated with 100 µl Griess reagent (6 mg/ml) at room temperature for 10 min and then NO concentration will be determined by the absorbance at 540 nm. The standard curve will be obtained using the known concentrations of sodium nitrite.

Total protein [16]

The assay is based on polypeptide chelation of cupric ion (colored chelate) in strong alkali tubes, Add 1 ml of homogenate to 4 ml biuret reagent. Incubate 20 min at room temperature. Read the absorbance at 550 nm against reagent blank.

Statistical analysis

Data will be expressed as means± standard deviations (SD). Statistical comparisons will be made with one-way analysis of variance (ANOVA). P <0.05 will be considered statistically significant.

RESULTS AND DISCUSSION

Plant extraction

The extraction of powdered Aerial part of *B.sensitivum* was carried out using methanol at 40-45˚C by soxhlet extraction method. The extract was obtained in dark greenish color and semisolid in nature. % yield of Aerial part extract of B.sensitivum was 15 % W/W.

Preliminary phytochemical investigation

The Methanolic extract of *Biophytum sensitivum* was subjected to phytochemical investigation. The results revealed the presence of tannins (phenolic compounds), steroids, flavonoids, carbohydrates and proteins.

LC- MS analysis of Methanolic extract of *B.sensitivum*

Mass data for bioactive compounds present in methanolic extract of *B.sensitivum* extract are shown in figure 1 and 2. During method development, ionization of analytes was tested in positive (Event-1) as well as negative (Event-2) ionizations modes. However, better sensitivity with higher response was achieved in positive electro spray ionization (ESI). The spectra showed at m/z 537 for Amentoflavone.

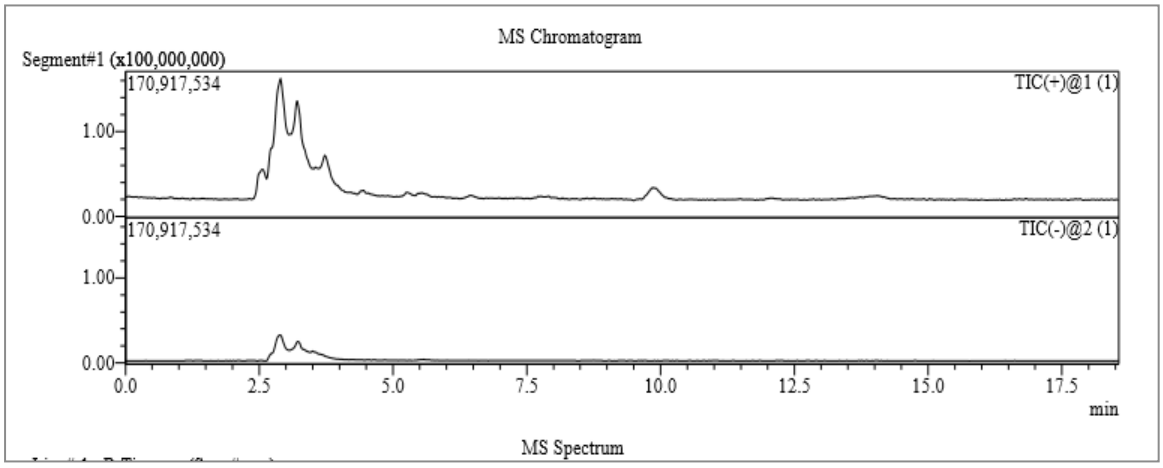


Figure 1: MS chromatogram of methanolic extract of *B.sensitivum*

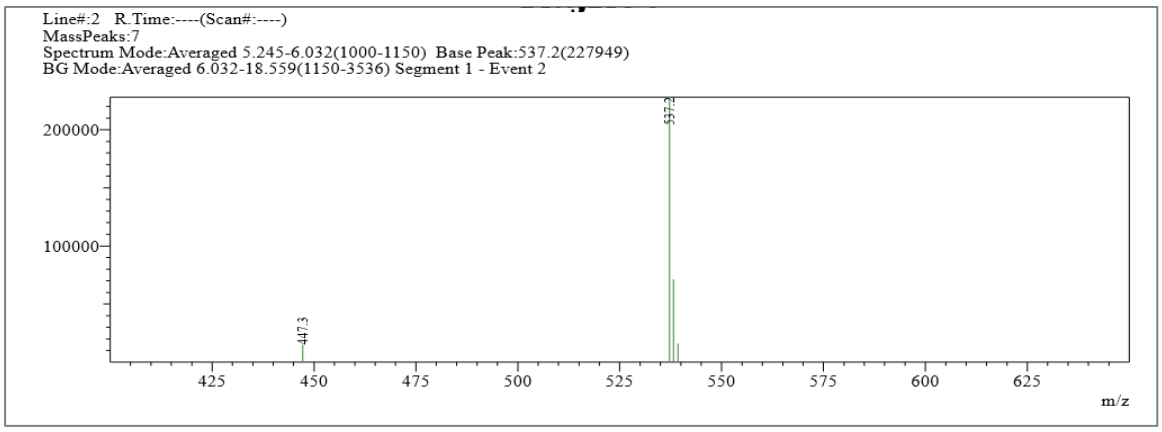


Figure 2: Mass Peak of constituents present in methanolic extract of *B.sensitivum*

In-vitro study of Mast cell Degranulation by Egg albumin

Mast cell degranulation is important in the initiation of immediate responses following exposure to allergens. Degranulated mast cells liberate mediators of inflammation such as histamine, leukotrienes, platelet activating factors and chemotactic factors for eosinophils, neutrophils etc. Egg albumin is an inciting allergen which induces the release of mediators such as histamine and proinflammatory cytokines and can be elicited by various stimuli. [17, 18] The degranulation of mast cell occur in response to the immunological stimuli in which antigen and antibody reactions are predominant.[19] However, mast cell degranulation observed in disease control group was significantly inhibited by administration of egg albumin by standard Dexamethasone and MBS (400mg/kg). Thus, it indicates that MBS has significant mast cell stabilizing activity. Methanolic extract of *Biophytum sensitivum* (400 mg/kg) significantly (P<0.05) protected rat mesentric mast cell degranulation as compare to disease control group as shown in table 1. Where, the mast cell granulation increase showed antiasthmatic activity.

Table 1: Effect of Methanolic extract of *B.sensitivum* on Egg Albumin induced Mast cell Degranulation

Groups	Granulated mast cells	Degranulated mast cells	%Protection of mast cells
Normal control	86±2.93	14±2.93	-
Disease control	22±3.33#	78±3.33#	-
STD-Dexamethasone(4mg/kg)	67±1.69*	33±1.69*	57.69%
MBS(100 mg/kg)	33±4.12*	67±4.12*	13.46%
MBS(200 mg/kg)	43±1.15*	57±1.15*	26.92%
MBS(400 mg/kg)	59±1.31*	41±1.31*	47.43%

All values are expressed as Mean± SEM (N=6) for each group.

#indicates significant difference from Normal control at P<0.05

*indicates significant difference from Model control at P<0.05

Effect of MBS on the Release of Inflammatory cells in Ovalbumin induced Asthma model.

The major feature, inflammation is associated with asthma, has received increased attention over the past decades.[20] Bronchial asthma is now no longer simply viewed as reversible airway obstruction or irritable airways. Rather it is now viewed primarily as an inflammatory illness that as a result has bronchial hyper reactivity and bronchospasm.

Table 2: Effect of Methanolic Extract of *B.Sensitivum* on Inflammatory Cells

Groups	Total cells ($\times 10^4$ Cells)	Eosinophils ($\times 10^4$ Cells)	Neutrophils ($\times 10^4$ Cells)
Normal control	2.25±0.17	0.45±0.03	0.085±0.03
Disease control	24.91±7.58#	9.73±0.93#	3.15±0.77#
STD-Dexamethasone(4mg/kg)	11.08±2.07*	5.63±0.73*	1.25±0.15*
MBS(100 mg/kg)	20.16±0.62*	9.06±1.07*	3.03±0.59*
MBS(200 mg/kg)	15.58±1.85*	8.30±0.67*	2.08±0.20*
MBS(400 mg/kg)	13.06±2.16*	6.43±1.01*	1.66±0.32*

All values are expressed as Mean±SEM for each group. (N=6)

#indicates significant difference from Normal control at P<0.05

*indicates significant difference from Model control at P<0.05

This airway inflammation is now also considered to be an immunologically initiated, mediator driven event, resulting in a cascade of immunologic events that includes antibodies, inflammatory cells and mediators.[21] Ovalbumin is a one type on pure protein obtained from chicken egg white, which play a role of allergen and produces the allergic inflammatory responses. It produces lung inflammation by produces the inflammatory cells like eosinophils, basophils total WBC etc.[22] Administration of Ovalbumin might lead to increased production of Total WBC, and differentiate WBC. In present study we show the inhibitory effect of MBS on inflammatory cells like Total WBC and others as compared to disease group, which suggest the inhibition of lung inflammation. This decreased lung inflammation directly inhibiting the asthmatic responses.

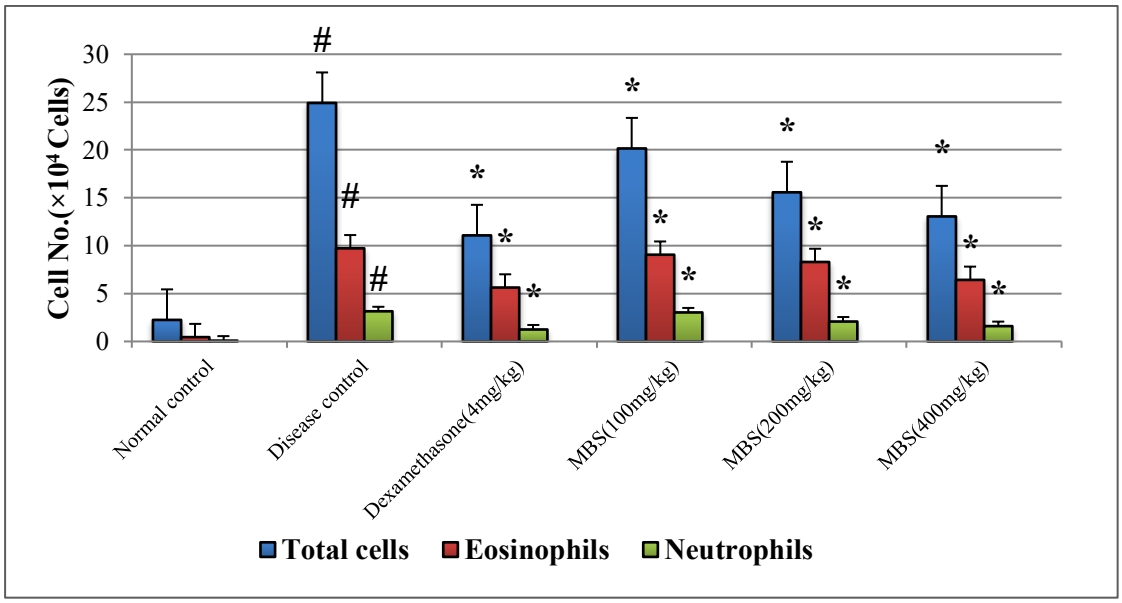


Figure 3: Effect of Methanolic extract of *B.Sensitivum* on Inflammatory cells

All values are expressed as Mean±SEM for each group. (N=6)

#indicates significant difference from Normal control at P<0.05

*indicates significant difference from Model control at P<0.05

Methanolic extract of *Biophytum sensitivum* (400 mg/kg) significantly (P<0.05) inhibited the inflammatory cells like Total WBC, Eosinophils and others as compare to disease control group as shown in table 2 and figure 3. Where, the inhibition of inflammatory cells shows inhibition of lung inflammation.

Effect of Methanolic extract of *B.Sensitivum* on Anti Oxidant Markers

Catalase is an important endogenous antioxidant enzyme in normal lung. It protects epithelial cell layer from inflammation and other damage. In lung inflammation epithelial cell layer gets damaged which shows the decreases catalase level. SOD enzyme is also a very useful antioxidant enzyme. SOD shows a very beneficial effect over inflammatory diseases like IBD, asthma. SOD catalyzes free radicals into ordinary molecules, which helps to protect from that free radicals.[13] MBS showed the significantly higher level of these enzyme as compared with Disease group.

Methanolic extract of *Biophytum sensitivum* (400mg/kg) significantly (P<0.05) showed higher Catalase level, SOD level and GSH level, but lower Nitrite level as compared with disease control group as shown in table 3 and Figure 4,5 6 and 7. It shows anti-oxidant activity of Methanolic extract of *B.sesnsitivum*.

Table 3: Effect of Methanolic extract of *B. sensitivum* on Anti-Oxidant Levels

Groups	CAT (U/mg of Protein)	SOD (U/mg of Protein)	GSH (µmol/mg of protein)	Nitrite (µmol/mg of protein)
Normal control	1.20±0.09	74.32±5.91	8.87±1.61	0.44±0.04
Disease control	0.31±0.03#	20.50±2.10#	2.82±0.19#	1.31±0.06#
STD -Dexamethasone(4mg/kg)	1.06±0.04*	53.59±6.00*	7.57±2.71*	0.50±0.02*
MBS(100mg/kg)	0.46±0.05*	25.44±3.75*	3.41±0.62*	0.98±0.12*
MBS(200mg/kg)	0.65±0.04*	27.93±7.46*	4.73±0.61*	0.67±0.06*
MBS(400mg/kg)	0.85±0.07*	38.19±12.38*	6.22±1.19*	0.60±0.09*

All values are expressed as Mean± SEM (N=6) for each group.

#indicates significant difference Normal control at P<0.05

*indicates significant from Model control at P<0.05

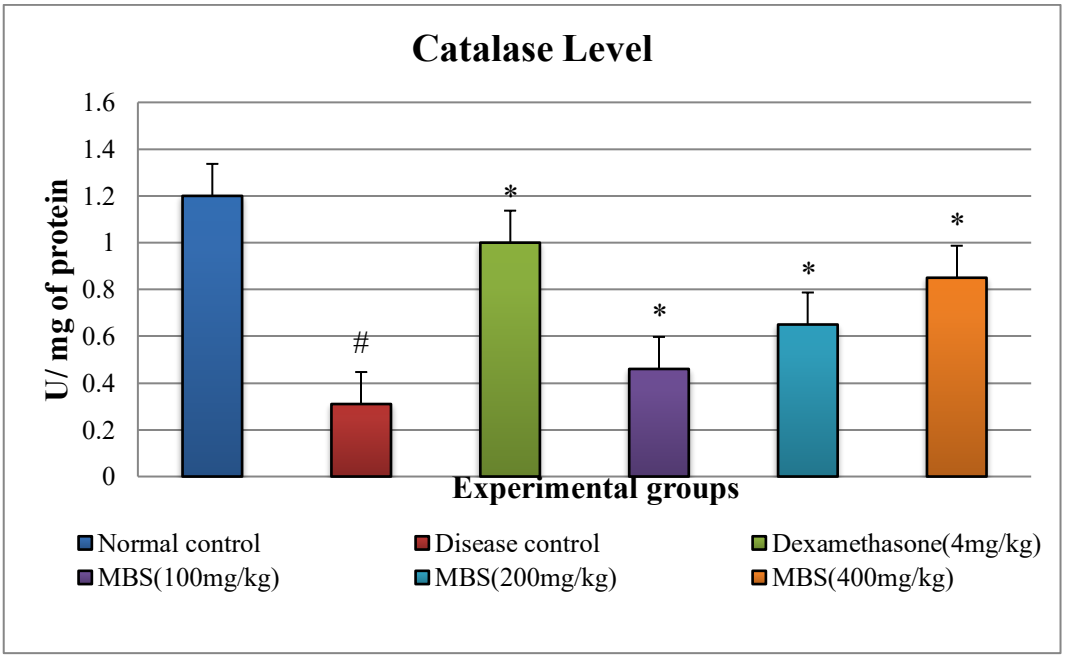


Figure 4: Effect of Methanolic extract of *B. Sensitivum* on Catalase level

All values are expressed as Mean± SEM (N=6) for each group.

#indicates significant difference Normal control at P<0.05

*indicates significant from Model control at P<0.05

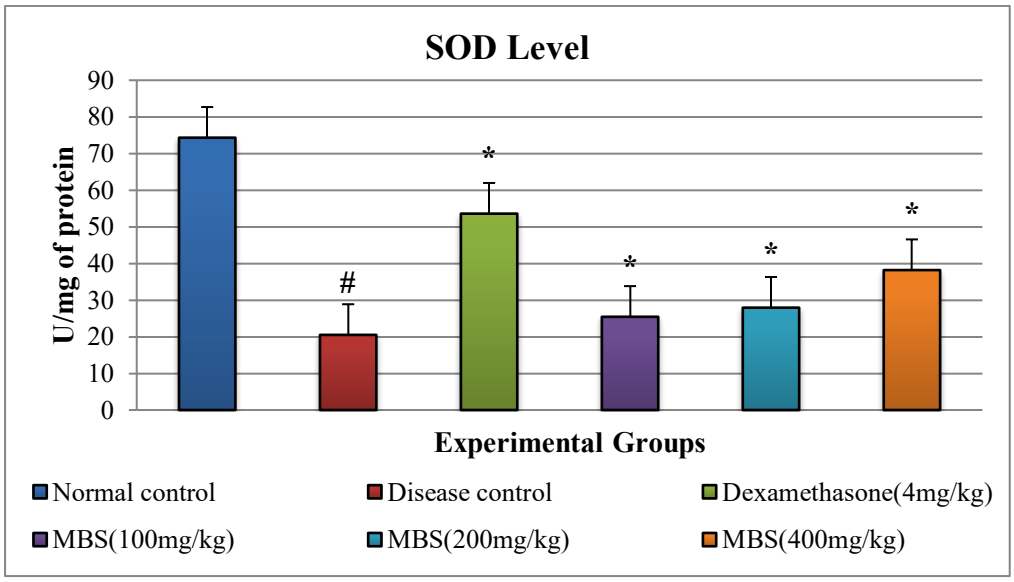


Figure 5: Effect of Methanolic extract of *B. Sensitivum* on SOD level

All values are expressed as Mean± SEM (N=6) for each group.

#indicates significant difference Normal control at P<0.05

*indicates significant from Model control at P<0.05

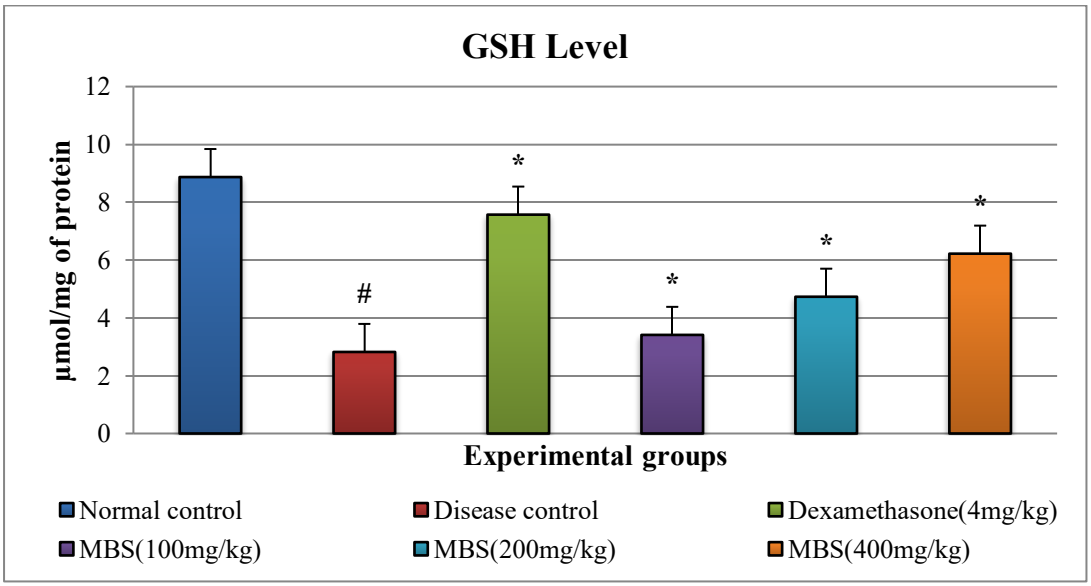


Figure 6: Effect of Methanolic extract of *B.Sensitivum* on GSH level

All values are expressed as Mean±SEM (N=6) for each group.

#indicates significant difference from Normal control at P<0.05.

*indicates significant difference from Model control at P<0.05

Administration of Ovalbumin might lead to increased production of nitrite which could be analyzed by above-described method. NO, nitrite release after cytokine stimulation is a potent agent in non-specific defense mechanisms.[23] However, it has been shown that its sustained production is deleterious since it plays an important role in various tissue injuries associated with

sub-acute and chronic inflammatory processes such as asthma other conditions. The marked decrease in NO level with MBS compared to the disease group reflects its role in anti-inflammatory activity.

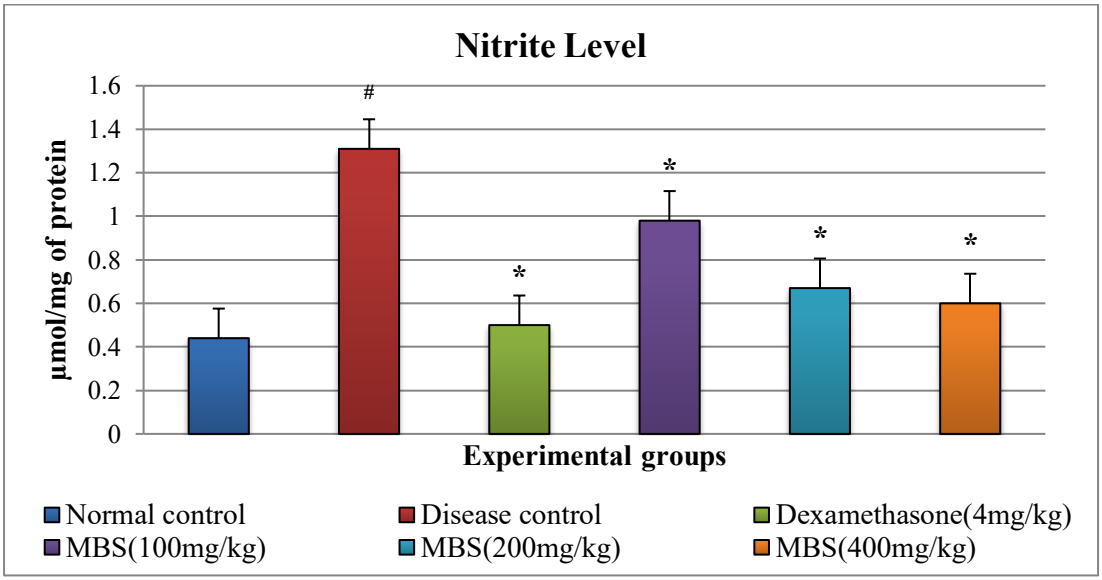


Figure 7: Effect of Methanolic extract of *B. Sensitivum* on Nitrite estimstion

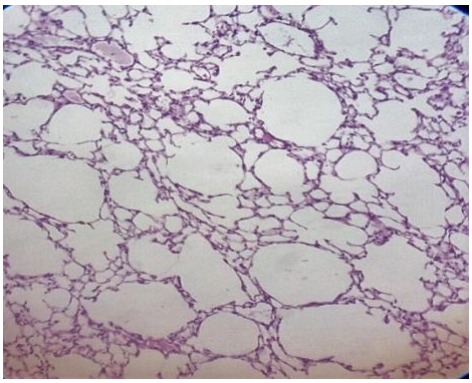
All values are expressed as Mean±SEM (N=6) for each group.

#indicates significant difference from Normal control at P<0.05.

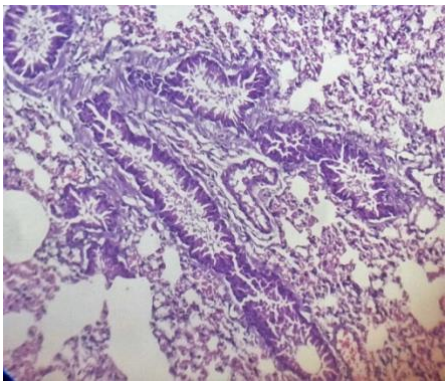
*indicates significant difference from Model control at P<0.05

Histopathology of Lungs:

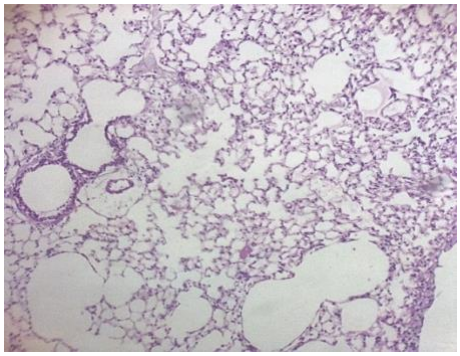
Histological finding of disease group showed hyperplasia of goblet cells, the mucus producing cells, and mucus within airway or bronchi, increased infiltration of polymorphonuclear Eosinophils, leukocytes, lymphocyte which is resemble histological feature of human asthma. MBS (400 mg/kg,) treated group and standard (4 mg/kg) treated group showed reversal of mucosal damage as compared to the disease group as evidence by less cell infiltration as compare to disease group.



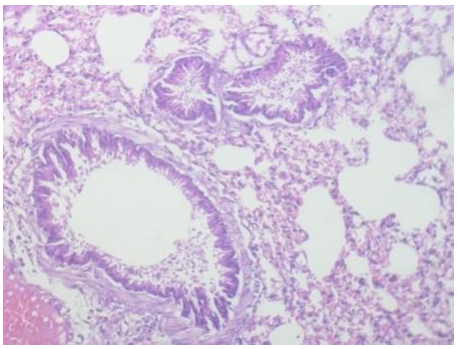
Normal Group
(Normal mucosal)



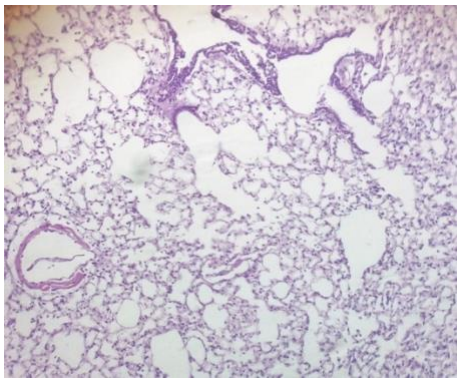
Disease Group
(Transmural inflammation, bronchial obstruction, cells infiltration)



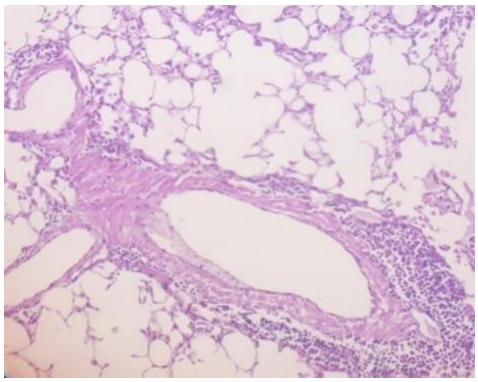
STD-Dexamethasone(4mg/kg)
(Epithelial damage, cells infiltration, hyperplasia of goblet cells)



MBS (100mg/kg)
(Hyperplasia of goblet cells, mucosal infiltration)



MBS(200mg/kg)
(Hyperplasia of goblet cells, mucosal infiltration, Cell infiltration)



MBS(400mg/kg)
(Normal structure with slight mucosal infiltration)

Figure 8: Histology of lungs

CONCLUSION

The present investigation reveals that methanolic extract of aerial part of *B.Sensitivum* is potentially benefited, as a protective agent in Ovalbumin induced asthma model. In present study,

Methanolic extract of *B.Sensitivum* showed Protective effect on cell infiltration. MBS possess the protective effect on Mast cell degranulation. MBS also possess good antioxidant activity. Histological studies also demonstrate the protective role of MBS in lung inflammation. Bioflavanoids like Amentoflavone showed the inhibitory effect on NF-κB. Phytochemical screening of methanolic extract of *B.Sensitivum* showed the presence of Amentoflavone.. So from this we concluded that the MBS might be inhibites the NF-κB and showed Protective role in lung inflammation. In the light of above finding and discussion, Methanolic extract of *Biophytum Sensiitivum* might have Protective effect on lung inflammation. Further studies are necessary in order to characterize the actual mechanism of action of MBS in the treatment of lung inflammation.

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