Vol 8|Issue 2| 2018 | 45-53.

ISSN 2249 - 7641
Print ISSN 2249 - 765X

International Journal of Pharmacology Research

www.ijprjournal.org

EVALUATION OF THE ANTI-ASTHMATIC POTENTIAL OF *BOERHAAVIA DIFFUSA (B.DIFFUSA)* IN EXPERIMENTAL ANIMALS

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ABSTRACT

Regardless of existing medical knowledge on allergy and asthma, the treatment of asthma still remains unsatisfactory owing to the limitations of current therapies and associated side effects. Aqueous root extract of *Boerhaavia diffusa (B.diffusa)*, possess anti inflammatory property and has been used traditionally in the treatment of asthma as mentioned in ayurvedic literature. Therefore, the present study was planned to evaluate and explore potential mechanism/s involved in the beneficial effects of *B.diffusa* in asthma using various animal models. The aqueous root extract *of B.diffusa* was studied at3different doses (200mg/kg, 400mg/kg, and 600 mg/kg) using 3 animal models. The models used for evaluating the antiasthma potential of *B.diffusa* were ovalbumin-induced allergic response in rats, clonidine-induced mast cell degranulation in rats, and model of bronchial hyperresponsiveness induced by histamine aerosol in guinea pigs. Each model consisted of 5 groups (Group 1: disease control; Group 2: positive control; Group 3: *B.diffusa* 200mg/kg; Group 4: *B. diffusa* 400 mg/kg, Group 5: *B. diffusa* 600 mg/kg. All the 3 doses of *B diffusa* showed significant reduction in total leucocyte count, eosinophils, neutrophils and mononuclear cell count as compared to disease control($p<0.001$). The reduction of peribronchial inflammation in both dexamethasone ($p<0.001$) and *B. diffusa*600 mg/kg ($p<0.05$) group was found to be significant compared to disease control group. The percentage of intact mast cells was found to be significantly higher in the sodium chromoglycate and *B. diffusa* 200, 400, and 600 mg/kg as compared to the disease control group ($p<0.001$).Increase in the preconvulsion time was observed with all 3 doses of *B.diffusa*, significant as compared to disease control group ($p<0.001$). These findings corroborate that *B.diffusa* suppresses the airway inflammation, demonstrates membrane stabilizing property and reduces airway hyperresponsiveness.

Keywords: Bronchial hyper reactivity, Bronchoalveolar lavage, Mast cell degranulation, Ovalbumin, Pre convulsion time.

INTRODUCTION

Asthma being one of the most common of all the allergic disorders contributes to considerable global burden due to its major socioeconomic impact. Worldwide, it is accountable for major loss of productivity and significant loss of disability adjusted life years (DALYs) [1].The pathophysiology of asthma is not yet completely understood, but definitely involves activation of immunological events such as mast cell activation, infiltration of eosinophils, and T helper 2 (Th2) lymphocytes in the airways [1,2]. The World Health Organization factsheet on bronchial asthma also states that India has an estimated 15-20 million asthmatics [3].

The current therapeutic options available for the treatment of asthma include drugs administered through both inhalational and systemic routes. Bronchodilators (eg, inhaled β_2-agonists, anticholinergic agents,

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methylxanthenes) and drugs that suppress airway inflammatory response (eg,glucocorticoids, leukotriene antagonists, and mast cell stabilizers) are effectively used for relieving bronchoconstriction and for reducing airway inflammation, respectively. The long term use of these drugs often results in significant local as well as systemic side effects. Above all, these drugs have limited efficacy on chronic use, significant adverse effects, poor patient compliance and are often empirical and symptomatic, with hardly any curative strategy[4].Owing to the limitations of drugs from modern medicine, researchers are now exploring the drugs from the alternative system of medicine being used clinically and validating their medicinal use with the help of well-defined scientific study designs. One such herb that may be promising in this regard is, *Boerhaavia diffusa* (*B. diffusa*), known as 'punarnava' in Sanskrit, a herbaceous member of Nyctaginaceae family [5].

Ayurvedic texts have claimed it to possess 'rasayana' (rejuvenating) properties. Many preclinical studies using *B. diffusa* extracts, have positively verified the 'rasayana' properties as well as analgesic and anti-inflammatory activities [6, 7]. One such study has demonstrated the antiinflammatory property of aqueous extract of root of *B. diffusa*. The authors have attributed the antiinflammatory property to the cell membrane stabilizing effect of *B. diffusa*, which in turn inhibits lysis and release of inflammatory mediators [7]. According to Ayurvedic literature leaf and the root extracts of this plant have been effectively used in the treatment of asthma [6,8].

However, due to the absence of precise scientific evidence of the mechanism by which *B. diffusa* modifies the disease process of bronchial asthma, we planned the present study so as to evaluate and explore the potential mechanism/s involved in the observed beneficial effects of *B.diffusa* in asthma using various animal models.

MATERIALS AND METHODS

Permission of the Institutional Animal Ethics Committee was obtained before the start of the experiments (EC/Pharma/4/2013). Animals bred in the animal house of the institute were used for the study. The guidelines laid down by the Committee for the Purpose of Control and Supervision of Experiments on Animals were followed throughout the study. Animals were housed in the animal house of our institute, in an air conditioned area with 12 – 15 filtered fresh air changes, temperature 22 ± 3° C, and relative humidity 30%to 70%. Three rats per cage and one guinea pig per cage were housed during treatment. Cages had a stainless steel top grill having facility to provide food and drinking water. Aquaguard pure drinking water was provided in polypropylene bottles with stainless steel sipper tube. Strict 12 hours of day and night cycles were maintained throughout the study duration.

Study Drugs and Chemicals

1. *Boerhaavia diffusa* (aqueous extract of roots) (provided by Shree Dhoothpapeshwar Pvt. Ltd.)
2. Sodium cromoglycate (provided by CIPLA Ltd.)
3. Chlorpheniramine maleate (CPM) (provided by CIPLA Ltd.)
4. Dexamethasone (purchased from Sigma Aldrich)
5. Clonidine(purchased from Sigma Aldrich)
6. Histamine hydrochloride (purchased from Sigma Aldrich)
7. Carboxymethylcellulose (CMC) (purchased from Sigma Aldrich)

Study Design and Procedures

Part 1

The objective of this phase was to assess the antiallergic and antiinflammatory activity of *B. diffusa* in a model of ovalbumin induced allergic response in Wistar rats. Thirty Wistar rats of either sex, weighing 250-300 g were divided into five groups of 6 animals each. All animals were sensitized by an intraperitoneal injection of 1 mL alum precipitate antigen containing 20µg of ovalbumin and 8mg of alum suspended in 0.9% sodium chloride solution. The day of first sensitization was considered Day 1.A booster injection of this alum-ovalbumin mixture was given 7 days later. Group 1 (disease control) received 5 mL of 5% CMC orally, daily for 15 days, starting from Day 1. Group 2 (positive control) received dexamethasone, 10 mg/kg intraperitoneally on Day 15, two hours before their sacrifice. Animals belonging to Groups 3,4, and 5 served as our test groups. Groups 3, 4, and 5 received aqueous extract of *B.diffusa* in the doses of 200, 400, and 600mg/kg orally, respectively, from Day 1 to Day 15.

On Day 15, (7 days after the booster injection of alum-ovalbumin mixture), these animals belonging to different groups were exposed to aerosolized ovalbumin (1%) for 30 min. The rats were then sacrificed using ketamine (100 mg/ kg) anesthesia and a tracheal catheter was inserted in trachea after dissection. Bronchoalveolar lavage (BAL) fluid was collected by lavaging the lung with 2 aliquotsof 5 mLof 0.9% sodium chloride solution. The total recovery volume of BAL fluid per rat was approximately 8mL. Immediately after collection, the BAL fluid was centrifuged at 3000 rotations per minute (rpm) at 10°C to 12°C. A pellet of white blood cells (WBC) which was obtained was further divided into 2 aliquots. The first aliquot was used to assess the total leukocyte count (TLC) using the Neubar's Chamber after the cells were mixed with WBC Counting Fluid. The second aliquot was used to prepare slides using a cytocentrifuge for differential leukocyte count. Two slides were obtained from each animal's BAL and microscopy was performed under 400× magnification for assessment of differential cell count. A total of 200 cells were counted from both the slides and average of the differential cell count of the 2 slides were then converted into percentage of neutrophils, eosinophils,

and mononuclear cells. The mononuclear cells were further divided into monocytes, macrophages and lymphocytes [9, 10, 11, 12, 13, 14].

After the recovery of the BAL fluid, the lungs were separated and stored in 10% buffered formalin for histopathological examination, which was performed by a veterinary pathologist. Lung tissue sections were stained with hematoxylin-eosin and examined microscopically. Peribronchial cells were counted using a 5-point scoring system to estimate the severity of leukocyte infiltration. The leukocyte infiltration score was examined in two independent fields of lung section from each rat.

The scoring system used was as follows [10]:

- Score 0-no cells;
- Score 1-a few cells;
- Score 2 - a ring of cells 1 cell layer deep;
- Score 3 - a ring of cells 2–4 cell layers deep;
- Score 4 - a ring of cells more than 4 cell layers deep.

Part 2

The objective of this phase was to assess the mast cell stabilizing property of *B.diffusa* in a model of clonidine - induced mast cell degranulation using wistar rats. Model was first standardized before actual experimentation.

Thirty Wistar rats of either sex, weighing 250-300 g were divided into five groups, each group containing 6 animals. Group 1 was disease control group which received 5 mL of 5% CMC on each of the 7 days of the experiment. Animals belonging to Group 2 served as positive control group that received 50 mg/kg of sodium cromoglycate intraperitoneally on Day 7. Groups 3,4,and 5 served as test groups and received aqueous extract of *B.diffusa* in the dose of 200, 400, 600mg/kg, respectively, orally, from Day 1 to Day 7.On Day 7, all the groups received treatment 2 hours before they were sacrificed. On Day 7, the rats were sacrificed using ketamine (100 mg/kg) anesthesia. Normal saline (10 mL) was injected into the peritoneal cavity of the rats and abdomen was gently massaged for 90 s. The peritoneal cavity was carefully opened and the fluid containing mast cells was aspirated and collected in siliconized test tubes containing 7 to 10 mL of RPMI-1640 Medium (pH 7.2- 7.4). The mast cells were then washed thrice by centrifugation at low speed (400-500 rpm) at 10°C to 12°C and the pellet of mast cells was taken in the medium. The solution was further challenged with 0.5 µg/mLof clonidine solution in all the groups. The cells were further stained with 1% toluidine blue after a gap of 10 min and observed under high power microscope field (400× magnification). Total 100 cells were counted from different visual areas and the number of intact and degranulated mast cells were counted [15,16].

The percent protection was calculated using the following formula:

Percent protection = T2-T1/T2 × 100.

(T1 = Number of intact mast cells in the Disease Control group; T2 = Number of intact mast cells in the Test groups[15].

Part 3

The objective of this phase was to assess the bronchodilator property of *B.diffusa* using a model of histamine-induced bronchospasm in guinea pigs.

Thirty guinea pigs of either sex, weighing 250-300 grams were used for the experiment. On Day 1 (pretreatment screening), all the experimental animals were kept in a closed plexiglass histamine chamber and exposed to an aerosol of 2% histamine dihydrochloride. Further, changes in the respiratory rate and the involvement of the abdominal musculature wasseen as the exposure to histamine aerosol continued. As soon as dyspnealeading to convulsions occurred, the animal was removed from the chamber and placed in fresh air to recover from the dyspneic episode. The time from the start of the histamine aerosol exposure to the beginning of appearance of convulsions was measured and was called as preconvulsion time (PCT). The 30 guinea pigs were divided in to 5 groups of 6 animals each. Animals belonging to Group 1 served as disease control group that received 5 ml of 5% CMC on each of the 7 days of the experiment. Animals belonging to Group 2 served as positive control and received CPM ie, 2 mg/kg per-orally from Day 1 to Day 7. Animals belonging to Groups3,4, and 5 served as test groups that received aqueous extract of *B.diffusa* 200, 400, and 600mg/kg, respectively from Day 1 to Day 7 orally. The treatment in each of the 5 groups on Day 7 was given 2 hours before the conduct of the actual experiment.

On Day 7, all animals were once again exposed to aerosol of 2% histamine dihydrochloride and a post treatment PCT was obtained for all the groups [11,15,16,17,18,19,20].

Parameters of Assessment

Symptoms like increased breathing frequency, forced inspiration and asphyxic convulsions were observed.

PCT on Day 0 (T1) and Day 7 (T2) was obtained.

Percentage increase in PCT was then calculated using the following formula,

% Increase in PCT = 1-TI/T2 × 100.

(Where, T1 = PCT on Day 0, T2 = PCT on Day 7)[15].

Statistical analysis

Statistical analysis was carried outusing MS Excel 2013 and Graph Pad Instat 3.0.

Part1

The leucocyte and the differential leucocyte count in the BAL fluid of animals in study groups was compared by using One way ANOVA and *post hoc* Tukey's test and the peribronchial lung inflammation in study groups were

compared using Kruskal-Wallis test and *post hoc* Dunn's test.

Part 2 and Part 3

The percent protection of mast cells from clonidine -induced degranulation (Part 2) and percent increase in the PCT (Part 3) between the study groups were compared by using One way ANOVA and *post hoc* Tukey's test. '*p*' value < 0.05 was considered to be statistically significant for all the results.

RESULTS

Part 1

This section includes the results of the experiment performed on all 5 groups. The Day 15 TLC in BAL fluid was found to be significantly lower in the dexamethasone group, *B. diffusa* 200, 400,and 600 mg/kg groups compared to the disease control ($p< 0.001$). But, the TLC was significantly lower in the dexamethasone group compared to all the test groups, namely *B. diffusa*2 00, 400 and 600 mg/kg. *B. diffusa* in the dose of 600 mg/kg showed greater reduction in TLC compared to both400 mg/kg ($p< 0.05$) and 200 mg/kg of *B.diffusa* ($p< 0.001$)(Refer Table 1).

Similar findings were observed with dexamethasone and *B.diffusa* 600 mg/kg when analysed for differential leukocyte count (DLC) i.e. neutrophil (%), eosinophil (%), and mononuclear cell (%) ($p< 0.001$). Additionally, *B. diffusa* in the dose of 400 mg/kg produced a significant reduction in the neutrophil (%) and mononuclear cell (%) count as compared to the *B. diffusa*200 mg/kg Group ($p< 0.05$).

The monocyte count (%) significantly increased in the dexamethasone group, *B. diffusa* 200, 400 and 600 mg/kg groups when compared to the disease control group ($p< 0.001$). The increase in percentage of monocytes was significantly greater in dexamethasone group as compared to *B. diffusa*200, 400 and 600 mg/kg groups ($p< 0.001$). On comparing the monocyte counts with different doses of *B.diffusa*, it was found that *B.diffusa* increased the monocyte count in a dose dependent manner.

The low macrophage and lymphocyte cell counts (%) in the disease control group is because of compensatory increase of the neutrophil (%) and eosinophil (%) counts. The treatment groups that received 200, 400, and 600 mg/kg of *B. diffusa* showed a significantly higher macrophage and lymphocyte counts (%) in comparison to both the disease control and dexamethasone groups, with a higher macrophage count (%) in *B.diffusa*200 and 400 mg/kg groups as compared to *B.diffusa*600 mg/kg group ($p< 0.001$).

The Histopathological grade of peribronchial inflammation in both the dexamethasone group ($p< 0.001$) and the *B. diffusa*600 mg/kg group ($p< 0.05$) was significantly lower as compared to the disease control group (which showed the highest median grade of inflammationie,Grade4) (Table 2).

Part 2

The percentage of intact mast cells was found to be significantly higher in the sodium cromoglycate group, *B.diffusa* 200, 400 and 600 mg/kg groups compared to the disease control group ($p< 0.001$). Moreover, in sodium cromoglycate group, the number of intact mast cells were significantly higher as compared to all the 3 doses of *B. diffusa* ($p< 0.001$). The 3 doses of *B. diffusa* were comparable to each other(Table 3).

Part 3

The post treatment PCT was found to be significantly higher in the CPM group and*B. diffusa* 200, 400, and 600 mg/kg groups compared to the disease control group ($p< 0.001$). However, the post treatment PCT was found to be significantly higher in CPM group when compared with all the 3 doses of *B.diffusa* ($p< 0.001$). All the 3 doses of *B. diffusa* were comparable to each other with respect to increase in PCT ($p> 0.05$) (Table 4).

Table 1. Effect of *B.diffusa* and dexamethasone on total and differential leukocyte counts after ovalbumin exposure

Groups(n=6)	Total Leukocyte Count (cells/uL)	Differential Leukocyte Count		
		Neutrophil Count (%)	Eosinophil Count (%)	Mononuclear Cell Count (%)
DiseaseControl (5% CMC)	616.67± 40.8	57± 3.1	25.33± 3.1	17.67± 4.7
Dexamethasone (10mg/kg)	241.67± 37.6$^{*\#}$	23.33± 2.8$^{*\#}$	9.33± 2.7$^{*\#}$	65.67± 3.8$^{*\#}$
B.diffusa (200 mg/kg)	445.83± 10.2*	41± 3.0*	19.83± 1.3*	39.17± 1.8*
B.diffusa (400 mg/kg)	404.17± 10.2*	35± 3.3$^{*\$}$	17.17± 3.5*	47.83± 1.5$^{*\$}$
B.diffusa (600 mg/kg)	354.17± 10.2$^{*@}$	31.17± 2.6$^{*@}$	14± 1.4$^{*@}$	54.83± 3.1$^{*@}$

CMC=carboxymethylcellulose

Values are expressed as Mean ± SD. Statistical analysis done by using ANOVA followed by post-hoc Tukey Test

* p< 0.001, compared to disease control group; #p< 0.001, compared to *B.diffusa* groups; $p< 0.001, compared to *B.diffusa*200 mg/kg; @p< 0.05, significant compared to*B.diffusa* 200 mg/kg

Table 2. Effect of *B.diffusa* on histological grading of lung parenchyma after exposure to ovalbumin

Groups (n=6 per group)	Median	Range
Disease control (5% CMC)	4	3-4
Dexamethasone(10mg/kg)	1**	1-2
B.diffusa(200mg/kg)	3	2-4
B.diffusa(400mg/kg)	3	2-3
B.diffusa(600mg/kg)	2*	2-3

Statistical analysis done by using Kruskal Wallis test followed by post-hoc Dunn's Multiple Comparison Test
* p< 0.05, compared to disease control group; ** p< 0.001, significant compared to disease control group

Table 3. Effect of *B.diffusa* on clonidine induced mast cell degranulation in rats

Groups (n=6 per group)	Intact Mast Cells (%)
Disease Control (5% CMC)	22.5 ± 3.4
Sodium cromoglycate(50mg/kg)	64.3 ± 4.3*#
B.diffusa(200 mg/kg)	41.7 ± 3.5*
B.diffusa(400 mg/kg)	42.7 ± 3.8*
B.diffusa(600 mg/kg)	43.3 ± 2.0*

Values are expressed as mean ± SD. Statistical analysis done by using ANOVA followed by post-hoc Tukey Test
*p<0.001, significant compared to disease control group; #p< 0.001, significant compared to *B.diffusa* 200, 400, and 600 mg/kg groups.

Table 4. Effect of *B.diffusa* on histamine induced bronchoconstriction in guinea pigs

Groups(n=6 per group)	Pre treatment PCT (sec)	Post treatment PCT (sec)	Percent increase in PCT (%protection)
Disease control (5% CMC)	55.17 ± 3.4	55.5 ± 4.2	0.72%
CPM (2mg/kg)	56.83 ± 3.8	306.67 ± 9.4*#	81.53%
B.diffusa(200 mg/kg)	58.5 ± 3.9	163.17 ± 5.1*	64.11%
B.diffusa(400 mg/kg)	54.17 ± 2.9	171.5 ± 5.7*	68.39%
B.diffusa(600 mg/kg)	54 ± 2.5	180.5 ± 4.6*	70.08%

CMC=carboxymethylcellulose; CPM-chlorpheniramine maleate; PCT=preconvulsion time
Values are expressed as mean ± SD. Statistical analysis done by using ANOVA followed by post-hoc Tukey test. * p< 0.001, significant compared to disease control group; # p< 0.001, significant compared to *B.diffusa* 200 mg/kg, *B.diffusa* 400 mg/kg and *B.diffusa* 600 mg/kg groups

DISCUSSION

The current therapeutic options available for the treatment of asthma target pathophysiological processes involved in the disease state. These pathophysiological processes include airway hyperresponsiveness and exaggerated airway narrowing on exposure to various allergens. This inflammatory response may be initially driven by allergen exposure, but it appears to become autonomous so that asthma is essentially incurable. The inflammation may be orchestrated by dendritic cells that regulate Th2 cells that drive eosinophilic inflammation and also IgE formation by B lymphocytes. The mast cells, alveolar macrophages, and epithelial cells of bronchial tissue are also involved in the production of various inflammatory cytokines and play a significant role in the pathogenesis of airway inflammation [8].

On thorough review of literature available it was realized that *B. diffusa* was a plant which is being used traditionally in the form of dhoomapana (smoke inhalation) for relieving asthma [5].A study carried out using aqueous extract of roots of this plant by Odadele *et al* (2011) concluded that it possesses antiinflammatory properties which the author referred could be attributed to its cell membrane stabilizing effect inhibiting the red cell lysis and release of the proinflammatory mediators [7]. Hence, we planned this study to ascertain its antiinflammatory property using experimental models and also to explore the antiallergic potential of this plant so as to provide the scientific rationale for its use in the treatment of asthma.

The selection of models was important; as there are numerous models for evaluating allergic conditions and asthma [11,12,13, 15, 21].Therefore, the selection of

experimental models was based on evaluating the prominent mechanisms involved in asthma, such as the effect on inflammatory cell components, mast cell stabilizing property and bronchial hyperresponsiveness. Also, feasibility, resources and funds available were considered for selection of models.

As the main objective of the first two parts of the experiment were to study the effect of *B.diffusa* on the inflammation and mast cell stabilizing potential, we selected Wistar rats because the pathological changes in the lungs, early as well as late phase changes of inflammation and inflammatory cells in the lungs can be rapidly induced following challenge with an allergen in an experimental setting [11].

The first model used was the ovalbumin (allergen) induced asthma to evaluate the antiinflammatory and antiallergic potential of *B.diffusa*. This model of asthma has been widely used for investigating antiasthmatic potential of drugs in preclinical studies [11,13,22,23,24,25]. This model provides information regarding the level and extent of lung inflammation and allergy, after allergen exposure. Following an allergen challenge, the changes in the various inflammatory cells found in the BAL fluid show an increase in TLC as well as increase in the differential counts. In our study, ovalbumin challenge of sensitized rats elicited an increase in number of total cells, eosinophils, and neutrophils in the BAL fluid compared to that of nonsensitized control group. The results were consistent with the study findings of Alves *et al* (2013), Arora *et al* (2016), and Schster *et al* (2000) which showed similar decrease in the number of total leukocyte count, neutrophils, and eosinophils and attributed this response to possible migration of these cells from blood to bronchial fluid after allergen exposure [23,24,26,].This increase in the inflammatory cells in the BAL fluid was significantly reduced by dexamethasone administered for 15 days. Oral treatment with *B. diffusa* in 3 different doses (200,400, and 600mg/kg)for 15 days also significantly reversed the ovalbumin induced infiltration of all the inflammatory cellsparticularly total leukocyte, neutrophil, and eosinophil counts, the result similar to dexamethasone but not comparable to dexamethasone. There was a dose dependent reduction in the number of inflammatory cells, maximum effect was seen with the dose of 600mg/kg.This dose particularly reduced neutrophil and eosinophil counts in the rat airways, implying the possible role of extract in allergen mediated eosinophilic interventions. The assessment of mononuclear cell count showed that treatment with dexamethasone and the 3 doses of *B.diffusa* had shown a significant increase in the cell count ($p< 0.001$) on comparison with disease control group.

A similar study with repeated exposures of ovalbumin in rats, conducted by Sajida *et al* (2011)also attributed the antiinflammatory activity of the *Ravannapas* plant extract owing to the reduction of leukocytes, eosinophilic and specific down regulation of Th2 responses, inhibiting the expression of intracellular adhesion molecule 1 and hemeoxygenase1 in lung tissue [10].Our results on the TLC and DLC were in accordance with the findings of all these studies thereby supporting the antiinflammatory property of *B. diffusa.*

Histopathological grading of the lung tissue was done with respect to severity of peribronchial infiltration of leukocytes based on cell counts. Dexamethasone group showed maximum suppression of the parenchymal inflammatory infiltrate, ($p< 0.001$) compared to all the treatment groups.The600 mg/kg dose of *B.diffusa* exhibited a significant reduction in airway inflammation versus disease control ($p< 0.05$) but this effect was less in comparison to dexamethasone group, implicating its potential antiinflammatory activity which was less as compared to that of dexamethasone.

The second model used provided an indirect measure of inhibition of the release of inflammatory mediators demonstrating the mast cell stabilizing potential of *B.diffusa*. The two agents which are used for mast cell degranulation include clonidine and compound 48/80, both of which cause dynamic expulsion of granules from mast cells without causing any damage to the cell wall [11,14,27,28, 29,30].It is known that disodium cromoglycate, a mast cell stabilizer prevents degranulation of mast cells and suppresses the release of histamine, eosinophils, neutrophils, and chemotactic factors including leukotrienesC_4,D_4, and E_4, prostaglandins, and platelet activating factor, which are responsible for the severe bronchoconstriction and airway inflammation [11,29,30].In our study, pretreatment with *B.diffusa* in 3 different doses followed by subsequent *ex-vivo* challenge with clonidine showed that all the 3doses provided significant protection when compared to the vehicle control group ($p< 0.01$). All the 3 doses of *B.diffusa* provided a similar degree of protection. Sodium cromoglycate however exerted the highest degree of percentage protection owing to its known mast cell stabilizing property. This finding corroborates with the findings of previous studies and suggests that *B.diffusa* exhibits mast cell stabilizing potential which could be responsible for its bronchodilator property.[24,29]. Several studies that have used this model to evaluate the mast cell stabilizing potential of various compounds having antiasthma activity have obtained similar results and have attributed this effect to the inhibition of histamine release from mast cells [27,28, 29, 30].The third model selected was to evaluate the protective effect of *B.diffusa* on bronchial hyperresponsiveness induced by histamine aerosol in guinea pigs. This is a very old and established model and there are numerous studies done with modifications and improvised techniques over the last few years [15, 16,20,32,33]. Inhalation of histamine causes hypoxia and that leads to subsequent convulsions in guinea pigs. This severe bronchoconstriction observed in guinea pigs is primarily due to the prominent effect of histamine that causes asphyxia.

A recent study published by Suralkar *et al* (2012) on antihistaminic potential of *B. diffusa* also puts some light on its possible mechanism of action. In this study, the authors used a bioassay model of goat tracheal chain contractions for evaluating the bronchodilator potential of an ethanolic extract of *B. diffusa* in guinea pigs [19].The study findings demonstrated that ethanolic extract of *B.diffusa* in the doses of 100, 200, and 400 mg/kg had a significant increase in the PCT of guinea pigs after 1 hour, 4 hours, and 24 hours after treatment with the extract. But in this study there was a possibility of getting the false positive result as the tissue was repeatedly exposed to histamine (1 hour, 4 hours and 24 hours). In such scenario, it is possible that the animals would get desensitized from the effect of histamine, which by itself could increase the PCT by 30% to 50%. However, in our study, after the first histamine aerosol exposure, the second exposure was given to the animal only after a gap of 7 days to prevent desensitization, which was recommended in previous study [33].

The experiment performed on goat tracheal chain by Suralkar *et al* (2012) only corroborates the findings of our experiment on guinea pig bronchial hyper-reactivity [19].

Our study demonstrated comparable increase in the preconvulsion time (PCT) with all the three doses of *B.diffusa*, which was found to be significant as compared to disease control group ($p<0.001$).

Histamine is a major product of mast cell and also a cause of mast cell degranulation. On the other hand it is also a major contributor for bronchial hyperresponsiveness both in guinea pigs as well as humans [34, 35]. This highlights the role of *B. diffusa*in inhibiting the release of histamine from the inflammatory cell mediators, which plays a major roles in an asthmatic episode. This increase in PCT following treatment with *B.diffusa* further supports its bronchodilator and antihistaminic effect as described by other studies reporting similar findings [28, 31].

CONCLUSION

The findings of our present study on 3different models reflects that the aqueous extract of *B. diffusa* holds potential as an antiallergic and anti-inflammatory agent which could be further validated by using animal models specific for evaluating antiasthma drugs and also conducting specific assays for cytokines (interleukin 5, 7, etc.) associated with asthma.

The experiments performed in the 3 models in our study have their own limitations. The use of manual technique instead of an automated technique for assessing differential leukocyte count was one of the drawbacks in the study. Moreover, currently there are many advancements in the evaluation of antiasthma drugs worldwide. There are numerous models which assess the cytokine levels, IgE antibodies and other newer sophisticated techniques [36, 37 38].Such techniques perhaps can give better understanding of the molecular mechanisms involved in the pathogenesis of the disease and also direct in delineating the exact mechanism of action of *B. diffusa.*

CONFLICT OF INTEREST

None

ACKNOWLEDGEMENTS

We are grateful to Diamond Jubilee Society Trust, Seth GS Medical College & KEM Hospital for the financial support. We are also thankful to Shri Dhoothpapeshwar Pvt. Ltd. and Cipla Ltd. Dr. Swapna Bolegave for assisting in manuscript writing.

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