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Evaluation of anti-asthmatic and antioxidant potential of *Boerhavia procumbens* in toluene diisocyanate (TDI) treated rats

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Abstract

Aim of the study: Asthma is an ailment of airways characterized by activation of the T helper (Th) 2 lymphocytes and subsequent movement of inflammatory cells. *Boerhavia procumbens* of family Nyctaginaceae is locally used for the treatment of asthma, cough, hemorrhoids, dropsy, cardiac, eyes and kidney problems. We have evaluated its methanol extract (BPM) as a therapeutic candidate for asthma against toluene diisocyanate (TDI) allergic model in rat.

Materials and methods: The BPM extract was obtained from the whole plant of *B. procumbens* in methanol. Sprague-Dawley male 36 rats (200-250 g) were categorized in to 6 groups having six rats in each category. The animals were provoked (10%) and sensitized (5%) by TDI. Animals of group I-III were; vehicle control (ethyl acetate), diseased control (TDI) and reference control (TDI + dexamethasone {2.5 mg/kg bw}), respectively. Animals of group IV (TDI + 200 mg/kg bw) and group V (TDI + 400 mg/kg bw) were administered with BPM whereas group VI was administered with 400 mg/kg bw alone of BPM. Protective effects of BPM were determined by counting the number of leucocytes and estimation of interleukines in blood, bronchoalveolar lavage (BAL) and in *in vitro* culture of spleen cells. Estimation of antioxidant enzymes, lipid peroxides and H₂O₂ and histopathology of lungs were carried out for antioxidant potential of plant extract used.

Results: Methanol extract of *B. procumbens* suppressed the asthmatic symptoms and inhibited the infiltration of eosinophils and lymphocytes in lungs of TDI provoked rats. Administration of BPM to TDI provoked rats, dose dependently, inhibited the release of interleukins (IL)-2 in serum and IL-4, IL-6 interferon gamma (IFN- γ) in bronchoalveolar lavage (BAL) and in *in vitro* culture of spleen cells, and ameliorated the oxidative stress in lung tissues. Quantitative scoring of the lung histopathology exhibited protective effects of BPM and the inflammation, mucus, thickening of peribronchial smooth muscle layer and subepithelial deposition of collagen induced with TDI were ameliorated.

Conclusion: The BPM has the anti-inflammatory properties that may be used to treat the asthma and inflammatory related ailments.

Keywords: *Boerhavia procumbens*; toluene diisocyanate; asthma; anti-inflammatory; eosinophils; interleukins.

Introduction

Asthma is a respiratory disease caused by chronic inflammation that is mediated by the influx and activation of various inflammatory cells. Its symptoms usually include the reversible airflow obstruction. Two stages; early and late, have been recognized on the basis of mediators and the associated cells. During early phase mast cell degranulation occur while the late phase is characterized by the obstruction of airways (Elliot et al., 2009). During the development of asthma pro-inflammatory cytokines such as IL-4, IL-5 and IL-13 secreted by CD4⁺ Th2 cells play a critical role in the initial phase (Holgate, 2008). IL-4 is reported to be involved in the recruitment of eosinophils via the upregulation of endothelial vascular cell adhesion molecule -1 (VCAM-1). Further, CD4⁺ Th1 cells augment the situation by release of IFN- γ that inhibits the differentiation of Th2 cells (Dai et al., 2009). Ultimately the accumulation of macrophages, neutrophils, eosinophils, lymphocytes, and mast cells in airway takes place leading to airway hyperresponsiveness.

Release of reactive oxygen species (ROS) during the development of asthmatic response further deteriorated the situation while causing lung injuries in diseased animals. Hyperactivity of airways induced with ROS elicits the histamine release from mast cells and mucous secretion from epithelial cells (Lee et al., 2011). During acute asthma exacerbation these changes are associated with cell shedding, enhanced release of arachidonic acid and chemo-attractants (Calabrese et al., 2000) and increased release of hydrogen peroxide and nitrous oxide during exhale (Wewel et al., 2006; Nagaraja et al., 2012).

Asthmatic response is triggered through various agents including viral infections, air pollutants and allergens. Toluene diisocyanate (TDI) is used in various industries and is considered as low molecular weight occupational sensitizer. TDI exposure in both acute and chronic is toxic to human. Malfunctioning and severe injuries are being induced with acute exposure to TDI in the respiratory, gastrointestinal and central nervous systems. It also induces severe irritation of the skin and eyes. However, its chronic exposure may provoke inflammatory response of airways that may develop in to asthma-like symptoms (Pisati et al., 1993; Padoan et al., 2003).

To combat the oxidative stress various non-enzymatic antioxidants; vitamin C, glutathione, vitamin E and albumin exist in the lung lining fluid. In mild form of asthmatic response concentration of the antioxidants is enhanced due to epithelial permeability. Aside from non-enzymatic antioxidants the crucial enzymatic antioxidant are also involved to ameliorate the

oxidative stress. Among the enzymatic antioxidant catalase, superoxide dismutase, peroxidase and glutathione peroxidase are the key biomolecules to combat and neutralize the deleterious effects of ROS. However, during exacerbation episodes of asthma antioxidant system of the lungs is suppressed. Plant species having antioxidant constituents can be added to the system to scavenge the free radicals. Diverse reports have indicated the beneficial effects of fruits, vegetables and medicinal plants in stress related ailments in experimental animals (Sahreen et al., 2014).

Boerhavia procumbens Banks ex Roxb. of family Nyctaginaceae is used in the local system of medicine to treat various ailments. Katewa and Galav (2005) reported the decoction of roots as eye tonic and the use of root paste as poultice in swelling of body, joints and in scorpion sting. Whole plant of *B. procumbens* is used in dysmenorrhea (Qureshi et al., 2008). Traditional healers use the whole plant of *B. procumbens* in anemia, bronchitis, piles, asthma, night blindness, jaundice and in inflammation (Akhtar and Begum, 2009). Its roots are used to cure jaundice (Hussain et al., 2010; Shaheen et al., 2012). The paste of whole plant is externally applied as poultice to treat paralysis (Shaheen et al., 2012). Infusion of whole plant is used to treat dropsy, menstrual flow dysregulation and gonorrhea (Srivastava et al., 2012). Ahmad et al. (2012) reported that whole plant of *B. procumbens* is used as a decoction and infusion in kidney failure, hematuria, as blood purifier and in septic conditions. Hussain et al. (2012) reported the use of whole plant of *B. procumbens* in asthma, edema, eye diseases, heart disease, hemorrhoids jaundice and in various other disorders. The decoction of the root is taken as a remedy in cough, asthma, hernia, dropsy, chest-pain, piles, and swellings. It is also used for gonorrhoea, and internal inflammations (Hussain et al., 2012; Nisar et al., 2014). Kayani et al. (2014) reported that local healers use the whole plant, leaves and roots for the treatment of asthma and cough.

Its phytoconstituents have been determined in the methanol extract and its derived fractions (Bokhari et al., 2015). HPLC analysis of *B. procumbens* was found to be comprised of rutin, kaempferol, catechin, quercetin, caffeic acid and myricetin. Experimentally the plant has shown the antioxidant in various *in vitro* free radical scavenging assays and anti-inflammatory potential in carrageenan induced paw edema in rat (Bokhari et al., 2015). On account of folklore use of whole plant of *B. procumbens* by traditional healers in asthma we have evaluated its anti-asthmatic potential against the toluene diisocyanate (TDI) induced allergic asthma in rat. In this regard we have provoked the rats with TDI and assessed the anti-inflammatory effects of crude

methanol extract of *B. procumbens* by assessing the infiltration of leucocytes, to determine the amount of various cytokines and through antioxidant effects in lung tissues.

Material and methods

Plant extract

In August-September 2010 the whole plant of *B. procumbens* was collected from the campus of Quaid-i-Azam University Islamabad and deposited (#059133) at the herbarium of Pakistan Museum of Natural History, Islamabad. The plant after shade drying was pulverized at 60-mesh size (5 kg) and extracted twice in 95% methanol at 25 °C for 48 h. The filtrate obtained was concentrated on rotary evaporator at 40 °C to get the extract (BPM) 3.5 % of the dry powder.

Animal treatment

The experiment was conducted on 36 Sprague-Dawley male rats (200-250 g); divided equally into 6 different groups (06 animals) and were maintained at room temperature (25 ±3 °C) with 12 h dark/light cycle. The animals had free access to standard laboratory feed and water given *ad libitum*. The experimental use of rats was approved by the Ethical Committee of Quaid-i-Azam University, Islamabad.

Animals of Group I were treated with intranasal ethyl acetate for two rounds of 7 days with middle 7 days of rest. After a week of rest these animals were provoked by administering 5 µl of 5% TDI for 7 days (vehicle control). Animals of Group II-V were treated for 7 consecutive days by dropping 5 µl of 10% TDI in to each nostril. Having a rest of 7 days these animals were again sensitized for 7 days. For provocation 5 µl of 10% TDI was administered after a week of 2nd sensitize course. Rats of Group-II were remained as such (diseased control); whereas 2.5 mg/kg body weight (bw) dexamethasone was administered to Group III animals (reference control). Animals of Group IV and Group V were treated with BPM (200 mg/kg bw and 400 mg/kg bw) respectively. In order to evaluate toxicity induced with BPM; rats of Group VI were administered with BPM (400 mg/kg bw; orally) alone. Animals of the respective groups were administered the drug and the plant sample on daily basis orally a week prior of the experiment and it remained continue for the rest of the experimental period. The last treatment was administered 2 h prior of the provocation dose.

Cytokines determination in serum

After provocation of asthmatic response the blood was collected from the cervical vein of animals; anesthetized with intraperitoneal injection of sodium phenobarbital. For the estimation of cytokines, serum was prepared from the blood and stored at -80°C .

Determination of cell count and cytokines in bronchoalveolar lavage (BAL)

To determine the cytokine concentration and the accumulation of leukocytes in the fluid of lungs, rats were sacrificed, bronchoalveolar lavage (BAL) was collected thrice via trachea with 1 ml of PBS. Cells were separated after centrifugation of the BAL fluid at $500 \times g$ (4°C). The cell pellet was suspended in 1000 μl of PBS to determine the total cell and differential count. The leukocytes were stained with hematoxylin and eosin (Sigma-Aldrich, MO, USA). Total cell number and differential cell count was made using a Neubauer hemocytometer. The supernatant was used for estimation of cytokines.

3.15. Spleen cell culture

From each animal spleen was excised under aseptic conditions and single cell suspension was prepared by pressing between two slide glasses. The suspension was treated with Tris ammonium chloride (5 g/L) to lyse the red blood cells; centrifuged at $200 \times g$ for 5 min at 4°C to get the cell pellet. The cell pellet was washed three times in RPMI 1640. Lastly cell pellet was resuspended in RPMI 1640 supplemented with 100 U/ml penicillin, 10% heat-inactivated fetal bovine serum (Hyclone, UT, USA), 100 $\mu\text{g}/\text{ml}$ streptomycin, and 2 mM 2-mercaptoethanol, 2 mM L-glutamine and 2 $\mu\text{g}/\text{ml}$ concanavalin A (Con A; Sigma-Aldrich, MO, USA). Total cell count was determined after checking cell viability with trypan blue and cells were cultured (5×10^5 cells/well) for 48 h at 37°C . Cytokines were determined in the supernatant.

Cytokine assay

The concentration of different cytokines; IL-2, IL-4, IL-6 and IFN- γ was determined with commercial rat ELISA kits; in serum, BAL fluid and in splenocyte supernatant. Level of IL-2, IL-4, IL-6 and IFN- γ was estimated with kits from OptEIA rat set according to manufacturer's instructions. Intra- and inter-assay coefficient of variation was determined for all the four cytokines studied in this experiment. For this purpose blood was drawn from the tail of rat and subdivided in to three portions and assayed for the four cytokines. Likewise the blood of the same rat was drawn on three different days and assayed for the four cytokines (Table 1). Intra- and inter- assay variations for IL-2 were 5.26% and 5.00% respectively. Intra- and inter- assay

coefficient of variation were obtained for IL-4 (3.12%, 4.72%), IL-6 (4.44%, 4.53%) and for IFN- γ were 5.88% and 8.33% respectively.

Estimation of antioxidant enzymes in lung tissues

1. Protein estimation

The protocol of Lowry et al. (1951) was used to estimate the total soluble proteins present in the tissue. To 0.1 ml of each supernatant sample, 1 ml of alkaline solution was added afterwards, it was mixed thoroughly by vortex machine. The optical density was recorded at 595 nm after an incubation of 30 min. Bovine serum albumin curve was used to figure out the concentration of total serum proteins present in the sample.

2. Catalase assay (CAT)

CAT activity determination is based on the process which depends on the decomposition of H_2O_2 . Lung samples were homogenized with homogenizer and 10X homogenate were prepared by the addition of potassium phosphate buffer (pH 7.0). The homogenate was centrifuged at 4 °C for 10 min at $1500 \times g$. The supernatant was used to determine the catalase activity. A mixture of H_2O_2 and potassium phosphate buffer was prepared by adding 100 μ l of 5.9 mM H_2O_2 and 625 μ l of 50 mM potassium phosphate buffer (pH 5.0). To the mixture 25 μ l of lung tissue homogenate was added. Due to the presence of catalase in the supernatant disappearance of H_2O_2 by catalase occur and it was monitored by a decline in absorbance at 240 nm for 1 min. Absorbance change of 0.01 as units/ min defines one unit CAT activity (Chance and Maehly, 1955).

3. Peroxidase assay (POD)

Chance and Maehly (1955) method was used to determine POD activity spectrophotometrically with minor modifications. POD activity in the lung samples was determined in the supernatant as described earlier. Reaction mixture was prepared by addition of 25 μ l of lung tissue supernatant to 25 μ l of 20 mM guaiacol, 75 μ l of 40 mM H_2O_2 and 625 μ l of 50 mM potassium phosphate buffer (pH 5.0). Change in absorbance of the reaction mixture at 470 nm was recorded for 1 min. One unit of enzyme corresponded to a decrease of 0.01 absorbance / min caused by the POD in the supernatant.

4. Superoxide dismutase assay (SOD)

Using the protocol of Kakkar et al. (1984) SOD activity was determined in various samples of lung. Tissue homogenates were first centrifuged at $1500 \times g$ for 10 min and then for 15 min at

10000 × g. Supernatant then obtained was used to determine the SOD activity in various lung samples. Reaction mixture was prepared by adding 50 µl of 186 µM phenazine methosulphate and 600 µl of 0.052 mM sodium pyrophosphate buffer (pH 7.0). To the reaction mixture 150 µl of lung supernatant was added. In order to start the reaction 100 µl of 780 µM NADH was added and then 500 µl of glacial acetic acid was added after 1 min to stop the reaction. Quantity of chromogen that was formed during the reaction was then evaluated by color intensity which was measured at 560 nm. A system devoid of enzymes served as control. One unit of enzyme activity was determined as the concentration of enzyme required to inhibit the optical density of chromogen by 50% in 1 min and was expressed as units/ mg protein.

5. *Glutathione-S-transferase assay (GST)*

According to Habig et al. (1974) activity of Glutathione-S-transferase is based on the formation of conjugate between reduced glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB). A complete assay mixture without enzyme was used as control. The assay mixture contained 150 µl of lung tissue supernatant, 12.5 µl of 1mM CDNB, 100 µl of 1mM reduced glutathione (GSH) and 720 µl of sodium phosphate buffer (pH 6.5). The CDNB was pre mixed with the sodium phosphate buffer (pH 6.5) and was incubated at 37 °C for 10 min. the reaction was started by adding the GSH followed immediately by the addition of the lung supernatant. The rate increased in absorbance was recorded at 340 nm for 10 min against a complete assay mixture without enzyme and substituted by distilled water. With the help of the molar extinction coefficient (9.6×10^3 /M/cm), enzymatic activity (GST) was determined and expressed as nM CDNB conjugate formed/min/mg protein.

6. *Glutathione reductase assay (GR)*

This method is based on the reduction of glutathione disulfide to reduced glutathione at the expense of NADPH. GSR activity was determined in the lung tissue supernatant at 25 °C in 0.1 M sodium phosphate buffer (pH 7.6) by monitoring of NADPH absorbance decline at 340 nm. The volumes of solutions were 50 µl of lung tissue supernatant, 25 µl of 1mM oxidized glutathione (GSSG), 50 µl of 0.5mM EDTA and 825 µl of 0.1M sodium phosphate buffer (pH 7.6). The reaction was started by the addition of 50 µl of 0.1mM NADPH to the cuvette. The decrease in absorbance at 340 nm (25 °C) was monitored spectrophotometrically for 20 min. The results were presented as dependence of absorbance on time. With the help of the molar

extinction coefficient ($6.22 \times 10^3/\text{M}/\text{cm}$), enzymatic activity (GSR) was expressed as nM NADPH oxidized/min/mg protein (Carlberg and Mannervik, 1975).

7. *Glutathione peroxidase assay (GPx)*

According to Mohandas et al. (1984), GPx activity in lung tissues was determined by monitoring the oxidation of NADPH at 340 nm at 25 °C. Briefly; lung tissue supernatant 50 µl was added to a reaction mixture consisting of 50 µl of 1mM sodium azide, 50 µl of 1mM EDTA, 25 µl of glutathione reductase (1 unit/ml), 25 µl of 1mM GSH, 5 µl of 0.25 mM H_2O_2 and 740 µl of 0.1M sodium phosphate buffer (pH 7.4). The reaction was initiated by the addition of 50 µl of 0.2 mM NADPH to the mixture against the blank containing distilled water. The disappearance of NADPH decreased the absorbance and was recorded at 340 nm for 20 min. With the help of the molar extinction coefficient ($6.22 \times 10^3/\text{M}/\text{cm}$), enzymatic activity (GPx) was expressed as nM of NADPH oxidized/min/mg protein.

Estimation of biomolecules in lung tissues

1. *Reduced glutathione assay (GSH)*

Glutathione reductase potential was evaluated by following the protocol of Jollow et al. (1974). The solution comprised of PBS (1.65 ml), EDTA (0.5 mM, 0.1 ml), 0.1 M, pH 7.6, oxidized glutathione (1 mM, 0.05 ml), NADPH (0.1 mM, 0.1ml) and 10% PMS (0.1 ml) making whole volume up to 2 ml. Enzyme potential was checked at 25°C by observing fading of NADPH at 340 nm, estimated as nM NADPH oxidized min^{-1} mg of protein by means of molar extinction factor ($6.22 \times 10^3/\text{M cm}$).

2. *Lipid peroxidation assay (TBARS) estimation*

Procedure described by Iqbal et al. (1996) was used to measure TBARS (thiobarbituric acid reactive substances) using TBA in the lung tissue homogenate. The reaction mixture consisted of 100 µl of lung tissue homogenate, 10 µl of 100 mM FeCl_3 , 100 µl of 100 mM ascorbic acid and 290 µl of sodium phosphate buffer (pH 7.4). For the duration of 1 h, the mixture was incubated in a shaking water bath at 37 °C. In order to stop the reaction 500 µl of 10% TCA was added. After addition of 500 µl of 0.67% TBA the tubes were placed in boiling water bath for 15 min. Then shifted on crushed ice for 5 min and allowed to centrifuge at $2500 \times g$ for 10 min. In order to determine the amount of TBARS formed, the absorbance of the supernatant was recorded at 535 nm. With the help of the molar extinction coefficient ($1.560 \times 10^5/\text{M}/\text{cm}$), lipid peroxidation activity (TBARS) was expressed as TBARS formed/min/mg tissue.

3. Hydrogen peroxide assay (H_2O_2)

Pick and Keisari (1981) protocol was followed to perform hydrogen peroxide (H_2O_2) assay. The oxidation of phenol red was carried out by H_2O_2 -mediated horseradish peroxidase enzyme. Briefly, the reaction mixture was prepared by the addition of 1 ml of phenol red (0.28 nM) solution, 2.0 ml of lung tissue homogenate, 5.5 nM dextrose, 0.05 M phosphate buffer (pH 7.0). the reaction was initiated by the addition of horseradish peroxidase (8.5 units) and incubated for 60 min at 37 °C. To stop the reaction 0.01 ml of 10 N NaOH was added and centrifugation was done at $800 \times g$ for 5-10 min. The absorbance of the sample was noted at 610 nm by using the reagent as a blank. The concentration of H_2O_2 was given as nM H_2O_2 /min/mg tissue based on the standard curve of H_2O_2 oxidized phenol red.

Histological studies

The tissues that were selected for histology were then weighed and a specific portion of it was chosen to be studied. So the tissue pieces were fixed in fixative sera for 3-4 h, then with the help of ascending grades of alcohol (80%, 90%, and 100%) tissues are dehydrated and finally shifted in cedar wood oil. Tissues after becoming clear were embedded in paraplast and the blocks were prepared for further microtomy. Thin slices (3-4 μm) were prepared with the help of the microtome and then after removing wax, it was stained with hematoxylin-eosin stain and examined by light microscopy. Histopathological changes of the lungs were assessed by means of a quantitative histological score. The degrees of inflammation, airway mucus production, thickening of peribronchial smooth muscle layer, subepithelial collagen deposition were scored from 0 (absent) to 3 (intense). These studies were performed by two pathologists who were unaware of the origin of material during scoring.

Statistical analysis

The values were expressed as means $\pm$ standard deviation (SD) of six observations in each group. To determine the difference among various treatments for *in vivo* studies in rat one way analysis of variance was estimated by using the Graph Pad Prism 5 software. Multiple comparisons among various treatments were determined using Bonferroni *post hoc* comparison test. A P value < 0.05 was considered significant. The histological scores were analyzed by the Kruskal–Wallis test and multiple comparison procedures. When comparing asthmatic rats with dexamethasone and the BPM dosages Student's *t*-test was employed by using the software Statistix 8. A P value < 0.05 was considered significant.

Results

Anti-asthmatic effect of *B. procumbens*

Effect of *B. procumbens* on airway hyperreactivity symptoms

All the animals of different groups were examined after the completion of experimental protocol. Animals treated with TDI showed severe symptoms of asthma; cough, had their mouth open, difficulty in breathing. They also exhibited prolong expiratory phase, sneezing, irritability, and aggression. These exertional symptoms were recorded in all TDI provoked rats. Animals of group treated with dexamethasone exhibited very mild symptoms of asthmatic response whereas those of plant sample treated rats, dose dependently, manifested mild to very mild asthmatic response.

Effect of *B. procumbens* on total leucocyte number in BAL fluid

Provocation of rats with TDI increased the total cell count and each leukocyte subtype in BAL fluid to that of control group. Increase in number of total leukocytes was significant ($P < 0.01$) in TDI sensitized rats to that of the control group (Table 2). Dexamethasone treatment to rats along with TDI restored the total number of leukocytes in the BAL fluid and this decrease in total leukocyte count was significant ($P < 0.05$) as compared to that of the TDI group. Further, co-administration of BPM was able to decrease ($P < 0.05$) the total number of leukocytes in the BAL fluid in TDI sensitized rats. A marked increase in the count of all subtypes was determined in TDI sensitized rats as compared to that of the control group. A significant increase in the number of eosinophils, lymphocytes ($P < 0.05$), neutrophils and monocytes ($P < 0.05$) was exhibited in TDI sensitized rats compared to those of control animals. Co-administration of dexamethasone ameliorated the sensitivity of rats and the count of eosinophils, lymphocytes, monocytes ($P < 0.05$) and neutrophils ($P < 0.05$) was restored near to the control animals (Table 1). Administration of BPM to rats along with TDI significantly reduced the elevated count of these parameters and these effects were more pronounced at the higher dose of BPM (200 mg/kg bw).

Effect of *B. procumbens* on cytokine production in serum

Concentration of IL-4, IL-6 ($P < 0.05$) and IFN- γ ($P < 0.05$) in serum of TDI sensitized rats was elevated significantly compared to control rats. However, the level of IL-2 ($P > 0.05$) in serum was not statistically affected by the administration of TDI to rats as compared to those of control rats. Dexamethasone and BPM administration to rats along with TDI restore the production of

IL-4, IL-6 ($P < 0.05$), IL-2 ($P < 0.05$) towards the control rats (Table 3). However, the level of IFN- γ was non significantly decreased ($P > 0.05$) in serum by the administration of dexamethasone or BPM to rats.

Effect of *B. procumbens* on cytokine production in BAL fluid

In TDI sensitized rats the concentration of IL-4 and IL-6 in BAL fluid was recorded significantly ($P < 0.05$) higher as compared to the control group. The concentration of IFN- γ was elevated to those found in the control rats ($P < 0.05$) but the increase in cytokine concentration IL-2 was not enough to reach the statistical significance (Table 4). Co-treatment of dexamethasone, various doses of BPM with the TDI provocation; the level of all the above parameters were returned towards their normal level. The protective effects of BPM were more evident at the higher dose of 200 mg/kg bw. The administration of BPM alone did not induce significant changes in the level of above parameters.

Effect of *B. procumbens* on cytokine production in spleen cell culture

The results obtained for cytokine production for spleen cell cultured *in vitro* of TDI-sensitized rats produced significantly higher level of IL-4 and IL-6 whereas the level of IL-2 and IFN- γ was elevated ($P > 0.05$) to those of spleen cells from control rats (Table 5). The co-administration of BPM along with TDI ameliorated the toxic effects of TDI and the level of these parameters was recorded near to the control group. Similarly the administration of dexamethasone guarded the change in these parameters in TDI co-treated rats. The level of IL-2, IL-4, IL-6 and IFN- γ with BPM alone did not statistically ($P > 0.05$) change to those of the spleen cells from control rats.

Effect of *B. procumbens* on antioxidant enzymes of lungs

Activity level of catalase, peroxidase, superoxide dismutase and concentration of protein was decreased significantly in lung tissues compared to the control animals (Table 6). Similarly the activities of GPx, GST, and GR were diminished in the lung tissues of rat sensitized with TDI to those of control rats (Table 5). Co-administration of rats with dexamethasone, various doses of BPM separately along with TDI reduced the asthmatic response in rats thereby elevated the level of protein, and all other antioxidant enzymes in the direction of control rats.

Effect of *B. procumbens* on biomolecules of lungs

The provocation of rats with TDI has significantly elevated the level of lipid peroxides and hydrogen peroxide concentration in lung tissues to those of control rats. However, the concentration of GSH in lung tissues of TDI treated rats was significantly lowered as compared

to the control rats. The rats treated with BPM and dexamethasone in combination with TDI restored the level of these parameters towards the control group (Table 7). The protective effects of BPM were recorded in a dose response scheme. The level of lipid peroxides, H_2O_2 and GSH statistically were not altered in BPM alone treated rats to those of control animals.

Effect of *B. procumbens* on pulmonary histopathology

Administration of TDI provoked the asthmatic responses in rats as illustrated by the accumulation of various leucocytes most prominent were the eosinophils in the central and peripheral airways. These changes were accompanied with the infiltration of goblet cells, bronchial lavage and edema in bronchial system (Figure 1). Co-administration of dexamethasone along with TDI alleviated the changes of pulmonary system. Similarly the treatment of BPM at various doses showed protective effects and these changes were exhaustively reduced only with mild pathological changes.

The histological scores obtained for various treatment groups of rat (inflammation, mucus, thickening of peribronchial smooth muscle layer and subepithelial collagen deposition) are presented in Table 8. The comparison of the six groups for histological scoring of various parameters; inflammation, mucus, thickening of peribronchial smooth muscle layer and subepithelial collagen deposition by Kruskal-Wallis test exhibited significant differences among groups ($P = 0.0001$, $P = 0.0007$, $P = 0.0001$, $P = 0.0001$). The histological scores were compared by Student's *t* test and found significant differences ($P < 0.05$) of the asthmatic group with the dexamethasone and the BPM (200 and 400 mg/kg) treated group.

Discussion

In recent years the importance of ethnobotanicals has increased due to a rich storehouse of secondary metabolites. Array of molecules present in plant species have diverse functionality and value to mankind. The ethnobotanical *B. procumbens* is reported to be used in the treatment of various ailments including asthma (Qureshi et al., 2010). Asthma is generally associated with the inflammation of airway passage and its severity is enhanced by the induction of oxidative stress. The *B. procumbens* possesses phytoconstituents endowed with antioxidant and anti-inflammatory properties (Bokhari et al. 2015). The rigorous use of *B. procumbens* in our local system of medicine for the treatment of asthma, prompted us to evaluate the methanol extract of *B. procumbens* for its anti-asthmatic and antioxidant potential in a rat model of toluene diisocyanate induced asthma.

Exposure of rats to TDI increased the inflammatory response of the airway passages; such as irritability, sneezing and troublesome breathing. These conditions are very similar to asthma patients. Activated eosinophils release mediators such as cysteinyl leukotriene C₄ has a role in the smooth muscle contraction of airways thereby exacerbate the breathing process. Dexamethasone, a glucocorticoid is an effective remedy to manage asthma. Symptoms induced with TDI were eliminated with dexamethasone in the experimental rats. However, there are reports that the use of dexamethasone in severe conditions had no effect or possesses side effects (Mjaanes et al., 2006). Herbal medicines have been used for centuries and their diverse physiological effects have been endorsed (Lee et al., 2011; Sahreen et al., 2014; Bokhari et al., 2015; Afsar et al., 2015). The asthmatic symptoms were ameliorated by the administration of BPM to TDI induced asthmatic rats. The rats treated at the highest dose of BPM were normal whereas mild asthmatic symptoms were recorded with low dose of BPM. Similar anti-asthmatic potential of ethnobotanicals have been reported in other studies (Lee et al., 2011).

Activated eosinophils have the ability to infiltrate in various tissues. In asthma patients there is an increased count of eosinophils in the respiratory system; contributing towards the inflammatory condition and dysfunction of lungs. Recruited eosinophils may synthesize many factors including basic proteins and neurotoxins from their granules. These factors enhance the responsiveness and induce damages to the lining of lungs. Further, infiltration of eosinophils is enhanced by the increased vascular permeability causing more severe damages. In this study the count of eosinophils and leukocytes was elevated with TDI exposure in the lining of lung tissues of rat. The treatment of dexamethasone to rats diminished the inflammatory response; the movement of eosinophils and leucocytes in the lining in lung tissues was restored to the normal level. Herbal medicines having anti-inflammatory potential reduced the recruitment of eosinophils in lungs. Statistically similar anti-inflammatory response of BPM at the highest dose to that of dexamethasone was recorded; showing evident potential to treat asthma. These results correlates the anti-inflammatory potential of BPM for carrageenan induced paw edema in rat (Bokhari et al., 2015). These results indicate the remedial potential of BPM against asthma by preventing the recruitment of eosinophils and release of injury causing asthma mediators.

In TDI sensitized rats the count of neutrophils was elevated in the lining of airways. It is suspected that the high level of neutrophil infiltration in airway passages elevated the severity and disease process. Neutrophils have a substantial role to mediate the allergic responses and in

the production of antibodies (Puga et al., 2011). Activated type 2 T-helper (Th2) cells release several cytokines; interleukine (IL)-2, IL-4, IL-6 and IL-13. Among these cytokines; IL-2 plays a pivotal role in the proliferation and maturation of T cells. Switching of B cells to secrete immunoglobulin E (IgE), infiltration of eosinophils and the mucus secretion in airway passages is regulated by IL-4. Provocation of rats with TDI elevated the IL-4 in BAL and in *in vitro* spleen cell culture. Increased infiltration of neutrophils in lungs has been documented in asthma patients (Quint and Wedzicha, 2007). The use of BPM in TDI sensitized rats has prevented the infiltration of neutrophils and suppressed the release of cytokines in BAL and in *in vitro* spleen cell culture. These results indicated that use of *B. procumbens* may be beneficial in asthmatic conditions.

The cytokine interferon gamma (IFN- γ) might also play an important role in asthma. In this study the level of IFN- γ was enhanced in TDI treated rats in the serum, BAL as well as in splenocyte culture but not so to reach the significance level. It has been demonstrated that the level of IFN- γ correlated with clinical symptoms such as responsiveness and obstruction of airways in atopic asthmatic patients (ten Hacken et al., 1998). Increase level of IFN- γ producing CD4⁺ T cells was demonstrated in the peribronchial lymph nodes. Therefore, it was suspected that IFN- γ might contribute towards the steroid resistance in asthma patients (Kumar et al., 2006). Low level of IFN- γ in BPM treated rats in serum, BAL and in *in vitro* culture of splenocytes suggested its beneficial role in the management of asthmatic conditions.

It has been reported that inflammation plays a significant role in the onset of oxidative stress, leading to severe pathogenesis of the inflamed tissue. In this experiment activities of antioxidant enzymes were suppressed whereas concentration of lipid peroxides and H₂O₂ was elevated in lungs of TDI sensitized rats. Catalase and SOD have a role to detoxify H₂O₂ and superoxide anion, respectively. Enhanced generation of lipid peroxides in the cells, damage the biomembranes and simultaneously generate more active free radicals. Glutathione peroxidase (Gpx) plays an important role to protect the cell by scavenging of H₂O₂. Alternatively, increased concentration of H₂O₂ in a cell may have the ability to react with superoxide anion to produce more lethal hydroxyl radicals. Reduced glutathione (GSH) and a variety of antioxidant enzymes of phase II play a pivotal role in scavenging of free radicals and xenobiotics (Sahreen et al., 2014). Decreased concentration of GSH and antioxidant enzymes with TDI were restored by the co-administration of BPM in lung tissues of rats indicating protective potential against oxidative

stress ailments.

Conclusion

The results obtained in this study suggest that *B. procumbens* possesses anti-inflammatory and antioxidant constituents. The use of BPM ameliorated the increase in inflammation and congestion induced with TDI. The protective effects of BPM were also associated with the restoration of cytokines. Its use in asthma and other inflammation related diseases might provide a promising therapeutic candidate.

Conflict of Interest: The authors declare no competing interest.

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Table 1: Intra- and inter- assay (CV=coefficient of variation. Intra assay CV calculated for blood of rat processed simultaneously in three separate tubes. Inter assay CV was determined from the same rat on three separate days. Blood was drawn from the tail vein).

Cytokine	Intra-assay variation				Inter-assay variation			
	1	2	3	CV%	1	2	3	CV%
IL-2	19	20	18	5.26	21	19	20	5.00
IL-4	32	33	31	3.12	34	32	31	4.72
IL-6	36	34	33	4.44	32	34	35	4.53
INF- γ	18	17	16	5.88	17	20	18	8.33

Interleukine-2 (IL-2), interleukine-4 (IL-4), interleukine-6 (IL-6), interferon gamma (INF- γ).

Table 2: Effect of BPM on cell count in BAL fluid of rats

Group	Eosinophils %	Lymphocytes %	Neutrophils %	Monocytes %	Total Leukocyte count (10^4 /ml)
Control	0.00 $\pm$ 0.00 ^a	5.91 $\pm$ 2.68 ^a	2.88 $\pm$ 1.21 ^a	75.03 $\pm$ 4.20 ^c	6.933 $\pm$ 0.96 ^a
Asthma	28.56 $\pm$ 2.15 ^c	22.03 $\pm$ 1.9 ^c	5.56 $\pm$ 1.52 ^b	88.0 $\pm$ 6.77 ^d	14.76 $\pm$ 1.27 ^c
Dexa	17.36 $\pm$ 3.71 ^b	11.01 $\pm$ 1.72 ^b	3.03 $\pm$ 1.54 ^a	66.76 $\pm$ 2.49 ^a	8.60 $\pm$ 1.24 ^a
A + BPM 200	21.71 $\pm$ 0.94 ^b	13.72 $\pm$ 3.61 ^b	2.43 $\pm$ 0.85 ^a	74.62 $\pm$ 3.75 ^{bc}	9.367 $\pm$ 1.23 ^b
A+ BPM 400	18.81 $\pm$ 1.86 ^b	10.09 $\pm$ 1.15 ^b	2.50 $\pm$ 1.39 ^a	74.09 $\pm$ 2.80 ^{bc}	6.85 $\pm$ 0.51 ^{ab}
BPM 400	0.00 $\pm$ 0.00 ^a	5.39 $\pm$ 1.82 ^a	3.19 $\pm$ 1.38 ^a	71.91 $\pm$ 3.71 ^b	6.25 $\pm$ 0.47 ^a

Mean $\pm$ SD (06). Means not sharing the same alphabet in the same column are significantly different at $P < 0.05$. Asthma; TDI sensitized rats; Dexa; Dexamethasone treated rats; A; Asthmatic; BPM; *B. procumbens* methanol extract.

Table 3: Effect of BPM on cytokines in blood serum of rats

Group	IL-2 pg/ml	IL-4 pg/ml	IL-6 pg/ml	INF- γ pg/ml
Control	19.0 $\pm$ 1.78 ^a	31.5 $\pm$ 2.58 ^a	34.5 $\pm$ 2.34 ^{ab}	18.33 $\pm$ 1.86 ^a
Asthma	21.16 $\pm$ 1.72 ^a	50.83 $\pm$ 2.78 ^c	42.0 $\pm$ 2.96 ^c	22.16 $\pm$ 1.47 ^b
Dexa	18.16 $\pm$ 2.40 ^b	31.33 $\pm$ 2.50 ^a	32.16 $\pm$ 2.31 ^a	18.83 $\pm$ 1.47 ^a
A + BPM 200	20.0 $\pm$ 1.78 ^a	41.0 $\pm$ 1.78 ^b	36.0 $\pm$ 2.89 ^b	20.0 $\pm$ 2.09 ^a
A+ BPM 400	20.50 $\pm$ 1.87 ^a	33.0 $\pm$ 2.82 ^a	34.67 $\pm$ 1.96 ^{ab}	18.67 $\pm$ 1.86 ^a
BPM 400	21.0 $\pm$ 1.41 ^a	30.33 $\pm$ 2.58 ^a	31.67 $\pm$ 2.42 ^a	18.17 $\pm$ 1.94 ^a

Mean $\pm$ SD (06). Means not sharing the same alphabet in the same column are significantly different at $P < 0.05$. Asthma; TDI sensitized rats; Dexa; Dexamethasone treated rats; A; Asthmatic; BPM; *B. procumbens* methanol extract. Interleukine-2 (IL-2), interleukine-4 (IL-4), interleukine-6 (IL-6), interferon gamma (INF- γ).

Table 4: Effect of BPM on cytokines in BAL fluid of rats

Group	IL-2 pg/ml	IL-4 pg/ml	IL-6 pg/ml	INF- γ pg/ml
Control	20.0 $\pm$ 2.60 ^a	22.83 $\pm$ 2.48 ^a	20.50 $\pm$ 2.16 ^a	20.67 $\pm$ 2.33 ^a
Asthma	22.0 $\pm$ 1.54 ^b	32.0 $\pm$ 2.82 ^b	50.50 $\pm$ 3.33 ^d	24.33 $\pm$ 2.33 ^b
Dexa	18.67 $\pm$ 2.16 ^a	21.67 $\pm$ 2.65 ^a	24.33 $\pm$ 2.94 ^b	21.67 $\pm$ 2.25 ^a
A + BPM 200	20.67 $\pm$ 2.06 ^a	23.17 $\pm$ 1.32 ^a	35.67 $\pm$ 3.20 ^c	23.67 $\pm$ 2.06 ^a
A+ BPM 400	19.33 $\pm$ 2.42 ^a	22.17 $\pm$ 1.72 ^a	25.50 $\pm$ 3.50 ^b	22.33 $\pm$ 1.75 ^a
BPM 400	21.0 $\pm$ 1.67 ^{ab}	21.83 $\pm$ 1.47 ^a	20.17 $\pm$ 2.48 ^a	21.0 $\pm$ 1.41 ^a

Mean $\pm$ SD (06). Means not sharing the same alphabet in the same column are significantly different at $P < 0.05$. Asthma; TDI sensitized rats; Dexa; Dexamethasone treated rats; A; Asthmatic; BPM; *B. procumbens* methanol extract. Interleukine-2 (IL-2), interleukine-4 (IL-4), interleukine-6 (IL-6), interferon gamma (INF- γ).

Table 5: Effect of BPM on cytokines in *in vitro* culture of spleen cells

Group	IL-2 pg/ml	IL-4 pg/ml	IL-6 pg/ml	INF- γ pg/ml
Control	45.0 $\pm$ 1.60 ^b	41.83 $\pm$ 1.16 ^a	48.50 $\pm$ 1.94 ^b	24.33 $\pm$ 1.14 ^b
Asthma	49.83 $\pm$ 1.81 ^c	108.37 $\pm$ 1.78 ^d	107.67 $\pm$ 1.94 ^d	30.17 $\pm$ 1.60 ^c
Dexa	43.0 $\pm$ 0.96 ^a	44.33 $\pm$ 1.42 ^a	44.83 $\pm$ 1.40 ^a	24.33 $\pm$ 1.11 ^b
A + BPM 200	47.50 $\pm$ 1.33 ^{bc}	80.17 $\pm$ 1.83 ^c	81.67 $\pm$ 1.67 ^c	26.16 $\pm$ 1.16 ^b
A+ BPM 400	45.17 $\pm$ 0.90 ^b	48.17 $\pm$ 1.95 ^b	50.50 $\pm$ 1.58 ^b	21.67 $\pm$ 1.30 ^a
BPM 400	42.67 $\pm$ 0.95 ^a	42.83 $\pm$ 1.01 ^a	48.50 $\pm$ 1.17 ^b	22.50 $\pm$ 1.28 ^{ab}

Mean $\pm$ SD (06). Means not sharing the same alphabet in the same column are significantly different at $P < 0.05$. Asthma; TDI sensitized rats; Dexa; Dexamethasone treated rats; A; Asthmatic; BPM; *B. procumbens* methanol extract. Interleukine-2 (IL-2), interleukine-4 (IL-4), interleukine-6 (IL-6), interferon gamma (INF- γ).

Table 6: Effects of BPM on antioxidant enzymes in lungs of rat

Group	CAT (U/min)	POD (U/min)	SOD (U/mg protein)	GST (nM/min /mg protein)	GPx (nM/min /mg protein)	GR (nM/min /mg protein)
Control	4.28±0.32 ^a	10.04±0.43 ^a	3.46±0.24 ^a	152.82±3.73 ^b	117.21±3.29 ^a	243.27±5.32 ^{ab}
Asthma	2.13±0.51 ^d	5.72±0.72 ^d	1.10±0.07 ^d	76.29±3.48 ^e	65.08±2.57 ^e	130.38±3.64 ^e
Dexa	4.19±0.41 ^a	10.17±0.51 ^a	3.53±0.31 ^a	159.43±3.31 ^a	123.48±4.12 ^b	238.72±4.62 ^b
A+BPM 200	3.60±0.36 ^c	7.53±0.42 ^d	1.76±0.48 ^c	100.48±2.03 ^d	85.40±1.28 ^d	173.51±4.82 ^d
A+BPM 400	3.92±0.17 ^{bc}	9.60±0.27 ^b	2.77±0.08 ^b	138.28±2.51 ^c	94.02±1.56 ^c	203.52±3.35 ^c
BPM 400	4.25±0.45 ^a	10.15±0.48 ^a	3.48±0.16 ^a	155.73±3.02 ^{ab}	115.74±3.63 ^b	246.82±2.84 ^a

Mean ± SD (06). Means not sharing the same alphabet in the same column are significantly different at $P < 0.05$. Asthma; TDI sensitized rats: Dexa; Dexamethasone treated rats: A; Asthmatic: BPM; *B. procumbens* methanol extract. Catalase (CAT), peroxidase (POD), superoxide dismutase (SOD), glutathione-S-transferase (GST), glutathione peroxidase (GPx), glutathione reductase (GR).

Table 7: Effects of BPM on tissue proteins and biomolecules in lungs of rat

Group	Protein (µg/mg tissue)	GSH (µM/g tissue)	TBARS (nM/min /mg protein)	H ₂ O ₂ (nM/min /mg tissue)
Control	2.07±0.145 ^a	25.62±1.5 ^a	3.39±0.42 ^d	1.23±0.162 ^d
Asthma	1.99±0.118 ^a	22.91±1.8 ^b	3.32±0.13 ^d	1.13±0.153 ^e
Dexa	0.71±0.111 ^d	08.23±0.9 ^c	5.27±0.68 ^a	2.43±0.165 ^a
A + BPM 200	0.93±0.082 ^c	13.65±0.2 ^d	4.91±0.11 ^b	2.01±0.133 ^b
A+ BPM 400	1.69±0.169 ^b	18.90±0.6 ^c	3.89±0.15 ^c	1.81±0.141 ^c
BPM 400	2.03±0.118 ^a	23.26±1.5 ^{ab}	3.34±0.21 ^d	1.29±0.181 ^d

Mean ± SD (06). Means not sharing the same alphabet in the same column are significantly different at $P < 0.05$. Asthma; TDI sensitized rats: Dexa; Dexamethasone treated rats: A; Asthmatic: BPM; *B. procumbens* methanol extract. Reduced glutathione (GSH), thiobarbituric acid reactant substances (TBARS), hydrogen peroxide (H₂O₂).

Table 8: Effect of BPM on lung histology score

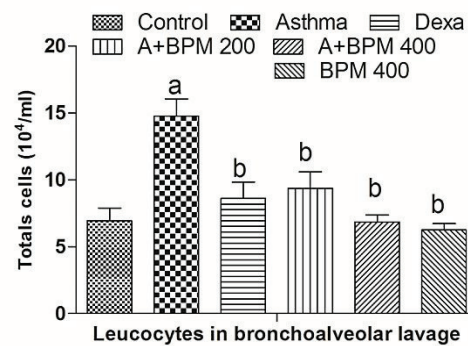
Control	Asthma	Dexa	A + BPM 200	A + BPM 400	BPM 400	P* value
Inflammation						
0	3	1	2	1	0	0.0001
0	2	1	2	1	0	
0	3	2	2	1	1	
0	2	1	2	1	0	
0	3	1	2	2	0	
0	2	1	1	2	0	
Airway mucus production						
1	3	1	2	1	1	0.0007
1	3	2	2	1	1	
0	2	1	1	0	0	
0	3	2	1	2	0	
1	2	0	1	1	0	
0	3	2	2	2	0	
Thickening of peribronchial smooth muscle layer						
1	2	2	2	2	0	0.0001
0	3	0	2	1	1	
1	3	1	2	2	1	
1	3	1	2	1	0	
1	2	2	2	1	0	
1	2	1	2	1	0	
Subepithelium collagen deposition						
0	1	1	1	1	0	0.0004
0	2	1	1	2	0	
0	2	1	2	1	0	
0	2	0	1	1	1	
0	3	1	2	1	1	
0	1	0	1	1	1	

P* value for comparison of six groups (Kruskal Wallis test)

Asthma; TDI sensitized rats: Dexa; Dexamethasone treated rats: A; Asthmatic: BPM; *B. procumbens* methanol extract.

Figure 1: Histopathological alterations and effect of *B. procumbens* in sections of lung and bronchioles. A) Shows normal view of lung pathology, (B) Shows typical damaged lung tissue with leukocyte infiltration, thickening of peribronchial smooth muscle layer, subepithelial collagen deposition (C) Shows less leukocyte infiltration by dexamethasone treatment (D) Shows less leukocyte infiltration by *B. procumbens*, E) Almost near to normal pathology with very less infiltration, F) normal lung pathology

Graphical Abstract



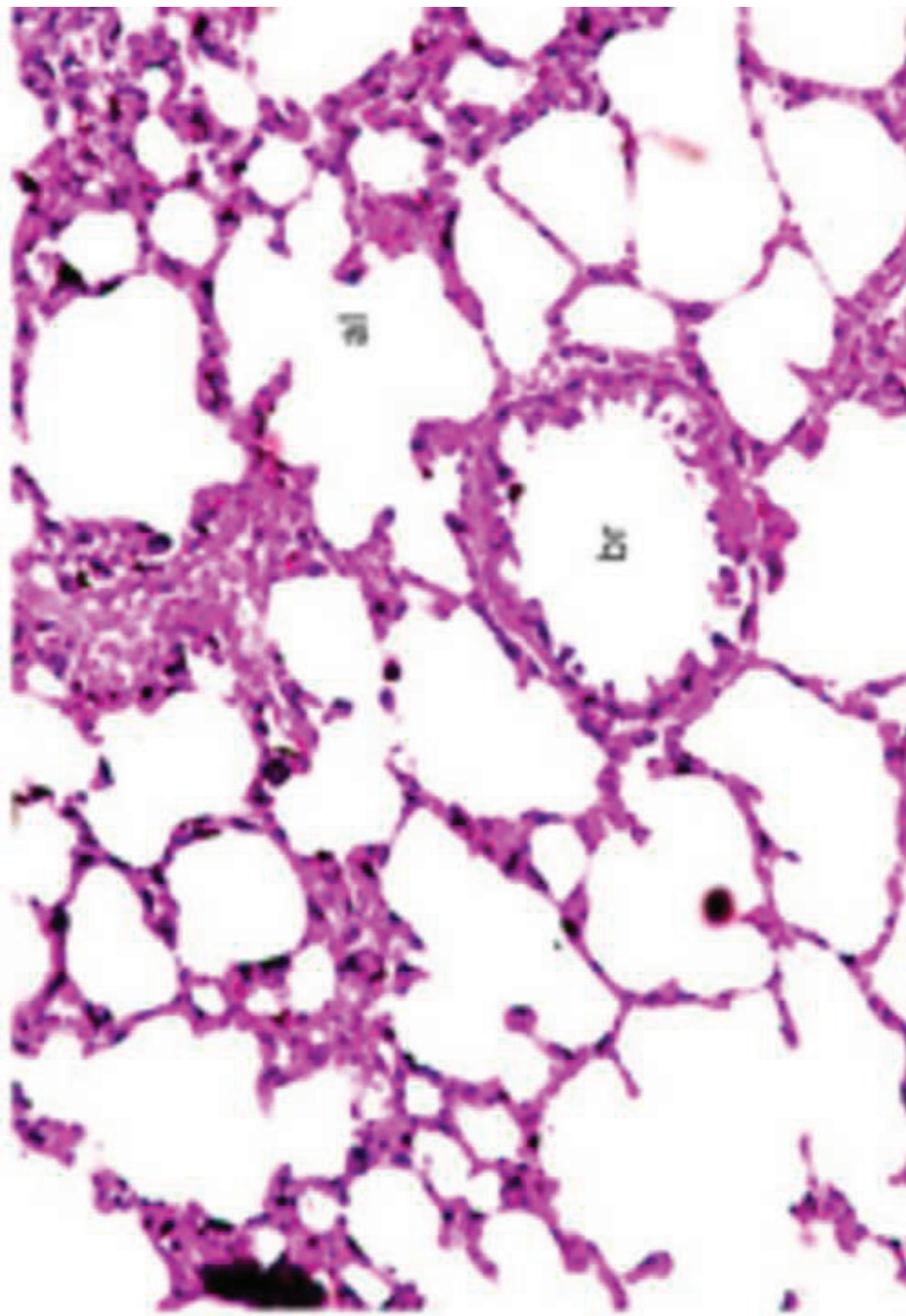


Figure 1A



Figure 1B

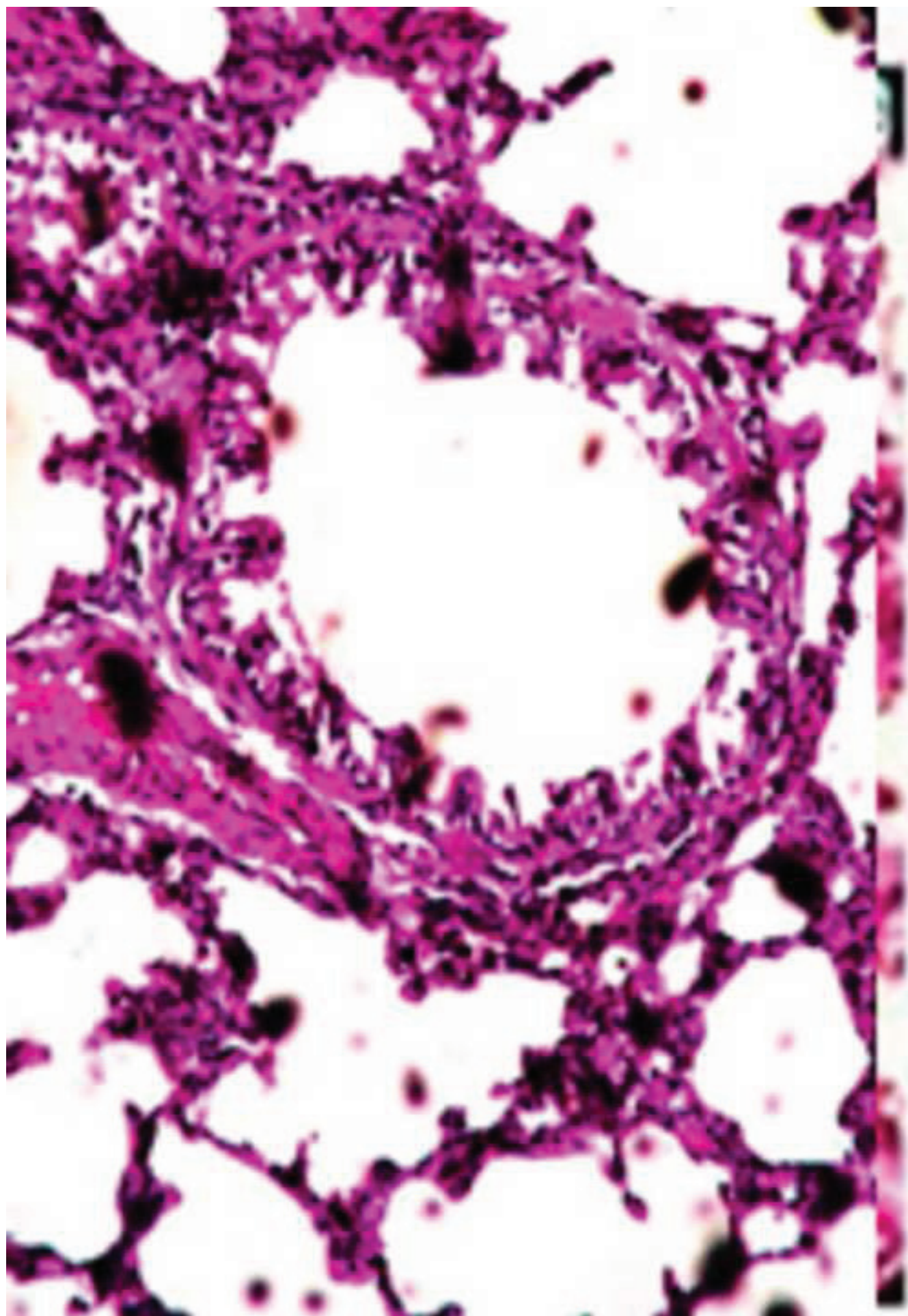


Figure 1C

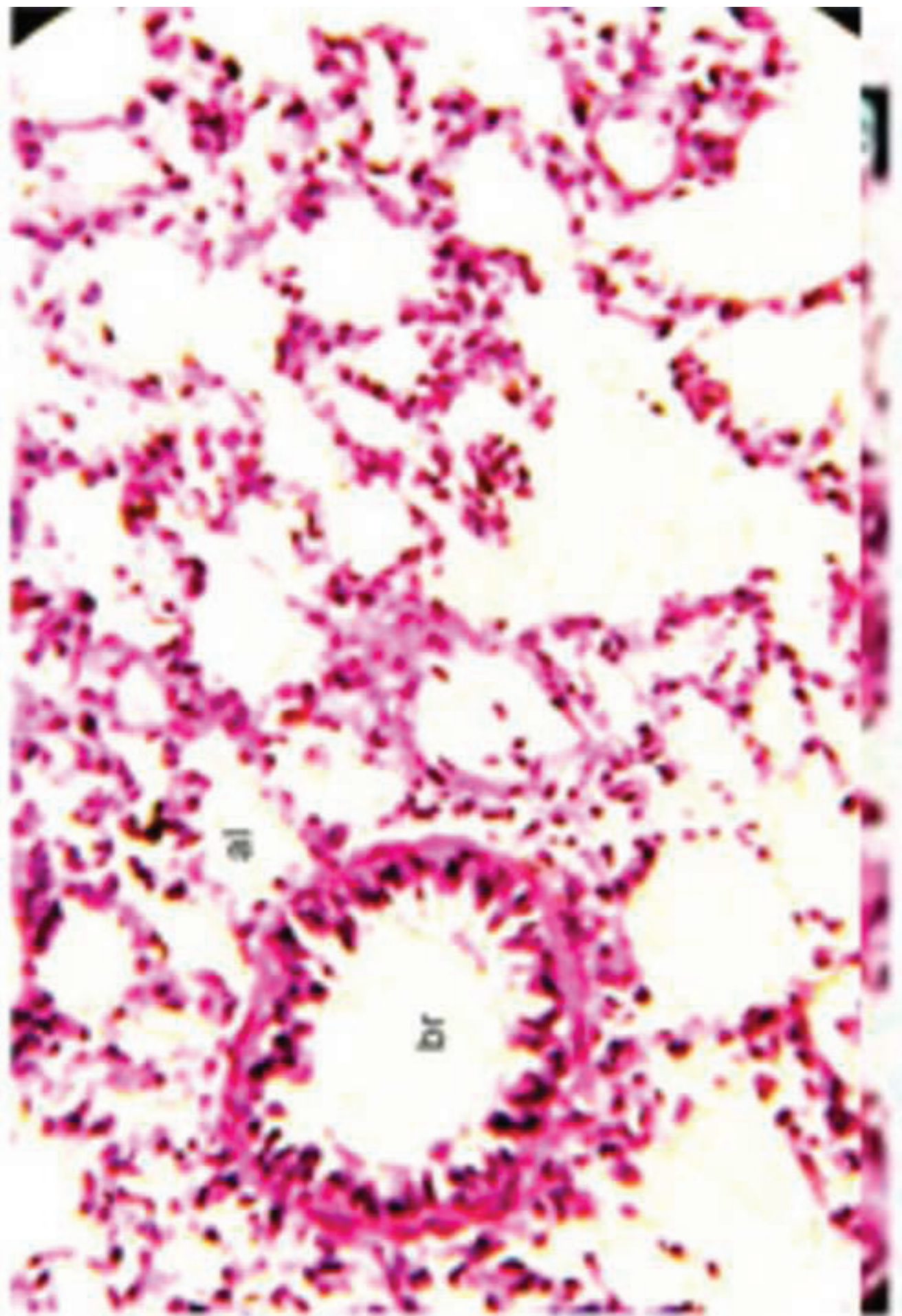


Figure 1D

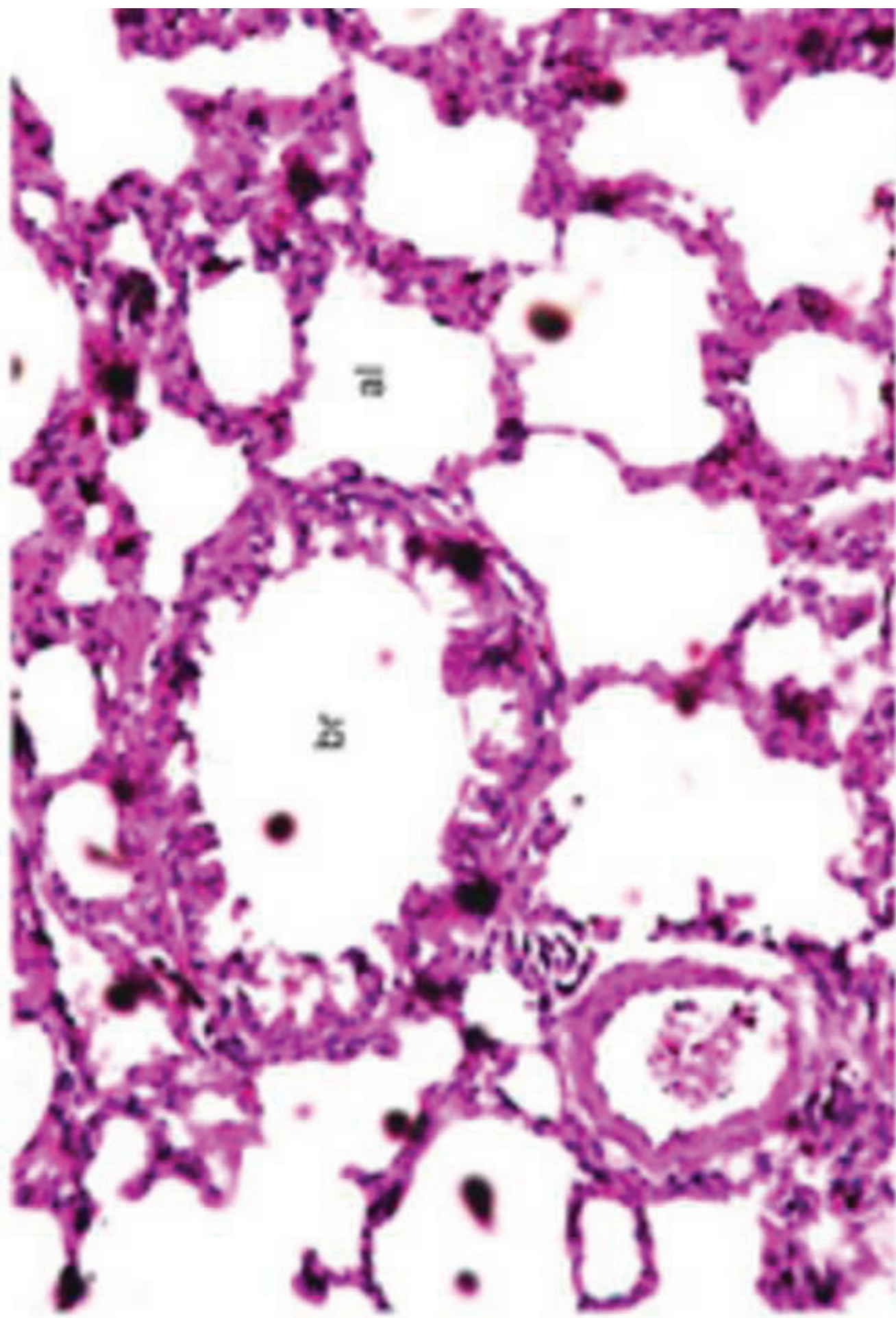


Figure 1E

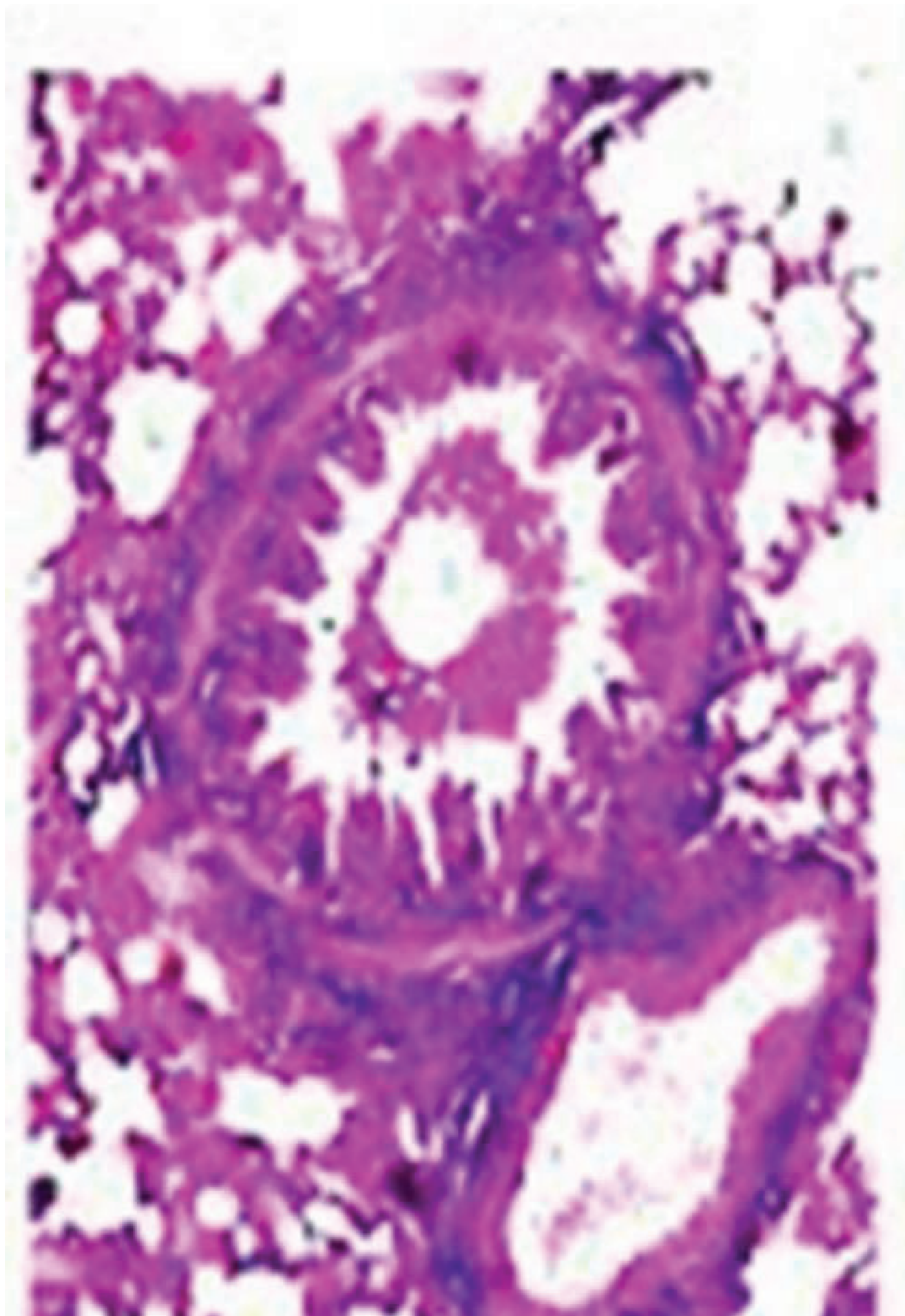


Figure 1F

Evaluation of anti-asthmatic and antioxidant potential of *Boerhavia procumbens* in toluene diisocyanate (TDI) treated rats

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Abstract

Aim of the study: Asthma is an ailment of airways characterized by activation of the T helper (Th) 2 lymphocytes and subsequent movement of inflammatory cells. *Boerhavia procumbens* of family Nyctaginaceae is locally used for the treatment of asthma, cough, hemorrhoids, dropsy, cardiac, eyes and kidney problems. We have evaluated its methanol extract (BPM) as a therapeutic candidate for asthma against toluene diisocyanate (TDI) allergic model in rat.

Materials and methods: The BPM extract was obtained from the whole plant of *B. procumbens* in methanol. Sprague-Dawley male 36 rats (200-250 g) were categorized in to 6 groups having six rats in each category. The animals were provoked (10%) and sensitized (5%) by TDI. Animals of group I-III were; vehicle control (ethyl acetate), diseased control (TDI) and reference control (TDI + dexamethasone {2.5 mg/kg bw}), respectively. Animals of group IV (TDI + 200 mg/kg bw) and group V (TDI + 400 mg/kg bw) were administered with BPM whereas group VI was administered with 400 mg/kg bw alone of BPM. Protective effects of BPM were determined by counting the number of leucocytes and estimation of interleukines in blood, bronchoalveolar lavage (BAL) and in *in vitro* culture of spleen cells. Estimation of antioxidant enzymes, lipid peroxides and H₂O₂ and histopathology of lungs were carried out for antioxidant potential of plant extract used.

Results: Methanol extract of *B. procumbens* suppressed the asthmatic symptoms and inhibited the infiltration of eosinophils and lymphocytes in lungs of TDI provoked rats. Administration of BPM to TDI provoked rats, dose dependently, inhibited the release of interleukins (IL)-2 in serum and IL-4, IL-6 interferon gamma (IFN- γ) in bronchoalveolar lavage (BAL) and in *in vitro* culture of spleen cells, and ameliorated the oxidative stress in lung tissues. Quantitative scoring of the lung histopathology exhibited protective effects of BPM and the inflammation, mucus, thickening of peribronchial smooth muscle layer and subepithelial deposition of collagen induced with TDI were ameliorated.

Conclusion: The BPM has the anti-inflammatory properties that may be used to treat the asthma and inflammatory related ailments.

Keywords: *Boerhavia procumbens*; toluene diisocyanate; asthma; anti-inflammatory; eosinophils; interleukins.

Introduction

Asthma is a respiratory disease caused by chronic inflammation that is mediated by the influx and activation of various inflammatory cells. Its symptoms usually include the reversible airflow obstruction. Two stages; early and late, have been recognized on the basis of mediators and the associated cells. During early phase mast cell degranulation occur while the late phase is characterized by the obstruction of airways (Elliot et al., 2009). During the development of asthma pro-inflammatory cytokines such as IL-4, IL-5 and IL-13 secreted by CD4⁺ Th2 cells play a critical role in the initial phase (Holgate, 2008). IL-4 is reported to be involved in the recruitment of eosinophils via the upregulation of endothelial vascular cell adhesion molecule -1 (VCAM-1). Further, CD4⁺ Th1 cells augment the situation by release of IFN- γ that inhibits the differentiation of Th2 cells (Dai et al., 2009). Ultimately the accumulation of macrophages, neutrophils, eosinophils, lymphocytes, and mast cells in airway takes place leading to airway hyperresponsiveness.

Release of reactive oxygen species (ROS) during the development of asthmatic response further deteriorated the situation while causing lung injuries in diseased animals. Hyperactivity of airways induced with ROS elicits the histamine release from mast cells and mucous secretion from epithelial cells (Lee et al., 2011). During acute asthma exacerbation these changes are associated with cell shedding, enhanced release of arachidonic acid and chemo-attractants (Calabrese et al., 2000) and increased release of hydrogen peroxide and nitrous oxide during exhale (Wewel et al., 2006; Nagaraja et al., 2012).

Asthmatic response is triggered through various agents including viral infections, air pollutants and allergens. Toluene diisocyanate (TDI) is used in various industries and is considered as low molecular weight occupational sensitizer. TDI exposure in both acute and chronic is toxic to human. Malfunctioning and severe injuries are being induced with acute exposure to TDI in the respiratory, gastrointestinal and central nervous systems. It also induces severe irritation of the skin and eyes. However, its chronic exposure may provoke inflammatory response of airways that may develop in to asthma-like symptoms (Pisati et al., 1993; Padoan et al., 2003).

To combat the oxidative stress various non-enzymatic antioxidants; vitamin C, glutathione, vitamin E and albumin exist in the lung lining fluid. In mild form of asthmatic response concentration of the antioxidants is enhanced due to epithelial permeability. Aside from non-enzymatic antioxidants the crucial enzymatic antioxidant are also involved to ameliorate the

oxidative stress. Among the enzymatic antioxidant catalase, superoxide dismutase, peroxidase and glutathione peroxidase are the key biomolecules to combat and neutralize the deleterious effects of ROS. However, during exacerbation episodes of asthma antioxidant system of the lungs is suppressed. Plant species having antioxidant constituents can be added to the system to scavenge the free radicals. Diverse reports have indicated the beneficial effects of fruits, vegetables and medicinal plants in stress related ailments in experimental animals (Sahreen et al., 2014).

Boerhavia procumbens Banks ex Roxb. of family Nyctaginaceae is used in the local system of medicine to treat various ailments. Katewa and Galav (2005) reported the decoction of roots as eye tonic and the use of root paste as poultice in swelling of body, joints and in scorpion sting. Whole plant of *B. procumbens* is used in dysmenorrhea (Qureshi et al., 2008). Traditional healers use the whole plant of *B. procumbens* in anemia, bronchitis, piles, asthma, night blindness, jaundice and in inflammation (Akhtar and Begum, 2009). Its roots are used to cure jaundice (Hussain et al., 2010; Shaheen et al., 2012). The paste of whole plant is externally applied as poultice to treat paralysis (Shaheen et al., 2012). Infusion of whole plant is used to treat dropsy, menstrual flow dysregulation and gonorrhea (Srivastava et al., 2012). Ahmad et al. (2012) reported that whole plant of *B. procumbens* is used as a decoction and infusion in kidney failure, hematuria, as blood purifier and in septic conditions. Hussain et al. (2012) reported the use of whole plant of *B. procumbans* in asthma, edema, eye diseases, heart disease, hemorrhoids jaundice and in various other disorders. The decoction of the root is taken as a remedy in cough, asthma, hernia, dropsy, chest-pain, piles, and swellings. It is also used for gonorrhoea, and internal inflammations (Hussain et al., 2012; Nisar et al., 2014). Kayani et al. (2014) reported that local healers use the whole plant, leaves and roots for the treatment of asthma and cough.

Its phytoconstituents have been determined in the methanol extract and its derived fractions (Bokhari et al., 2015). HPLC analysis of *B. procumbens* was found to be comprised of rutin, kaempferol, catechin, quercetin, caffeic acid and myricetin. Experimentally the plant has shown the antioxidant in various *in vitro* free radical scavenging assays and anti-inflammatory potential in carrageenan induced paw edema in rat (Bokhari et al., 2015). On account of folklore use of whole plant of *B. procumbens* by traditional healers in asthma we have evaluated its anti-asthmatic potential against the toluene diisocyanate (TDI) induced allergic asthma in rat. In this regard we have provoked the rats with TDI and assessed the anti-inflammatory effects of crude

methanol extract of *B. procumbens* by assessing the infiltration of leucocytes, to determine the amount of various cytokines and through antioxidant effects in lung tissues.

Material and methods

Plant extract

In August-September 2010 the whole plant of *B. procumbens* was collected from the campus of Quaid-i-Azam University Islamabad and deposited (#059133) at the herbarium of Pakistan Museum of Natural History, Islamabad. The plant after shade drying was pulverized at 60-mesh size (5 kg) and extracted twice in 95% methanol at 25 °C for 48 h. The filtrate obtained was concentrated on rotary evaporator at 40 °C to get the extract (BPM) 3.5 % of the dry powder.

Animal treatment

The experiment was conducted on 36 Sprague-Dawley male rats (200-250 g); divided equally into 6 different groups (06 animals) and were maintained at room temperature (25 ±3 °C) with 12 h dark/light cycle. The animals had free access to standard laboratory feed and water given *ad libitum*. The experimental use of rats was approved by the Ethical Committee of Quaid-i-Azam University, Islamabad.

Animals of Group I were treated with intranasal ethyl acetate for two rounds of 7 days with middle 7 days of rest. After a week of rest these animals were provoked by administering 5 µl of 5% TDI for 7 days (vehicle control). Animals of Group II-V were treated for 7 consecutive days by dropping 5 µl of 10% TDI in to each nostril. Having a rest of 7 days these animals were again sensitized for 7 days. For provocation 5 µl of 10% TDI was administered after a week of 2nd sensitize course. Rats of Group-II were remained as such (diseased control); whereas 2.5 mg/kg body weight (bw) dexamethasone was administered to Group III animals (reference control). Animals of Group IV and Group V were treated with BPM (200 mg/kg bw and 400 mg/kg bw) respectively. In order to evaluate toxicity induced with BPM; rats of Group VI were administered with BPM (400 mg/kg bw; orally) alone. Animals of the respective groups were administered the drug and the plant sample on daily basis orally a week prior of the experiment and it remained continue for the rest of the experimental period. The last treatment was administered 2 h prior of the provocation dose.

Cytokines determination in serum

After provocation of asthmatic response the blood was collected from the cervical vein of animals; anesthetized with intraperitoneal injection of sodium phenobarbital. For the estimation of cytokines, serum was prepared from the blood and stored at -80°C.

Determination of cell count and cytokines in bronchoalveolar lavage (BAL)

To determine the cytokine concentration and the accumulation of leukocytes in the fluid of lungs, rats were sacrificed, bronchoalveolar lavage (BAL) was collected thrice via trachea with 1 ml of PBS. Cells were separated after centrifugation of the BAL fluid at $500 \times g$ (4°C). The cell pellet was suspended in 1000 μ l of PBS to determine the total cell and differential count. The leukocytes were stained with hematoxylin and eosin (Sigma-Aldrich, MO, USA). Total cell number and differential cell count was made using a Neubauer hemocytometer. The supernatant was used for estimation of cytokines.

3.15. Spleen cell culture

From each animal spleen was excised under aseptic conditions and single cell suspension was prepared by pressing between two slide glasses. The suspension was treated with Tris ammonium chloride (5 g/L) to lyse the red blood cells; centrifuged at $200 \times g$ for 5 min at 4°C to get the cell pellet. The cell pellet was washed three times in RPMI 1640. Lastly cell pellet was resuspended in RPMI 1640 supplemented with 100 U/ml penicillin, 10% heat-inactivated fetal bovine serum (Hyclone, UT, USA), 100 μ g/ml streptomycin, and 2 mM 2-mercaptoethanol, 2 mM L-glutamine and 2 μ g/ml concanavalin A (Con A; Sigma-Aldrich, MO, USA). Total cell count was determined after checking cell viability with trypan blue and cells were cultured (5×10^5 cells/well) for 48 h at 37°C. Cytokines were determined in the supernatant.

Cytokine assay

The concentration of different cytokines; IL-2, IL-4, IL-6 and IFN- γ was determined with commercial rat ELISA kits; in serum, BAL fluid and in splenocyte supernatant. Level of IL-2, IL-4, IL-6 and IFN- γ was estimated with kits from OptEIA rat set according to manufacturer's instructions. Intra- and inter-assay coefficient of variation was determined for all the four cytokines studied in this experiment. For this purpose blood was drawn from the tail of rat and subdivided in to three portions and assayed for the four cytokines. Likewise the blood of the same rat was drawn on three different days and assayed for the four cytokines (Table 1). Intra- and inter- assay variations for IL-2 were 5.26% and 5.00% respectively. Intra- and inter- assay

coefficient of variation were obtained for IL-4 (3.12%, 4.72%), IL-6 (4.44%, 4.53%) and for IFN- γ were 5.88% and 8.33% respectively.

Estimation of antioxidant enzymes in lung tissues

I. Protein estimation

The protocol of Lowry et al. (1951) was used to estimate the total soluble proteins present in the tissue. To 0.1 ml of each supernatant sample, 1 ml of alkaline solution was added afterwards, it was mixed thoroughly by vortex machine. The optical density was recorded at 595 nm after an incubation of 30 min. Bovine serum albumin curve was used to figure out the concentration of total serum proteins present in the sample.

2. Catalase assay (CAT)

CAT activity determination is based on the process which depends on the decomposition of H_2O_2 . Lung samples were homogenized with homogenizer and 10X homogenate were prepared by the addition of potassium phosphate buffer (pH 7.0). The homogenate was centrifuged at 4 °C for 10 min at $1500 \times g$. The supernatant was used to determine the catalase activity. A mixture of H_2O_2 and potassium phosphate buffer was prepared by adding 100 μ l of 5.9 mM H_2O_2 and 625 μ l of 50 mM potassium phosphate buffer (pH 5.0). To the mixture 25 μ l of lung tissue homogenate was added. Due to the presence of catalase in the supernatant disappearance of H_2O_2 by catalase occur and it was monitored by a decline in absorbance at 240 nm for 1 min. Absorbance change of 0.01 as units/ min defines one unit CAT activity (Chance and Maehly, 1955).

3. Peroxidase assay (POD)

Chance and Maehly (1955) method was used to determine POD activity spectrophotometrically with minor modifications. POD activity in the lung samples was determined in the supernatant as described earlier. Reaction mixture was prepared by addition of 25 μ l of lung tissue supernatant to 25 μ l of 20 mM guaiacol, 75 μ l of 40 mM H_2O_2 and 625 μ l of 50 mM potassium phosphate buffer (pH 5.0). Change in absorbance of the reaction mixture at 470 nm was recorded for 1 min. One unit of enzyme corresponded to a decrease of 0.01 absorbance / min caused by the POD in the supernatant.

4. Superoxide dismutase assay (SOD)

Using the protocol of Kakkar et al. (1984) SOD activity was determined in various samples of lung. Tissue homogenates were first centrifuged at $1500 \times g$ for 10 min and then for 15 min at

10000 × g. Supernatant then obtained was used to determine the SOD activity in various lung samples. Reaction mixture was prepared by adding 50 µl of 186 µM phenazine methosulphate and 600 µl of 0.052 mM sodium pyrophosphate buffer (pH 7.0). To the reaction mixture 150 µl of lung supernatant was added. In order to start the reaction 100 µl of 780 µM NADH was added and then 500 µl of glacial acetic acid was added after 1 min to stop the reaction. Quantity of chromogen that was formed during the reaction was then evaluated by color intensity which was measured at 560 nm. A system devoid of enzymes served as control. One unit of enzyme activity was determined as the concentration of enzyme required to inhibit the optical density of chromogen by 50% in 1 min and was expressed as units/ mg protein.

5. *Glutathione-S-transferase assay (GST)*

According to Habig et al. (1974) activity of Glutathione-S-transferase is based on the formation of conjugate between reduced glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB). A complete assay mixture without enzyme was used as control. The assay mixture contained 150 µl of lung tissue supernatant, 12.5 µl of 1mM CDNB, 100 µl of 1mM reduced glutathione (GSH) and 720 µl of sodium phosphate buffer (pH 6.5). The CDNB was pre mixed with the sodium phosphate buffer (pH 6.5) and was incubated at 37 °C for 10 min. the reaction was started by adding the GSH followed immediately by the addition of the lung supernatant. The rate increased in absorbance was recorded at 340 nm for 10 min against a complete assay mixture without enzyme and substituted by distilled water. With the help of the molar extinction coefficient (9.6×10^3 /M/cm), enzymatic activity (GST) was determined and expressed as nM CDNB conjugate formed/min/mg protein.

6. *Glutathione reductase assay (GR)*

This method is based on the reduction of glutathione disulfide to reduced glutathione at the expense of NADPH. GSR activity was determined in the lung tissue supernatant at 25 °C in 0.1 M sodium phosphate buffer (pH 7.6) by monitoring of NADPH absorbance decline at 340 nm. The volumes of solutions were 50 µl of lung tissue supernatant, 25 µl of 1mM oxidized glutathione (GSSG), 50 µl of 0.5mM EDTA and 825 µl of 0.1M sodium phosphate buffer (pH 7.6). The reaction was started by the addition of 50 µl of 0.1mM NADPH to the cuvette. The decrease in absorbance at 340 nm (25 °C) was monitored spectrophotometrically for 20 min. The results were presented as dependence of absorbance on time. With the help of the molar

extinction coefficient ($6.22 \times 10^3/\text{M}/\text{cm}$), enzymatic activity (GSR) was expressed as nM NADPH oxidized/min/mg protein (Carlberg and Mannervik, 1975).

7. Glutathione peroxidase assay (GPx)

According to Mohandas et al. (1984), GPx activity in lung tissues was determined by monitoring the oxidation of NADPH at 340 nm at 25 °C. Briefly; lung tissue supernatant 50 μl was added to a reaction mixture consisting of 50 μl of 1mM sodium azide, 50 μl of 1mM EDTA, 25 μl of glutathione reductase (1 unit/ml), 25 μl of 1mM GSH, 5 μl of 0.25 mM H_2O_2 and 740 μl of 0.1M sodium phosphate buffer (pH 7.4). The reaction was initiated by the addition of 50 μl of 0.2 mM NADPH to the mixture against the blank containing distilled water. The disappearance of NADPH decreased the absorbance and was recorded at 340 nm for 20 min. With the help of the molar extinction coefficient ($6.22 \times 10^3/\text{M}/\text{cm}$), enzymatic activity (GPx) was expressed as nM of NADPH oxidized/min/mg protein.

Estimation of biomolecules in lung tissues

1. Reduced glutathione assay (GSH)

Glutathione reductase potential was evaluated by following the protocol of Jollow et al. (1974). The solution comprised of PBS (1.65 ml), EDTA (0.5 mM, 0.1 ml), 0.1 M, pH 7.6, oxidized glutathione (1 mM, 0.05 ml), NADPH (0.1 mM, 0.1ml) and 10% PMS (0.1 ml) making whole volume up to 2 ml. Enzyme potential was checked at 25°C by observing fading of NADPH at 340 nm, estimated as nM NADPH oxidized min^{-1} mg of protein by means of molar extinction factor ($6.22 \times 10^3/\text{M cm}$).

2. Lipid peroxidation assay (TBARS) estimation

Procedure described by Iqbal et al. (1996) was used to measure TBARS (thiobarbituric acid reactive substances) using TBA in the lung tissue homogenate. The reaction mixture consisted of 100 μl of lung tissue homogenate, 10 μl of 100 mM FeCl_3 , 100 μl of 100 mM ascorbic acid and 290 μl of sodium phosphate buffer (pH 7.4). For the duration of 1 h, the mixture was incubated in a shaking water bath at 37 °C. In order to stop the reaction 500 μl of 10% TCA was added. After addition of 500 μl of 0.67% TBA the tubes were placed in boiling water bath for 15 min. Then shifted on crushed ice for 5 min and allowed to centrifuge at $2500 \times g$ for 10 min. In order to determine the amount of TBARS formed, the absorbance of the supernatant was recorded at 535 nm. With the help of the molar extinction coefficient ($1.560 \times 10^5/\text{M}/\text{cm}$), lipid peroxidation activity (TBARS) was expressed as TBARS formed/min/mg tissue.

3. Hydrogen peroxide assay (H_2O_2)

Pick and Keisari (1981) protocol was followed to perform hydrogen peroxide (H_2O_2) assay. The oxidation of phenol red was carried out by H_2O_2 -mediated horseradish peroxidase enzyme. Briefly, the reaction mixture was prepared by the addition of 1 ml of phenol red (0.28 nM) solution, 2.0 ml of lung tissue homogenate, 5.5 nM dextrose, 0.05 M phosphate buffer (pH 7.0). the reaction was initiated by the addition of horseradish peroxidase (8.5 units) and incubated for 60 min at 37 °C. To stop the reaction 0.01 ml of 10 N NaOH was added and centrifugation was done at $800 \times g$ for 5-10 min. The absorbance of the sample was noted at 610 nm by using the reagent as a blank. The concentration of H_2O_2 was given as nM H_2O_2 /min/mg tissue based on the standard curve of H_2O_2 oxidized phenol red.

Histological studies

The tissues that were selected for histology were then weighed and a specific portion of it was chosen to be studied. So the tissue pieces were fixed in fixative sera for 3-4 h, then with the help of ascending grades of alcohol (80%, 90%, and 100%) tissues are dehydrated and finally shifted in cedar wood oil. Tissues after becoming clear were embedded in paraplast and the blocks were prepared for further microtomy. Thin slices (3-4 μm) were prepared with the help of the microtome and then after removing wax, it was stained with hematoxylin-eosin stain and examined by light microscopy. Histopathological changes of the lungs were assessed by means of a quantitative histological score. The degrees of inflammation, airway mucus production, thickening of peribronchial smooth muscle layer, subepithelial collagen deposition were scored from 0 (absent) to 3 (intense). These studies were performed by two pathologists who were unaware of the origin of material during scoring.

Statistical analysis

The values were expressed as means $\pm$ standard deviation (SD) of six observations in each group. To determine the difference among various treatments for *in vivo* studies in rat one way analysis of variance was estimated by using the Graph Pad Prism 5 software. Multiple comparisons among various treatments were determined using Bonferroni *post hoc* comparison test. A P value < 0.05 was considered significant. The histological scores were analyzed by the Kruskal–Wallis test and multiple comparison procedures. When comparing asthmatic rats with dexamethasone and the BPM dosages Student's *t*-test was employed by using the software Statistix 8. A P value < 0.05 was considered significant.

Results

Anti-asthmatic effect of *B. procumbens*

Effect of *B. procumbens* on airway hyperreactivity symptoms

All the animals of different groups were examined after the completion of experimental protocol. Animals treated with TDI showed severe symptoms of asthma; cough, had their mouth open, difficulty in breathing. They also exhibited prolong expiratory phase, sneezing, irritability, and aggression. These exertional symptoms were recorded in all TDI provoked rats. Animals of group treated with dexamethasone exhibited very mild symptoms of asthmatic response whereas those of plant sample treated rats, dose dependently, manifested mild to very mild asthmatic response.

Effect of *B. procumbens* on total leucocyte number in BAL fluid

Provocation of rats with TDI increased the total cell count and each leukocyte subtype in BAL fluid to that of control group. Increase in number of total leukocytes was significant ($P < 0.01$) in TDI sensitized rats to that of the control group (Table 2). Dexamethasone treatment to rats along with TDI restored the total number of leukocytes in the BAL fluid and this decrease in total leukocyte count was significant ($P < 0.05$) as compared to that of the TDI group. Further, co-administration of BPM was able to decrease ($P < 0.05$) the total number of leukocytes in the BAL fluid in TDI sensitized rats. A marked increase in the count of all subtypes was determined in TDI sensitized rats as compared to that of the control group. A significant increase in the number of eosinophils, lymphocytes ($P < 0.05$), neutrophils and monocytes ($P < 0.05$) was exhibited in TDI sensitized rats compared to those of control animals. Co-administration of dexamethasone ameliorated the sensitivity of rats and the count of eosinophils, lymphocytes, monocytes ($P < 0.05$) and neutrophils ($P < 0.05$) was restored near to the control animals (Table 1). Administration of BPM to rats along with TDI significantly reduced the elevated count of these parameters and these effects were more pronounced at the higher dose of BPM (200 mg/kg bw).

Effect of *B. procumbens* on cytokine production in serum

Concentration of IL-4, IL-6 ($P < 0.05$) and IFN- γ ($P < 0.05$) in serum of TDI sensitized rats was elevated significantly compared to control rats. However, the level of IL-2 ($P > 0.05$) in serum was not statistically affected by the administration of TDI to rats as compared to those of control rats. Dexamethasone and BPM administration to rats along with TDI restore the production of

IL-4, IL-6 ($P < 0.05$), IL-2 ($P < 0.05$) towards the control rats (Table 3). However, the level of IFN- γ was non significantly decreased ($P > 0.05$) in serum by the administration of dexamethasone or BPM to rats.

Effect of *B. procumbens* on cytokine production in BAL fluid

In TDI sensitized rats the concentration of IL-4 and IL-6 in BAL fluid was recorded significantly ($P < 0.05$) higher as compared to the control group. The concentration of IFN- γ was elevated to those found in the control rats ($P < 0.05$) but the increase in cytokine concentration IL-2 was not enough to reach the statistical significance (Table 4). Co-treatment of dexamethasone, various doses of BPM with the TDI provocation; the level of all the above parameters were returned towards their normal level. The protective effects of BPM were more evident at the higher dose of 200 mg/kg bw. The administration of BPM alone did not induce significant changes in the level of above parameters.

Effect of *B. procumbens* on cytokine production in spleen cell culture

The results obtained for cytokine production for spleen cell cultured *in vitro* of TDI-sensitized rats produced significantly higher level of IL-4 and IL-6 whereas the level of IL-2 and IFN- γ was elevated ($P > 0.05$) to those of spleen cells from control rats (Table 5). The co-administration of BPM along with TDI ameliorated the toxic effects of TDI and the level of these parameters was recorded near to the control group. Similarly the administration of dexamethasone guarded the change in these parameters in TDI co-treated rats. The level of IL-2, IL-4, IL-6 and IFN- γ with BPM alone did not statistically ($P > 0.05$) change to those of the spleen cells from control rats.

Effect of *B. procumbens* on antioxidant enzymes of lungs

Activity level of catalase, peroxidase, superoxide dismutase and concentration of protein was decreased significantly in lung tissues compared to the control animals (Table 6). Similarly the activities of GPx, GST, and GR were diminished in the lung tissues of rat sensitized with TDI to those of control rats (Table 5). Co-administration of rats with dexamethasone, various doses of BPM separately along with TDI reduced the asthmatic response in rats thereby elevated the level of protein, and all other antioxidant enzymes in the direction of control rats.

Effect of *B. procumbens* on biomolecules of lungs

The provocation of rats with TDI has significantly elevated the level of lipid peroxides and hydrogen peroxide concentration in lung tissues to those of control rats. However, the concentration of GSH in lung tissues of TDI treated rats was significantly lowered as compared

to the control rats. The rats treated with BPM and dexamethasone in combination with TDI restored the level of these parameters towards the control group (Table 7). The protective effects of BPM were recorded in a dose response scheme. The level of lipid peroxides, H_2O_2 and GSH statistically were not altered in BPM alone treated rats to those of control animals.

Effect of *B. procumbens* on pulmonary histopathology

Administration of TDI provoked the asthmatic responses in rats as illustrated by the accumulation of various leucocytes most prominent were the eosinophils in the central and peripheral airways. These changes were accompanied with the infiltration of goblet cells, bronchial lavage and edema in bronchial system (Figure 1). Co-administration of dexamethasone along with TDI alleviated the changes of pulmonary system. Similarly the treatment of BPM at various doses showed protective effects and these changes were exhaustively reduced only with mild pathological changes.

The histological scores obtained for various treatment groups of rat (inflammation, mucus, thickening of peribronchial smooth muscle layer and subepithelial collagen deposition) are presented in Table 8. The comparison of the six groups for histological scoring of various parameters; inflammation, mucus, thickening of peribronchial smooth muscle layer and subepithelial collagen deposition by Kruskal-Wallis test exhibited significant differences among groups ($P = 0.0001$, $P = 0.0007$, $P = 0.0001$, $P = 0.0001$). The histological scores were compared by Student's *t* test and found significant differences ($P < 0.05$) of the asthmatic group with the dexamethasone and the BPM (200 and 400 mg/kg) treated group.

Discussion

In recent years the importance of ethnobotanicals has increased due to a rich storehouse of secondary metabolites. Array of molecules present in plant species have diverse functionality and value to mankind. The ethnobotanical *B. procumbens* is reported to be used in the treatment of various ailments including asthma (Qureshi et al., 2010). Asthma is generally associated with the inflammation of airway passage and its severity is enhanced by the induction of oxidative stress. The *B. procumbens* possesses phytoconstituents endowed with antioxidant and anti-inflammatory properties (Bokhari et al. 2015). The rigorous use of *B. procumbens* in our local system of medicine for the treatment of asthma, prompted us to evaluate the methanol extract of *B. procumbens* for its anti-asthmatic and antioxidant potential in a rat model of toluene diisocyanate induced asthma.

Exposure of rats to TDI increased the inflammatory **response** of the airway passages; such as irritability, sneezing and troublesome breathing. These conditions are very similar to asthma patients. Activated eosinophils release mediators such as cysteinyl leukotriene C₄ has a role in the smooth muscle contraction of airways thereby exacerbate the breathing process. Dexamethasone, a glucocorticoid is an effective remedy to manage asthma. Symptoms induced with TDI were eliminated with dexamethasone in the experimental rats. However, there are reports that the use of dexamethasone in severe conditions had no effect or possesses side effects (Mjaanes et al., 2006). Herbal medicines have been used for centuries and their diverse physiological effects have been endorsed (Lee et al., 2011; Sahreen et al., 2014; Bokhari et al., 2015; Afsar et al., 2015). The asthmatic symptoms were ameliorated by the administration of BPM to TDI induced asthmatic rats. The rats treated at the highest dose of BPM were normal whereas mild asthmatic symptoms were recorded with low dose of BPM. Similar anti-asthmatic potential of ethnobotanicals have been reported in other studies (Lee et al., 2011).

Activated eosinophils have the ability to infiltrate in various tissues. In asthma patients there is an increased count of eosinophils in the respiratory system; contributing towards the inflammatory condition and dysfunction of lungs. Recruited eosinophils may synthesize many factors including basic proteins and neurotoxins from their granules. These factors enhance the responsiveness and induce damages to the lining of lungs. Further, infiltration of eosinophils is enhanced by the increased vascular permeability causing more severe damages. In this study the count of eosinophils and leukocytes was elevated with TDI exposure in the lining of lung tissues of rat. The treatment of dexamethasone to rats diminished the inflammatory response; the movement of eosinophils and leucocytes in the lining in lung tissues was restored to the normal level. Herbal medicines having anti-inflammatory potential reduced the recruitment of eosinophils in lungs. Statistically similar anti-inflammatory response of BPM at the highest dose to that of dexamethasone was recorded; showing evident potential to treat asthma. These results correlates the anti-inflammatory potential of BPM for carrageenan induced paw edema in rat (Bokhari et al., 2015). These results indicate the remedial potential of BPM against asthma by preventing the recruitment of eosinophils and release of injury causing asthma mediators.

In TDI sensitized rats the count of neutrophils was elevated in the lining of airways. It is suspected that the high level of neutrophil infiltration in airway passages elevated the severity and disease process. Neutrophils have a substantial role to mediate the allergic responses and in

the production of antibodies (Puga et al., 2011). Activated type 2 T-helper (Th2) cells release several cytokines; interleukine (IL)-2, IL-4, IL-6 and IL-13. Among these cytokines; IL-2 plays a pivotal role in the proliferation and maturation of T cells. Switching of B cells to secrete immunoglobulin E (IgE), infiltration of eosinophils and the mucus secretion in airway passages is regulated by IL-4. Provocation of rats with TDI elevated the IL-4 in BAL and in *in vitro* spleen cell culture. Increased infiltration of neutrophils in lungs has been documented in asthma patients (Quint and Wedzicha, 2007). The use of BPM in TDI sensitized rats has prevented the infiltration of neutrophils and suppressed the release of cytokines in BAL and in *in vitro* spleen cell culture. These results indicated that use of *B. procumbens* may be beneficial in asthmatic conditions.

The cytokine interferon gamma (IFN- γ) might also play an important role in asthma. In this study the level of IFN- γ was enhanced in TDI treated rats in the serum, BAL as well as in splenocyte culture but not so to reach the significance level. It has been demonstrated that the level of IFN- γ correlated with clinical symptoms such as responsiveness and obstruction of airways in atopic asthmatic patients (ten Hacken et al., 1998). Increase level of IFN- γ producing CD4⁺ T cells was demonstrated in the peribronchial lymph nodes. Therefore, it was suspected that IFN- γ might contribute towards the steroid resistance in asthma patients (Kumar et al., 2006). Low level of IFN- γ in BPM treated rats in serum, BAL and in *in vitro* culture of splenocytes suggested its beneficial role in the management of asthmatic conditions.

It has been reported that inflammation plays a significant role in the onset of oxidative stress, leading to severe pathogenesis of the inflamed tissue. In this experiment activities of antioxidant enzymes were suppressed whereas concentration of lipid peroxides and H₂O₂ was elevated in lungs of TDI sensitized rats. Catalase and SOD have a role to detoxify H₂O₂ and superoxide anion, respectively. Enhanced generation of lipid peroxides in the cells, damage the biomembranes and simultaneously generate more active free radicals. Glutathione peroxidase (Gpx) plays an important role to protect the cell by scavenging of H₂O₂. Alternatively, increased concentration of H₂O₂ in a cell may have the ability to react with superoxide anion to produce more lethal hydroxyl radicals. Reduced glutathione (GSH) and a variety of antioxidant enzymes of phase II play a pivotal role in scavenging of free radicals and xenobiotics (Sahreen et al., 2014). Decreased concentration of GSH and antioxidant enzymes with TDI were restored by the co-administration of BPM in lung tissues of rats indicating protective potential against oxidative

stress ailments.

Conclusion

The results obtained in this study suggest that *B. procumbens* possesses anti-inflammatory and antioxidant constituents. The use of BPM ameliorated the increase in inflammation and congestion induced with TDI. The protective effects of BPM were also associated with the restoration of cytokines. Its use in asthma and other inflammation related diseases might provide a promising therapeutic candidate.

Conflict of Interest: The authors declare no competing interest.

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Table 1: Intra- and inter- assay (CV=coefficient of variation. Intra assay CV calculated for blood of rat processed simultaneously in three separate tubes. Inter assay CV was determined from the same rat on three separate days. Blood was drawn from the tail vein).

Cytokine	Intra-assay variation				Inter-assay variation			
	1	2	3	CV%	1	2	3	CV%
IL-2	19	20	18	5.26	21	19	20	5.00
IL-4	32	33	31	3.12	34	32	31	4.72
IL-6	36	34	33	4.44	32	34	35	4.53
INF- γ	18	17	16	5.88	17	20	18	8.33

Interleukine-2 (IL-2), interleukine-4 (IL-4), interleukine-6 (IL-4), interferon gamma (INF- γ).

Table 2: Effect of BPM on cell count in BAL fluid of rats

Group	Eosinophils %	Lymphocytes %	Neutrophils %	Monocytes %	Total Leukocyte count (10^4 /ml)
Control	0.00 $\pm$ 0.00 ^a	5.91 $\pm$ 2.68 ^a	2.88 $\pm$ 1.21 ^a	75.03 $\pm$ 4.20 ^c	6.933 $\pm$ 0.96 ^a
Asthma	28.56 $\pm$ 2.15 ^c	22.03 $\pm$ 1.9 ^c	5.56 $\pm$ 1.52 ^b	88.0 $\pm$ 6.77 ^d	14.76 $\pm$ 1.27 ^c
Dexa	17.36 $\pm$ 3.71 ^b	11.01 $\pm$ 1.72 ^b	3.03 $\pm$ 1.54 ^a	66.76 $\pm$ 2.49 ^a	8.60 $\pm$ 1.24 ^a
A + BPM 200	21.71 $\pm$ 0.94 ^b	13.72 $\pm$ 3.61 ^b	2.43 $\pm$ 0.85 ^a	74.62 $\pm$ 3.75 ^{bc}	9.367 $\pm$ 1.23 ^b
A+ BPM 400	18.81 $\pm$ 1.86 ^b	10.09 $\pm$ 1.15 ^b	2.50 $\pm$ 1.39 ^a	74.09 $\pm$ 2.80 ^{bc}	6.85 $\pm$ 0.51 ^{ab}
BPM 400	0.00 $\pm$ 0.00 ^a	5.39 $\pm$ 1.82 ^a	3.19 $\pm$ 1.38 ^a	71.91 $\pm$ 3.71 ^b	6.25 $\pm$ 0.47 ^a

Mean $\pm$ SD (06). Means not sharing the same alphabet in the same column are significantly different at $P < 0.05$. Asthma; TDI sensitized rats; Dexa; Dexamethasone treated rats; A; Asthmatic; BPM; *B. procumbens* methanol extract.

Table 3: Effect of BPM on cytokines in blood serum of rats

Group	IL-2 pg/ml	IL-4 pg/ml	IL-6 pg/ml	INF- γ pg/ml
Control	19.0 $\pm$ 1.78 ^a	31.5 $\pm$ 2.58 ^a	34.5 $\pm$ 2.34 ^{ab}	18.33 $\pm$ 1.86 ^a
Asthma	21.16 $\pm$ 1.72 ^a	50.83 $\pm$ 2.78 ^c	42.0 $\pm$ 2.96 ^c	22.16 $\pm$ 1.47 ^b
Dexa	18.16 $\pm$ 2.40 ^b	31.33 $\pm$ 2.50 ^a	32.16 $\pm$ 2.31 ^a	18.83 $\pm$ 1.47 ^a
A + BPM 200	20.0 $\pm$ 1.78 ^a	41.0 $\pm$ 1.78 ^b	36.0 $\pm$ 2.89 ^b	20.0 $\pm$ 2.09 ^a
A+ BPM 400	20.50 $\pm$ 1.87 ^a	33.0 $\pm$ 2.82 ^a	34.67 $\pm$ 1.96 ^{ab}	18.67 $\pm$ 1.86 ^a
BPM 400	21.0 $\pm$ 1.41 ^a	30.33 $\pm$ 2.58 ^a	31.67 $\pm$ 2.42 ^a	18.17 $\pm$ 1.94 ^a

Mean $\pm$ SD (06). Means not sharing the same alphabet in the same column are significantly different at $P < 0.05$. Asthma; TDI sensitized rats; Dexa; Dexamethasone treated rats; A; Asthmatic; BPM; *B. procumbens* methanol extract. Interleukine-2 (IL-2), interleukine-4 (IL-4), interleukine-6 (IL-4), interferon gamma (INF- γ).

Table 4: Effect of BPM on cytokines in BAL fluid of rats

Group	IL-2 pg/ml	IL-4 pg/ml	IL-6 pg/ml	INF-γ pg/ml
Control	20.0±2.60 ^a	22.83±2.48 ^a	20.50±2.16 ^a	20.67±2.33 ^a
Asthma	22.0±1.54 ^b	32.0±2.82 ^b	50.50±3.33 ^d	24.33±2.33 ^b
Dexa	18.67±2.16 ^a	21.67±2.65 ^a	24.33±2.94 ^b	21.67±2.25 ^a
A + BPM 200	20.67±2.06 ^a	23.17±1.32 ^a	35.67±3.20 ^c	23.67±2.06 ^a
A+ BPM 400	19.33±2.42 ^a	22.17±1.72 ^a	25.50±3.50 ^b	22.33±1.75 ^a
BPM 400	21.0±1.67 ^{ab}	21.83±1.47 ^a	20.17±2.48 ^a	21.0±1.41 ^a

Mean ± SD (06). Means not sharing the same alphabet in the same column are significantly different at $P < 0.05$. Asthma; TDI sensitized rats; Dexa; Dexamethasone treated rats; A; Asthmatic; BPM; *B. procumbens* methanol extract. Interleukine-2 (IL-2), interleukine-4 (IL-4), interleukine-6 (IL-6), interferon gamma (INF-γ).

Table 5: Effect of BPM on cytokines in *in vitro* culture of spleen cells

Group	IL-2 pg/ml	IL-4 pg/ml	IL-6 pg/ml	INF-γ pg/ml
Control	45.0±1.60 ^b	41.83±1.16 ^a	48.50±1.94 ^b	24.33±1.14 ^b
Asthma	49.83±1.81 ^c	108.37±1.78 ^d	107.67±1.94 ^d	30.17±1.60 ^c
Dexa	43.0±0.96 ^a	44.33±1.42 ^a	44.83±1.40 ^a	24.33±1.11 ^b
A + BPM 200	47.50±1.33 ^{bc}	80.17±1.83 ^c	81.67±1.67 ^c	26.16±1.16 ^b
A+ BPM 400	45.17±0.90 ^b	48.17±1.95 ^b	50.50±1.58 ^b	21.67±1.30 ^a
BPM 400	42.67±0.95 ^a	42.83±1.01 ^a	48.50±1.17 ^b	22.50±1.28 ^{ab}

Mean ± SD (06). Means not sharing the same alphabet in the same column are significantly different at $P < 0.05$. Asthma; TDI sensitized rats; Dexa; Dexamethasone treated rats; A; Asthmatic; BPM; *B. procumbens* methanol extract. Interleukine-2 (IL-2), interleukine-4 (IL-4), interleukine-6 (IL-6), interferon gamma (INF-γ).

Table 6: Effects of BPM on antioxidant enzymes in lungs of rat

Group	CAT (U/min)	POD (U/min)	SOD (U/mg protein)	GST (nM/min /mg protein)	GPx (nM/min /mg protein)	GR (nM/min /mg protein)
Control	4.28±0.32 ^a	10.04±0.43 ^a	3.46±0.24 ^a	152.82±3.73 ^b	117.21±3.29 ^a	243.27±5.32 ^{ab}
Asthma	2.13±0.51 ^d	5.72±0.72 ^d	1.10±0.07 ^d	76.29±3.48 ^e	65.08±2.57 ^e	130.38±3.64 ^e
Dexa	4.19±0.41 ^a	10.17±0.51 ^a	3.53±0.31 ^a	159.43±3.31 ^a	123.48±4.12 ^b	238.72±4.62 ^b
A+BPM 200	3.60±0.36 ^c	7.53±0.42 ^d	1.76±0.48 ^c	100.48±2.03 ^d	85.40±1.28 ^d	173.51±4.82 ^d
A+BPM 400	3.92±0.17 ^{bc}	9.60±0.27 ^b	2.77±0.08 ^b	138.28±2.51 ^c	94.02±1.56 ^c	203.52±3.35 ^c
BPM 400	4.25±0.45 ^a	10.15±0.48 ^a	3.48±0.16 ^a	155.73±3.02 ^{ab}	115.74±3.63 ^b	246.82±2.84 ^a

Mean ± SD (06). Means not sharing the same alphabet in the same column are significantly different at $P < 0.05$. Asthma; TDI sensitized rats: Dexa; Dexamethasone treated rats: A; Asthmatic: BPM; *B. procumbens* methanol extract. Catalase (CAT), peroxidase (POD), superoxide dismutase (SOD), glutathione-S-transferase (GST), glutathione peroxidase (GPx), glutathione reductase (GR).

Table 7: Effects of BPM on tissue proteins and biomolecules in lungs of rat

Group	Protein (µg/mg tissue)	GSH (µM/g tissue)	TBARS (nM/min /mg protein)	H ₂ O ₂ (nM/min /mg tissue)
Control	2.07±0.145 ^a	25.62±1.5 ^a	3.39±0.42 ^d	1.23±0.162 ^d
Asthma	1.99±0.118 ^a	22.91±1.8 ^b	3.32±0.13 ^d	1.13±0.153 ^e
Dexa	0.71±0.111 ^d	08.23±0.9 ^e	5.27±0.68 ^a	2.43±0.165 ^a
A + BPM 200	0.93±0.082 ^c	13.65±0.2 ^d	4.91±0.11 ^b	2.01±0.133 ^b
A+ BPM 400	1.69±0.169 ^b	18.90±0.6 ^c	3.89±0.15 ^c	1.81±0.141 ^c
BPM 400	2.03±0.118 ^a	23.26±1.5 ^{ab}	3.34±0.21 ^d	1.29±0.181 ^d

Mean ± SD (06). Means not sharing the same alphabet in the same column are significantly different at $P < 0.05$. Asthma; TDI sensitized rats: Dexa; Dexamethasone treated rats: A; Asthmatic: BPM; *B. procumbens* methanol extract. Reduced glutathione (GSH), thiobarbituric acid reactant substances (TBARS), hydrogen peroxide (H₂O₂).

Table 8: Effect of BPM on lung histology score

Control	Asthma	Dexa	A + BPM 200	A + BPM 400	BPM 400	P* value
Inflammation						
0	3	1	2	1	0	0.0001
0	2	1	2	1	0	
0	3	2	2	1	1	
0	2	1	2	1	0	
0	3	1	2	2	0	
0	2	1	1	2	0	
Airway mucus production						
1	3	1	2	1	1	0.0007
1	3	2	2	1	1	
0	2	1	1	0	0	
0	3	2	1	2	0	
1	2	0	1	1	0	
0	3	2	2	2	0	
Thickening of peribronchial smooth muscle layer						
1	2	2	2	2	0	0.0001
0	3	0	2	1	1	
1	3	1	2	2	1	
1	3	1	2	1	0	
1	2	2	2	1	0	
1	2	1	2	1	0	
Subepithelium collagen deposition						
0	1	1	1	1	0	0.0004
0	2	1	1	2	0	
0	2	1	2	1	0	
0	2	0	1	1	1	
0	3	1	2	1	1	
0	1	0	1	1	1	

P* value for comparison of six groups (Kruskal Wallis test)

Asthma; TDI sensitized rats: Dexa; Dexamethasone treated rats: A; Asthmatic: BPM; *B. procumbens* methanol extract.

Figure 1: Histopathological alterations and effect of *B. procumbens* in sections of lung and bronchioles. A) Shows normal view of lung pathology, (B) Shows typical damaged lung tissue with leukocyte infiltration, thickening of peribronchial smooth muscle layer, subepithelial collagen deposition (C) Shows less leukocyte infiltration by dexamethasone treatment (D) Shows less leukocyte infiltration by *B. procumbens*, E) Almost near to normal pathology with very less infiltration, F) normal lung pathology