Research Article

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# MECHANISTIC EVALUATION OF *BUTEA MONOSPERMA* USING IN VITRO AND IN VIVO MURINE MODELS OF BRONCHIAL ASTHMA

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**ABSTRACT**
The present investigation aimed to elucidate the probable mechanism of antiasthmatic action of *Butea monosperma.* Bioactive fractionations of methanolic extract of *B. monosperma* (MBM) were carried out to get, ethyl acetate fraction (EBM) and butanolic fraction (BBM) of *Butea monosperma*. All fractions were evaluated mechanistically for its antiasthmatic actions in vitro using lipoxygenase assay. The lipoxygenase inhibitory effect of the extract followed the order BBM > MBM > EBM. Based on in vitro lipoxygenase assay BBM was selected for in vitro compound 48/80 induced mast cell degranulation assay and in vivo lipopolysaccharide induced inflammation in rats. BBM 50, 100 and 200µg/ml inhibited compound 48/80 induced mast cell degranulation. BBM exhibited dose dependent (50, 100 and 200mg/kg i.p.) inhibition of lipopolysaccharide-induced increased in total cell count, differential leukocyte count, nitrate-nitrite, total protein and albumin levels in bronchoalveolar fluid (BALF) and myeloperoxidase (MPO) levels in lungs homogenate.
**Keywords**: Anti-inflammatory; *Butea monosperma;* lipoxygenase; Lipopolysaccharide

## INTRODUCTION

Asthma is a chronic inflammatory disease characterized by airway hyper responsiveness and recurrent reversible airway obstruction caused by activation of many inflammatory cells (e.g. eosinophils, T cells, mast cells) and structural cells (airway epithelial cells, smooth muscle cells, endothelial cells, fibroblasts)[1,2]. The inflammation causes an associated increase in airway responsiveness to various stimuli[3]. Bronchoconstriction, cough, mucus production and airway hyperreactivity are the main clinical manifestations of asthma and these features correlate well with the severity of the disease. Contemporary treatment of this disease addresses the dual need for rapid symptom relief (reliever therapy) while also attacking the inflammatory component of asthma (controller therapy)[4]. Given the complexity of the disease process involved in asthma and activities of mediators linked to the disease, the case for a single mediator approach to asthma therapy is difficult to establish[5].

The folklore uses flowers and leaves of *Butea monosperma* are anticonvulsant, antioxidant, antistress, and memory and behaviour stimulant, antigout, diuretic, anti-inflammatory, antiulcer, astringent and antihepatotoxic[6,7]. The plant is known to have hepatoprotective[8], anti-inflammatory[9], anti-diabetic[10], anti-stress, anti-diarrheal, aphrodisiac, diuretic, febrifuge, anti-arthritic and anti-estrogenic[11] activities. *Butea monosperma* flowers contain butin and butrin, isobutrin, palasitrin, coreipsin and isocoreipsin, chalcones, and aurones[12]. Methanolic extract of the *Butea monosperma* exhibited anti-inflammatory and analgesic activity in carrageenin induced paw edema and acetic acid induced writhing test cotton pellet induced granuloma formation[13]. However no compelling evidences are available for its usefulness in treatment of bronchial asthma. Thus, to scientifically evaluate the biological activity this plant was selected for further preclinical evaluations in allergic and inflammatory conditions in lungs.

## MATERIALS AND METHODS

### Plant materials

The fresh shed dried flowers of *Butea monosperma* used in the study were collected from local market of Mumbai and authenticated by Dr. Ganesh Iyer, Department of Life Science, R. Ruia College, Mumbai, India.

### Reagents

o-dianisidine, nitrate reductase, naphthylethylenediamide were purchased from Sigma-Aldrich Co., (USA). LDH, FAD and NADPH were purchased from SRL Diagnostics Ltd, India. Other reagents were reagent grade and were purchased from SD fine chemicals Ltd. Mumbai, India.

### Extraction, Fractionation and characterization of methanolic extract of *Butea monosperma* flowers

### Extraction and Fractionation

The shed dried coarse powders of the flowers of *Butea monosperma* (100 g) were extracted with methanol (1L) in soxhlet extractor for 24 hrs at 50°C. The extracts were concentrated in a rotary vacuum evaporator (Buchi, Switzerland) at temperature not higher than 50°C to get methanolic extract (MBM). The procedure was repeated several times to collect sufficient amount of extract. The crude MBM (20 gm) was suspended in distilled water (50 ml) and sonicated for 5 min. The mixture was then transferred to a 1-L separating funnel and sequentially fractionated with ethyl acetate (3 x 50 ml), and n-butanol (3 x 50 ml). The resulting fractions were then concentrated on a rotary evaporator under reduced pressure to get ethyl acetate fraction (EAF) and n-butanol fraction (BBM). The fractionation process was repeated; the fractions were pooled and preserved in airtight glass

container at 4°C until use. Percentage yield was found to be MBM (24 %), BBM (24.9%) and EBM (6%).

**Characterization of methanolic extract and its fractions using HPTLC fingerprinting**

MBM and its BBM and EBM (5 mg/ml) were subjected to HPTLC analyses for fingerprinting. The samples were spotted on silica gel F254 pre-coated aluminium TLC plates. The plate was developed by ascending technique in chamber saturated with solvent system ethyl acetate: methanol: water: formic acid (4:0.8:0.2:0.2) until the solvent moved through a distance of 8 cm. After development of the plate, it was scanned at different wavelength i.e. 254 and 366; $R_f$ values and area under curve was determined.

HPTLC analysis of MBM and BBM shows presence of substance 1 and substance 2 at $R_f$ values 0.28 and 0.37 respectively. HPTLC analysis of EBM shows presence of substance 2 at $R_f$ value 0.37. UV-visible spectrum of substance 1 shows λmax at 279 nm and 313 nm, which matches with reported λmax of butrin[14]. UV-visible spectrum of substance 2 shows λmax at 264 nm and 373 nm, which matches with reported λmax of isobutrin and monospermoside[14]. HPTLC analysis of MBM and BBM shows presence of above flavonoids as the major constituents, which was established by post derivatization of HPTLC plate with 1% aluminium chloride reagent showing florescent colour spots corresponding to flavonoids. (Figure 1 and 2)

**Animals**

Adult, Sprague Dawley female rats weighing 180-200g were housed in standard polypropylene cages with wire mesh top and maintained at 23 ± $2^0$C and relative humidity 60 ± 5% with 12-h light-dark cycle. Rats were fed with commercially available standard rodent pellet diet and water was provided *ad libitum*. The animal experimentation protocol was approved prior to experimentation from 'Institutional Animal Ethics Committee' of Bombay College of Pharmacy, Mumbai and the studies were performed in accordance with 'Committee for the Purpose of Control and Supervision on Experiments on Animals' (CPCSEA) guidelines (Ethical clearance number 23 & 24/2009).

**Lipoxygenation assay**

Aqueous solutions of MBM, BBM and EBM were prepared with concentrations 10 to 100µg/ml. The substrate solution (2.0 ml) was mixed with 0.9 ml of buffer solution. MBM (0.05ml), to obtain final concentrations of 10-100 µg/ml, were preincubated with the enzyme (0.05 ml) for 5 minutes. Lipoxygenation was started by addition of the enzyme solution to the substrate solution and change in absorbance was determined per minute at 234 nm using spectrophotometer for a time period of 5 minutes. Phenidone (10-100) was used as reference standard for a comparison[15]. The percent inhibition was calculated using equation:

$$\% \text{ inhibition} = (A_{control} - A_{test}) \times 100 / A_{control.}$$

**Effect of *Butea monosperma* on compound 48/80 induced degranulation of mast cells**

Female rats (180-200g) were anaesthetized with ether and 20ml normal saline containing 5 units/ml of heparin was injected in the peritoneal cavity. After a gentle abdominal message, the peritoneal fluid containing mast cells was collected in centrifuge tubes placed over ice. Peritoneal fluid of 4 - 5 rats was collected and pooled and centrifuged at 2,000 rpm for 5 min. Supernatant solution was discarded and the cells were washed twice with saline and resuspended in 1 ml of saline. Peritoneal cell suspension (0.1 ml) was transferred to 6 test tubes and was treated as follows.

Group 1 & 2 – Saline, Group 3 to 5- 0.1 ml BBM solutions in saline at different concentrations (50, 100 and 200µg/ml, respectively), Group 6 - 0.1 ml of 10 µg/ml of Ketotifen fumarate. Each group was incubated for 15 min at 37°C and then Compound 48/80 (0.1 ml, 10µg/ml) was added to each test tube except test tube no. 1. After further incubation for 10 min at 37 $^0$C, the cells were stained with 0.1% toluidine blue solution made in saline and examined using Motic microscope (Motic SD digital microscope). Percent protection of the mast cells in the control group and the treated groups were calculated by counting the number of degranulated mast cells from total of at least 100 mast cells counted.

**Acute oral toxicity of methanolic extract and fractions**

Acute oral toxicity of methanolic extract and fractions was carried out as per OECD Guideline 423[16].

**In vivo evaluation of *Butea monosperma***

**Effect of *Butea monosperma* on Lipopolysaccharide induced inflammation in rats**

Thirty six female Sprague Dawely rats, weighing 150-170 g were divided randomly in six groups of six animals each. n-Butanolic fraction of *Butea monosperma* (BBM) 50, 100 and 200 mg/kg was administered per orally for four days to animals and one hour prior to LPS administration. Roflumilast (1mg/kg p.o.) was used as a standard drug for comparison.

Solution of LPS (*E coli,* O111:B4; 1 mg/ml) was prepared in sterile phosphate buffered saline (PBS) just prior to administration to animals. LPS solution was maintained cold by keeping in ice throughout the administration procedure. Bronchoalveolar Lavage fluid (BALF) was evaluated for total cell count, differential cell count, MPO activity, nitrate/ nitrite, total proteins and albumin as described earlier.

**Assessment of bronchoalveolar lavage**

**Total Cell count**

An aliquote of 100 µl was taken for with equal volume of Turk's solution and total cell were counted using Neubaur's chamber.

**Differential Cell Count**

The cell pellet after centrifugation was suspended in rat serum (harvested previously) and smears were prepared for differential cell count (DLC). Cells were stained with Giemsa stain and DLC of 200 cells was performed using standard morphological criteria.

**Measurement of Myeloperoxidase activity**

The lung was rinsed with ice-cold saline, blotted dry, weighed and minced. Minced tissue was homogenized in 10 volumes of ice-cold potassium phosphate buffer (pH 7.4), using Remi tissue homogenizer (RQ-127A). The homogenate was centrifuged at 3500 rpm for 30 min at 4 $^0$C (Remi centrifuge C23). The supernatant was discarded. A 10 ml of ice-cold 50mM potassium phosphate buffer (pH 6.0), containing 0.5% hexadecyl trimethyl ammonium bromide (HETAB) and 10mM EDTA was added to the pellet. It was subjected to one cycle of freezing and thawing and brief period (15 s) of sonication. After sonication, the solution was centrifuged at 15,000 rpm for 20 min (Remi centrifuge, C23). Myeloperoxidase activity was measured spectrophotometrically as follows.

A 0.1 ml of supernatant was combined with 2.9 ml of 50mM phosphate buffer containing 0.167 mg/ml O-dianisidine hydrochloride and 0.0005% $H_2O_2$. The change in absorbance was measured spectrophotometrically (Shimadzu UV 160A UV-VIS spectrophotometer), at 460 nm. One unit of MPO activity is defined as the change in absorbance per minute by 1.0 at room temperature, in the final reaction[17].

MPO activity was calculated as:

MPO activity (U/g) =X/ wt of the piece of tissue taken
Where, X = (10 × change in absorbance per minute)/ Volume of supernatant taken in the final reaction.

**Measurement of nitrite/nitrate**

Nitrite/nitrate (NOx) production, an indicator of NO synthesis, was measured in BALF[18]. To reduce nitrate to nitrite, nitrate in BALF supernatant was incubated with nitrate reductase (0.1 U/ml) and NADPH (1 mM) and FAD (50 µM) at 37°C for 15 min followed by another incubation with LDH (100 U/ml) and sodium pyruvate (10 mM) at 37°C for 5 min. Nitrite in the samples was measured by Griess reaction, briefly by adding 100 µl of Griess reagent (0.1 % w/w naphthylethylenediamide dihydrochloride in water and 1% sulphanilamide w/w in 5 % concentrated $H_2PO_4$; mixed in volumes (1:1) to 100 µl samples. The optical density at 550 nm was measured using microplate reader (Modulus Microplate reader, Turner Biosystem). Nitrate concentrations were calculated by comparison with the absorbance of standard solution of sodium nitrate prepared in saline solution.

**Total protein assay**

As a measure of epithelial injury and lung permeability, total protein concentration in BALF was measured [19]. For assay 100µl BALF sample was mixed with 2ml Lowery reagent (Reagent A: 0.1N NaOH, 2g Sodium carbonate and 20 mg sodium potassium tartarate in 100ml distilled water Reagent B: $CuSO_4$ 1g in 100ml distilled water. Mix Reagent A 100ml and B 2.5ml) and incubated for 10min at room temperature. To this mixture 0.2ml of Folin's reagent (1:2 dilution of commercially available Folin's Reagent) was added and incubated at 30 $^0$C. The optical density at 660 nm was measured using microplate reader (Modulus Microplate reader, Turner Biosystem) and total protein was quantified using a standard plot of Bovine serum albumin (0.1 to 1 mg/ml).

**Albumin assay**

Albumin measurements in BALF were done with the bromocresol green method[20]. Briefly, aliquots of BALF supernatant (50 µl) were incubated for 10 min at room temperature with 500µl of albumin working reagent (Bromocresol green 0.08mmol/L, Succinate buffer pH 4.2 ± 0.1 at 25 $^0$C and sodium azide 1g/L). Absorbance was read at 630 nm (Modulus Microplate reader, Turner Biosystem) and albumin was quantified using a standard plot of albumin (0.1 to 1 mg/ml).

**Histopathological evaluation of lungs**

The lower lobes of the right lungs were removed, fixed in 10% neutral buffered formalin for at least 24 h, processed routinely. After being embedded in paraffin, the tissues were cut into 5 µM thick sections and stained with hematoxylin and eosin (HE) before examination under a light microscope.

**Table 1: Compound 48/80 induced mast cell degranulation**

| Treatment | Mast cells degranulation (Mean ± SEM) | % Inhibition of degranulation |
|---|---|---|
| Negative Control | 5.16 ± 0.96 | - |
| Positive Control | 78.21± 1.4$^{\#}$ | - |
| BBM 50 (µg/ml) | 60.03 ± 1.28$^{*}$ | 23.25 |
| BBM 100 (µg/ml) | 37.24 ± 0.89$^{*}$ | 52.39 |
| BBM 200 (µg/ml) | 21.90 ± 0. 61$^{*}$ | 71.99 |
| Ketotifen 10 (µg/ml) | 14.10 ± 0.34$^{*}$ | 81.97 |

#p < 0.001, when compared with control, $^{*}$p < 0.05 when compared with positive control using ANOVA followed by Dunnett's test.

**Table 2: Effect of BBM and Roflumilast on differential cell count in Bronchoalveolar Lavage fluid in LPS induced lung inflammation in rats**

| Treatment | Neutrophils | Mast cells | Monocytes | Macrophages |
|---|---|---|---|---|
| **Saline treatment** | 17.27 ± 2.54 | 18.29 ± 2.78 | 23.52±3.03 | 40.93 ± 3.15 |
| **Positive control** | 75.69 ± 2.672$^{\#}$ | 7.12 ± 1.9$^{\#}$ | 6.26 ± 2.48$^{\#}$ | 11 ±1.28$^{\#}$ |
| **BBM 50** | 34.32 ± 2.73$^{*}$ | 12.71±4.25$^{*}$ | 22.06 ± 4.85$^{*}$ | 30.91 ± 4.94$^{*}$ |
| **BBM 100** | 27.61±2.18$^{*}$ | 12.04±2.97$^{*}$ | 25.07±3.26$^{*}$ | 35.28±3.69$^{*}$ |
| **BBM 200** | 19.28±1.87$^{*}$ | 9.95±3.05 | 29.04±3.44$^{*}$ | 41.73±2.98$^{*}$ |
| **Roflumilast 1** | 22 ± 3.032$^{*}$ | 7.99±1.85 | 20.03±3.115$^{*}$ | 49.98±3.67$^{*}$ |

Results are expressed as Mean ± SD; n = 6; $^{\#}$: significant w. r. t. saline control; $^{*}$: significant w. r. t. LPS control at p < 0.0001

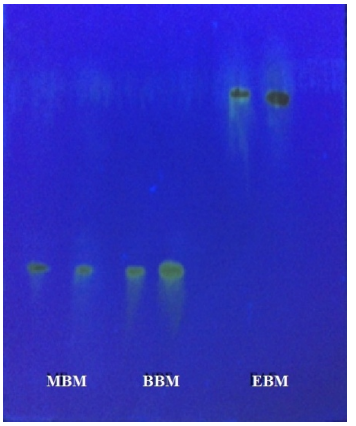



**Figure 1: HPTLC chromatogram of MBM, EBM and BBM**

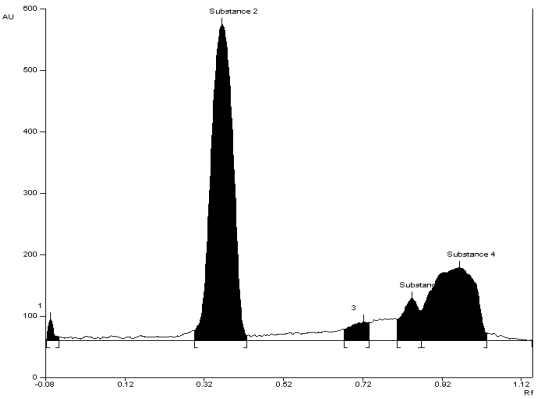



**Figure 2: HPTLC scan of BBM at 366 nm**

## Influence of BBM on LPS induced lung inflammation in rats

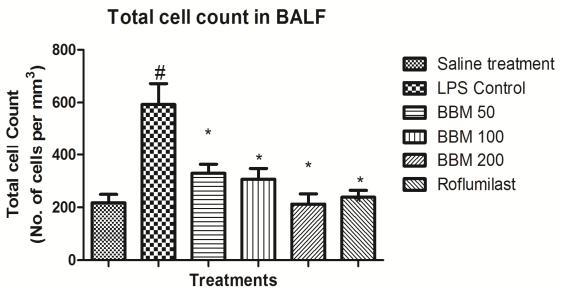



**Figure 3: Effect of BBM and Roflumilast on total cell count in Bronchoalveolar Lavage fluid in LPS induced lung inflammation in rats**

Results are expressed as Mean ± SD; n = 6; #: significant w.r.t. saline control; *: significant w. r. t. LPS control at $p < 0.0001$

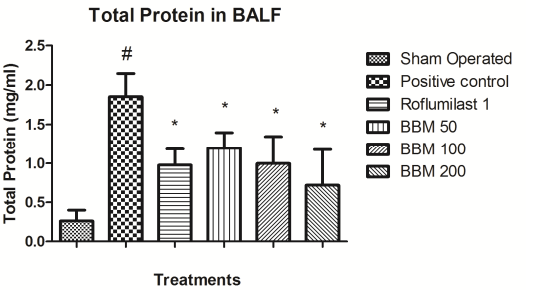



**Figure 4: Effect of BBM and Roflumilast on total protein levels in Bronchoalveolar Lavage fluid in LPS induced lung inflammation in rats**

Results are expressed as Mean ± SD; n = 6; #: significant w.r.t. saline control; *: significant w. r. t. LPS control at $p < 0.05$

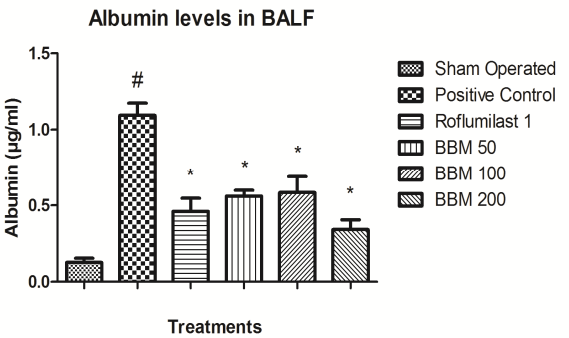



**Figure 5: Effect of BBM and Roflumilast on albumin levels in Bronchoalveolar Lavage fluid in LPS induced lung inflammation in rats**

Results are expressed as Mean ± SD; n = 6; #: significant w. r.t. saline control; *: significant w. r. t. LPS control at $p < 0.05$

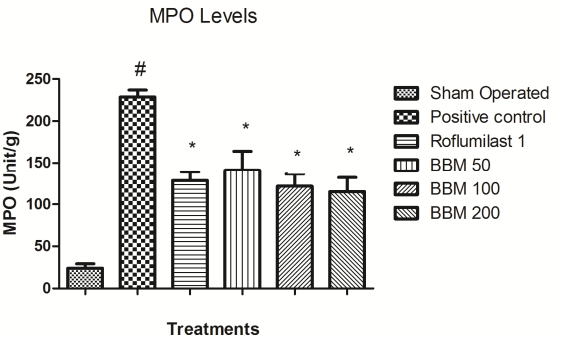



**Figure 6: Myeloperoxidase activity in Bronchoalveolar Lavage fluid in LPS induced lung inflammation in rats**

Results are expressed as Mean ± SD; n = 6; #: significant w. r. t. saline control; *: significant w. r. t. LPS control at $p < 0.05$

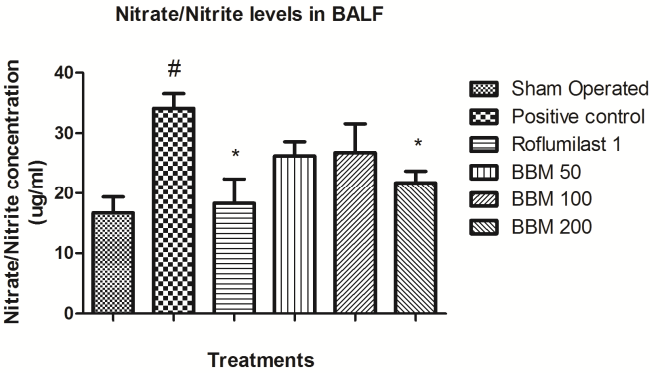
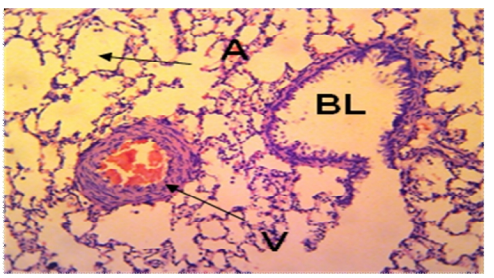
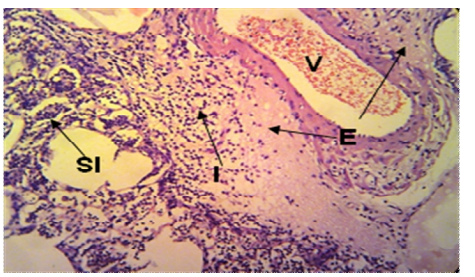


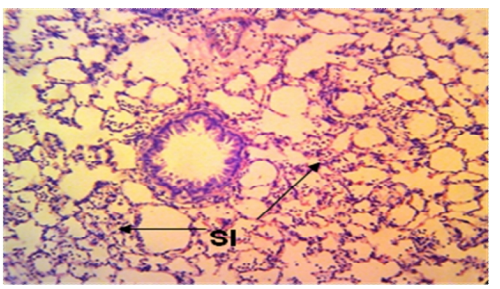

**Figure 7: Effect of BBM and Roflumilast on Nitrate/nitrite levels in Bronchoalveolar Lavage fluid in LPS induced lung inflammation in rats**
Results are expressed as Mean ± SD; n = 6; #: significant w. r.t. saline control; *: significant w. r.t. LPS control at $p < 0.05$



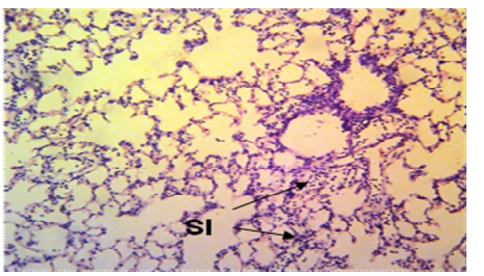

**Negative Control**



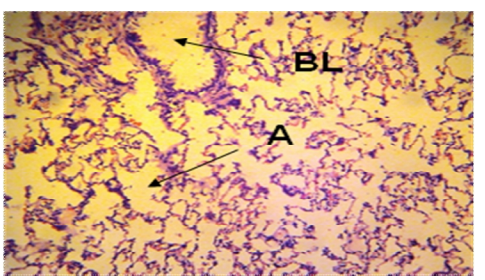

**Positive control**



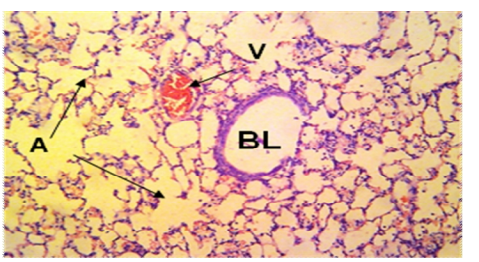

**BBM 50 mg/kg, i.p.**




**BBM 100 mg/kg, i.p.**




**BBM 200 mg/kg, i.p.**




**Roflumilast 1 mg/kg**

**Figure 8: Histopathological microphotographs of lungs in LPS induced lung inflammation in rats**

Note: A: Alveoli; AS: inter alveolar Septum; BL: Bronchial lumen, BE: bronchial epithelium; V: Blood Vessel; SI: Interalveolar septal infiltration; E: edema; HB: hyperplasia of BALT (Bronchial associated Lymphoid tissues); FP: Fibrous connective tissue Proliferation and Li: Alveolar luminal infiltration; PI: Peribronchiolar infiltration.

**Statistical analysis**

All Values are expressed as Mean ± S.D. Significance was determined by One-Way ANOVA followed by Bonferroni's Multiple Comparison Test: $^{*}p < 0.05$ when compared with positive control group and $^{\#}p < 0.05$ when compared with vehicle control group were considered significant**.** Statistics was applied using Graph pad Prism 5 software (Graph pad Software Inc).

## RESULTS

**Lipoxydase inhibitory activity**

The lipoxydase inhibitory effect of the extract/fractions followed the order BBM > MBM > EBM, with $IC_{50}$ values of 76.5 µg/ml, 87.9 µg/ml and 163.8 µg/ml, respectively, indicating that the BBM was found to be have more lipoxydase inhibitory ability than the MBM and EBM. The $IC_{50}$ of a reference standard phenidone was found to be 7.8144ng/ml.

**Effect of *Butea monosperma* on compound 48/80 induced mast cell degranulation**

Compound 48/80 (10 µg/ml) produced 78.21 ± 1.4 % disruption of mast cells compared to saline control. Pre-treatment with BBM (50, 100 and 200µg/ml) significantly reduced mast cell degranulation to 60.03 ± 1.28%, 37.24 ± 0.89% and 21.91 ± 0.61% respectively. Ketotifen (10µg/ml) significantly reduced mast cell degranulation to 14.10 ± 0.34. BBM (200µg/ml) and Ketotifen (10µg/ml) exhibited 71.99% and 81.97% protection of compound 48/80 induced mast cell degranulation (Table 1).

**Acute oral toxicity of methanolic extract and fractions**

A single oral administration of 2000 mg/kg of extract/fractions did not produce any sign of acute toxicity in the animals. There was no mortality observed and no significant changes were observed in body weight. The quantity of food consumed by the extract/fractions and control animals were found to be comparable. Thus, extract/fractions were found to be safe at the single oral dose of 2000 mg/kg. The $LD_{50}$ of extract/fractions could be > 2000 mg/kg (p.o.) in animals.

***In vivo* Evaluation of *Butea monosperma* on Lipopolysaccharide induced inflammation in rats**

**Effect of BBM on Total cell Count in BALF**

Intratracheal administration of LPS challenge caused significant increase in cell count in the BALF in comparison to saline treatment ($p< 0.05$). Treatment with BBM 50, 100 and 200mg/kg demonstrated dose dependant inhibition of 55.48%, 51.60% and 35.75% of total cell count respectively. Roflumilast 1 mg/kg treatment demonstrated a significant reduction of 40.13% in total cell count in comparison to LPS control treatment. The influence of BBM 30mg/kg was found to be comparable with Roflumilast 1mg/kg treatment (Figure 3).

**Effect of BBM on Differential leukocyte count in BALF**

Intratracheal LPS challenge resulted in significant increase in number of neutrophils in BALF. BBM 50, 100 and 200mg/kg, i.p. treatment exhibited a significant decrease in neutrophil count to 34.32 ± 2.73%, 27.61 ± 2.18 and 19.28 ± 1.87% respectively. Treatment with Roflumilast (1 mg/kg, p.o.) significantly lowered the neutrophil count in BALF to 22 ± 3.30% in comparison to LPS control treatment. Percent of neutrophils in Roflumilast treated group was close to percentage of neutrophils found in saline control.

Mast cells count in BALF of LPS control animals was found to be 7.12 ± 1.9%. It was found to be significantly lower in comparison to saline control treatment. BBM 50, 100 and 200mg/kg, i.p. exhibited mast cell count of 12.71 ± 4.25%, 12.04 ± 2.97% and 9.95 ± 3.05 % respectively in the BALF. Only BBM 50 and 100mg/kg, i.p. exhibited significant increase in mast cell count of all the treatment. Roflumilast 1 mg/kg, i.p. exhibited mast cell count of 7.99 ± 1.85% which was again not significant from LPS control treatment.

The macrophage count in BALF of saline treatment animals was found to be 40.93 ± 3.15%. LPS treatment caused significant reduction of macrophage count to 11 ± 1.28%. Treatment with BBM demonstrated macrophage count of 30.91 ± 4.94%, 35.28 ± 3.69% and 41.73 ± 2.98 % respectively in the BALF. Thus BBM failed to demonstrate significant difference in macrophage count in BALF in comparison to LPS control treatment. Roflumilast treatment however exhibited the macrophage count of 49.98 ± 3.61%. This increase in count was significantly different from LPS control treatment and similar to saline control treatment.

The monocyte count in the BALF of saline treated animals was found to be 23.52 ± 3.025%. However LPS treatment caused significant reduction in monocyte count to 6.26 ± 2.475%.

Treatment with BBM 50, 100 and 200mg/kg demonstrated monocyte count of 22.06 ± 4.85%, 25.07 ± 3.26 % and 29.04 ± 3.44 % respectively in the BALF. Roflumilast treatment exhibited monocyte count of 20.03 ± 3.115%. All the treatments thus showed significant increase in monocyte count in comparison to LPS control treatment (Table 2).

**Effect of BBM on Total protein in BALF**

The protein concentration in BALF in saline treated rats was found to be 0.2604 ± 0.1397mg/ml. LPS treatment significantly increased protein levels in BALF to 1.8538 ± 0.2925mg/ml in comparison to LPS control treatment. BBM 50, 100 and 200mg/kg treatment causes significant reduction in protein concentration to 1.1970 ± 0.1996, 0.9995 ± 0.3450 and 0.7193 ± 0.4602 mg/ml respectively in comparison to LPS control treatment. BBM demonstrated the dose dependant reduction in protein levels. The BALF protein level of Roflumilast treatment was found to be 0.9785 ± 0.2076 mg/ml (Figure 4).

**Effect of BBM on Albumin content in BALF**

The albumin concentration in BALF in saline treated rats was found to be 0.1273 ± 0.0541 mg/ml. LPS treatment significantly increased albumin levels in BALF to 1.0938 ± 0.1805 mg/ml in comparison to LPS control treatment. Roflumilast 1.3mg/kg significantly reduced the albumin levels to 0.4604 ± 0.1940 mg/ml in comparison to LPS control treatment. BBM 50, 100 and 200mg/kg treatment causes significant reduction in albumin concentration to 0.5612 ± 0.07754, 0.5841 ± 0.2344 and 0.3417 ±

0.1573mg/ml respectively in comparison to LPS control treatment. BBM demonstrated the dose dependant reduction in protein levels. The BALF protein levels of BBM treated group at all tested dose levels were comparable with Roflumilast treatment (0.4604 ± 0.1940mg/ml) (Figure 5).

**Effect of BBM on MPO activity**

MPO activity of BALF of saline control animals was found to be 24.1623 ± 8.7912 units/g. Intratracheal LPS administration caused significantly increased in MPO activity of 228.6638 ±16.7408 units/g. Treatment with BBM 50, 100 and 200mg/kg significantly reduced the MPO levels in the BALF to 141.2 ± 45.35, 121.5 ± 35.93 and 115.2 ± 37.38 units/g respectively in comparison to LPS control treatment. MPO activity of BBM treated groups was comparable to MPO activity of Roflumilast 128.4439 ± 22.9554units/g (Figure 6).

**Effect of BBM on Nitrate/nitrite content in BALF**

The nitrate/nitrite concentration in BALF was significantly increased to 34.10 ± 4.917 µM of LPS control treatment in comparison to saline control treatment (16.6926 ± 5.9284 µM). Roflumilast 1.3mg/kg significantly reduced the nitrate/nitrite concentration in BALF to 18.2864 ± 7.0953 µM in comparison to LPS control treatment.

BBM 50, 100 and 200mg/kg treatment causes reduction in nitrate/nitrite concentration to 26.23 ± 4.708, 26.77 ± 9.546 and 21.72 ± 3.898 µM respectively in comparison to LPS control treatment.

BBM demonstrated the dose dependant reduction in nitrate/nitrite levels. Nitrate/nitrite concentration in BBM 30 mg/kg group was found to be similar to that of in Roflumilast treated group (Figure 7).

**Histopathological Evaluation**

Intratrachea instillation of LPS induces much severed peribronchiolar, interalveolar septal and perivascular infiltration of inflammatory cells (majority were neutrophils) and edema (++++). The inflammatory cells were present in bronchiolar and alveolar lumen. There was hyperplasia of BALT (++) and severe proliferation of FCT was observed.

Treatment with BBM showed mild alveolar infiltration of inflammatory cells (+) in rats treated with BBM 50 mg/kg, i.p. interalveolar septal infiltration of neutrophils (+) in animals treated with BBM 100 mg/kg, i.p. However no abnormality was detected in the rats treated with BBM 200 mg/kg, i.p. (Figure 8).

**DISCUSSION**

Cells involved in the asthmatic process have capacity for producing reactive oxygen species (ROS), activating eosinophils, neurophils, monocytes, and macrophages to generate superoxides ($O_3^-$) via membrane associate NADPH dependant complex. ROS also activate NF-kB, which orchestrates the expression of multiple inflammatory genes that undergo increased expression in asthma, thereby amplifying the inflammatory response[2]. Thus there is a need of more potent antioxidants to be developed which can be used to treat clinical manifestation of asthma.

Antiallergic activity of MBM and BBM were evaluated using *in vitro* mast cell degranulation studies. Mast cells play a critical role in immediate hypersensitivity and allergic reactions when activated through immunglobulin E (IgE) by specific antigens. Antigen antibody-induced degranulation requires an active mast cell response[21]. Uvnas (1969)[21] studied the mast cell degranulation and its correlation with the release of histamine after administration of compound 48/80, the mast cell degranulating agent. After activation, mast cells exert their biological effects by releasing preformed and *de novo*-synthesized mediators, such as histamine, leukotrienes, and various cytokines/chemokines. Biogenic amines and lipid mediators cause rapid leakage of plasma from blood vessels, vasodilatation, and bronchoconstriction. Several other mediators like tryptase, chymase, activin A platelet-derived growth factors, Transforming Growth Factor-β, Amphiregulin and Plasminogen activator inhibitor-1 have been reported to have deleterious effects in airway remodelling[22]. Compound 48/80, act through the dynamic expulsion of granules without causing any damage to the cell wall[23]. These mediators released initiate rapid vascular permeability, leading to plasma extravasations and tissue edema, bronchoconstriction, mucus overproduction and leukocyte recruitment. Mast cells are also implicated in the late phase response in asthma. A prolonged secretion of chemoattractive and immunomodulatory molecules contribute to continuing tissue edema, cellular influx and inflammation observed hours after initial mast cell activation[24]. It is known that Disodium cromoglycate a standard mast cell stabilizer prevents degranulation of the mast cells by raising the cAMP[25]. BBM was thus evaluated for antiallergic activity following mast cell degranulation studies. BBM markedly reduced the mast cell degranulation induced by compound 48/80, a calcium channel inophore. The activity exhibited was comparable with Ketotifen. The flavonoids also inhibited the histamine release induced by compound 48/80, a major basic polyamine can induce mast cell secretion to a site on the cell membrane, which seems to be associated with an influx of $Ca^{2+}$ into the cell[26]. Plants containing flavonoids have been reported to possess the antihistaminic and mast cell degranulation properties [27, 28].

Selective human 5-lipoxygenase inhibitor, Zileuton, has been approved by the FDA for the treatment of asthma[29]. Lipoxygenase (LOX) inhibitory assay was used to elucidate the possible contribution of the radical scavenging effect on the lipoxygenase inhibitory mechanism of MBM and its fractions (BBM and EBM). The study was carried out according to Shinde et al. (1999) using soyabean lipooxygenase as enzyme and linoleic acid as substrate[15]. It was found that the highest amount of lipooxygenase inhibitory effect was observed in BBM and MBM. Inhibition of a number of other enzymes like cyclo-oxygenase (COX) and lipooxygenase by terpenoids and flavonoids like compounds has also been reported. This *in vitro* lipooxygenase inhibitory activity could partly explain the anti-inflammatory effect of MBM and its fractions. So BBM was used for further investigations.

*In vitro* studies made a strong reservation for evaluation of BBM for its anti-inflammatory and anti-allergic activity *in vivo*.

The methanolic extract of flowers of *Butea monosperma* inhibited the carrageenan induced paw edema and cotton pellet induced granuloma formation suggesting potent anti inflammatory activity[13]. Butein isolated from BM flowers significantly inhibited COX-2 expression and phospho-mitogen activated protein kinase followed by inhibition in total activity of PKC suggesting the anti-inflammatory and anti-cancer activity[30]. BBM was evaluated for anti-inflammatory and antiallergic potential in experimental models of asthma in comparison with roflumilast, a PDE4 inhibitor. Roflumilast possess broad range of pharmacological activities of potential use in the treatment of airway disorders. It not only potently modulates the activity of various inflammatory cells *in vitro*[31] but also blocks inflammatory and pathophysiological cascades in complex *in vivo* settings.

Bochner BS[32] reported infiltration of eosinophils, neutrophils, macrophages and lymphocytes into the lung. Recruitment of these inflammatory cells from the blood to the site of inflammation is regarded as a critical event in the development and persistence of airway inflammation.

*In vivo* evaluation of BBM was carried out in LPS induced acute lung inflammation (neutrophilia). LPS is a macromolecular cell surface bacterial antigen which, when applied *in vivo* triggers a network of inflammatory responses. One of the primary events is the activation of mononuclear phagocytes through a receptor mediated process, leading to the release of a number of cytokines, including tumour necrosis factor-α (TNF- α)[33]. TNF-α is a multifunctional proinflammatory $TH_1$ cytokine which has been suggested to play a critical role in the initiation, maintenance and progression of airway inflammation in asthma. TNF-α is stored in granules and is known to be released during allergic responses both from mast cells and macrophages via Ig-E dependant mechanisms [34]. Many other cell types that play a contributing role in the pathogenesis of asthma are also significant sources of TNF- α, including eosionphils[35], epithelial cells[36] and T cells[37].

The increased adherence of neutrophils to endothelial cell induced by TNF- α leads to a massive infiltration in the pulmonary space [38]. TNF- α production and neutrophils infiltration and activation, are the main features associated with this model which simulates the disease conditions in chronic obstructive pulmonary disease (COPD).

Intraperitoneal injection of bacterial LPS are the most common routes of administration used to develop acute injury in animal models. Pulmonary lung inflammation can be measured by albumin extravasation or neutrophils myeloperoxidase activity in the lung parenchyma[39]. In the present study *in vivo* intra-tracheal administration of LPS in SD female rats induced a time dependant invasion of the airway lumen with neutrophils. The chronology of this inflammatory response in lungs was evaluated by the analysis of inflammatory cell count in the branchoalveloar lavage, reflecting their extravasation from the vascular lumen to the airway airspace, and by the measurement of myeloperoxidase activity in the bronchoalveolar Lavage reflecting the activation of neutrophils.

LPS (0.1mg/animal) when instilled into the lung, induced optimal acute pulmonary inflammation. The preliminary results obtained from the time course experiment showed that an acute neutrophilia could be achieved as soon as 4 hours after the LPS challenge (80-85% neutrophilia). According to earlier reports neutrophils in bronchoalveolar Lavage fluid (BALF) return to baseline levels 5-6 days after LPS administration and the animal recovers on their own from the induced trauma[40]. The results of the present investigation showed that intratracheal administration of low amount of LPS in SD rats resulted in an acute and well controlled pulmonary neutrophilia. Apart from massive influx of neutrophils, which represent about 72.95% of the total cells in the Bronchoalveolar Lavage, a small but significant increase in lymphocytes was also observed. Macrophages did not increase in significantly manner after LPS instillation. In contrast, a significant decrease was observed following LPS challenge only at the earlier time point (4hours). This observation was in agreement with a previous report[41] and probably reflects macrophages adherence to the alveolar surface. Infiltrating neutrophils were also activated after the LPS challenge as evidenced by the increased levels of MPO activity. However, despite the massive neutrophils influx into the airway space of (Sprague Dawley) SD rats and their activation status, no bronchoalveolar lavage hemorrhage was evident. Negligible amount of haemoglobin was detected in the BALF of LPS treated animals (unreported data) indicating minimal lung damage due to the infiltrating neutrophils.

BBM was found to attenuate LPS induced neutrophilia in rats. BBM significantly and dose dependently inhibited leukocyte infiltration as a measure of total cell count in BALF. Significant decrease in albumin extravasation was seen on albumin and protein levels in BALF of BBM treated rats. Decrease in leukocyte infiltration was associated with significant reduction in neutrophil count and myeloperoxidase activity in BALF.

BBM was also evaluated for the effect on NO levels in BALF. Endogenous nitric oxide (NO) produced by inducible NO synthase (iNOS), is well known for its possible role in inducing airway diseases including asthma[42]. NO strongly promotes the chemotaxis of inflammatory cells in lung and favors the development of Th2 response [43]. This is good evidence that NO inhibitors suppress airway inflammation by suppressing the recruitment of inflammatory cells, and mucus secretion in the lungs[2]. In the present investigation NO levels in the BALF of BBM treated animals were found to be significantly lower than positive control animals, demonstrating the inhibitory influence of BBM on NO in LPS induced lungs inflammation in rats.

NFκβ is known important regulator of iNOS, TNFα and IL-1β expression in LPS-activated inflammatory cells, such as macrophages and microglias[44,45]. In the present study roflumilast treatment showed significant reduction in NO levels which were in earlier results that roflumilast inhibit the production of several inflammatory mediators such as NO, TNF-α, and IL-1β in RAW264.7 cells via inhibition of NF-κβ activation and phosphorylation of SAOK/JNK and p38 MAP kinase[46]. It could be hypothesized that like roflumilast, inhibition of NF-κβ pathway directly or indirectly via inhibition of PDE

isoenzyme could be plausible mechanism of action for action of BBM anti-inflammatory potential.

Histopathologic findings were in line with our data. The lungs of rats treated with LPS presented severe infiltration of inflammatory cells (majority were neutrophils) and edema. There was a hyperplasia bronchial associated lymphoid tissue and prolifereation fibrous connective tissue in LPS treated rats. BBM dose dependently reduced all of these inflammatory changes, and thus, ameliorated the inflammatory conditions of bronchial asthma.

Butrin, isobutrin, and butein form *Butea monosperma* flowers significantly reduced the phorbol 12- myristate 13-acetate and calcium ionophore A23187-induced inflammatory gene expression and production of TNF-α, IL-6, and IL-8 in HMC-1 cells by inhibiting the activation of NF-kB suggesting anti inflammatory activity[47]. Butein isolated from flowers significantly inhibited COX-2 expression and phospho-mitogen activated protein kinase followed by inhibition in total activity of PKC suggesting the anti-inflammatory and anti-cancer activity[30]. Thus the mechanism of anti-inflammatory potential of BBM in bronchial asthma could be due to directly influencing through iNOs and via NF-κβ activation or indirectly via PDE isoenzyme inhibition.

Airway microvascular leakage and edema are other inflammatory events frequently observed in asthmatic airway which has been hypothesized to contribute directly to disease process[48]. The increase protein content detected in the airways following antigen challenge was reduced after pre-treatment with BBM. The increase in BAL protein content after antigen challenge might reflect increase in vascular permeability or increase protein secretion. Increase protein extravasation would be expected to result from the severe antigen antibody reaction. Plasma leakage from the capillary venules observed in asthmatic airways contribute to the disease process by augmenting airway resistance[49,50] showed a correlation between plasma exudation and airway resistance in the late phase reaction in the sensitized guinea pigs after antigen challenge. Ultrastructural studies of airway biopsies have revealed bronchial mucosa and vasculature of asthmatics presence of large number of endothelial gaps after allergen exposure presumably due to release of various pro-exudative mediators such as histamine, prostaglandins, leukotriene, platelet-activating factor and bradykinin. Increase concentration of these spasmogens and vasodilator mediators in BAL fluid from asthmatic airways makes them likely candidate for causing edema and airway hyper-responsiveness[51].

Treatment with BBM a flavonoid rich fraction effectively inhibits both acute and chronic inflammation as well as angiogenesis in bronchial asthma. The mechanism of effect of *Butea monosperma* appears to be mediated by influencing through iNOs and NF-κβ activation or indirectly via PDE isoenzyme inhibition. Thus the findings support the notion that *Butea monosperma* could be of value in the treatment of asthma, either as alternative or as adjunct to conventional therapy.

Asthma is a heterogeneous disorder immunologically, physiologically and biochemically and its aetiology is multifactorial. The present study was planned in such a way to cover various aspects of asthma like eosinophilia, lipoxidase activity, angiogenesis, mast cell degranulation and allergy associated with inflammation using various *in vitro* and *in vivo* animal models. The study revealed that the herbs examined have different mode of action. Thus in conclusion, since asthma involves several biochemical substances, a herbal formulation containing plants antagonizing the effects of various biochemical substances would be beneficial for the patient.

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