

NATURAL DRUGS

EFFECT OF METHANOL EXTRACT OF *BOMBAX MALABARICUM* LEAVES
ON NITRIC OXIDE PRODUCTION DURING INFLAMMATIONEMDAD HOSSAIN¹, DEBJANI SARKAR², MITALI CHATTERJEE³, SAIKAT CHAKRABORTY⁴,
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Abstract: In different parts of India, *Bombax malabaricum* DC. (Family: Bombacaceae), a lofty deciduous tree with large leaves, is traditionally used in inflammation. The aim of the present study was to confirm its anti-inflammatory activity and to search for the possible mechanism of action for methanol extract of *Bombax malabaricum* leaves (MEBM). The anti-inflammatory activity of MEBM was evaluated in a carrageenan-induced model of acute inflammation. As inflammation usually involves increased nitric oxide (NO) production, effect of MEBM on lipopolysaccharide-induced NO production in mouse peritoneal macrophages was studied to evaluate its possible mechanism of action. It was found that MEBM was non-toxic up to a dose of 2 g/kg for mice and rats, orally. MEBM (100, 200, and 400 mg/kg) significantly reduced carrageenan-induced rat paw edema ($p < 0.05$, $p < 0.01$, $p < 0.001$, respectively). In mice peritoneal macrophages, the IC_{50} for MEBM was $258.33 \pm 6.96 \mu\text{g/mL}$ and it was non-toxic up to 125 $\mu\text{g/mL}$. MEBM (0-100 $\mu\text{g/mL}$) reduced lipopolysaccharide-induced NO production in macrophages in a dose-dependent fashion ($p < 0.001$). Hence, MEBM possesses anti-inflammatory activity, mediated through inhibition of NO production.

Keywords: anti-inflammatory activity, *Bombax malabaricum*, macrophage, paw edema, nitric oxide

Inflammation occurs in response to the tissue injury through edema formation due to fluid exudation and cellular influx. It is a complex process associated with the release of different mediators like bradykinin, nitric oxide, vasoactive amines (histamines, serotonin, adenosine), interleukins 1, platelet activating factor, tumor necrosis factor (TNF) and eicosanoids (prostaglandins, thromboxanes, leukotrienes, lipoxins) (1, 2). Chronic inflammation contributes to the pathogenesis of series of diseases including gastritis, rheumatoid arthritis, atherosclerosis, inflammatory bowel disease and cancer (3). The reactive free nitric oxide (NO) possesses very important role for the inflammatory responses. NO is biosynthesized by inducible NO synthase (iNOS) and excessive production of NO can result in the development of inflammatory diseases like rheumatoid arthritis and autoimmune disorders (2). Compound possessing inhibitory effect

over the synthesis of excessive production of NO, may be considered as potential anti-inflammatory drug (4). Although there are synthetic anti-inflammatory medicines available in the market, they often possess severe toxic side effects and furthermore, reappearance of inflammatory diseases occurs with discontinuation of the medication (1). In contrast, plants are rich sources of structurally diverse secondary metabolites many of which have indication of possessing potential anti-inflammatory activity affecting different molecular targets (3). Thus, screening of such natural products for anti-inflammatory activity is worth trying for developing newer drugs having less chances of above side effects (5).

In the family Bombacaceae, *Bombax malabaricum* DC. (Semal, Silk cotton tree) [Synonym: *Bombax ceiba* L.; *Salmalia malabaricum* Schott & Endl.], is important for its traditional medicinal use

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and its specific role in economy for cotton and timber. It is a lofty deciduous tree up to 40 m tall; young stems and branches are covered with stout, hard prickles; the leaves are large, spreading, glabrous, digitate, leaflets 5-7, lanceolate, 10-20 cm long; flowers numerous, large, fleshy, bright crimson, yellow or orange, clustered at the end of the branches; capsules oblong-ovoid containing many seeds, containing long, dense silky hairs. It is distributed in the tropics and subtropics of the world. (6).

According to literature, the leaves are used in inflammation and for cutaneous trouble (6, 7). The leaves extract exhibited significant antifungal activities against ringworm causing fungus. The bark gum contains catechutannic acid (8). Antioxidant and analgesic mangiferin was isolated from leaves of this plant, *Bombax malabaricum* (9).

Bombamalones A-D, bombamaloside, isohemigossypol-1-methyl ester, 5,2-*O*-methylisohemigossylic acid lactone, 6-bombaxquinone B and lacinilene C were isolated from the root (10). Petal contains orange-red anthocyanin pelargonidin-5- β -D-glucopyranoside and cyanidine-7-methyl ether-3- β -glucopyranoside. The root-bark contains lupeol, β -sitosterol, isohemigossypol-1-methyl ether, 7-hydroxycadalene (6). Seshadri et al. (11) isolated lupeol, β -sitosterol and β -sitosterol-D-glucoside from stem bark and two naphthoquinones were obtained from root bark of the plant along with lupeol and β -sitosterol. Lupeol from stem bark possesses antiangiogenic activity (12). Lin et al. (13) reported the anti-inflammatory activity of Taiwan folk medicine 'Mu-mien' which contains root, xylem of stem of *Bombax malabaricum*.

The purpose of the present work with methanol extract of *Bombax malabaricum* leaves (MEBM) was to confirm experimentally its anti-inflammatory activity as well as to establish its possible mechanism of action.

EXPERIMENTAL

Plant material

The fresh leaves of *Bombax malabaricum* were collected from the city of Azamgarh, Uttar Pradesh, India during May 2008 (Collection reference no. BM/08/02) and botanical identification was done by Dr. H. J. Chowdhery, a taxonomist and Joint Director, Central National Herbarium (CNH), Botanical Survey of India, Howrah, West Bengal, India and a voucher specimen was deposited in the herbarium of Central National Herbarium, Botanical Survey of India, Howrah, India for future reference (Voucher specimen no. CNH/1-I(314)/2009-Tech

II/356/346). The shade dried leaves were powdered and stored in air tight container.

Preparation of extracts

The powdered leaves were extracted in a Soxhlet apparatus using methanol as solvent. The solvent was removed under vacuum to get solid mass (15.48% w/w) and the crude extract was kept in a desiccator. Crude MEBM was suspended in 1% Tween 80 prior to each animal experiment. For cytotoxicity study and nitric oxide evaluation, MEBM (500 mg) was solubilized in 1 mL of DMSO and diluted in sterile distilled water to get required concentration range 0-500 μ g/mL.

Test animals

Wistar albino rats of either sex weighing 120-140 g were used. They were acclimatized for one week before the experiment and maintained under controlled conditions (temperature, $25 \pm 2^\circ\text{C}$ along with a light/dark cycle of 12 h each) and fed a standard pellet diet along with water *ad libitum*. The study had received prior approval from the Institutional Animal Ethical Committee, Pharmacy College, Azamgarh, India (937/c/06/CPCSEA).

Chemicals

Methanol, naphthyl ethylenediamine dihydrochloride (NED), sulfanilamide, phenazine methosulfate (PMS) and lipopolysaccharide (LPS) were obtained from CDH (India), Loba Chemie (India), SRL (India), Promega (USA) and Sigma (USA), respectively. Other chemicals utilized were of analytical grade.

Toxicity study

Acute toxicity and determination of LD₅₀ (upstairs and downstairs method) was performed as previously described (14). Further toxicity study was done with rats for a single fixed dose of 2 g/kg body weight (b.w.) according to the OECD guidelines (No. 420).

Carrageenan-induced paw edema in rats

Animals were divided into five groups containing six animals in each group. Control group (Group I) received 1% Tween 80. Crude MEBM as suspended in 1% Tween 80 and administered orally to the test animals in three doses, 100, 200, and 400 mg/kg b.w. (Group II, III, and IV). In another group (Group V), rats received indomethacin [10 mg/kg b.w. in 1% Tween 80 (4)].

Pedal inflammation was produced according to the method described by Winter et al. (15). Briefly,

rats were injected subcutaneously with 0.1 mL of freshly prepared 1% carrageenan solution in 0.9% w/v sodium chloride into the sub-plantar region of the right hind paw. One hour prior to carrageenan injection animals received saline with 1% Tween 80 (Group I), MEBM (Group II, III and IV) and indomethacin (Group V). Paw volumes were measured immediately before and 1-5 h following carrageenan injection using a plethysmometer with slight modification according to Roy et al. (16). In addition, the paw volume was measured 24 h later to observe the long term effect of the test substances. The percentage of inhibition was calculated as follows (4):

$$\text{Percent inhibition} = \frac{E_c - E_t}{E_c} \times 100$$

where E_c is the edema volume of the control group and E_t is the edema volume of the treated group.

Preparation of mouse peritoneal macrophages

Swiss albino mice were used for collection of peritoneal macrophages as previously described (4). Each mouse was injected *i.p.* with 2 mL of 2% starch solution in phosphate buffered saline (PBS), 0.02 M, pH 7.2. After three days, the peritoneal cells comprising of macrophages principally, were lavaged with sterile PBS (10 mL). The exudate was centrifuged (4000 rpm \times 10 min). The resultant cell pellet was washed with PBS and finally resuspended with RPMI-1640 phenol red free medium supplemented with 10% fetal calf serum (Medium A) (4). Macrophage viability (> 95%) was confirmed by the Trypan blue dye exclusion technique.

Cytotoxicity assay

The viability of macrophages against MEBM in DMSO was determined by re-suspending macrophages (1×10^6 cells/mL in Medium A) in 96-well sterile tissue culture plates. It was incubated with MEBM (0-500 $\mu\text{g/mL}$) at 37°C in a CO₂ incubator for 48 h. At the end of the drug exposure, the MTS ([3-(4,5-diethylthiazol-2-yl)-5-(carboxymethoxyphenyl)]-2-(4-sulfophenyl)-2H-tetrazolium) assay was performed. Briefly 20 μL of MTS (2 mg/mL in PBS) and PMS (0.92 mg/mL in PBS) mixture (ratio 20 : 1, v/v) were added to each well (4). The plates were then incubated at 37°C for 3 h and absorbance was measured at 490 nm using a Multiskan ELISA reader (BioRad, USA). DMSO, the vehicle, was reported to be non-toxic up to 5% (4); subsequently all the experiments were performed at a concentration lower than the said level.

Measurement of nitric oxide (NO) production in macrophages

Peritoneal macrophages were seeded (2×10^6 cells/mL in Medium A) in 6 well plates and incubated at 37°C in 5% CO₂ incubator for 4 h. Cells were then treated with LPS (10 mg/mL) in the presence of MEBM (0-100 mg/mL) for 48 h. At the end of 48 h, the Griess assay was performed (17) by taking equal volumes of the Griess reagent and incubated in the dark at room temperature (25-30°C) for 10 min. Griess reagent is a mixture of NED (0.1% in water) and sulfanilamide (1% in 5% phosphoric acid) of 1 : 1 ratio. The nitrite levels were assayed using a standard curve generated with sodium nitrite

Table 1. Effect of MEBM on carrageenan induced rat paw edema.

Group	Edema volume in mL (% inhibition) [#]					
	1 h	2 h	3 h	4 h	5 h	24 h
Control	0.3175 ± 0.019	0.4645 ± 0.0209	0.5553 ± 0.0373	0.6605 ± 0.0244	0.7142 ± 0.0466	0.8858 ± 0.0491
MEBM 100 mg/kg	0.3022* ± 0.0112 (4.82)	0.3955* ± 0.0118 (14.85)	0.4302* ± 0.006 (22.53)	0.4783* ± 0.007 (27.59)	0.4967* ± 0.011 (30.45)	0.6938* ± 0.0343 (21.68)
MEBM 200 mg/kg	0.2822** ± 0.0252 (11.12)	0.371** ± 0.0208 (20.13)	0.4043** ± 0.0255 (27.19)	0.445** ± 0.0224 (32.63)	0.4683** ± 0.0214 (34.43)	0.634** ± 0.0412 (28.43)
MEBM 400 mg/kg	0.2572*** ± 0.013 (18.99)	0.330*** ± 0.0205 (28.96)	0.375*** ± 0.0513 (32.47)	0.4168*** ± 0.0256 (36.9)	0.4292*** ± 0.0275 (39.9)	0.5945*** ± 0.0426 (32.89)
Indomethacin 10 mg/kg	0.204*** ± 0.0228 (35.75)	0.2357*** ± 0.0178 (49.26)	0.197*** ± 0.0214 (64.52)	0.1785*** ± 0.0185 (72.98)	0.1717*** ± 0.0257 (75.96)	0.3358*** ± 0.0263 (62.09)

[#] Edema volume and % inhibition was measured as described in Materials and Methods. Each value is the mean \pm SD (n = 6). *p < 0.05; **p < 0.01; ***p < 0.001 as compared to controls.

as standard and *N*-monomethyl L-arginine (NMMA, 100 μ M) was used to confirm the assay specificity.

Statistical analysis

All results are expressed as the mean \pm SEM. The data were analyzed for statistical significance by one-way analysis of variance (ANOVA) followed by Tukey test using computerized GraphPad Prism, version 4.03 software (Graph Pad Software Inc.). Values of $p < 0.05$ were considered statistically significant.

RESULTS

Toxicity study

In the LD₅₀ study, all mice survived with a dose up to 2.0 g/kg b.w. in oral administration. No death was also observed with rats at 2.0 g/kg b.w. There were no signs of toxicity, change in color of eye, skin and mucous membrane and also no changes were observed in their respiratory rate during the 14 days observation period. Minor enhancement of sleeping time was found on second day. There was no tremor, convulsion, diarrhea observed during the study period.

Anti-inflammatory activity

MEBM at a lower dose of 100 mg/kg demonstrated significant anti-inflammatory activity ($p <$

0.05); improved activity was seen with 200 mg/kg ($p < 0.01$) and most significant activity ($p < 0.001$) was observed with MEBM at a 400 mg/kg dose range as compared to untreated control (Table 1) and the maximum activity was observed at 5th h for all the test groups after carrageenan injection. Indomethacin 10 mg/kg demonstrated potent anti-inflammatory activity ($p < 0.001$) maximum at 5th h (75.96%). 22.53, 27.19, and 32.47% inhibition was found with MEBM at a dose of 100, 200, and 400 mg/kg, respectively, at 3rd h (Table 1).

Cytotoxicity assay

In peritoneal macrophages, the IC₅₀ for MEBM was 258.33 μ g/mL. It was observed that up to 125 μ g/mL, no cell death occurred and therefore, in all studies, concentration below 125 μ g/mL was used.

Effect of MEBM on nitric oxide production in macrophages

Murine peritoneal macrophage when stimulated with LPS translated into a 15.13 fold increase in NO production ($24.170 \pm 0.196 \mu$ M vs. $1.597 \pm 0.087 \mu$ M in control). With the addition of MEBM (25, 50, 75 and 100 μ g/mL), a dose dependent decrease in NO production occurred being 22.37 ± 0.08 , 21.44 ± 0.51 , 18.14 ± 0.13 , and $16.01 \pm 0.13 \mu$ M, respectively ($p < 0.001$). *N*-monomethyl L-arginine (NMMA, 100 μ M) served as the positive

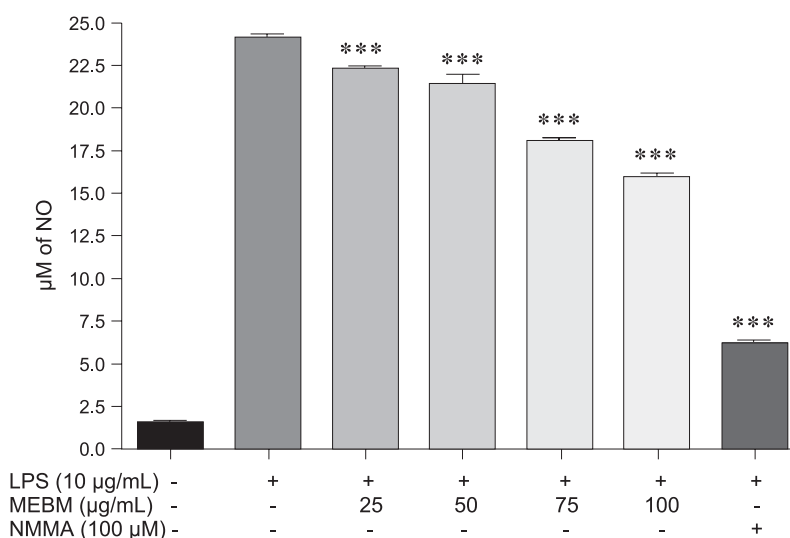


Figure 1. Effect of MEBM and NMMA on production of NO in macrophages. Mice peritoneal macrophages (2×10^6 cells/mL in RPMI-1640 phenol red free medium supplemented with 10% FCS) were incubated with LPS (10 μ g/mL) in the presence of MEBM (25, 50, 75, and 100 μ g/mL) or NMMA (100 μ M) at 37°C, 5% CO₂ for 48 h and assayed for levels of extra-cellular NO as described in Experimental. Each point represents the mean \pm SD of NO (μ M) of at least three experiments in duplicate. *** $p < 0.001$ as compared to LPS control

control and caused $6.2 \pm 0.12 \mu\text{M}$ in NO production ($p < 0.001$) (Fig. 1).

DISCUSSION AND CONCLUSION

In the present study, the anti-inflammatory activity of MEBM was found to be significant and dose dependent. The activity increased with time reaching a maximum at the 5th h, which might be due to delayed oral absorption. Thus, this study established the anti-inflammatory activity of MEBM on carrageenan induced acute phase of inflammation and thus, could justify experimentally the folklore use of the leaves of *Bombax malabaricum* in inflammation. It was further observed that maximum activity (39.90%) was observed with MEBM at a dose of 400 mg/kg b.w. The highest increased rate of anti-inflammatory activity was found between first and second hour in all the cases of MEBM. The activity persisted even up to 24 h and beyond after carrageenan injection like indomethacin probably due to protein binding. Indomethacin also demonstrated the maximum activity (75.96%) at 5th h.

It is understood that the subcutaneous injection of carrageenan into the rat paw produces biphasic edema formation (4). It is characterized by increased water and plasma protein exudation with neutrophil extravasation as well as by increased formation of metabolites of arachidonic acid *via* the cyclooxygenase and lipoxygenase enzyme pathways (18). It is expected that anti-inflammatory effect of the test samples might be due to its inhibition of any of the inflammatory mediators like histamines, kinins, prostaglandins, cytokines, nitric oxide (NO), etc.

NO is an important mediator for host defense and inflammatory response. During inflammatory disease, excessive amount of NO formation takes place in macrophages. It is produced as free radical by iNOS through catalyzing the oxidation of guanidine of L-arginine with formation of L-citrulline. LPS, derived from cell walls of Gram negative bacteria, activates multiple signaling pathways in macrophages to enhance production of inflammatory mediators like NO. It couples with superoxide to form peroxynitrite, which is responsible for the production of prostaglandins through prostaglandin endoperoxide synthase pathway (2, 19). The experimental data (Fig. 1) show that in a dose dependent manner, the MEBM reduces the production of NO.

In phytochemical screening of the extract (MEBM), certain phytochemicals like steroids, carbohydrates, tannins, triterpenoids, flavonoids and coumarin glycosides were found to be present (20). Inhibition of the synthesis and/or release of inflam-

matory mediators such as NO, caused by certain phytochemical present in MEBM, may be the main mechanism(s) of action of the extract. So far, anti-inflammatory activity of lupeol which produced only 11% in 3 h at concentration of 400 mg/kg in carrageenan induced paw edema has been reported (21). Anti-inflammatory potentials of steroids, tannins, triterpenoids, flavonoid and coumarin glycosides have been reported in various studies (22-24). Therefore, antipyretic activity of MEBM may be due to the presence of steroids, tannins, triterpenoids, flavonoid and coumarin glycosides.

The results obtained have demonstrated significant anti-inflammatory activity of methanol extract of *Bombax malabaricum* leaves in dose dependent fashion. It was found that MEBM possesses the anti-inflammatory activity by reduced secretion of prostaglandins due to inhibition of the production of one of the principal mediators for inflammation like NO. Further experimentation is under way in our laboratory to isolate active molecules and to establish their role in the anti-inflammatory activity of MEBM and in possible mechanism of action. Such study might help to obtain better anti-inflammatory drugs with specific mechanism of action in the future.

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