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Stem bark extraction of *Ficus bengalensis* Linn for anti-inflammatory and analgesic activity in animal models

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In the present study, anti-inflammatory and analgesic effect of aqueous extract of *Ficus bengalensis* (AEFB) and methanolic extract of *F. bengalensis* (MEFB) was evaluated in animal models. Preliminary results indicated that MEFB treatment possesses significant anti-inflammatory potential as compared to AEFB. The anti-inflammatory activity of MEFB exhibited in both acute (carrageenan induced hind paw edema and acetic acid induced vascular permeability) and sub-chronic (cotton pellet-induced granuloma) models of inflammation was found to be significant. In addition, the extract also showed significant analgesic activity in acetic acid induced writhing. Pretreatment with MEFB during inflammatory condition (both acute and sub-chronic) prevented increase in malondialdehyde (MDA) formation and myeloperoxidase activity in edematous as well as granulomatous tissue. Further, serum marker enzymes (AST, ALT and ALP) increased in inflammatory conditions were also inhibited with MEFB treatment. Hence, the anti-inflammatory activity observed in the present study with MEFB could be attributed largely to its antioxidant and lysosomal membrane stabilizing effects.

Keywords: Analgesic activity, Anti-inflammatory, Biochemical parameters, *Ficus bengalensis*

Ayurvedic practitioners in India are using the milky juice (latex) of stem bark of *F. bengalensis* for the treatment of rheumatism and other inflammatory diseases[1]. Extract of *F. bengalensis* has shown antioxidant[2], antidiabetic[3], immunomodulatory[4], hypolipidemic[5-6], antiasthamatic[7], and wound healing[8] activity in experimental animals.

Phytochemical study has revealed that *F. bengalensis* contains β-sitosterol, flavonoids, tannins, 5,7-dimethyl ether of leucopelargonidin 3-0-α-L rhamnoside, 5,3'dimethyl leucocyanidin 3-0-β-galactosyl cellobioside[2].

The present study was designed to demonstrate the anti-inflammatory activity of methanolic extract of *F. bengalensis* (MEFB) and efforts were made to understand the possible mechanism(s) of anti-inflammatory activity of MEFB.

Materials and Methods

Plant material—Stem bark of *F. bengalensis* was collected from Hindustan Antibiotic colony Pimpri, Pune in the month of October 2007, dried below 40°C in hot air oven. The plant material was authenticated and deposited at Agharkar Research Institute, Pune, India (Voucher no. Auth 07-096).

Extraction process—The dried bark of *F. bengalensis* was pulverized and extracted with water (AEFB) and methanol (100 % v/v; MEFB) separately for 72 h by maceration process, which was repeated for 3 times to ensure the complete extraction of chemical constituents from the bark.

Chemicals—Carrageenan (Sigma-Aldrich MO, USA.), diclofenac sodium (J.B.Chemicals pharmaceutical Ltd. Mumbai.), standard reagents kit (Nirmal Lab, India) for determination of aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP) were procured from respective producer.

Animals—Wistar rats (150-200 g) and Swiss albino mice (20-25g) were purchased from National Toxicological Centre, Pune, India. Animals were housed under standard condition of temperature (24° ±1°C), relative humidity (65±10 %), light and dark cycle (12:12 h) and fed with standard pellet food, (Amrut Laboratory animal feed diet, Pune, India) and water *ad libitum.*

Preparation and route of drug administration—The reference drug diclofenac sodium, AEFB and MEFB

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was prepared in 1 % (w/v) gum acacia as a uniform suspension using mortar and pestle. The dosages of suspension were adjusted so that maximum volume of drug administered was within the range of 1 ml/100 g. Diclofenac sodium, AEFB and MEFB were administered orally to rats and mice for all experiments.

Institutional ethics committee approval—The experimental design and research plan along with animal's handling and disposal procedure were placed before the institutional ethics committee. The committee granted approval after carefully evaluating research project during their meeting held in January 2008. Animal House Registration No. 198/99/CPCSEA.

Acute toxicity study—Acute toxicity study of AEFB and MEFB was carried out in mice according to OECD guidelines[9]. Different doses of AEFB and MEFB were administered upto 2000 mg/kg, (po) and animals observed for a period of 72 h for behavioral changes, toxic reactions and mortality.

Dose response study in carrageenan-induced paw edema rats[10]—Rats were randomly divided into 4 groups (6 rats/ group) and to all the groups, hind paw edema was induced by injecting 0.1 ml of 1 % (w/v) carrageenan subcutaneously (sc) into the planter region of the hind paws of rats. Diclofenac sodium (10 mg/kg) and MEFB [100, 200, 300, 400 and 500 mg/kg (po)] were administered 1 h prior to carrageenan injection. One group of rats served as vehicle treated control.

Time course study in carrageenan-induced paw edema rats—The hind paw edema volume was measured by volume displacement method using plethysmometer (UGO Basile 7140, Italy) by immersing the paw till the level of *lateral malleolus,* at various time intervals (0, 1, 2, 3, and 6 h) after carrageenan injection. Results were expressed as percentage inhibition of edema by comparing with the vehicle treated control group.

Rats were sacrificed at 6 h after carrageenan injection (after measuring the paw volume) under light ether anesthesia. Blood was collected by cardiac puncture and serum was separated by centrifugation (Remi, USA) at 2500 rpm below 30°C for 30 min and used for the assay of marker enzymes (AST, ALT and ALP). The edematous tissues of hind paw was separated under controlled temperature (0-10°C) and used for the determination of malondialdehyde (MDA) formation and myeloperoxidase activity (MPO).

Cotton pellet-induced granuloma in rats[11]—Four sterilized cotton pellets (10 mg) were implanted on either side (2 on each side) of the ventral region of rats. Cotton pellet inserted rats were randomly divided into 4 groups (6 rats/ group). Different groups of rats were administered with MEFB (200 and 400 mg/kg, po) and diclofenac sodium (10 mg/kg, po) daily for 8 days. The control group received vehicle, gum acacia (1% w/v, 1ml/100g, po) for the same period. On the day 9, rats were sacrificed under ether anesthesia. Blood was collected by cardiac puncture and allowed to clot for 30 min at room temperature. The serum was separated by centrifugation (Remi, USA) at 2500 rpm below 30°C for 30 min and used the assay of marker enzymes (AST, ALT and ALP). The cotton pellets were removed and freed from extraneous tissue. Two cotton pellets from each rat were used for the separation of granular tissues for biochemical studies. In remaining 2 cotton pellets of each rat was used for determination of granular tissue formation. Granular tissue formation was studied by drying cotton pellets at 60 °C for 6 h or till the weight of the pellet remains constant. The dry weight was calculated after deducting cotton pellet weight and taken as a measure of granular tissue formation.

Acetic acid-induced vascular permeability[12]—The mice were divided into 4 groups, (6 mice/group) and allowed free access to water *ad libitum*. Group 1 and 2 of mice were treated with MEFB 200 and 400 mg/kg, po respectively, group 3 treated with diclofenac (10 mg/kg, po) and group 4 (control) received vehicle 1% (w/v) 1ml/100 g, (po)

After 1 h of administration of MEFB and diclofenac sodium, mice were injected with 0.25 ml of 0.6 % (v/v) solution of acetic acid intraperitoneally (ip). Immediately, 10 ml/kg of 10 % (w/v) Evan's blue was injected intravenously via tail vain. After 30 min of Evan's blue injection, the animals were anaesthetized with ether anesthesia and sacrificed. The abdomen was cut open and exposed viscera. The animals were held by a flap of abdominal wall over a petri dish. The peritoneal fluid (exhudate) collected, filtered and made up the volume to 10 ml using normal saline solution. The dye leaking out into the peritoneal cavity was measured by reading the diluted peritoneal fluid at 610nm on a spectrophotometer (Shimadzu 1700, Japan).

Acetic acid induced writhing in mice[13]—A group of mice were injected (ip) with 0.1ml/10g of 0.3 % (v/v) acetic acid. The mice exhibiting the writhing

movements (stretching of hind limbs and bending of trunk) were selected for the study. These mice were randomly divided into 4 groups (6 mice/ group). These mice were administered with MEFB (200 and 400 mg/kg, po) and diclofenac sodium (10 mg/kg, po) 1 h prior to acetic acid injection. The numbers of writhings movements were counted for 30 min following acetic acid injection.

Biochemical studies—The biochemical changes were investigated after 6 h and on day 9 in carrageenan edema and in cotton pellets granuloma, respectively. Rats were anaesthetized under ether anesthesia and blood was collected by cardiac puncture and serum was separated by centrifugation (Remi, USA) at 2500 rpm below 30°C for 30 min and marker enzymes AST, ALT and ALP were determined[14,15] using kits (Nirmal Lab, Jalgaon, India).

Edematous and granulomatous tissues homogenates (10% w/v) were prepared in phosphate buffer (*p*H 7.6). Quantitative estimation of MDA formation was done by determining the concentration of thiobarbituric acid reactive substances (TBARS) in 10% homogenates of edematous and granulomatous using the method[16] as described earlier. Amount of MDA formed was quantified by reaction with thiobarbituric acid and used as an index of lipid peroxidation. The results were expressed as nmole of MDA/g of wet tissue using molar extinction coefficient of chormophore (1.56×10^{-5} /M/cm) and 1, 1, 3, 3-tetraethoxypropane as standard.

The MPO activity was assayed[17] in the same homogenate by the reaction with hexaethyltrimethylammonium bromide (HTAB) and ortho-dinisidene dihydrochloride.

Statistical analysis—Data are expressed as mean±SEM. Statistical analysis was carried out by using one-way ANOVA followed by Dunnett's test. The values at $P<0.05$ were considered as significant.

Results

Extraction process—Percentage yield by AEFB and MEFB was found to 1.6 and 2.1% (w/w), respectively. Preliminary phytochemical analysis for extract indicated the presence of flavonoids (sulphuric acid test and ferric chloride test) presence of glycoside, carbohydrates (molisch reagent test and benedict reagent) presence of tannins, amino acid (ninhydirn reagent test positive) and sterols. The other physicochemical and analytical profiles are presented in Table 1.

Acute toxicity studies—LD_{50} value by oral route could not be determined as no mortality was observed upto 4 g/kg dose level. No toxic reactions were observed at none of the doses employed.

Dose response study in carrageenan-induced paw edema rats—Based on preliminary studies, MEFB showed significant anti-inflammatory activity at 200 and 400 mg/kg in carrageenan hind paw edema in rats than AEFB (Fig. 1). Hence, only MEFB was selected for further anti-inflammatory activity evaluation.

In dose response study in carrageenan edema, MEFB treatment induced a dose related inhibition. However, the extent of edema inhibition in rats treated with MEFB (400 mg/kg) showed lesser inhibition as compared to diclofenac sodium (10 mg/kg) treated group (Table 2). In time course study, rats treated with MEFB (400 mg/kg) and diclofenac sodium (10 mg/kg) elicited maximum inhibition of edema formation at 6 h after carrageenan injection (Fig. 2).

Table 1—Physicochemical and analytical profile of MEFB

Test	Observation
Physical appearance	Brown Amorphous powder
Melting point	Decomposes above 90°C
Solubility	Freely soluble in methanol, ethanol, Sparingly soluble in water, ether, chloroform
UV MeOH λmax nm ($E^{1\%}$1 cm)	268, 211
R_f value (TLC) *of MEFB	0.52
R_f value (HPTLC) **of MEFB	At 268 nm-0.82, 0.89, 0.88 At 211 nm- 0.54, 0.43, 0.42, 0.41

* Solvent system: n-hexane: ethyl acetate (4:1)
** Solvent system: n-hexane: ethyl acetate (9:1), n-hexane: ethyl acetate (4:1),

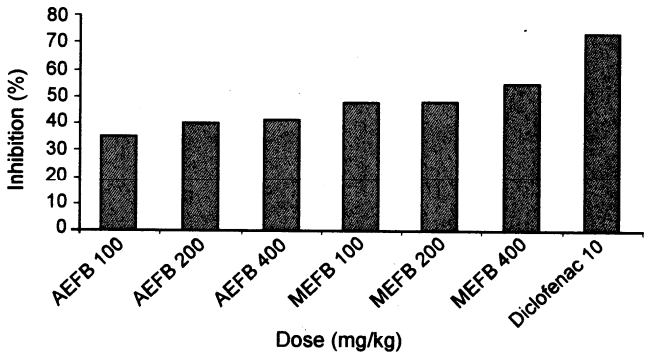

Fig. 1—Effect of extracts of F. bengalensis (AEFB and MEFB) at different doses on inhibition of carrageenan induced hind paw edema in rats after 6 h of treatment [Values are mean ± SEM of 6 animals]

Cotton pellet-induced granuloma in rats—MEFB treatment (orally) inhibited both the exudatory and granulatory phases of inflammation and its inhibitory effect was significant and dose related. Diclofenac sodium treatment also showed similar inhibitory effect on both the phases. It was observed that diclofenac sodium induced greater inhibitory effect than the MEFB treatment (Table 3).

Acetic acid-induced vascular permeability—Administration of acetic acid induces increase in vascular permeability resulting into a greater leakage of dye into peritoneal fluid. Pretreatment with the MEFB inhibited significantly increased leakage of dye into peritoneal fluid due to acetic acid at both the doses (200 and 400 mg/kg). Diclofenac sodium (10 mg/kg) treatment showed greater inhibitory effect than MEFB (Table 4).

Acetic acid induced writhing in mice—Pretreatment with MEFB (200 and 400 mg/kg) prevented acetic acid induced writhing movements in mice. Inhibitory effect of diclofenac sodium (10 mg/kg) on acetic acid induced writhing was greater than MEFB (400 mg/kg) effect. (Table 5).

Table 2—Effects of *F. bengalensis* Linn on carrageenan induced hind pawedema in rats

[Values are mean±SEM of 6 rats]

Treatments and dose(mg/kg, po)	Volume of paw edema (ml) at various time interval			
	0 h	1 h	3 h	6 h
Vehicle (1ml/100g)	2.4±0.1	4.1±0.1	5.9±0.1	7.5±0.2
Diclofenac sodium (10)	1.9±0.1	3.3±0.1	2.9±0.1**	2.0±0.1**
MEFB (200)	3.2±0.1	4.1±0.1	4.0±0.1**	3.7±0.1**
MEFB (400)	3.1±0.1	4.0±0.1	3.8±0.1**	3.4±0.1**

Significance level ***P*<0.01was calculated by comparing with vehicle treated control group

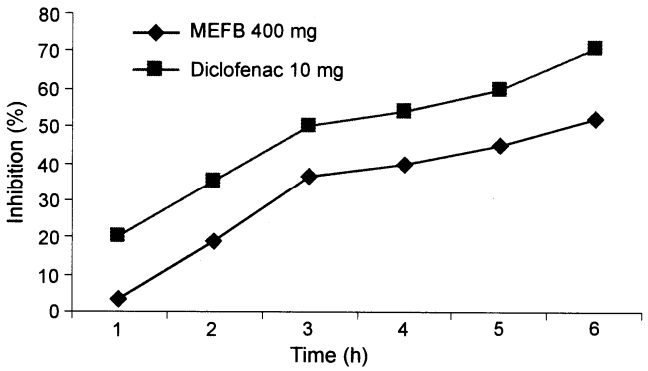

Fig. 2—Effect of MEFB and diclofenac on carrageenan edema at different time interval) [Values are mean ± SEM of 6 animals]

Biochemical studies—Marker enzymes (AST, ALT and ALP) in serum and MDA formation, myeloperoxidase activity in edematous tissue were elevated significantly during carrageenan induced hind paw edema in rats. Pretreatment with MEFB (200 and (400 mg/kg) and diclofenac sodium (10 mg/kg) significantly prevented the elevated myeloperoxidase level and MDA formation in edematous tissue and marker enzymes in serum. The increased level of marker enzymes in serum and MDA formation in granulomatous tissue prevented significantly at both the doses of MEFB (200 and 400 mg/kg) and diclofenac sodium 10 mg/kg (Table 6).

Table 3—Effects of *F. bengalensis* Linn on cotton-pellet granuloma in rats

[Values are mean±SEM of 6 rats]

Treatments and dose (mg/kg, po)	Granuloma weight (mg)	
	Wet	Dry
Vehicle (1ml/100g)	271.3±15.1	144.4±9.9
Diclofenac sodium (10)	125.2±9.6**	79.96±6.2**
MEFB (200)	226.7±7.5*	117.9±3.3**
MEFB (400)	174.5±10.2**	89.2±5.5**

Significance level **P*<0.05, ***P*<0.01was calculated by comparing with vehicle treated control group

Table 4—Effect of *F. bengalensis* Linn on acetic acid induced vascular permeability in mice

[Values are mean±SEM of 6 mice]

Treatment and dose of drug (mg/kg, po)	Amount of dye leakage (ug/ml)	Inhibition of dye leakage (%)
Vehicle (1ml/100 g)	20.10±1.0	-
Diclofenac sodium (10)	6.62±1.0**	67.1
MEFB (200)	14.59±0.9**	27.4
MEFB (400)	8.07±0.6**	59.9

Significance level **P*<0.05, ***P*<0.01was calculated by comparing with vehicle treated control group

Table 5—Effects of *F. bengalensis* Linn on acetic acid-induced writhing in mice

[Values are mean±SEM of 6 mice]

Treatments and dose (mg/kg, po)	Number of writhing			
	0-10 min	10-20 min	20-30 min	Total
Vehicle (1ml/100g)	18.22±1.2	21.33±1.2	8.00±0.6	47.55±2.9
Diclofenac sodium (10)	8.20±1.0**	11.66±1.2**	3.16±1.2**	23.48±2.1**
MEFB (200)	10.00±1.0	17.5±0.6*	5.1±0.6*	32.76±2.2*
MEFB (400)	9.60±1.1*	12.0±0.7**	4.16±0.4**	25.76±2.1**

Significance level **P*<0.05, ***P*<0.01was calculated by comparing with vehicle treated control group

Table 6—Effect of MEFB on serum marker enzymes, edematous , granulomatous MDA formation and edematous MPO level in experimental inflammation in rats

[Values are mean ± SEM of 6 rats]

Type of tissue	Biochemical parameters	Carrageenan-induced paw edema				Cotton pellet-induced granuloma			
		Vehicle	Diclofenac sodium	MEFB		Vehicle	Diclofenac sodium	MEFB	
		(1ml/100)	(10)	(200)	(400)	(1ml/100)	(10)	(200)	(400)
Serum	AST(U/ml)	122.0±3.7	88.0±2. 0**	106.0±6.4	99.3±2.9**	79.0±5.8	34.0±2.4**	51.83±5.0*	43.0±2.3**
	ALT(U/ml)	58.0±1.2	41.0±4.0**	56.0±2.0**	52.0±1.2*	115.33±12.6	33.66±5.5**	85.33±5.7*	55.7±7.0*
	ALP (U/ml)	71.5±3.2	56.3±3.0*	65.2±1.7	62.3±2.1	91.08±5.0	54.42±1.4**	66.60±3.7*	56.8±1.0**
Tissue	MDA formation (nmole of MDA/g of wet tissue)	82.20±4.5	30.58±0.6**	65.3±0.7	50.0±0.8*	90.01±4.2	45.77±2.6**	77.47±1.8*	46.3±1.9*
	MPO level (U/mg of tissue)	1.14±0.1	0.69±0.9*	1.13±0.02	0.73±0.9*	-	-	-	-

Significance level *p<0.05, **p<0.01was calculated by comparing with vehicle treated control group

Discussion

Carrageenan induced inflammation has been reported to be a useful model for screening of clinically effective anti-inflammatory agents[18]. Edema formation due to carrageenan in rat is a biphasic event. The initial phase of edema is attributed to the release of histamine and serotonin and the second phase of edema is due to the release of prostaglandins, protease and lysosomal enzymes. Further, it has been demonstrated that the second phase is sensitive to the most clinically effective anti-inflammatory drugs[19].

In the present experiments, MEFB inhibited the carrageenan edema in a dose related manner at 3 and 6 h. Hence, it is likely that MEFB might elicits its anti-inflammatory activity by inhibiting synthesis and release of prostaglandins, proteases and lysosomal enzymes like nonsteriodal anti-inflammatory drugs[20].

During the repair process of inflammation, there is proliferation of macrophages, neutrophils, fibroblasts and multiplication of small vessels, which are the basic sources of forming a highly vascularised reddish mass termed granular tissue[21]. In sub-chronic rat model of inflammation (cotton pellet granuloma), MEFB inhibited both exudatory and granulatory phase of inflammation in a dose related manner. This ihhibitory effect can be attributed to its multilple actions on targets like (a) mediators of inflammation, (b) lysosomal enzymes, (c) oxidative stress and (d) capillary permeability. There are documented reports that lysosomal enzymes play an important role in the development of acute and chronic inflammation[22-23]. In the present experiments, MEFB treatment (200 and 400 mg/kg) prevented the increased serum marker enzymes both in acute as well as sub-chronic inflammatory condition, thereby suggesting its membrane stabilising potential. Most of the anti-inflammatory drugs exert their beneficial effect either by inhibiting the release of lysosomal enzymes or by stabilizing lysosomal membrane[24]. Furthermore, it was observed that membrane stabilising effect of MEFB can be correlated to its anti-inflammatory activity.

Myeloperoxidase (MPO) is an enzyme present in neutrophils, monocytes and macrophages at a much lesser concentration. It has been demonstrated that, the level of MPO activity is directly proportional to neutrophils concentration in the inflamed tissue[17]. Hence, the measurement of the MPO activity has been considered a sensitive index of chemotaxis and neutrophils infiltration into the inflammation site. Pretreatment with MEFB significantly decreased MPO activity in edematous tissue. The extent of inhibition of MPO is well correlated with the reduction of edema formation. Similarly, MDA formation (an index of lipid peroxidation) during inflammation, could also serve as an important paradigm for the assessment of the antioxidant activity of anti-inflammatory agents. Furthermore, a good correlation was observed between biochemical effects like inhibitory effect on MDA formation, inhibition in serum marker enzymes; MPO activity); and pharmacological activity (inhibition of edema formation). Hence, it is suggested that antioxidant activity of MEFB might contribute to a great extent to its anti-inflammatory activity observed in the present study. MEFB treatment had also demonstrated its

ability to prevent the increase in serum marker enzymes during carrageenan edema and cotton pellet granuloma, which might be attributed to its membrane stabilization effect.

Acetic acid is known to cause irritation in the peritoneum, resulting in vasodilatation through liberation of mediators, like histamine and nitric oxide. This event is followed by increased permeability of the microvasculature that facilitates to escape of protein-rich fluid into the extravascular compartment[25]. Ability of MEFB to reduce vascular permeability was reflected by significant reduction of dye leakage into the peritoneal cavity in a dose related manner. It is quite possible that inhibition of vascular permeability by MEFB likely to contribute significantly to its anti-inflammatory activity observed in the present study in both acute and sub-chronic inflammation.

Acetic acid-induced writhing is highly sensitive and documented model of visceral pain for screening of analgesic drugs[26]. MEFB reduced the acetic acid writhing movements in mice significantly, thereby indicating its analgesic activity. Since pain is an integral part of inflammation, the analgesic activity shall certainly a beneficial factor during inflammatory condition.

Since MEFB contains singificant amount of flavonoids and tannins which are known to elicit anti-inflammatory activity through their effect on oxidative stress and membrane stabilization[27].

In conclusion our findings, using both the pharmacological and biochemical parameters in different animal models suggest that MEFB is a promising anti-inflammatory and analgesic agent and may be useful for the treatment of inflammatory conditions. However, studies are required on human subjects to prove its clinical efficacy as an anti-inflammatory-analgesic agent.

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