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Research Article

ANALGESIC AND ANTI-INFLAMMATORY ACTIVITY OF DIFFERENT FRACTIONS OF *HIPTAGE BENGHALENSIS* (*LINN*)

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ABSTRACT

The present study was carried out to evaluate the analgesic and anti-inflammatory activity of various fractions of toluene, ethyl acetate, butan-2-one and n-butyl alcohol of *Hiptage benghalensis (Linn)* (Malpighiace). The fractions of the plant material were evaluated for the analgesic activity by writhing test, Eddy's Hot plate test in mice and formalin-induced test and anti-inflammatory activity was screened by Carrageenan-induced rat paw edema and cotton pellet induced granuloma formation in rat models. The test fractions were showed analgesic and anti-inflammatory effect in dose dependent manner and ethyl-acetate fraction was found to be most potent among the test fractions. At150mg/Kg b.w.p.o dose Ethyl-acetate fraction significantly inhibited 70.39% writhing response and 83.64, 82.71% Formalin induced analgesic in mice. The fraction with same dose showed significant 68.50% inhibition of Carrageenan induced rat paw edema and 41.36, 36.95% anti-proliferative effect of cotton pellet in rats and also different fractions of *Hiptage benghalensis (Linn)* showed prompt analgesic and anti-inflammatory activity due to dual inhibition properties on COX-2 and 5-LOX.

Keywords: *Hiptage benghalensis,* Acetic acid-induced writhing, Carrageenan-induced rat paw edema, Cotton pellet induced granuloma.

INTRODUCTION

Hiptage benghalensis is a large, evergreen, climbing shrub with brownish bark peeling off in flakes, young parts silky. Leaves opposite, coriaceous 9-18 cm long and 4-8 cm wide, lanceolate to elliptic-oblong, apex acute to acuminate, base rounded to cuneate, margins entire, lower surface finely hairy, venation reticulate, petioles thick 1.2-2 cm across, fragrant, white with a yellow or pink tinge, borne in erect, finely to mentose terminal and axillary racemes about as long as the leaves. In India the plant is widely distributed in forest at an altitude of 1800 m, except in dry regions.

And also found in moist habitats such as along riverbanks and in ravines. The plant occur in Sri Lanka, Andaman Islands, Bangladesh, and Myanmar (Burma) to Southern China. The bark, leaves and flowers are aromatic in nature and are commanly used as refrigerant, expectorant, cardiotonic, Anti-inflammtory and insecticidal. They are used in burning sensation, wounds, ulcers, leprosy, cardiac debility, rheumatism and hyperdipsia **(**Chatterjee and Chandrapakrashi, 1994; Vaidyaranam, 1995**)**.

In Ayurveda the leaves and barks are considered vulnerary, the leaves are highly regarded for treating skin diseases. The leaf juice possesses insecticidal properties and is used an external application for scabies. The plant is also used in the treatment of chronic rheumatism and asthma (Parrotta, 2001).

MATERIALS AND METHODS

Plant material

The leaves of *Hiptage benghalensis (Linn)* (Malpighiace) was collected in the month of June from Warangal, Andhra Pradesh, India. The selected plants were authenticated by Prof. Raju S. Vastavaya, Department of Botany, Kakatiya University, Warangal and voucher specimens were being maintained in the herbarium of University College of Pharmaceutical Sciences, Kakatiya University, Warangal.

Preparation of Extracts

Leaves of *Hiptage benghalensis* (4kg) were made free from the adherent foreign material and air-dried. Then they were coarsely powdered and 2kg of each was macerated with methanol in a round bottom flask for 7 days separately. The content of the flask were stirred intermittently to ensure the efficiency of the extraction. After a week, they were filtered and concentrated under reduced pressure to yield corresponding extracts, and the extracts were kept in a desiccator to remove moisture and stored properly until used.

The methanolic extracts of *Hiptage benghalensis (Linn)* (MHB) were dispersed in sufficient amount of distilled water separately and fractionated with toluene, ethyl acetate, butan-2-one and n-butyl alcohol in succession. The obtained fractions and the aqueous residues were concentrated under reduced pressure to yield corresponding extracts.

Chemicals

The chemicals were purchased from the following companies: Carrageenan-S.D Fine chemicals, Mumbai. Diclofenac sodium - Dr. Reddys laboratories, Hyderabad. Pentazocine - Pure Pharma Ltd. Mumbai. Methanol - Ranbaxy laboratories, Mumbai. Toluene, Ethyl acetate, Butanone and Butanol - Merck, Germany), Formaldehyde-S.D Fine chemicals, Mumbai). Indomethacin - S.D Fine chemicals, Mumbai. All other chemicals and solvents used were of analytical grade.

Animals

Swiss albino mice (25-30 g) and Wistar albino rats (180-225 g) of either sex were purchased from M/S Mahaveera agencies, Hyderabad and maintained in the animal house of University College of Pharmaceutical Sciences, Warangal. Animals were provided with standard rodent pellet diet and the food was withdrawn 18-24 h before the experiment, water was allowed *ad libutum*. They were maintained at ($27^0C \pm 2$) 12h light and dark cycle throughout the period of acclimatization and experiment. All the animal experimental protocols were duly approved by the institutional animal ethics committee (Reg No.169/1998/CPCSEA).

Acute toxicity study

Wistar albino mice of either sex (25-30 g) were divided into ten Groups of six animals each. Acute toxicity study was carried out according to the method described in the literature (Palanichamy and Nagarajan, 1990). Fractions of *Hiptage benghalensis (Linn)*, were suspended in 5% gum acacia in doses of 100, 200, 400, 800, 1000, 1200, 1400, 1800 and 2000 mg/kg b.w.p.o were administered orally to albino mice. The animals were observed continuously for any change in autonomic or behavioral responses for first few hours and later at 24h intervals for a period of 48h. At the end of this period, the mortality if any, in different dose Groups were noted. The various fractions of MHB were also found to be safe for further studies as no lethality was observed upto a dose level of 1500 mg/kg b.w.p.o. in mice. Since, LD_{50} of the individual fractions was greater than 1500 mg/kg b.w.p.o., the investigation on these fractions were

carried out with a dose i.e. 50,100 and 150 mg/kg b.w.p.o., considered as safe.

Evaluation of analgesic activity

Eddy's Hot plate test in mice

The central analgesic activity of the extracts was determined by hot plate method (Eddy and Leimback, 1953). Swiss albino mice of either sex weighing between 16-25 g consisting of 6 in each Group were selected for the study. The mice which reacted within 3-8 sec (normal reactions) and which did not show large variation when tested on 4 separated occasions on Eddy's hot plate were selected. Group I received 2% gum acacia (10 ml/kg b.w.p.o) as control and Group II received standard drug, pentazocine (10 mg/kg, i.p). Remaining Groups were administered with different doses of *Hiptage benghalensis (Linn)* (leaves). Mice were screened by placing them on a hot plate maintained at 55 ± 0.50 C and recorded the reaction time in sec for blowing or licking of hind paw or jumping off the surface (Turner, 1965). The reaction time was recorded initially at '0' h (before the treatment) and later at 60, 120, 180 and 240 min after oral administration of the vehicle/standard/extracts. The percentage variation in reaction time of each Group was calculated by using the following formula:

Percentage variation = Drug latency- Baseline latency/ Baseline latency. x 100

Acetic acid induced Writhing test in mice

The peripheral analgesic activity of the extracts was determined by acetic acid- induced writhing test (Koster et al., 1959). In this test, Swiss albino mice were divided into different Groups. Abdominal writhing in animals was induced by the intraperitoneal administration of 0.7% acetic acid (10 ml/kg). The control (2% gum acaia), standard drug diclofenac sodium (20mg/kg, b.w) and the fractions of different doses were administered orally, 30 min prior to the injection of acetic acid. The mice were placed in a transparent box after the administration of acetic acid injection and the number of writhes was counted 5 min after administration of acetic acid for a period of 20 min. A significant reduction in the level of writhing by any treatment compared to acetic acid treated control animals was considered as a positive analgesic response. The percent protection against writhing was taken as an index of analgesia and was calculated according to following formula:

Percentage inhibition = [(Nc – Nt) / Nc] x 100

Nc = Number of writhes in control animals

Nt = Number of writhes in treated animals

Formalin induced paw licking in mice

The formalin induced pain test was used for evaluation of analgesic activity (Hunskaar and Hole, 1997). Mice were injected with 20 µl of 2.5% formalin into the dorsal surface of the left hind paw and placed immediately in a transparent box for observation. The duration of paw licking was determined between 0-5 min (first phase) and 20-25 min (second phase) after formalin injection. The time in sec spent in licking and biting responses of the injected paw was noted. Animals were treated orally with different fractions of varying doses, 30 min prior to administration of formalin. Pentazocine (10 mg/kg, i.p) was used as standard reference. Control animals received 2% gum acacia, 10 ml/kg as vehicle. The paw licking time of the animals was compared to the toxicant control Group and represented as percent inhibition.

Evaluation of anti-inflammatory activity *in vivo* studies

Carrageenan-induced rat paw edema:

a. Preparation of carrageenan suspension

1% suspension of carrageenan sodium salt was prepared by sprinkling 100 mg of carrageenan powder on 10 ml of saline (0.9% NaCl) solution and set aside to soak for 1h. A homogeneous suspension was then obtained by thorough mixing with a magnetic stirrer.

b. Experimental procedure

The anti-inflammatory effect was evaluated in carrageenan-induced edema model in rats (Winter et al., 1962). The animals were randomly divided into different Groups with 6 rats in each Group and pretreated with extract or standard drug, diclofenac sodium (20 mg/kg, b.w), 30 min before the injection with 0.1 ml of 1% carrageenan (in distilled water) suspension into the sub plantar region of right hind paw. Paw volume was measured at '0' min by dipping the right hind paw into mercury column up to the tibiotarsal junction and noticing the mercury displacement by using plethysmograph (Inco Lab, Ambala, India) immediately after carrageenan injection and at 1, 2, 3 and 4 h time intervals. A significant reduction in the paw volume compared to carrageenan-treated control animals were considered as anti-inflammatory response.

Percentage inhibition of edema was calculated by using the following formula:

Percentage of Inhibition =(VT-VO) control – (VT-VO) treated/(VT – VO) control. x 100

VO= paw volume of the rat before administration of carrageenan.

VT= paw volume of the rat after administration of carrageenan at different time intervals.

Cotton pellet-induced granuloma

The cotton pellet-induced granuloma in rats was studied according to the method of (D'Arcy et al., 1960). The animals were divided into 5 Groups of 6 animals in each Group. The rats were anaesthetized and sterile cotton pellets weighing 10 ± 1 mg were implanted subcutaneously into both sides of the groin region of each rat. Group I was served as control and received only the vehicle (2% gum acacia). Group 2 received the standard drug, indomethacin (10 mg/kg b.w) and the other Groups were administered with methanolic extract and their fractions orally for 7 consecutive days from the day of cotton pellet implantation. On the 8th day the animals were anaesthetized and the pellets together with granuloma tissues were carefully removed and made free from extraneous tissues. The wet pellets were weighed and then dried in an oven at 60^0C for 24 h to constant weight, after that the dried pellets were weighed again. Increment in the dry weight of the pellets was taken as a measure of granuloma formation. The anti-proliferative effect of all extracts, fractions and standard drug were compared with control.

Evaluation of anti-inflammatory activity *in vitro* studies

TMPD assay method

Cayman's colorimetric COX (ovine) inhibitory screening assay kit was used to carry out the invitro anti-inflammatory activity (Kulmacz and Lands, 1983).

Procedure of the assay

1. Preparation of Background Wells: A mixture containing 150 µl of Assay Buffer, 10 µl of heme, and 10 µl of inactive sample was prepared and transferred to three wells per sample.

2. 100% Initial Activity Wells: A mixture containing 150 µl of Assay Buffer, 10 µl of heme and 10 µl of enzyme (COX-2) was prepared and transferred to three wells.

3. Inhibitor Wells: 150 µl of Assay Buffer was added to 10 µl of heme, and 10 µl of enzyme (COX-2) were added to three wells.

4. COX Standard Wells: 150 µl of Assay Buffer was added to 10 µl of heme and 10 µl of standard per well in the designated wells on the plate.

5. Sample Wells: 150 µl of Assay Buffer was added to 10 µl of heme, and 10 µl of sample to three wells. To obtain reproducible results, the amount of COX added to the well should fall within the range of the assay. If necessary, samples can be diluted with Assay Buffer (dilute). The concentrations of the selected fractions of MHB were chosen between 100-500 µg/ml.

6. The plates were shaken carefully for few seconds to mix and incubated for 5 min at 25° C.

7. 20 µl of Colorimetric substrate solution was added to every well.

8. The reactions were initiated by adding 20 µl of AA solution to all the wells in use. The plate was shaken carefully for few seconds to mix and incubated for 5 min at 25° C.

9. Read the absorbance at 590nm using a plate reader.

Calculations

1. Determine the average absorbance of all the samples.

2. Subtract the absorbance of the background wells from absorbances of the 100% intial activity and the inhibitor wells.

3. Substract each inhibitor samples from the 100% intial activity sample, then divide by the 100% intial activity sample and multiply by 100 to give the percent inhibition.

***In vitro* 5-Lipoxygenase inhibition:** 5-LOX enzyme inhibitory activity of *Hiptage benghalensis (Linn)* fractions was measured using the method of (Reddanna *et al*.) modified by (Ulusu *et al*.) The assay mixture contained 80 mM linoleic acid and 10 µl potato 5-LOX in 50 mM phosphate buffer (pH 6.3). The reaction was initiated by the addition of enzyme buffer mix to linoleic acid and the enzyme activity was monitored as the increase in absorbance at 234 nm. The reaction was monitored for 120 sec and the inhibitory potential of the test substances was measured by incubating various concentrations of test substances for two minutes before addition of linoleic acid.

All assays were performed in triplicate. Percentage inhibition was calculated by comparing slope of test substances with that of enzyme activity.

Calculation

Percentage Inhibition = Control O.D. – Test O.D./ Control O.D. x 100

RESULTS AND DISCUSSION

The following acute and sub acute models were selected to evaluate the anti-inflammatory activity. Carrageenan-induced rat paw edema model has been commonly used as an experimental animal model for acute inflammation and is believed to be biphasic. The early phase (1-2 h) of the carrageenan model is mainly mediated by histamine, serotonin and increased synthesis of prostaglandins in the damaged tissue surroundings.The inflammation (edema) volume reaches its maximum approximately between 3-4 h post treatment after which it begins to decline.The late phase (after 2h) is sustained by prostaglandin release and mediated by bradykinin, leukotrienes, polymorphonuclear cells and prostaglandins produced by tissue macrophages (Brito and Antonio, 1998). The inhibitory activity shown by MHB fractions over a period of 3h in carrageenan-induced paw inflammation was quite similar to that exhibited by the Group treated with diclofenac sodium. Moreover, it is known that diclofenac sodium reduces inflammation, swelling and arthritic pain by inhibiting prostaglandin synthesis and/or production (Mahgoub, 2002; Skoutakis et al., 1988). There is evidence that compounds inhibiting the carrageenan induced edema have also been found effective against the cyclooxygenase enzymes (Selvam et al., 2004). Based on these reports it can be inferred that the inhibitory effect of tested plant fractions on the carrageenan induced inflammation seen maximum at 3 h is possibly mediated by arachidonic acid metabolites, which produce an edema dependent on neutrophil mobilization (Just et al., 1998).

Table 1: Analgesic effect of fractions of *Hiptage benghalensis* using Eddy's hot plate model in mice

Dose (mg/kg)	Reaction time (sec) after				
	0min	60 min	120 min	180 min	240 min
Control	4.27±0.41	4.30±0.51	3.98±0.69	4.17±0.52	4.23±0.52
Pentazocine10	4.15±0.60	7.75±0.76**	13.42±0.49**	9.12±0.78**	6.97±0.39**
T-MHB50	4.28±0.54	5.30±0.28*	6.05±0.34**	5.08±0.38*	4.02±0.10
T-MHB100	4.30±0.49	5.73±0.59*	7.13±0.46**	5.83±0.26*	4.77±0.26
T-MHB150	4.45±0.41	6.22±0.32**	8.25±0.29**	6.05±0.23**	4.85±0.19
EA-MHB50	4.37±0.50	6.85±0.52**	7.60±0.57**	7.50±0.31**	4.63±0.28
EA-MHB100	4.40±0.58	7.75±0.47**	9.92±0.58**	8.68±0.63**	5.25±0.26*
EA-MHB150	4.83±0.43	9.08±0.68**	10.17±0.68**	9.13±0.45**	5.88±0.45*
BN-MHB50	4.33±0.41	5.52±0.43*	6.87±0.33**	5.48±0.28*	4.22±0.38
BN--MHB100	4.48±0.41	6.03±0.33**	7.33±0.33**	6.65±0.54**	4.98±0.13
BN-MHB150	4.48±0.56	7.20±0.76**	9.83±0.19**	8.12±0.43**	5.07±0.32*
BL-MHB50	4.12±0.52	5.75±0.69*	7.63±0.37**	6.20±0.55**	5.22±0.35*
BL-MHB100	4.07±0.36	6.93±0.47**	8.55±0.39**	7.28±0.25**	5.78±0.96*
BL-MHB150	4.67±0.53	8.88±0.77**	11.25±0.76**	8.68±0.40**	6.27±0.75**

All the values were expressed as mean±SD (n=6), *p<0.05**p<0.01 vs control

Table 2: Analgesic effect fractions of *Hiptage benghalensis* leaves using Acetic Acid-induced writhing model in mice

Dose (mg/kg)	No of Writhing	% Inhibition
Control	73.17±6.85	---
Diclofenac Sod 20	19.67±1.63**	73.12
T-MHB50	46.83±2.64**	35.99
T-MHB100	40.50±2.07**	44.65
T-MHB150	27.00±2.00**	63.1
EA-MHB50	43.17±3.06**	41.00
EA-MHB100	31.50±1.64**	56.95
EA-MHB150	21.67±1.63**	70.39
BN-MHB50	45.67±3.08**	37.59
BN--MHB100	34.5±3.33**	52.85
BN-MHB150	24.83±3.60**	66.06
BL-MHB50	43.00±6.36**	41.23
BL-MHB100	30.67±5.79**	58.09
BL-MHB150	29.83±3.66**	59.23

All the values were expressed as mean±SD (n=6), **p<0.01 vs control

Table 3: Analgesic effect of fractions of *Hiptage benghalensis* using formalin-induced pain model in mice

Dose (mg/kg)	Licking Time(sec) First Phase	Licking Time(sec) Second Phase	%Inhibition First Phase	%Inhibition Second Phase
Control	176.33±2.35	119.11±1.39		
Pentazocine10	86.95±2.03**	42.22±1.68**	50.69	64.55
T-MHB50	47.78±1.08**	42.76±0.04**	72.42	64.1
T-MHB100	43.68±1.10**	39.33±0.15**	74.79	66.98
T-MHB150	40.78±1.20**	32.55±0.16**	76.46	72.67
EA-MHB50	40.67±1.19**	33.34±2.05**	76.53	72.01
EA-MHB100	33.32±2.10**	29.45±0.04**	80.77	75.27
EA-MHB150	28.34±2.06**	20.59±1.03**	83.64	82.71
BN-MHB50	60.89±1.20**	40.24±1.19**	64.86	66.22
BN--MHB100	55.17±1.18**	33.59±1.07**	68.16	71.8
BN-MHB150	45.81±0.09**	28.77±0.02**	73.56	75.85
BL-MHB50	63.34±1.19**	42.49±0.15**	63.44	64.33
BL-MHB100	57.99±1.06**	38.89±1.19**	66.53	67.35
BL-MHB150	47.77±1.20**	30.19±1.16**	72.43	74.65

All the values were expressed as mean±SD (n=6), *p<0.05**p<0.01 vs control

Table 4: Anti-inflammatory activity of fractions of *Hiptage benghalensis* using carrageenan-induced rat paw edema model

Group	Dose (mg/kg)	Paw edema volume (ml)							
		1hr	%IPE	2hr	%IPE	3hr	%IPE	4hr	%IPE
1	control	2.05±0.08		2.02±0.17		2.12±0.13		2.15±0.15	
2	diclofenac20	0.97±0.05**	52.85	0.77±0.05**	61.98	0.55±0.05**	74.02	0.70±0.14**	67.44
3	T-MHB50	1.63±0.12*	20.33	1.40±0.09**	30.58	1.07±0.08**	49.61	1.58±0.17**	26.36
4	T-MHB100	1.48±0.08**	27.64	1.30±0.06**	35.54	0.85±0.05**	59.84	1.57±0.10**	27.13
5	T-MHB150	1.48±0.10**	30.08	1.98±0.10**	51.24	0.77±0.14**	63.78	1.37±0.12**	36.43
6	EA-MHB50	1.52±0.16**	26.02	1.22±0.12**	39.67	0.90±0.09**	57.48	1.32±0.16**	38.76
7	EA-MHB100	1.37±0.05**	33.33	1.03±0.05**	48.76	0.82±0.10**	61.42	1.27±0.10**	49.09
8	EA-MHB150	1.20±0.13**	41.46	0.85±0.14**	57.85	0.67±0.10**	68.50	1.02±0.08**	52.71
9	BN-MHB50	1.62±0.10*	21.14	1.35±0.16**	33.06	1.17±0.14**	44.88	1.50±0.19**	30.23
10	BN--MHB100	1.48±0.15**	27.64	1.25±0.22**	38.02	1.05±0.08**	50.39	1.42±0.15**	34.11
11	BN-MHB150	1.47±0.05**	28.46	1.00±0.06**	50.41	0.87±0.05**	59.06	1.45±0.10**	32.56
12	BL-MHB50	1.57±0.05*	23.58	1.25±0.05**	38.02	1.12±0.13**	47.24	1.67±0.08**	22.48
13	BL-MHB100	1.35±0.12**	34.15	1.05±0.08**	47.93	0.92±0.10**	56.69	1.30±0.06**	39.53
14	BL-MHB150	1.32±0.10**	35.77	0.95±0.05**	52.89	0.73±0.08**	65.35	1.05±0.05**	51.16

All the values were expressed as mean±SD (n=6), *p<0.05**p<0.01 vs control

Table 5: Anti-inflammatory activity of fractions of *Hiptage benghalensis* using cotton pellet-induced granuloma in rats

Dose (mg/kg)	Wet weight of Cotton pellet	%Inhibition	Dry weight of Cotton pellet	%Inhibition
Control	159.17±15.12		67.67±8.50	
Indomethacin10	82.50±11.27**	48.17	37.33±4.25**	44.83
T-MHB50	120.00±12.97*	24.61	53.00±5.30*	21.67
T-MHB100	113.50±13.02**	28.69	50.50±4.17**	25.37
T-MHB150	96.33±11.51**	39.48	42.50±4.64**	37.19
EA-MHB50	113.83±13.19**	28.48	52.83±5.32*	21.92
EA-MHB100	103.50±10.83**	34.97	46.17±4.41**	31.77
EA-MHB150	93.33±8.76**	41.36	42.67±5.56**	36.95
BN-MHB50	126.00±12.61*	20.84	56.00±5.16*	17.24
BN--MHB100	119.83±13.06*	24.71	53.50±3.07*	20.94
BN-MHB150	109.33±12.80**	31.31	48.67±4.98**	28.08
BL-MHB50	122.67±10.08*	22.93	57.17±7.49*	15.52
BL-MHB100	107.67±9.34**	32.36	49.33±6.94**	27.09
BL-MHB150	98.33±8.40**	38.22	44.67±3.97**	33.99

All the values were expressed as mean±SD (n=6), *p<0.05**p<0.01 vs control

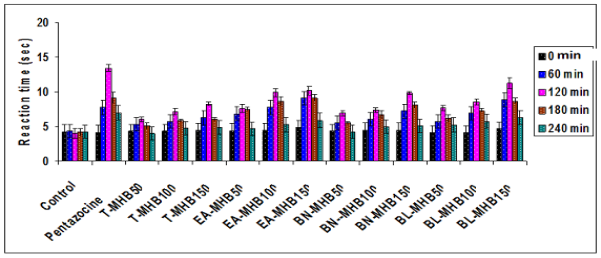

Fig. 1: Effect of fractions of MHB using Eddy's hot plate model in mice

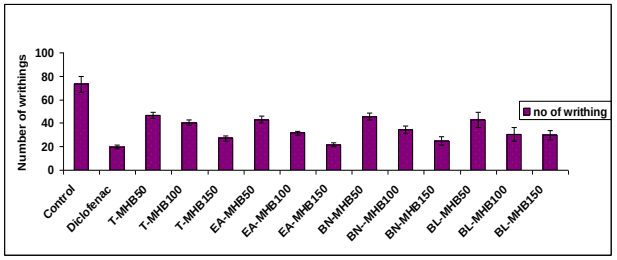

Fig. 2: Effect of fractions of MHB using Acetic Acid-induced writhing model in mice

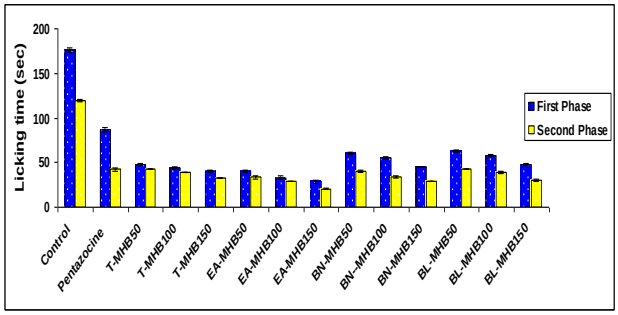

Fig. 3: Effect of fractions of MHB using formalin-induced pain model in mice

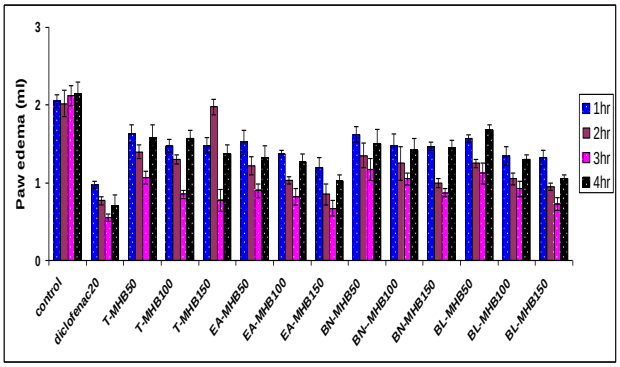

Fig. 4: Effect of fractions of MHB using carrageenan-induced rat paw edema model

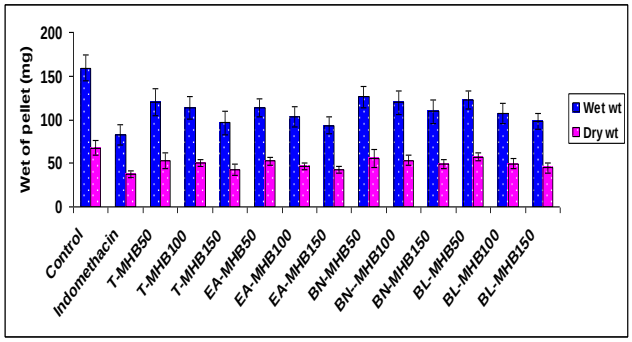

Fig. 5: Effect of fractions of MHB using cotton pellet-induced granuloma in rats

Table 6: Selected Fractions of MHB Cox2 inhibitory activity

Compound	IC50 (µg/ml)
Celecoxib	8.5
T-MHB	43
EA-MHB	33.6
BN-MHB	70.5
BL-MHB	58.3

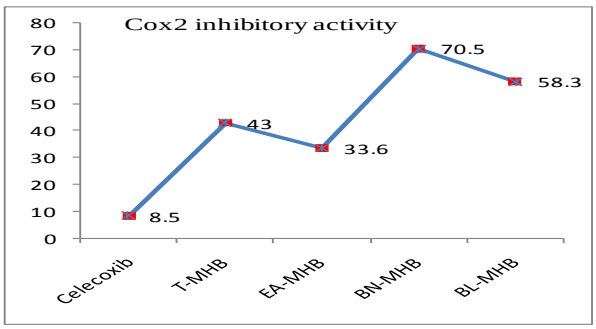

Fig. 6: Selected Fractions of MHB Cox-2 Inhibitory Activity

Table 7: Selected Fractions of MHB 5-LOX inhibitory activity

Compound	IC50 (µg/ml)
STD (Zileuton)	4.11
T-MHB	30.5
EA-MHB	19.3
BN-MHB	45
BL-MHB	49.7

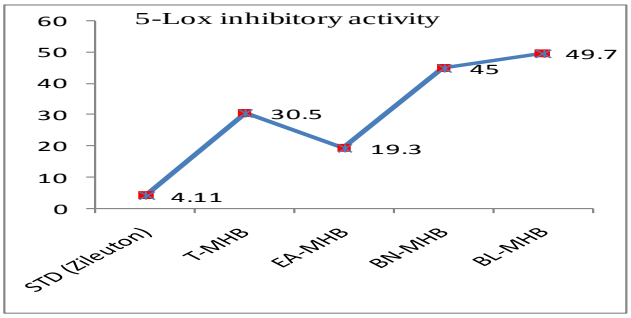

Fig. 7: Selected Fractions of MHB 5-LOX inhibitory Activity

In order to assess the efficacy against proliferative phase of inflammation cotton pellet granuloma model is selected in which tissue degeneration and fibrosis occur. During the repair process of inflammation, there is proliferation of macrophages, neutrophils, fibroblasts and multiplication of small blood vessels, which are the basic sources of forming a highly vascularised reddish mass, termed granuloma tissue. It is used to evaluate the transudative and proliferative components of chronic inflammation. The wet weights of the cotton pellet correlates with transudate; the dry weight of the cotton pellet correlates with the amount of the granulomatous tissue (Olajide et al., 1999). Hence the decrease in the weight of granuloma indicates that the proliferative phase was effectively suppressed by MHB fractions and exhibited significant anti-inflammatory activity. These results reflected their efficacy in inhibiting the increase in the number of fibroblasts and synthesis of collagen and mucopolysaccharides during formation of granuloma tissue. The methods used for investigation of analgesic activity with MHB fractions are selected in such a way that both peripherally and centrally mediated effects can be evaluated. The thermal test (hot plate test) was selected because the test is sensitive to strong analgesics and the experimental animals with a limited tissue damage because of a cut off time usually fixed to a time limit for the animals to present on the hot plate. This method is considered to be selective for opioid like compounds in several animal species, but other centrally acting drugs including sedatives and muscle relaxants too show activity in this test (Hiruma-Lima et al., 2000). All the fractions as mentioned above produced significant ($p<0.05$) and a dose dependent prolongation in latency time. Thus the results of the study confirm the centrally acting analgesic activity of leaves fractions.

Acetic acid-induced abdominal constriction method is widely used for the evaluation of agents with peripheral analgesic activity (Gene et al., 1998). Acetic acid is used to induce writhing, causes algesia by liberation of endogenous substances, which in turn sensitize the pain nerve endings (Taesotikul et al., 2003). Local peritoneal receptors are postulated to be partly involved in the abdominal constriction response (Bentley et al., 1983). This experiment is a sensitive procedure to establish peripherally acting analgesics and the response was thought to be involving local peritoneal receptors (Vasudevan et al., 2006). This method has been associated with prostanoids in general, e.g. increased levels of PGE2 and PGF2α in peritoneal fluids as well as LOX products (Derardt et al., 1980). According to the percentage of inhibition on the number of writhes obtained with different doses of MHB fractions, it was found that the intensity of the analgesic effect was similar to that of standard drug, diclofenac sodium. Thus the results confirm the peripheral analgesic activity of fractions. The formalin induced paw licking test is used for elucidating the mechanism of pain and algesia. It has been reported that formalin induced pain involves two distinct phases and different analgesics may act differentially in the early and late

phases of this test. Therefore, this test can be used to clarify the possible mechanism of antinociceptive effect of proposed analgesics (Tjolsen et al., 1992). In the first phase (neurogenic phase) the pain caused by formalin is due to direct stimulation of the sensory nerve fiber and the second or late phase (inflammatory phase) the pain is due to release of inflammatory mediators such as histamine, serotonin, prostaglandin and bradykinin (Hunskaar and Hole, 1987; Murray et al., 1988). Drugs which act centrally, such as narcotic analgesics inhibits both phases of pain in this model whereas peripherally acting drugs, such as aspirin or indomethacin, inhibit only the late phase. The effect of MHB fractions exhibited predominant effect on both phases of formalin-induced pain with more potent effect on the second phase than the first one.

These results provided a significant ($p<0.05$) inhibitory effect of respective fractions on both phases of pain, suggesting the involvement of both neurogenic and inflammatory mechanisms. The analgesic activity in the late phase might be mediated by arachidonic acid metabolites. The selected fractions were evaluated for in-vitro COX-2 and 5-LOX enzyme inhibition assay to elucidate the mechanism of action of all the fractions. The COX-2 inhibitory activities of selected fractions i.e. BN-MHB, BL-MHB, T-MHB, EA-MHB, were examined for the mechanism of their anti-inflammatory action. It was observed that the COX-2 inhibitory activity of MHB fractions were found as BN-MHB > BL-MHB>T-MHB > EA-MHB. Similar to the COX-2 inhibition, all the fractions significantly inhibited the 5-LOX enzyme.

The 5-LOX inhibitory activity of MHB fractions were found as BN-MHB > BL-MHB> T-MHB> EA-MHB. Therefore, dual inhibition of COX-2 and 5-LOX enzymes may provide therapeutic benefit with a greater degree of safety than currently available modalities (Martel-Pelletier et al., 2003**).** T-MHB and EA-MHB exhibited the desired dual inhibition properties on COX-2 and 5-LOX. These results confirm that the analgesic and anti-inflammatory activities are due to the inhibition of COX and LOX enzymes. Hence, the elevated AA metabolism is part of the etiology of arthritis, the inhibition of the production of these inflammatory mediators via dual inhibition of COX-2 and 5-LOX pathways may provide a way to manage arthritis safely with acceptable efficacy.

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