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Research article

Evaluation of Analgesic and Anti-Inflammatory activity of different fractions from *Leucas Cephalotes*

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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 *Leucas cephalotes*. The fractions of the plant material were evaluated for the analgesic activity by acetic acid-induced 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. At 150mg/Kg b.w.p.o dose Ethyl-acetate fraction significantly inhibited 68.98% writhing response and 73.52, 76.03% Formalin induced analgesic in mice. The fraction with same dose showed significant 65.35% inhibition of Carrageenan induced rat paw edema and 43.87, 40.64% anti-proliferative effect of cotton pellet in rats and also different fractions of *Leucas cephalotes* showed prompt analgesic and anti-inflammatory activity due to dual inhibition properties on COX-2 and 5-LOX.

Keywords: Acetic acid-induced writhing, Carrageenan-induced rat paw edema, Cotton pellet induced granuloma, Leucas cephalotes.

Introduction

Leucas cephalotes (Roxb.ex Roth) Spreng. (Lamiaceae) (Kirtikar and Basu,1988; Parrotta, 2001). An annual hairy and pubescent herb 0.6-0.9 m. high, stems and branches obtusely quardrangular, hairy with spreading hairs. Whole Plant has a pungent taste with a flavour, heating, indigestible causes "Vata" and "Pitta", laxative, antihelmentic, stimulant and diaphoretic, useful in bronchitis, jaundice, inflammations, asthma, dyspepsia, paralysis, leucoma. Leaves are also useful in fever and urinary discharges (Ayurveda). The fresh juice is used in certain localities as an external application in scabies. Leaves, in combination with other drugs, are prescribed for scorpion-sting (Vagghata), but they are not an antidote to scorpion-venom. Plant is indigenously grown in India, Nepal, Pakistan and Afghanistan. In India it is widely distributed in Punjab, Bengal, Assam, Himalaya, Rajputana Deseri, Kathiawar, Gujrat, all plain districts of Madras Presidency and at an altitude of 1800 m in the Himalayas (Kirtikar and Basu,1988).

The leaf juice, sometimes mixed with honey to treat coughs and colds among the Santhalis in southern Bihar and by rural inhabitants of Gujarat in India, where it is also used for the treating jaundice. It is valuable homoeopathic drug and such is used for the treatment of chronic malaria and asthma (Ghosh,

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1988). Dry leaves along with tobacco (1:3) are smoked to treat bleeding as well as itching piles (Khare, 2007). The ethyl extract of whole plant of *Leucas cephalotes* has been reported for its protective effects on CCl_4-induced hepatotoxicity in mice and rats (Singh *et al.*,1978). Nineteen compounds were isolated by chromatographic separation of 1-butanol-soluble fraction of the MeOH extract of *Leucas cephalotes* (Miyaichi *et al.*, 2006). The structures of known compounds were identified as oleanolic acid, 7-oxysitisterol (Greca *et al.*,1990; Pettit *et al.*,1972), 7-oxostigmasterol (Katsui *et al.*,1972), 7 alpha-hydroxysitosterol (Greca *et al.*,1990), 7 alpha-hydroxystigmasterol (Miyaichi *et al.*, 2006), stigmasterol (Kolak *et al.*, 2005), gonzalitosin (Dominguez *et al.*,1976), tricin (Fujii *et al.*, 1995) and apigenin 7-0-β-D-(6-O-p-coumaroyl) glucopyranoside (Itokawa *et al.*,1981).

Materials and Methods

Plant material

The whole plant of *Leucas cephalotes* was collected in the month of July 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

The whole plant of *Leucas cephalotes* (3kg) 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 *L cephalotes* (MLC) 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),

Formaldehyd 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 *L cephalotes*, 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 MLC 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 *Leucas cephalotes* (whole plant). 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:

$$\text{Percentage variation} = \frac{\text{Drug latency- Baseline latency}}{\text{Baseline latency}} \times 100$$

Acetic acid induced Writhing test in mice

The peripheral analgesic activity of the extracts was determined by acetic acid- induced writhing test (Whittle, 1964; 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 (Okoli et al., 2006).

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 (IncoLab, 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:

$$\text{Percentage of Inhibition} = \frac{(VT\text{-}VO)\ \text{control} - (VT\text{-}VO)\ \text{treated}}{(VT - VO)\ \text{control}} \times 100$$

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 (Kulmacz and Lands, 1983)

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

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 MLC 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 *Leucas cephalotes* 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

$$\text{Percentage Inhibition} = \frac{\text{Control O.D.} - \text{Test O.D.}}{\text{Control O.D.}} \times 100$$

Preliminary phytochemical tests

Chemical tests were carried out to know the nature of compounds present in the fractions of LC in order to identify the presence of various phytoconstituents present in them (Paech and Tracey, 1979).

Results and Discussion

Table 1. Analgesic effect of fractions of *Leucas cephalotes* 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-MLC50	4.40±0.37	5.83±0.21*	6.63±0.43**	5.17±0.68*	4.08±0.13
T-MLC100	4.33±0.50	6.27±0.39**	7.30±0.45**	5.92±0.38**	4.58±0.38
T-MLC150	4.48±0.37	7.62±0.63**	9.32±0.83**	6.37±0.64**	5.98±0.77**
EA-MLC50	4.23±0.45	5.92±0.58**	7.93±0.81**	5.97±0.48**	4.28±0.16
EA-MLC100	4.40±0.58	6.75±0.82**	8.87±0.96**	7.75±0.27**	5.10±0.20*
EA-MLC150	4.62±0.30	9.00±0.32**	10.50±0.55**	8.58±0.66**	5.42±0.26*
BN-MLC50	4.33±0.42	5.97±0.35**	6.87±0.33**	5.63±0.20*	4.22±0.38
BN--MLC100	4.37±0.29	6.13±0.90**	7.47±0.41**	6.62±0.52**	4.22±0.57
BN-MLC150	4.32±0.53	7.53±0.63**	10.12±0.44**	7.33±0.41**	4.33±0.52
BL-MLC50	4.40±0.50	5.58±0.74*	6.57±0.37**	6.03±0.39**	4.70±0.20
BL-MLC100	4.20±0.44	6.97±0.46**	8.52±0.45**	7.40±0.25**	5.43±0.46*
BL-MLC150	4.08±0.49	9.52±0.35**	11.33±0.82**	9.35±0.97**	6.33±0.55**

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

Figure 1. Effect of fractions of MLC using Eddy's hot plate model in mice

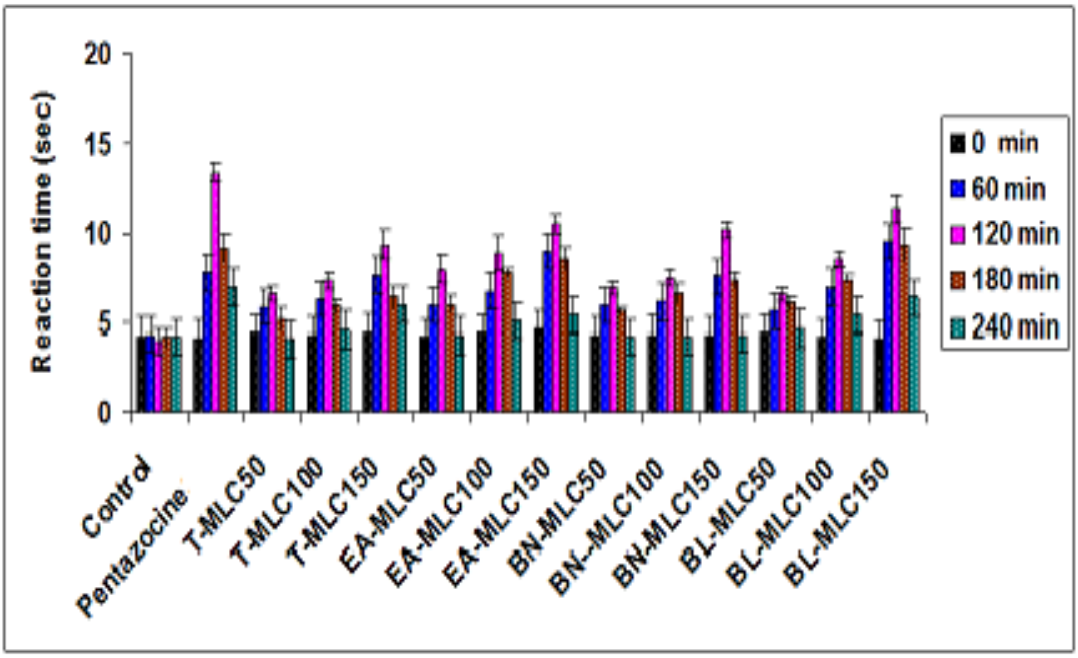


Table 2. Analgesic effect of methanolic extract of *Leucas cephalotes* using Acetic Acid-induced writhing model in mice.

Dose (mg/kg)	No of Writhing	% Inhibition
Control	73.17±6.85	--
Diclofenac20	19.67±1.63**	73.12
T-MLC50	44.67±1.63**	37.96
T-MLC100	35.33±1.86**	50.93
T-MLC150	23.00±1.79**	68.06
EA-MLC50	37.17±4.17**	48.38
EA-MLC100	27.33±1.75**	62.04
EA-MLC150	22.33±1.75**	68.98
BN-MLC50	48.00±2.10**	33.33
BN--MLC100	35.83±1.72**	50.23
BN-MLC150	28.50±2.43**	60.42
BL-MLC50	42.00±5.22**	41.67
BL-MLC100	28.83±3.25**	59.95
BL-MLC150	24.17±3.25**	66.44

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

Figure 2. Analgesic effect of methanolic extract of *Leucas cephalotes* using Acetic Acid-induced writhing model in mice

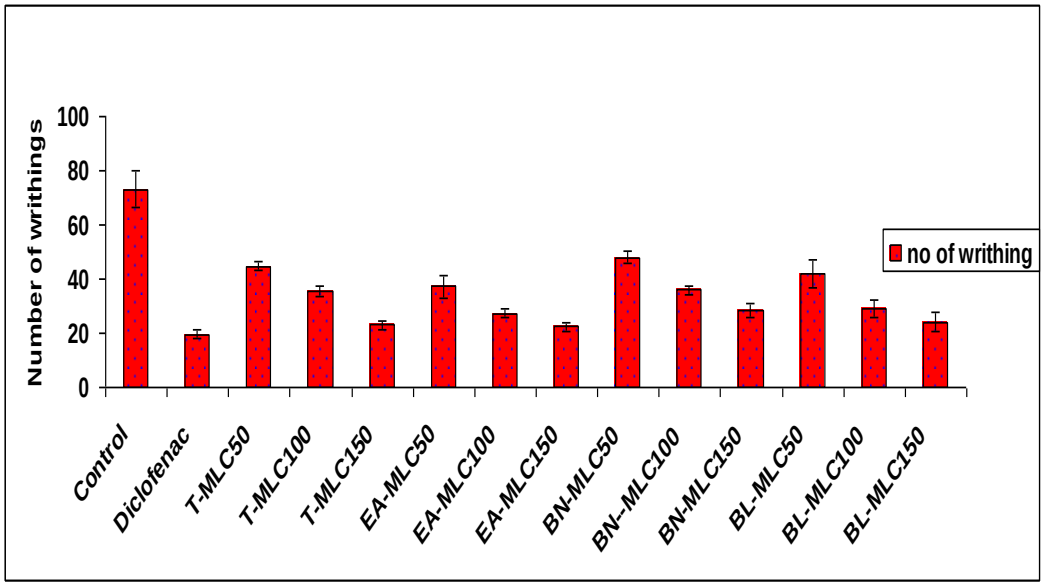


Table 3. Analgesic effect of fractions of *Leucas cephalotes* using formalin-induced pain model in mice.

Dose (mg/kg)	Licking Time(sec)	Licking Time(sec)	%Inhibition	
	First Phase	Second Phase	First Phase	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-MLC50	76.88±1.15**	47.77±0.18**	55.63	59.89
T-MLC100	58.49±2.08**	32.58±1.20**	66.24	72.65
T-MLC150	50.99±1.19**	29.19±2.09**	70.57	75.49
EA-MLC50	59.89±1.08**	34.17±1.08**	65.44	71.31
EA-MLC100	51.91±1.12**	30.66±2.08**	70.04	74.26
EA-MLC150	45.88±1.11**	28.55±1.11**	73.52	76.03
BN-MLC50	55.49±1.09**	35.66±1.11**	67.97	70.06
BN--MLC100	54.19±1.11**	33.49±1.09**	68.73	71.88
BN-MLC150	45.89±1.17**	28.78±1.12**	73.52	75.84
BL-MLC50	60.95±1.12**	40.88±1.10**	64.82	65.68
BL-MLC100	50.87±1.17**	32.84±1.16**	70.64	72.43
BL-MLC150	45.57±1.15**	28.79±1.19**	73.70	75.83

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

Figure 3. Effect of fractions of MLC using formalin-induced pain model in mice

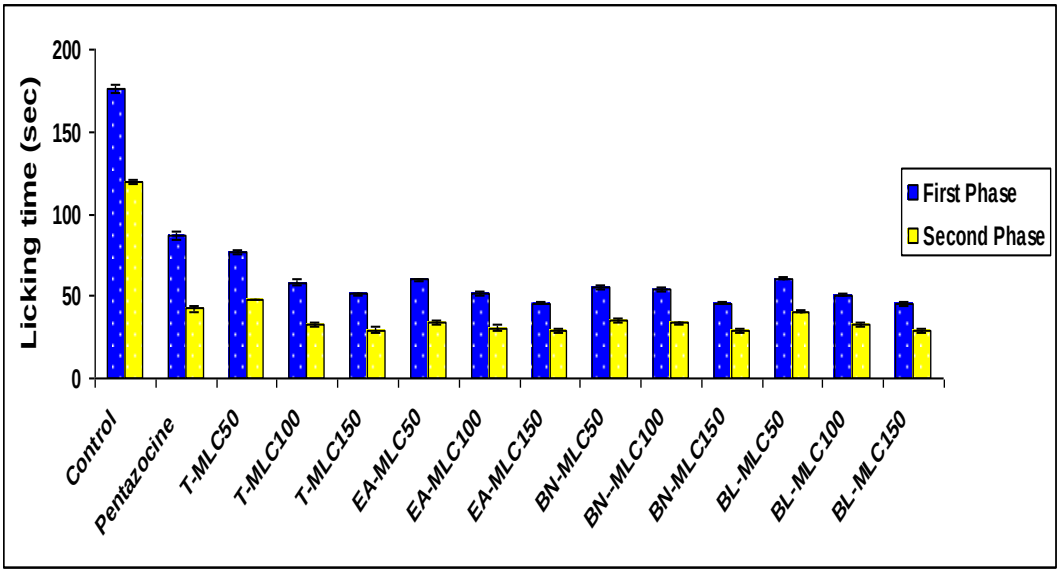


Table 4. Anti-inflammatory activity of fractions of *Leucas cephalotes* using carrageenan-induced rat paw edema model.

		Paw edema volume (ml)							
Group	**Dose (mg/kg)**	**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-MLC50	1.55±0.05*	24.39	1.53±0.08**	25.2	1.08±0.17**	48.82	1.22±0.12**	43.41
4	T-MLC100	1.45±0.10**	29.27	1.38±0.10**	32.52	0.87±0.15**	59.06	0.98±0.08**	54.26
5	T-MLC150	1.40±0.14**	30.58	1.08±0.13**	47.15	0.78±0.08**	62.99	0.93±0.08**	56.59
6	EA-MLC50	1.60±0.06*	20.66	1.38±0.04**	32.52	1.13±0.10**	46.46	1.23±0.14**	42.64
7	EA-MLC100	1.47±0.08**	27.27	1.28±0.08**	37.40	0.95±0.08**	55.12	1.03±0.05**	51.94
8	EA-MLC150	1.48±0.10**	26.45	1.07±0.05**	47.97	0.73±0.08**	65.35	0.88±0.10**	58.91
9	BN-MLC50	1.62±0.19	19.83	1.35±0.05**	34.15	1.03±0.05**	51.18	1.20±0.11**	44.19
10	BN--MLC100	1.57±0.10*	23.58	1.23±0.15**	39.84	0.92±0.15**	56.69	1.03±0.05**	51.94
11	BN-MLC150	1.45±0.08**	28.10	1.10±0.13**	46.34	0.77±0.10**	63.78	0.85±0.14**	60.57
12	BL-MLC50	1.55±0.19*	23.14	1.35±0.10**	34.15	1.12±0.18**	47.24	1.23±0.19**	42.64
13	BL-MLC100	1.33±0.08**	33.88	1.15±0.14**	43.9	0.83±0.08**	60.63	0.93±0.08**	56.59
14	BL-MLC150	1.27±0.08**	37.19	0.93±0.10**	54.47	0.80±0.11**	62.2	0.90±0.13**	58.14

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

Figure 4. Effect of fractions of MLC using carrageenan-induced rat paw edema model

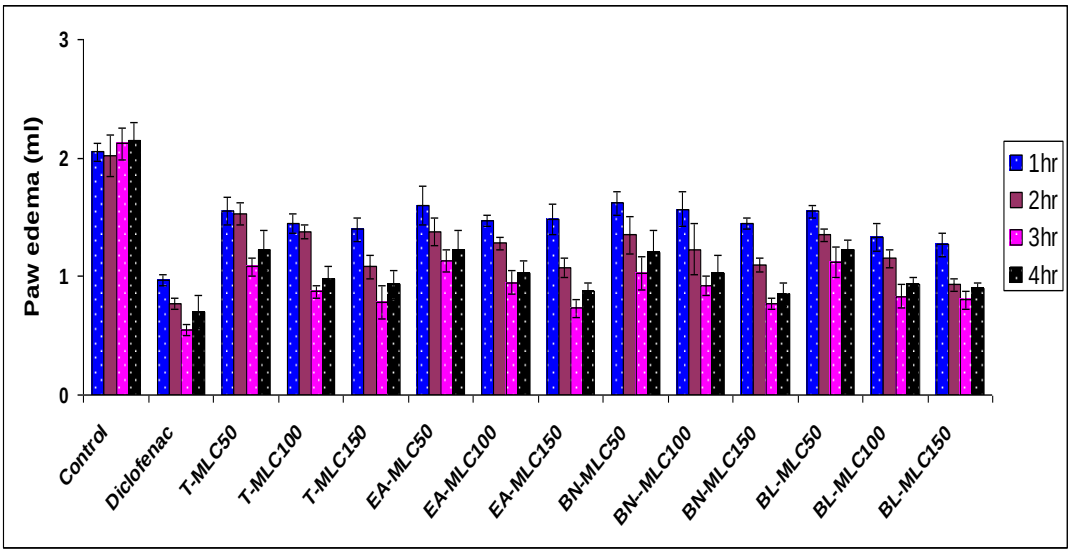


Table 5. Anti-inflammatory activity of fractions of *Leucas cephalotes* using cotton pellet-induced granuloma in rats.

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-MLC50	121.33±12.16*	23.77	51.17±3.23*	24.38
T-MLC100	110.17±9.91**	30.79	48.83±4.17**	27.83
T-MLC150	101.17±10.49**	36.44	44.33±3.58**	34.48
EA-MLC50	117.1±11.05**	26.39	51.50±4.59**	23.89
EA-MLC100	106.33±9.15**	33.19	45.50±5.21**	32.76
EA-MLC150	89.33±8.49**	43.87	40.17±3.83**	40.64
BN-MLC50	122.50±12.95*	23.04	54.83±5.56*	18.97
BN--MLC100	114.67±13.20**	27.96	50.83±4.64*	24.88
BN-MLC150	109.50±10.32**	31.20	48.50±3.56**	28.33
BL-MLC50	124.50±11.22*	21.78	54.67±6.08*	19.21
BL-MLC100	107.67±9.09**	32.36	47.33±4.34**	30.05
BL-MLC150	91.17±7.43**	42.72	41.67±4.42**	38.43

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

Figure 5. Effect of fractins of MLC using cotton pellet-induced granuloma in rats

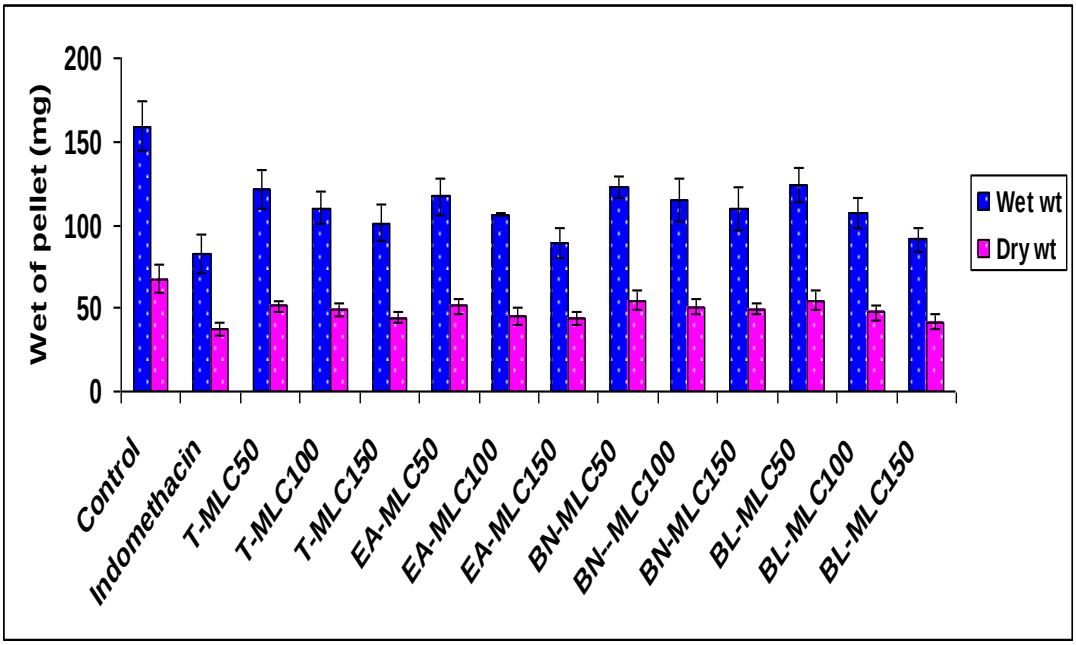


Table 6. Selected Fractions of MLC Cox2 inhibitory activity

Compound	IC50 (µg/ml)
Celecoxib	8.5
T-MLC	53.5
EA-MLC	22.8
BN-MLC	62.3
BL-MLC	68.5

Figure 6. Selected Fractions of MLC Cox-2 Inhibitory Activity

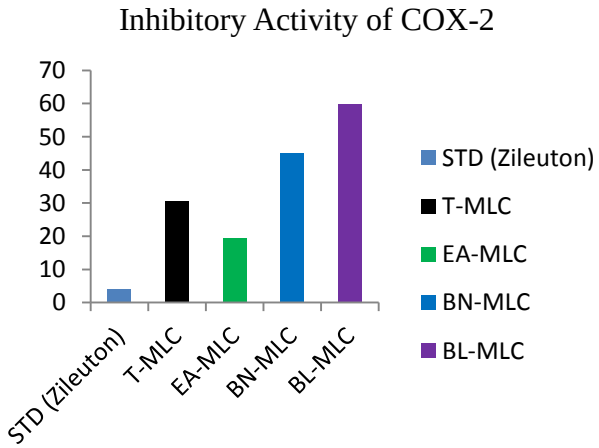
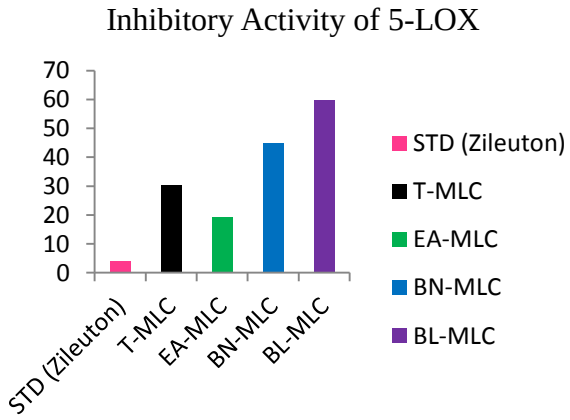


Table 7. Selected Fractions of MLC 5-LOX inhibitory activity

Compound	IC50 (µg/ml)
STD (Zileuton)	4.11
T-MLC	30.5
EA-MLC	19.3
BN-MLC	45
BL-MLC	59.7

Figure7.Selected Fractions of MLC 5-LOX inhibitory Activity



The methods used for investigation of analgesic activity with MLC 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 methanolic extracts as mentioned above and their fractions 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 whole plant 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; Dhara et al., 2000). According to the percentage of inhibition on the number of writhes obtained with different doses of MLC fractions, it was found that the intensity of the analgesic effect was similar to that of standard drug, diclofenac sodium. Therefore, the results of acetic acid induced writhing strongly suggest that the mechanism of action of MLC fractions may be linked partly with endogenous substances like lipoxygenase and/or cyclooxygenase. 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 MLC 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 methanolic extracts and their 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 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 (Garcia et al., 2004).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 MLC 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 extracts and their 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).

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 (Swingle, 1974). 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 MLC 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.

NSAIDs are of huge therapeutic benefit in the treatment of rheumatoid arthritis and various inflammatory conditions. NSAIDs bind to COX thereby inhibiting the production of prostaglandins from the substrate arachidonic acid. COX catalyzes the conversion of arachidonic acid into prostaglandins, which play a significant role in healthy and disease states in the gastrointestinal tract and in the renal, skeletal and ocular systems. COX exists in two forms i.e. COX-1 and COX-2. The inhibition of COX-1 results in some undesirable side effects, where as COX-2 inhibition provides therapeutic effects in pain, inflammation, cancer and neuropathalogic conditions like Alzeimer's disease and Parkinsonism (Gautam et al., 2010). 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-MLC, BL-MLC, T-MLC, EA-MLC, were examined for the mechanism of their anti-inflammatory action. It was observed that the COX-2 inhibitory activity of MLC fractions were found as BL-MLC > BN-MLC>T-MLC > EA-MLC. Similar to the COX-2 inhibition, all the fractions significantly inhibited the 5-LOX enzyme. The 5-LOX inhibitory activity of MLC fractions were found as BL-MLC > BN-MLC> T-MLC> EA-MLC.

There is considerable evidence that COX-2 inhibition associated with an increased risk for cardiovascular events, including edema, hypertension, stroke and myocardial infarction. This suggests that an excess of TXA2, which acts as a vasoconstrictive molecule in the cardiovascular system over reduced vasodilatory PGI2, as a consequence of selective COX-2 inhibition, is the primary reason for increased cardiovascular toxicity (Grosser et al.,2006). Evidence is also available that COX inhibition shunts AA metabolism toward the 5-LOX pathway generating LTB4, which attracts neutrophils producing toxicity in various organ systems. 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-MLC and EA-MLC 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.

Preliminary phytochemical screening of *Leucas cephalotes* fractions revealed the presence of carbohydrates, glycosides, alkaloids, steroids, saponins, phenolic compounds and flavonoids. Glycosides/steroids, phenolic compounds and flavonoids are present in majority of the fractions. Earlier the plants possessing carbohydrate, saponins, flavonoids, phenolic compounds and tannins were already reported for their analgesic and anti-inflammatory activities.

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