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Anti-inflammatory Activity of Ethanolic Extracts of Leaf and Stem Bark of *Calophyllum inophyllum* on Albino Wistar Rats

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ABSTRACT
The aim of the present study was to conduct phytochemical screening, perform acute oral toxicity effect and to evaluate the anti-inflammatory effect of ethanolic extracts of leaf and stem bark of *Calophyllum inophyllum* on albino Wistar rats. Carrageenan induced paw edema and cotton pellet granuloma techniques were applied to determine anti-inflammatory properties of the extracts. Extracts were administered orally. Acute oral toxicity studies were conducted using the OECD guidelines 423 Annexure – 2d. The results indicate the mortality was not observed during the toxicity studies and maximum safe does was determined. The anti-inflammatory effect of the extracts showed significant dose dependent effect (200 mg/kg b.w and 400 mg/kg b.w) on both acute and chronic models of inflammation i.e., carrageenan induced paw edema and cotton pellet granuloma respectively. Additionally, *Calophyllum inophyllum* leaves extract showed more activity compared to *Calophyllum inophyllum* stem bark extract.

Keywords: *Calophyllum inophyllum*, *Calophyllum inophyllum* leave extract, *Calophyllum inophyllum* stem bark extract, anti-inflammatory effect, carrageenan induced paw edema, cotton pellet induced granuloma.

INTRODUCTION

The genus Calophyllum belongs to the family Clusiaceae which are native to Tropical Asia and its geographical distribution area also includes Melanesia and Polynesia. It grows near the sea coast throughout India. [1] The Tamanu tree is 2-3 m high, and has a thick trunk covered with a rough, black and cracked bark. It has elliptical, shiny and tough leaves. Its flowers, arranged in axillary cymes, have a sweet, lime-like fragrance. The tree, which flowers twice a year, is said to attain a great age. [2] Inflammation is considered as an immunological defense mechanism that is elicited in response to mechanical injuries, burns, microbial infections, allergens and other noxious stimulus. [3] Treatment of inflammation is a debate as the conventional opioids and NSAIDS are commonest to cause Adverse Drug Reactions. [4] Hence there is ongoing research to develop safer and more effective drugs for the therapy for inflammation. The present study is to evaluate acute oral toxicity, anti-inflammatory effect and conduct phytochemical screening.

MATERIALS AND METHODS

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Plant Material

The plant *Calophyllum inophyllum* was collected from "Sri Kotla Vijaybhaskar Reddy Botanical Garden", Hyderabad, India. The plant was identified by a taxonomist (Annexure – I) and voucher specimens representing *Calophyllum inophyllum* (No. 0555) was deposited at the Department of Biology, Osmania University, Hyderabad, India.

Preparation of Plant Extracts

Leaves and stem bark of *Calophyllum inophyllum* were washed carefully to make them free from dust and foreign material. Then they were dried under shade at room temperature. After seven days of drying, the leaves and stem bark were powdered by grinding and passed through a sieve. The powdered leaves and stem bark of *Calophyllum inophyllum* were stored in air tight container for further use.

Leaf Extraction

Leaf powder (60 g) was subjected to Soxhlet extraction for 24 hours continuous extraction using pet ether. After the pet ether extraction was completed, the leaf powder was subjected to further extraction using ethanol as solvent for 24 hours. The plant material to solvents ratio (pet ether and ethanol) were taken as 1:5. Extract was subjected to dried heat treatment on a hot plate and the final product was obtained. Percentage yield of the semisolid material obtained was calculated and was stored at 4°C.

Stem Bark Extraction

Stem bark powder (123g) was subjected to Soxhlet extraction for 24 hours continuous extraction using ethanol. The plant material and the solvent were taken in the ratio 1:5. Extract was subjected to dried heat treatment on a hot plate and the final product was obtained. Percentage yield of the semisolid material obtained was calculated and was stored at 4°C. The ethanol extracts were concentrated in a rotary evaporator. The concentrated ethanol extracts were used for anti-inflammatory activity.

Experimental Animals

Healthy Albino Wistar rats of either sex, weighing 125-175 g, were used for present investigation. Animals were housed under standard environmental conditions of temperature and humidity (25±2°C) and 12 h light/dark cycle were utilized for studies. Rats were fed with standard pellet diet and water *ad libitum.* The ethical clearance was obtained from the 'Anwarul Uloom College of Pharmacy Animal Ethical Committee' for using animals in the present study (1534/PO/a/11/CPCSEA, India).

Acute Oral Toxicity Studies

Acute toxicity studies for ethanolic CILE and CISBE were conducted as per OECD guidelines 423 Annexure – 2d, using Albino Wistar rats. Females were selected as they were considered to be more sensitive. [5] 3 animals of a single sex per step were used. The dose level used as the starting dose was selected from one of four fixed levels, 5, 50, 300 and 2000 mg/kg body weight. The starting dose level should be that which is most likely to produce mortality in some of the dosed animals. 2000 mg/kg dose was selected as starting dose. Depending upon the mortality and the moribund bond status of the animal on an average 2 to 4 steps are necessary.

Anti-inflammatory Activity

Carrageenan Induced Paw Edema

Albino Wistar rats were subjected to 0.1 ml of 1% carrageenan after subjecting them to various treatments. [6] The paw volume is measured by plethysmometer (Orchid Scientifics) immediately after injection, again 1 hour and 2 h, 3 h and 4 h after challenge. The increase of paw volume after every hour is calculated as percentage compared with the volume measured immediately after injection of the irritant for each animal.

Experimental design: The rats were divided into 6 groups (n=6) and treated with the respective solutions as given below.

Group I (Control): CMC (1% w/v, 1ml, p.o.).
Group II (Standard): Indomethacin (10 mg/kg b.w, p.o.).
Group III (Test-I): CILE (200 mg/kg b.w, p.o.).
Group IV (Test-II): CILE (400 mg/kg b.w, p.o.).
Group V (Test-III): CISBE (200 mg/kg b.w, p.o.).
Group VI (Test-IV): CISBE (400 mg/kg b.w, p.o.).

Percentage inhibition (protection) against edema formation was taken as an index of acute anti-inflammatory activity. It was calculated by:

The percent inhibition of edema = 100 (Vc – Vt)/ Vc

Where, Vc = mean paw edema volume in the control group.
Vt = mean paw edema volume in the drug treated group.

Cotton Pellet Induced Granuloma

Albino wistar rats were subjected to cotton pellet induced granuloma technique. [7] The average weight of the dried cotton pellets of the control group as well as of the test group is calculated. The percent change of granuloma weight relative to vehicle control group is determined.

Experimental Design: The rats were divided into 6 groups (n=6) and treated with the respective solutions as given below.

Group I (Control): CMC (1% w/v, 1ml, p.o.).
Group II (Standard): Indomethacin (10 mg/kg b.w, p.o.).
Group III (Test-I): CILE (200 mg/kg b.w, p.o.).
Group IV (Test-II): CILE (400 mg/kg b.w, p.o.).
Group V (Test-III): CISBE (200 mg/kg b.w, p.o.).
Group VI (Test-IV): CISBE (400 mg/kg b.w, p.o.).

Statistical Analysis

The values are expressed as mean ± SEM. $p<0.05$ was considered statistically significant and $p<0.01$ was considered statistically highly significant. Data obtained was analyzed by one-way ANOVA test (parametric ANOVA) followed by Dunnett's multiple comparisons post-hoc test using Graph pad Instat version 3.05, 32 bit for windows.

RESULTS

Plant Yield

CILE: Total weight of dried ethanolic leaf extract of *Calophyllum inophyllum* was found to be 9.76 grams and percentage yield was calculated as 16.27%.

CISBE: Total weight of dried ethanolic stem bark extract of *Calophyllum inophyllum* was found to be 21.68 grams and percentage yield was calculated as 17.62%.

Preliminary Phytochemical Screening

Phytochemical investigation of ethanolic CILE and CISBE revealed the presence of flavonoids, alkaloids, glycosides, carbohydrates, tannins, steroids proteins and amino acids as major chemical constituents as shown in Table 1.

Table 1: Preliminary Phytochemical Screening

Phytoconstituents	CILE		CISBE
	Petroleum Ether	Ethanol	Ethanol
Alkaloids	-	+	+
Carbohydrates	+	+	+
Glycosides	-	+	+
Saponins	+	+	+
Tannins	-	+	+
Flavonoids	-	+	+
Proteins	+	+	+
Amino Acids	+	+	+
Steroids	-	+	+

Oral Acute Toxicity Studies

Oral acute toxicity studies showed that the ethanolic CILE and CISBE had shown no mortality at 2000 mg/kg. Therefore, 2000 mg/kg dose was considered as maximum safe dose.

Carrageenan Induced Paw Edema

From Table 2, the 200 mg/Kg doses of the ethanol CILE and CISBE significantly reduced the paw volume ($p<0.05$) at the 3 hour after administration. The 400 mg/Kg dose of the ethanol CILE significantly reduced the paw volume ($p<0.05$) at 2nd hour. All the doses of CILE and CISBE were highly significant at 4th hour (200 mg/Kg and 400 mg/Kg, $p<0.01$). CILE reduced paw volume more significantly at all respective doses compared to the CISBE.

From Table 3, the resultant inhibition of ethanol leaf extract v/s ethanol stem bark extract, the ethanol CILE shows high inhibition percentage than CISBE.

From Figure 1, shows the graph of effect of *Calophyllum inophyllum* on Carrageenan Induced Paw Edema in Rats. Figure 2 shows the graph of % Inhibition in Carrageenan induced paw edema.

Table 2: Measurement of Paw Volume

Time	Group I Control	Group II Standard	Group III CILE 200 mg/kg	Group IV CILE 400 mg/kg	Group V CISBE 200 mg/kg	Group VI CISBE 400 mg/kg
0 Hrs.	0.70 ± 0.007	0.56 ± 0.012**	0.68 ± 0.007	0.67 ± 0.008*	0.69 ± 0.007	0.67 ± 0.005
1 Hrs.	1.10 ± 0.016	0.69 ± 0.024**	0.99 ± 0.019	0.93 ± 0.019	1.01 ± 0.028	0.95 ± 0.020
2 Hrs.	1.41 ± 0.019	0.575 ± 0.024**	0.99 ± 0.019	0.87 ± 0.019*	1 ± 0.020	0.92 ± 0.016
3 Hrs.	1.48 ± 0.020	0.54 ± 0.022**	0.87 ± 0.020*	0.77 ± 0.019**	0.93 ± 0.021*	0.83 ± 0.015*
4 Hrs.	1.495 ± 0.019	0.49 ± 0.019**	0.86 ± 0.018**	0.70 ± 0.017**	0.91 ± 0.018**	0.78 ± 0.014**

* $p<0.05$, ** $p<0.01$

Table 3: % Inhibition in Carrageenan Induced Paw Edema

Time	% Inhibition of Treated Groups					
	Group I Control	Group II Standard	Group III CILE 200 mg/kg	Group IV CILE 400 mg/kg	Group V CISBE 200 mg/kg	Group VI CISBE 400 mg/kg
1 Hrs.	-	37.61%	12.99%	22.96%	11.18%	19.49%
2 Hrs.	-	59.22%	29.67%	38.18%	28.96%	35.11%
3 Hrs.	-	63.21%	40.86%	47.63%	36.91%	43.57%
4 Hrs.	-	67.34%	42.47%	53.29%	39.46%	48.05%

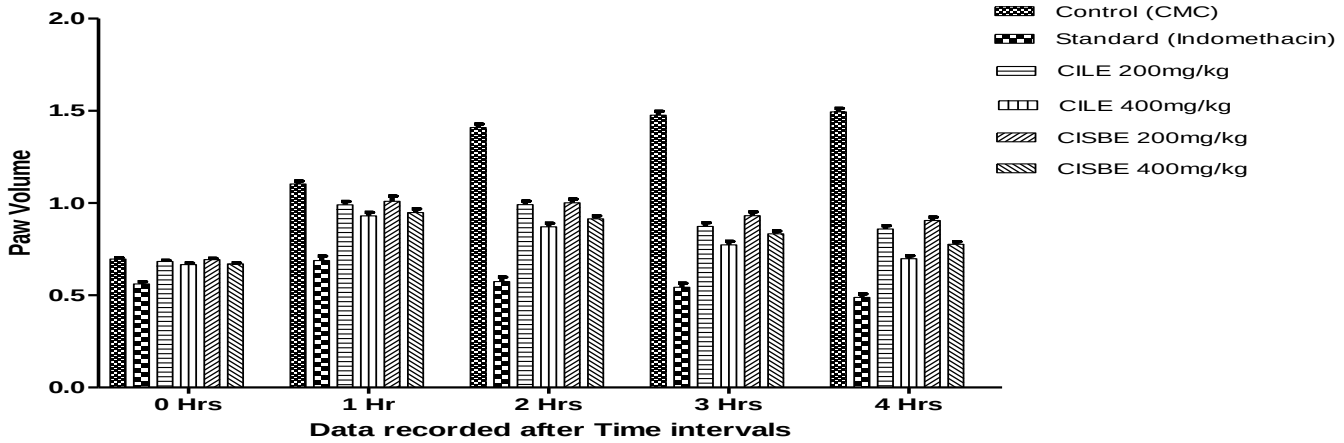

Fig. 1: Effect of CI on Carrageenan Induced Paw Edema in Rats

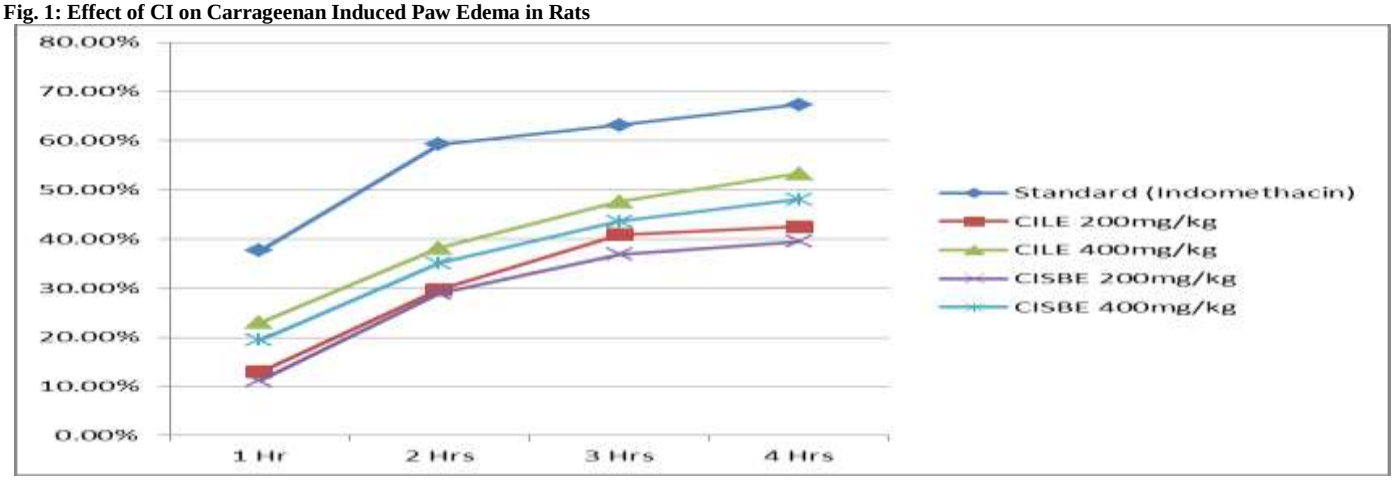

Fig. 2: % Inhibition in Carrageenan Induced Paw Edema

Cotton Pellet Induced Granuloma

From Table 4, CILE and CISBE doses, 200 mg/kg and 400 mg/kg, showed significant results ($p<0.01$) after the 7 days of oral dosing. The resultant inhibition of ethanol leaf extract v/s ethanol stem bark extract, the ethanol CILE shows high inhibition percentage than CISBE.

Figure 3 shows the graph of the effect of *Calophyllum inophyllum* on cotton pellet induced Granuloma. Figure 4 shows the graph of % Inhibition in Cotton Pellet Induced Granuloma.

DISCUSSION

The presence of edema is one of the prime signs of inflammation. [8] It has been documented that carrageenan induced rat paw edema is suitable *in vivo* model to study anti-inflammatory drugs both steroidal and non-steroidal since it involves several mediators. [9]

Non-steroidal anti-inflammatory drugs (NSAIDs) are the most commonly used drugs in the world today. Pain and fever are being the most common complaints associated with inflammation. The NSAIDs used in the inflammatory conditions do not cure and remove the underlying cause of the disease but they only modify the inflammatory response to the diseases. There is a market need for orally active molecules that can treat inflammatory disease processes, rather than just the symptoms, more effectively than currently available drugs. Therefore, new anti-inflammatory agents are in process. [10]

Different flavonoids with anti-inflammatory activity have been reported. The three flavonoids; 3-O-methylquercetin, 3,

7-O-dimethylquercetin and 3, 7-O-dimethylkaempferol from the ethanol extract of *Cistus laurifolius* L. [11], nepetin, jaceosidin and hispidulin isolated from dichloromethane extract of *Eupatorium arnottianum* Griseb [12] exhibited anti-inflammatory effect. Thus the presence of flavonoids may contribute towards anti-inflammatory effect.

Presence of xanthones may also be the factors contributing to the anti-inflammatory activity. [13]

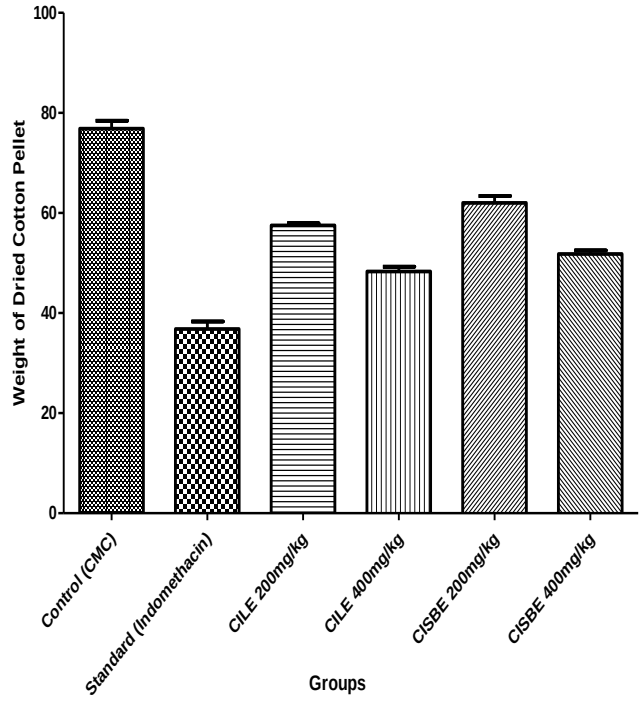

Fig. 3: Effect of CI on Cotton Pellet Induced Granuloma in Rats

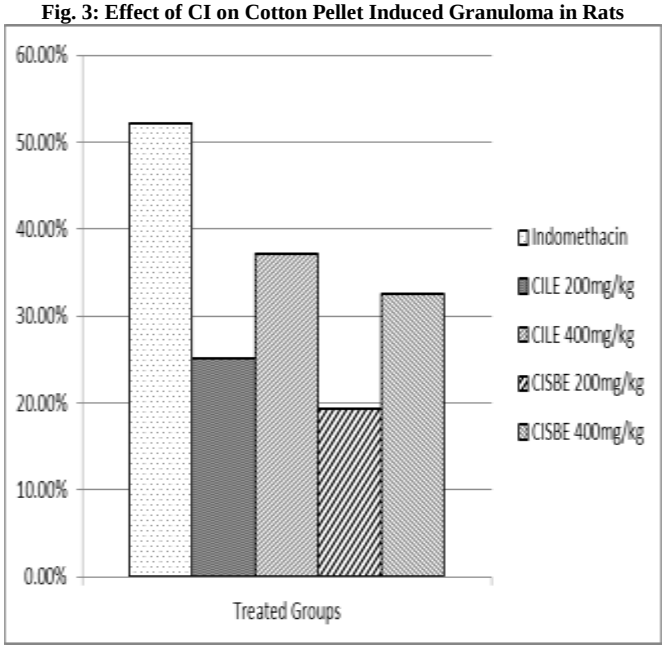

Fig. 4: % Inhibition in Cotton Pellet Induced Granuloma

Table 4: Cotton Pellet Induced Granuloma

Group	Treatment	Dose	Wt. of Dried Cotton Pellet (mg)	% Inhibition
I	**Control**	1 ml	76.83 ± 1.6	-
II	**Standard**	10 mg/kg	36.83 ± 1.47**	52.06%
III	**CILE**	200 mg/kg	57.5 ± 0.43**	25.16%
IV	**CILE**	400 mg/kg	48.3 ± 0.92**	37.09%
V	**CISBE**	200 mg/kg	62 ± 1.39**	19.31%
VI	**CISBE**	400 mg/kg	50.5 ± 0.70**	32.54%

***p*<0.01

The oral acute toxicity studies suggest that ethanolic CILE and CISBE doses are safe up to 2000 mg/kg in albino Wistar rats. The experimental data showed that the ethanolic CILE and CISBE possess significant anti-inflammatory activity at doses 200 mg/kg and 400 mg/kg. In addition, data suggests that ethanolic CILE showed more anti-inflammatory activity compared to ethanolic CISBE.

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