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Pharmacological Study of Medicinal Plant *Calophyllum inophyllum* L. on Swiss Albino Mice in the Management of Pain and Inflammation

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Authors' contributions

This work was carried out in collaboration between all authors. Author YB designed and supervised the study. Authors SA and MSH performed the experiments, managed the literature searches and wrote the manuscript. Author IJB performed the statistical analysis. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To investigate the analgesic and anti-inflammatory effect of ethanol extract of *Calophyllum inophyllum* (Family: clusiaceae) in Swiss albino mice.
Study Design: For the purpose of this experiment the leaf extract was divided into two concentrations, 100 and 200 mg/kg body weight respectively. And then they were used to evaluate the analgesic and anti-inflammatory activity of the plant.
Place and Duration of Study: Department of Pharmacy, Southeast University, Banani, Dhaka-1213, Bangladesh, within a period of one year.
Methodology: The analgesic activity was conducted using Acetic acid induced writhing test, formalin induced paw licking, tail immersion, eddy's hot plate method and the anti-inflammatory activity was evaluated by using carrageenan induced hind paw edema models. All studies were carried out in mice by the ethanol extract of *C. inophyllum* at the doses of 100 and 200 mg/kg body weight respectively.
Results: The results of the present investigation indicates that the ethanol extract of *C. inophyllum*

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displayed significant (*P* <.001) analgesic activity and showed maximum writhing inhibition (49.82%) at the dose of 200 mg/kg body weight compared to standard drug indomethacin (74.56%) in acetic acid induced writhing method. In the formalin induced paw licking method, *C. inophyllum* showed significant (*P* < .001) activity in both phases and the maximum inhibition was shown at the dose of 200 mg/kg body weight, 50.74% and 53.79% respectively at early and late phase. The leaf ethanol extract of *C. inophyllum* was highly significant (*P* < .001) and showed dose dependent reduction of pain in both tail immersion and eddy's hot plate method and maximum inhibition was found (55.88% and 54.17%) at 1 hour compared to standard drug. In the anti-inflammatory assay, both doses of ethanol extract of *C. inophyllum* showed significant (*P* < .001) activity after 3 hours of administration of plant extract against Carrageenan induced hind paw edema.

Conclusion: The investigated study concludes that *C. inophyllum* leaf extract has anti-inflammatory, central as well as peripheral analgesic effects which supports a significant scope to develop its medicinal practice.

Keywords: Calophyllum inophyllum; acetic acid induced writhing; analgesic; anti-inflammatory.

1. INTRODUCTION

Natural remedies can defeat pain without relying on the risky drugs. At present, plant parts are widely used traditionally or medicinally to avoid the side effects such as gastric lesions, ulcers, hypertension and cardiac abnormalities produced by Steroids, NSAIDs and Opiates. Lots of biological investigations are conducted to evaluate the herbal drugs as new analgesic and anti-inflammatory agents to improve the quality of life. In this context, our objective of the present study is to evaluate the analgesic and anti-inflammatory activities of the plant extract *C. inophyllum.*

C. inophyllum family clusiaceae, commonly known as "Indian laurel" or Alexandrian laurel, occurring above the high-tide mark along sea coasts of northern Australia and extending throughout Southeast Asia and southern India. It is an ornamental roadside tree having great economical and medicinal value. All parts of *C. inophyllum* are traditionally used in folk medicine for treating various health problems. The leaves are used in migraine, dermatosis, urticaria (hives) and eczema [1]. The oil extracted from the seeds known as tamanu oil which is analgesic and is used for neuritis, leprous neuritis, rheumatism and gout. Tamanu oil also has the ability to accelerate wound healing and render the skin healthy. The bark is used as an antiseptic, disinfectant externally whereas used internally in chronic bronchitis, phthisis and haemorrhage. In contrast, the root juice is used in headache [1-5].

Potential medicinal uses of *C. inophyllum* have proved the presence of many bioactive secondary metabolites as coumarins, xanthones, flavonoids, steroids and triterpenoid. It contains 4-hydroxyxanthone; 1, 5-dihydroxyxanthone; 1, 7-dihydroxyxanthone; 1, 3, 5-trihydroxy-2-methoxyxanthone; 6-deoxyjacareubin; amentoflavone; kaempferol-3-O-alpha-L-rhamnoside and quercetin-3-O-alpha-L-rhamnoside. it also has Caloxanthones A and B; 1,5-dihydroxyxanthone-6-desoxyjacareubin; 2-3-methylbut-2-enyl-1,3,5-trihydroxyxanthone; 2-3-methylbut-2-enyl-1,3,5,6-tetrahydroxyxanthone. From the leaves canophyllol, and canofilic acid; 4 tetracyclic phenyl-coumarins (Inofilum A, C, D, E) three tricyclic (calocoumarin A, calofiloid, apetatolide); two dimethylcyclopropyl (calocoumarin B and C) and a cinnamic phenolic acid and inofilum C and E were isolated. Beisdes the above mentioned compounds *C. inophyllum* has 3,4-secofreidelan-3,28-dioic acid; 27-hydroxyacetate canofilic acid and 3-oxo-27-hydroxyacetate-friedelan-28-oic acid. Most of these bioactive compounds possess significant anti-HIV, anti-bacterial, anti-tumor, anti-cancer, anticoagulant, anti-inflammatory, antiplatelet aggregation, cytotoxic activity [6-18].

2. MATERIALS AND METHODS

2.1 Plant Material Collection

The fresh mature leaves of *C. inophyllum* were collected from Narsingdi, Bangladesh in July, 2014 identified in the Bangladesh National Herbarium (DACB), Mirpur, Dhaka (Accession No. 39674) and a voucher specimen has been recorded in the herbarium for future reference.

2.2 Preparation of Leaf Extract

The leaves were washed with running water to remove adhering dirt, then air dried and

powdered with a mechanical grinder and stored properly in a container. The powdered material (1 kg) was then taken in a clean glass container and soaked in ethanol for seven days. The whole mixture was then filtered through clean, white cotton bed followed by Whatman filter paper number 1. The total filtrate was concentrated, *in vacuo* at 40°C to render the ethanolic extract (400 g).

2.3 Experimental Animals

Young Swiss-albino mice weighing between 30 to 35 gm of either sex were used for this study. The animals were collected from the Animal Resources Branch of ICDDR, B (International Centre for Diarrheal Disease and Research, Bangladesh) and housed in steel cages with food and water *ad libitum* formulated by ICDDR, B in standard environmental condition (at 24.0±0°C temperature, 55-65% relative humidity and 12 hour light/12 hour dark cycle). The animals were allowed to acclimatize to the environment for 7 days prior to experimental session. Throughout the experimental period, all animals received human care according to the criteria outlined in the 'Guide for the Care and Use of Laboratory Animals', 8th edition, prepared by the National Academy of Sciences and published by the National Institute of Health (US).

2.4 Drugs and Chemicals

Indomethacin and Diazepam were obtained from Square Pharmaceuticals Ltd., Bangladesh. Acetic acid and Carrageenan were obtained from Merck, Germany. Tween-80 was procured from BDH Chemicals, UK. Normal saline solution was purchased from Beximco Infusion Ltd., Bangladesh. All chemicals used were of analytical reagent grade.

2.5 Analgesic Activity Test

2.5.1 Acetic acid induced writhing method

The analgesic activity of the ethanolic extract of *C. inophyllum* leaves was evaluated using acetic acid-induced writhing method in mice [19]. At first, twenty animals were divided into four groups with five mice in each.

Group I (Control): Treated with vehicle (1% Tween 80 in water, 10 ml/kg body weight p.o.).

Group II (Standard): Received Indomethacin (10mg/kg) body weight (p.o.).

Group III (Leaf extract 100mg/kg): Treated with 100 mg/kg body weight (p.o.) of the extract.

Group IV (Leaf extract 200 mg/kg): Treated with 200 mg/kg body weight (p.o.) of the extract.

The test samples and vehicle were administered orally 30 minutes before intraperitoneal administration of 0.7% v/v acetic acid but indomethacin (Standard drug) was administered orally 15 minutes before injection of acetic acid. After an interval of 5 minutes, the mice were observed for specific contraction of body referred to as 'writhing' for the next 10 minute. Complete writhing was not always accomplished by the animal; this incomplete writhing was considered as half-writhing. Accordingly, two half-writhing were taken as one full writhing. The number of writhing in each treated groups was compared to that of a control group. Samples having analgesic activity will reduce number of writhes of treated rats. The percentage of analgesic activity was calculated by:

$$\text{Percentage analgesic activity} = [(A - B)/ A] \times 100\%$$

Where, A= average number of writhing of group-I (control group); B= average number of writhing of test group.

2.5.2 Formalin induced paw licking method

The antinociceptive activity of the drugs was determined using the formalin test described by Dubuission and Dennis [20]. Control group received 5% formalin. 20 µl of 5% formalin was injected into the dorsal surface of the right hind paw 30 min after administration of *C. inophyllum* leaf extract (100 and 200 mg/kg, p.o.) and 15 minutes after administration of Diclofenac sodium (10 mg/kg, i.p.). The mice were observed for 30 minutes after the injection of formalin, and the amount of time spent licking the injected hind paw was recorded. The first 5 minutes post formalin injection is referred to as the early phase and the period between 15 and 30 minutes as the late phase. The total time spent licking or biting the injured paw (pain behavior) was measured with a stop watch.

2.5.3 Eddy's hot plate method

The paws of mice are very sensitive to heat. The responses are jumping, withdrawal and licking of the paws. Eddy's hot plate method was followed to evaluate analgesic activity [21]. Albino mice

were introduced to a hot plate maintained at 55 ± 0.5°C. The reaction time to the thermal stimulus was recorded as the time interval from introduction of the animal to the plate until the first lick of the limbs or the first jump of the animals. The test groups received ethanolic extracts of *C. inophyllum* at 100 and 200 mg/kg dose levels prepared as suspension in 2% Tween 80 orally, the standard group received diclofenac (9 mg/kg-body weight) and control group received only 1 ml of 2% Tween 80 solution. The reaction times were determined before and after 30 minutes, 1 hour, 2 hours and 3 hours period with reference to the control group receiving only vehicle.

2.5.4 Tail immersion method

The test procedure is based on the typical tail-withdrawal reflex in mice induced by immersing the end of the tail in warm water of 55°C [22]. Group 1 treated with saline water (isotonic saline solution, 0.9%), Group 2 with standard drug indomethacine at the dose of 9 mg/kg-body weight as a reference drug and Group 3-4 with ethanol extracts at the dose of 100 mg/kg and 200 mg/kg body weight respectively. The lower 5 cm portion of the tail is marked. This part of the tail is immersed in to the water bath of exactly 55°C and the withdrawal of the tail from the hot water was counted as the reaction time. Cut off time was 20 seconds to avoid the damage of paw. After each determination the tail is carefully dried. The reaction time is determined before and periodically after oral administration of the test and standard substance.

2.6 Anti-inflammatory Activity

2.6.1 Carrageenan-induced hind paw edema in mice

Carrageenan induced hind paw edema mice models were used to evaluate anti inflammatory activity of *C. inophyllum leaf* extract [23]. Swiss albino mice (30-35 g) were divided into four groups of five animals each. The test groups received 100 and 200 mg/kg body weight of *C. inophyllum* leaf extract. The reference group received indomethacin (10 mg/kg body weight, p.o.) while the control group received 3 ml/kg body weight normal saline. After 30 min, 0.1 ml, 1% carrageenan suspension in normal saline was injected into the subplanatar tissue of the right hind paw. The paw volume was measured at 1, 2, 3 and 4 h after carrageenan injection using a micrometer screw gauge. The percentage inhibition of the inflammation was calculated from the formula:

$$\text{Percentage inhibition} = (1 - Dt/Do) \times 100$$

Where, Do was the average inflammation (hind paw edema) of the control group of mice at a given time, Dt was the average inflammation of the drug treated (i.e., extract or reference indomethacin) mice at the same time.

2.7 Statistical Analysis

All the values in the test are expressed as mean ± SEM. The data were statistically analyzed by ANOVA (Analysis of variance) followed by Dunnett's test with the Statistical Package for Social Sciences (SPSS 16.0, USA) program. A probability of P = .05 was considered as statistically significant compared to control group.

3. RESULTS

3.1 Analgesic Activity

3.1.1 Acetic acid induced writhing method

Ethanolic extracts of *C. inophyllum* demonstrated a significant analgesic activity by inhibiting pain caused by acetic acid induced writhing which has been showed in Table 1. The dose of 100 mg/kg of extract of *C. inophyllum* displayed 31.02% decrease in writhing and the dose of 200 mg/kg of extract of *C. inophyllum* displayed 49.82% decrease in writhing. The result at dose 200 mg/kg were highly significant (P < .01) compared to normal control group.

3.1.2 Formalin induced paw licking method

Formalin induced paw licking was significantly reduced by both the dose of 100 mg/kg and 200 mg/kg body weight of *C. inophyllum* which has been showed in Table 2. In the early phase, both doses produced 31.62% and 50.74% paw licking inhibition and in late phase inhibition was 35.17 and 53.79% respectively.

3.1.3 Tail immersion method

The result obtained from two doses of extract of *C. inophyllum* by tail immersion method for analgesic activity showed in the Table 3 and the percentage of inhibition of pain was given in Fig. 1. One hour after extract administration of 100 mg/kg and 200 mg/kg body weight reduced the

painful stimulation 55.88% and 38.24% respectively; it indicates that both doses were more effective at the 1 hour after administration.

3.1.4 Eddy's hot plate method

In Eddy's hot plate method, two doses of extract of *C. inophyllum* showed significant decrease in pain stimulus in a dose dependent manner, as shown in Table 4 and the percentage of inhibition of pain was given in Fig. 2. The maximum result was found at the dose of 200 mg/kg body weight which showed highest reaction time for the response against thermal stimuli 54.17% compared to Diclofenac 79.17%.

Table 1. Effects of ethanolic extract of *C. inophyllum* leaves on acetic acid induced writhing in mice

Treatment	Avg. no. of writhing	% Inhibition
Group- I (Control)	35.38±3.98	-
Group-II (Standard)	9.00±1.29***	74.56
Group-III (Leaf extract 100 mg/kg)	27.00±1.78	31.02
Group- IV (Leaf extract 200 mg/kg)	17.75±0.63**	49.82

*Values are reported as mean ± S.E.M. for group of four animals (n = 4). Values are analyzed as compared to control using one way ANOVA followed by dunnett's test. Asterisks indicated statistically significant values from control, * indicates P = .05, ** indicates P < .01 and *** indicates P < .001*

Table 2. Effects of ethanolic extract of *C. inophyllum* leaves on formalin induced paw licking method in mice

Treatment	Number of paw licking at different time (minutes)		% Inhibition at different time(minutes)	
	0-5	20-30	0-5	20-30
Group- I (Control)	34.00±1.83	36.25±1.377	-	-
Group-II (Standard)	9.25±0.85***	11.5±0.289***	72.791	68.276
Group-III (Leaf extract 100 mg/kg)	23.25±1.91**	26.00±2.121***	29.412	28.276
Group- IV (Leaf extract 200 mg/kg)	16.75±2.39**	18.00±0.816***	51.471	50.345

*Values are reported as mean ± S.E.M. for group of four animals (n = 4). Values are analyzed as compared to control using one way ANOVA followed by dunnett's test. Asterisks indicated statistically significant values from control, * indicates P = .05, ** indicates P < .01 and *** indicates P < .001*

Table 3. Effects of ethanolic extract of *C. inophyllum* leaves on tail immersion method in mice

Treatment	Reaction time (s)					
	0 h	1 h	2 h	3 h	4 h	5 h
Group- I (Control)	8.00±0.41	8.50±0.29	8.25±0.25	7.50±0.29	7.50±0.29	7.75±0.25
Group-II (Standard)	7.50±0.29	2.00±0.41***	2.00±0.41***	2.00±0.41***	2.25±0.25***	2.50±0.29***
Group-III (Leaf extract 100 mg/kg)	7.75±0.25	5.25±0.25***	6.00±0.85*	5.50±0.29**	6.50±0.65	7.00±0.41
Group- IV (Leaf extract 200 mg/kg)	7.70±0.65	3.75±0.48***	4.00±0.41***	4.00±0.41***	5.75±0.95	6.75±1.18

*Values are reported as mean ± S.E.M. for group of four animals (n = 4). Values are analyzed as compared to control using one way ANOVA followed by dunnett's test. Asterisks indicated statistically significant values from control, * indicates P = .05, ** indicates P < .01 and *** indicates P < .001*

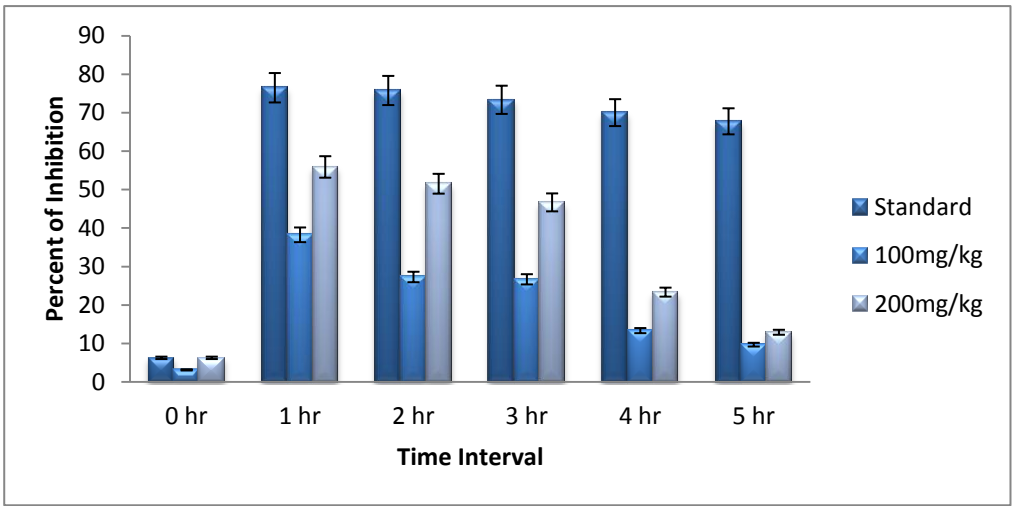

Fig. 1. Percentage of inhibition of pain at various time intervals in tail immersion method

Table 4. Effects of ethanolic extract of *C. inophyllum* leaves on Eddy's hot plate method in mice

Treatment	Reaction time (s)					
	0 h	1 h	2 h	3 h	4 h	5 h
Group- I (Control)	6.25±0.25	6.00±0.41	6.00±0.41	6.25±0.48	6.50±0.288	6.25±0.25
Group-II (Standard)	5.50±0.29	1.25±0.48***	1.50±0.29***	1.75±0.48***	2.00±0***	2.5±0.29**
Group-III (Leaf extract 100 mg/kg)	6.00±0.58	3.75±0.45*	4.00±0.41**	5.00±0.71	5.25±1.03	5.75±1.25
Group- IV (Leaf extract 200 mg/kg)	5.75±0.25	3.00±0.71**	3.25±0.25***	3.50±0.29**	4.75±0.63	5.50±0.29

*Values are reported as mean ± S.E.M. for group of four animals (n = 4). Values are analyzed as compared to control using one way ANOVA followed by dunnett's test. Asterisks indicated statistically significant values from control, * indicates P = .05, ** indicates P < .01 and *** indicates P < .001*

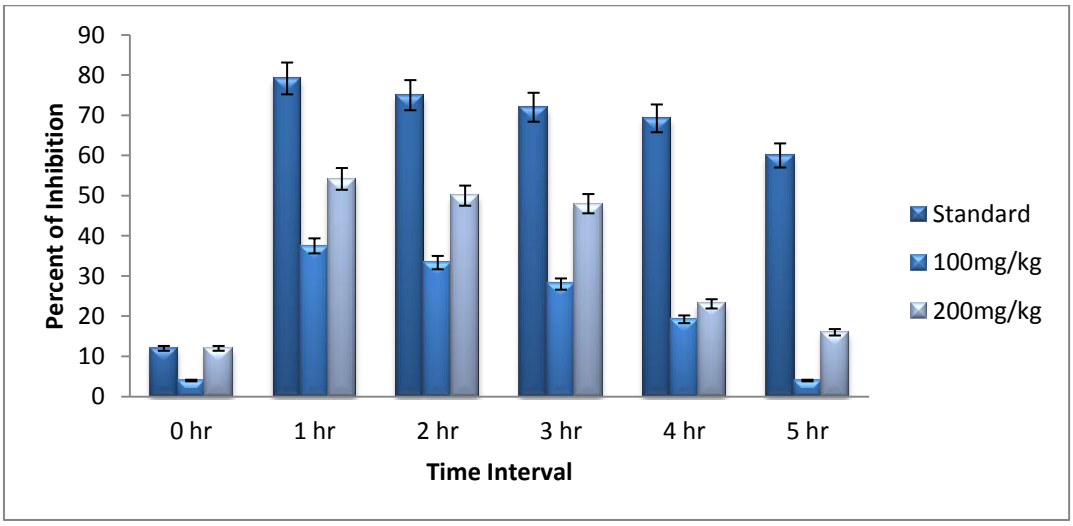

Fig. 2. Percentage of inhibition of pain at various time intervals in eddy's hot plate method

Table 5. Effects of ethanolic extract of *C. inophyllum* leaves on carragenan induced paw edema

Treatment	Paw volume (mm)				
	0 h	1 h	2 h	3 h	4 h
Group- I (Control)	17.50±0.61	13.88±0.51	13.38±0.55	12.25±0.43	11.75±0.32
Group-II (Standard)	15.5±0.29	8.25±0.25***	5.25±0.95***	4.25±0.25***	3.50±0.29***
Group-III (Leaf extract 100 mg/kg)	16.88±0.49	12.75±0.29	11.5±0.33	8.63±0.80***	7.38±0.72***
Group- IV (Leaf extract 200 mg/kg)	16.63±0.24	11.5±0.29**	8.5±0.65***	6.50±0.29***	5.75±0.63***

*Values are reported as mean ± S.E.M. for group of four animals (n = 4). Values are analyzed as compared to control using one way ANOVA followed by dunnett's test. Asterisks indicated statistically significant values from control, * indicates P = .05, ** indicates P < .01 and *** indicates P < .001*

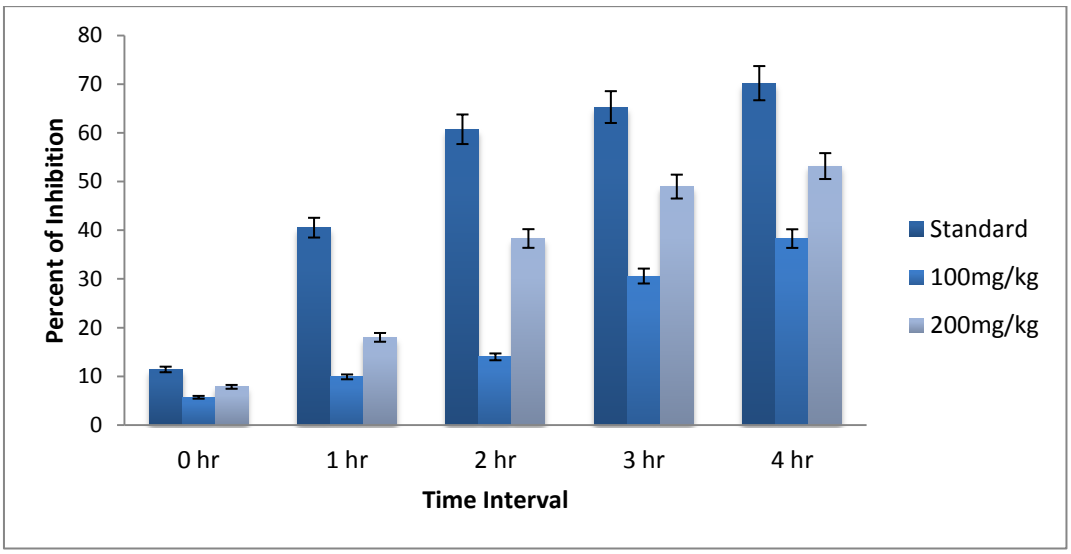

Fig. 3. Percentage of inhibition of inflammation at various time intervals in carrageenan induced hind paw edema

3.2 Carrageenan Induced Hind Paw Edema

From Table 5 above, it is clear that the development of paw edema induced by carrageenan was significantly reduced by both the doses of 100 mg/kg and 200 mg/kg and the percentage of inhibition of pain was given in Fig. 3 above. Maximum inhibition (53.19%) of carrageenan induced inflammation was observed at the dose 200 mg/kg after 3 hours of administration compared to Diclofenac which showed 70.21% inhibition.

4. DISCUSSION

Writhing is an overt response to the intense pain induced by irritant principles via nociceptors characterized by episodes of retraction of abdomen and stretching of hind limb [24]. Acetic acid-induced writhing is a sensitive method in evaluating for peripheral analgesic activity [25]. The experimental result of this method recommend that prostaglandin synthesis might be inhibited by this extract, a peripheral mechanism of pain reduction. Hot plate test and Tail immersion method has been used for screening the central nociceptive mechanism of plant in producing analgesia [26]. Both These methods produce pain sensation by thermal stimuli but differ from each other in their pathway to producing pain stimulus conducted through sensory nerves. Pain sensation induced in Tail immersion method is due to release of substance P and glutamate in the dorsal horn of the spinal cord while hot plate tests indicates supraspinally

integrated response [27,28,29]. Centrally acting analgesic exert their spinal analgesia by inhibiting the release of substance-P in the dorsal horn of the spinal cord and supraspinal analgesia by the activation of descending inhibitory impulses in the midbrain [30,31]. The ethanol extract caused significant increase in the percentages reaction time in both methods compared to centrally acting agents suggest the central analgesic activity of the plant via central mechanism. Formalin induced paw licking test conducted to clarify the possible mechanism of plant extract produces a distinct biphasic response including early phase and late phase. Pain sensation observed during early phase is supposed to reflect the neurogenic pain while late phase is believed to represent inflammatory pain [32]. In this test the delay in paw licking time in both phases further approve that the plant has analgesic activity with both pathway involving inflammatory and non-inflammory analgesia. All the above experimental analgesic methods reveal the potential central and peripheral antinociceptive activity of *C. inophyllum*. *C. inophyllum* widely used as a folklore medicine having many bioactive compounds like flavonoids which are responsible for the impairment of cyclooxygenase activities that may reduce the levels of prostaglandins producing analgesia [33].

Carrageenan-induced hind paw edema is a well established experimental model used largely to study acute inflammation/anti inflammatory activity. It comprises of two phases: the first phase (0-2 h) involves the release of histamine and serotonin (5-HT), while a second phase (>4 h) of swelling is mediated by PG release [34]. Plateau phase (3 h) in between first and delayed phase is maintained by a kinin like substance. In the study plant exhibited a pronounced inhibition of carrageenan induced rat paw inflammation by inhibiting the mediators of acute inflammation and indicating its anti - inflammatory activity. *C. inophyllum* is reach in triterpinoids that reduce histamine release from mast cells and exert anti-inflammatory activity [35]. This finding correlate the traditional anti inflammatory activity of this plant and prove consistent with the previous results.

5. CONCLUSION

The present study showed that ethanol extract obtained from the leaves of *C. inophyllum* possessed significant analgesic and anti-inflammatory activity compared with the control group. As a result of this potential analgesic and anti-inflammatory activity of the plant further studies needs to understand the mechanism of these activities.

CONSENT

It is not applicable.

ETHICAL APPROVAL

The experimental protocols were approved by the Ethical Committee for Animal Care and Use at Department of Pharmacy, Southeast University (2014/015-25).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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