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EVALUATION AND COMPARISON OF ANXIOLYTIC ACTIVITY OF ETHANOLIC AND AQUEOUS LEAF EXTRACTS OF *CALOPHYLLUM INOPHYLLUM* IN ALBINO WISTAR RATS

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ABSTRACT

The aim of the present study was to perform phytochemical screening, acute oral toxicity and to evaluate and compare the anxiolytic activity of ethanolic and aqueous leaf extracts of *Calophyllum inophyllum* on albino wistar rats. Elevated plus maze method and Light and Dark model were implemented to determine the anxiolytic activity of the leaf extracts. Ethanolic and aqueous leaf 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 acute oral toxicity studies and maximum safe does determined. The anxiolytic activity effect of the extracts showed that *Calophyllum inophyllum* leaf ethanolic extract doses 200mg/kg, 400mg/kg b.w and *Calophyllum inophyllum* leaf aqueous extract dose 400mg/kg b.w exhibited significant anxiolytic activity whereas *Calophyllum inophyllum* leaf aqueous extract dose 200mg/kg b.w did not show any significant activity. Additionally, the effects were more prominent in the ethanolic extract than the aqueous extract which may be due to successive solvent extraction.

KEYWORDS: ***Calophyllum inophyllum*, *Calophyllum inophyllum* leave ethanolic extract, *Calophyllum inophyllum* leaf aqueous extract, anxiolytic activity, Elevated plus maze method and Light and Dark model**

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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]. Anxiety disorders are marked by excessive fear (and avoidance), often in response specific objects or situation and in the absence too danger, and they are extremely common in the general population. According to a recent epidemiological study, the life time prevalence of any anxiety disorder is 28.8% [3]. Anxiety disorders are associated with impaired work place performance and hefty economic costs [4]. The present study is to evaluate and compare the anxiolytic activity of ethanolic and aqueous leaf extracts of *Calophyllum inophyllum* on albino wistar rats.

MATERIALS AND METHODS

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 in the Department of Biology, Osmania University, Hyderabad, India.

Preparation of plant extracts

The powdered material was subjected to successive extraction using Soxhlet Apparatus with solvents in increasing order of polarity. The solvents used were petroleum ether, ethanol and distilled water. In this process the substance, which is soluble in a solvent with particular range of polarity was extracted in the solvent and remaining marc was further extracted with next solvent. The 60g powdered drug was taken and subjected for successive solvent extraction. The extraction was carried until the solvent becomes colorless.

Preparation of ethanolic extract

The marc from the above process was repacked in a Soxhlet apparatus and extracted with 95% alcohol until a clear and colorless solvent appears in the siphon tube of the Soxhlet apparatus.

Preparation of aqueous extract

After ethanolic extraction, the remaining dried marc was extracted with water to get aqueous extract. For aqueous extract, the above dried marc was macerated for 3 days with distilled water and the residue was removed by filtration and filtrate was concentrated to obtain aqueous extract.

All the extracts were concentrated by distillation of the solvent and evaporating them to dryness on a water bath maintained at 45°C to get a solid mass. They were then weighed and the percentages of different extractive values were calculated in terms of air dried weight of the plant material. All these extracts were stored in an airtight container in a refrigerator below 100^0C.

Phytochemical Screening

Calophyllum inophyllum leaf ethanolic and aqueous extracts were tested for the presence of carbohydrate, steroids, saponins, flavonoids, tannins, alkaloids, proteins, amino acids and glycosides [5, 6].

Experimental animals

Healthy albino Wistar rats of either sex, weighing 180-200g, were used for the present investigation. Animals were housed under standard environmental conditions of temperature and humidity (25±2□C) and 12h 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 oral toxicity studies of *Calophyllum inophyllum* leaf ethanolic extract were previously performed and no mortality was observed at 2000mg/kg. Therefore, 2000mg/kg dose was considered as maximum safe dose. 2000mg/kg dose was considered as maximum safe dose [7]. *Calophyllum inophyllum* leaf aqueous extract was subjected to acute oral toxicity studies as per OECD guidelines 423 Annexure – 2d, using albino Wistar rats. Females were selected as they were considered to be more sensitive [8].

Anxiolytic Studies

Elevated Plus Maze

Locally fabricated elevated plus maze consisting of two open arms (50 × 10 cm) and two enclosed arms (50 × 10 × 40 cm) was used. The maze was elevated to the height of 50 cm. Sixty minutes after administration of drug/vehicle the rats were placed individually in the center of the EPM facing an enclosed arm. The time spent by the mouse during the next 05 min on the open and enclosed arm was recorded [9].

Experimental Design

Animals were randomly divided into six groups, each group containing 6 rats as follows:

Group I: Served as normal control (1ml p.o.; 1% CMC)

Group II: Receive only Diazepam (2mg/kg body weight p.o.) [10]

Group III: CILEE 200 mg/kg body weight p.o.

Group IV: CILEE 400 mg/kg body weight p.o.

Group V: CILAE 200 mg/kg body weight p.o.

Group VI: CILAE 400 mg/kg body weight p.o.

Light and Dark model

The apparatus consists of an open-top box with two distinct arenas, namely, a dark arena (20 × 30 × 35 cm) painted black and illuminated with a dim red light, and a bright arena (30 × 30 × 35 cm) painted white and brightly illuminated with a 100 W white light source placed 17 cms above the box. The two arenas are connected by a small open doorway (7.5×5 cms) located at floor level in the center of the partition. Sixty minutes after administration of treatment the animals were placed in the center of the brightly lit arena. During the 5 min test period the number of entries into light and dark arenas was recorded [9].

Experimental Design

Animals were randomly divided into six groups, each group containing 6 rats as follows:

Group I: Served as normal control (1ml p.o.; 1% CMC)

Group II: Receive only Diazepam (2mg/kg body weight p.o.) [10]

Group III: CILEE 200 mg/kg body weight p.o.

Group IV: CILEE 400 mg/kg body weight p.o.

Group V: CILAE 200 mg/kg body weight p.o.

Group VI: CILAE 400 mg/kg body weight 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 comparison post-hoc test using Graph pad Instat version 3.05, 32 bits for windows.

RESULTS

Preliminary phytochemical screening

The preliminary phytochemical evaluation of different extracts of *Calophyllum inophyllum* leaves revealed the presence of carbohydrates, saponins, triterpenoids, flavonoids, tannins, alkaloids, proteins, amino acid, glycosides and phenolic compounds. Table 1 shows the tests involved in the preliminary phytochemical studies.

Table 1
Preliminary Phytochemical Screening of Calophyllum inophyllum leaf extracts

Phyto-constituents	Test	Pet ether extract	Ethanolic extract	Aqueous extract
Carbohydrates	Fehling's Test	-	+	+
	Molisch's Test	-	+	+
Saponins	Froth Test	+	+	+
Steroids and Triterpenoids	Salkowski Test	-	+	-
	Libermann-Burchards Test	-	+	-
Flavonoids	Shinoda Test	-	+	+
	Alkaline reagent Test	-	+	+
Tannins and polyphenolic compounds	Ferric chloride Test	-	+	+
	Lead acetate Test	-	+	+
	Gelatin test	-	+	-
Alkaloids	Dragendroff's Test	-	+	+
	Mayer's Test	-	+	+
	Hager's Test	-	+	+
Proteins	Biuret's Test	+	+	+
Amino Acids	Ninhydrin Test	+	+	-
Glycosides	Baljet Test	-	+	-
	Keller-killiani Test	-	+	-
	Legal Test	-	+	-

(+) Indicates present (–) indicates absent

Oral Acute Toxicity Studies

Acute toxicity studies revealed that ethanolic and aqueous extract of *Calophyllum inophyllum* was safe at a maximum oral dose of 2000 mg/kg b.w in albino wistar rats and showed no lethal or toxic reactions in 24-h period. However, the animals were hypoactive and showed sedation after 1 h of both extract administration. Therefore, 2000mg/kg dose was considered as maximum safe dose.

Elevated plus maze

From the Table 2, CILEE 200mg/kg has significantly ($p<0.05$) increased the number of entries and time spent in open arm whereas, in closed arm the time spent was significantly ($p<0.05$) reduced when compared to control. CILEE 400mg/kg has significantly ($p<0.01$) increased the number of entries and time spent in open arm whereas, in closed arm number entries as well as the time spent was significantly ($p<0.01$) reduced when compared to control. CILAE 200mg/kg did not show any significant effect on any of the parameters. CILAE 400mg/kg has significantly ($p<0.05$) increased the number of entries and time spent in open arm whereas in closed arm the time spent was significantly ($p<0.05$) reduced when compared to control. Figures 1 and Figure 2 represents the number of entries in the closed and open arms, respectively. Figure 3 and Figure 4 represents time spent in closed and open arms, respectively.

Table 2
Effect of CILEE and CILAE on Elevated plus Maze Test in Rats

Groups	Closed arm entries	Open arm entries	Time spent in closed arm	Time spent in open arm
Control(1ml CMC)	6±0.37	2.17±0.31	221.50±6.38	20.83±2.83
Standard(DZP 2mg/kg b.w; p.o)	2.83±0.31**	8.00±0.37**	62.17±6.31**	118.50±7.79**
CILEE(200mg/kg b.w; p.o)	4.67±0.33*	3.83±0.40*	179.83±8.20*	51.00±5.17*
CILEE(400mg/kg b.w; p.o)	3.83±0.31**	6.00±0.52**	146.17±10.15**	84.17±6.09**
CILAE(200mg/kg b.w; p.o)	5.83±0.40	2.50±0.22	204.50±9.06	29.67±2.33
CILAE(400mg/kg b.w; p.o)	5.5±0.22	3.67±0.33*	184.33±11.86*	41.83±4.98*

Values are expressed as Mean ± S.E.M. (n=6) Significance vs control group: *P<0.05, **P<0.01

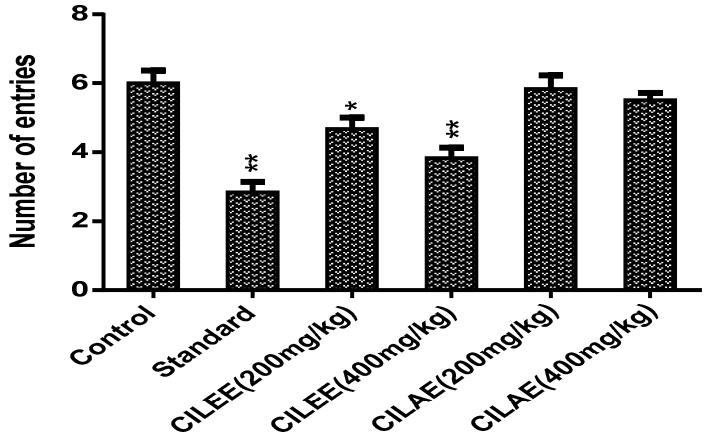

Figure 1
Number of Entries in Closed Arm

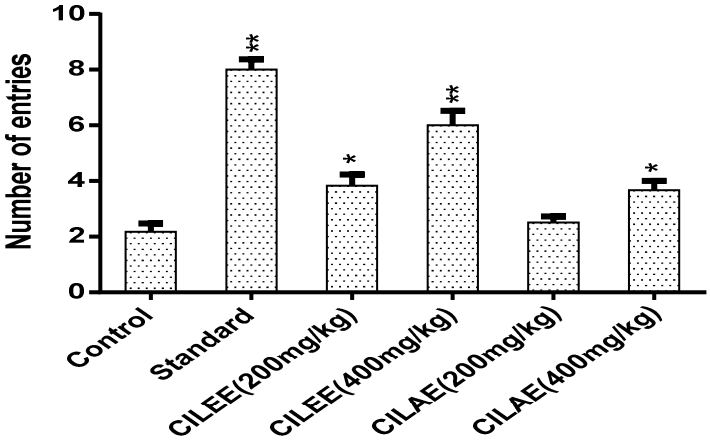

Figure 2
Number of Entries in Open Arm

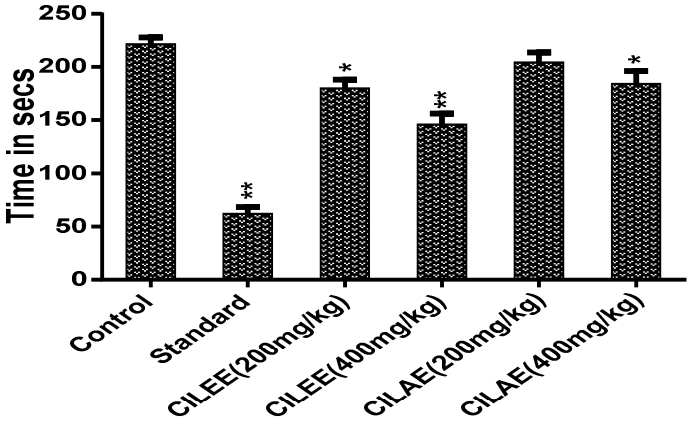

Figure 3
Time Spent In Closed Arm

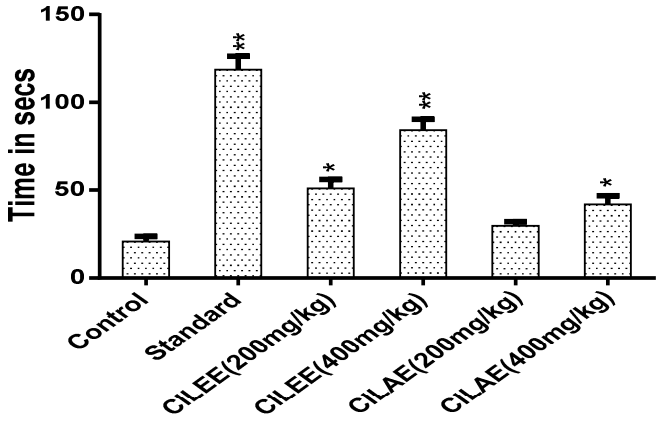

Figure 4
Time Spent In Open Arm

Light and dark test

From Table 3, CILEE 200mg/kg has significantly ($p<0.01$) increased the number of entries and time spent in light arena whereas, in dark arena number of entries ($p<0.01$) and time spent was significantly ($p<0.05$) reduced when compared to control. CILEE 400mg/kg has significantly ($p<0.01$) increased the number of entries and time spent in light arena whereas, in dark arena the time spent was significantly ($p<0.01$) reduced when compared to control. CILAE 200mg/kg did not show any significant effect on any of the parameters. CILAE 400mg/kg has significantly ($p<0.05$) increased the number of entries and time spent in light arena when compared to control, whereas in dark arena no significant effect was seen. Figure 5 and Figure 6 shows the number of entries in dark and light arenas, respectively. Figure 7 and Figure 8 shows the time spent in dark and light arenas, respectively.

Table 3
Effect of CILEE and CILAE on Light and Dark Model in Rats

Groups	Entries in Dark arena	Entries in Light arena	Time spent in Dark arena	Time spent in Light arena
Control(1ml p.o CMC)	8.17±0.40	3.17±0.31	236.00±5.94	26.83±1.85
Standard(DZP 2mg/kg b.w; p.o)	4.00±0.37**	13.00±0.58**	68.83±9.5**	131.17±11.16**
CILEE(200mg/kg b.w; p.o)	5.67±0.33**	5.83±0.60**	202.30±8.05*	59.50±6.15**
CILEE(400mg/kg b.w; p.o)	4.33±0.49**	8.00±0.58**	186.17±8.91**	72.33±7.21**
CILAE(200mg/kg b.w; p.o)	7.83±0.48	4.00±0.37	243.83±5.67	30.67±2.64
CILAE(400mg/kg b.w; p.o)	6.83±0.31	5.33±0.33*	215.33±7.93	53.50±5.77*

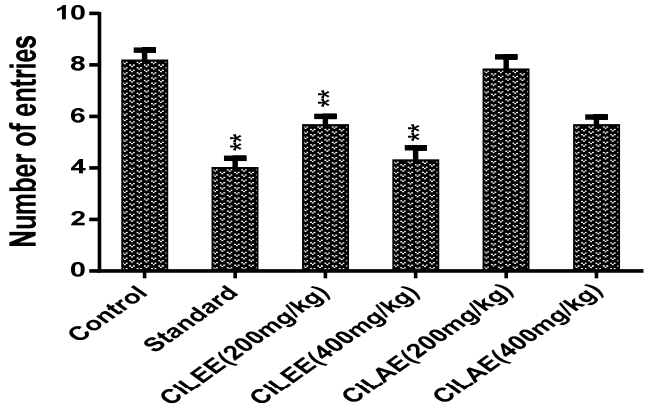

Figure 5
Number of Entries in Dark Arena

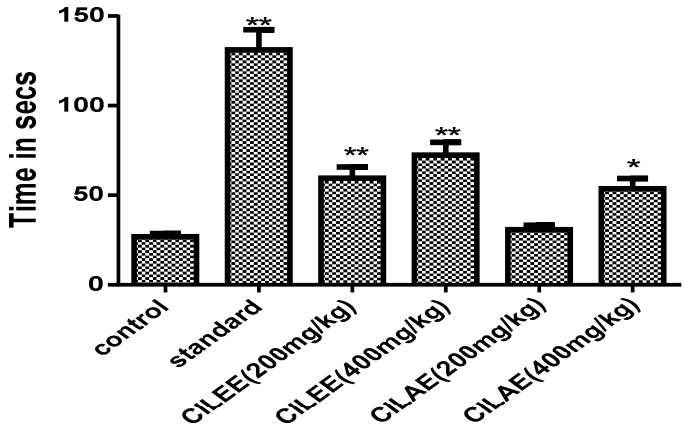

Figure 6
Number of Entries in Light Arena

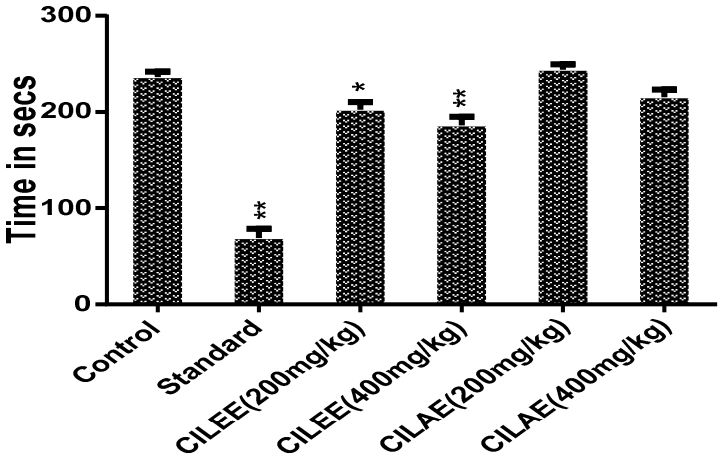

Figure 7
Time Spent In Dark Arena

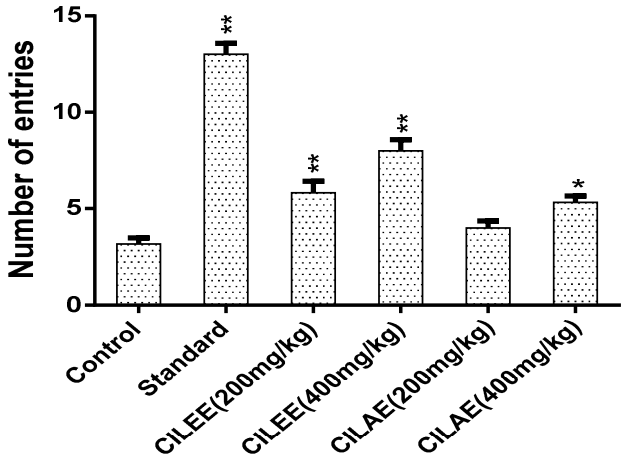

Figure 8
Time Spent In Light Arena

DISCUSSION

The etiology of most anxiety disorders is not fully understood, but various studies have shown the involvement of GABAergic serotonergic neurotransmission in etiology, expression and treatment of anxiety. The adrenergic and dopaminergic systems have also been shown to a role of in anxiety[11]. In EPM, naive rat will normally prefer to spend much of their etiology in the closed arms. This preference appears to reflect an aversion towards the open arms that is generated by fear of open spaces. Drugs that increase open arm exploration are considered as anxiolytics and the reverse holds true for anxiogenics [12]. In our study, we observed that CILEE (200 and 400 mg/kg) and CILAE (400mg/kg) induced significant increases in the both the number of entries and time spent in the open arms. The time spent in the closed arms was reduced in the extract-treated group as compared to the control group. These results obtained from EPM supports the anxiolytic activity of *C. inophyllum*. In the light - dark test, animals always try to spend more time in the dark compartment compared to light arena out of fear of exposure to the new environment. Transitions have been reported to be an index of activity exploration because of habituation over time and the time spent in each arena are reflection of aversion [13]. Anxiolytics reduce the natural aversion to light and increase the time spent in the light arena. In this model, compared to vehicle CILEE (200 and 400 mg/kg) and CILAE (400mg/kg) produced significant increase in the both the number of entries and time spent in the lighted box and decrease in the time spent in the dark box, thus demonstrating its anxiolytic activity.

CONCLUSION

Acute toxicity studies revealed that ethanolic and aqueous extract of *Calophyllum inophyllum* was safe at a maximum oral dose of 2000 mg/kg b.w in albino wistar rats. Thus, 2000mg/kg dose was considered as maximum safe dose. Anxiolytic activity was evaluated by elevated plus maze test and light/dark test in wistar rats. Experimental studies have shown that CILEE (200mg/kg, 400mg/kg b.w) and CILAE (400mg/kg b.w) exhibited significant anxiolytic whereas CILAE (200mg/kg b.w) did not show any significant activity. The effects were more prominent in the ethanolic extract than the aqueous extract which may be due to successive solvent extraction.

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