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Original Article

Investigation on in vitro antioxidant and in vivo neurobehavioral effects of Clerodendrum indicum leaf extract

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ABSTRACT: The aim of the study was to investigate the antioxidant, anxiolytic, and antidepressant activity of the ethanolic leaf extract of *Clerodendrum indicum*. Antioxidant potential of the extract was investigated using multiple in vitro assays at different concentrations. *In vivo* anxiolytic and antidepressant effects were evaluated by animal model at two test doses (200,400 mg/kg BW). A preliminary phytochemical screening was also done through conducting phytochemical group tests of the crude ethanolic extract of C. *indicum*. The IC50 values of the extract for DPPH radical scavenging was 7.89 µg/ml compared to the standard BHT with IC50 value of 3.25 µg/ml. The phenolic content and flavonoid content were found as 99.28±0.945 mg gallic acid equivalent/gm and 75.13±0.863 mg catechin equivalent/gm of dry plant extract respectively. Moreover, the extract showed remarkable total antioxidant and ferric reducing power activity found to be increase with concentration. The results of neuropharmacological assays demonstrated low antidepressant but significant ($p<0.05$) anxiolytic activity of the extract in a dose dependent manner compared to the normal control group. Phytochemical analyses were found to be positive for alkaloid, phenol, flavonoid, tannin, glycoside and steroid. The present results support that the ethanolic extract of C.*indicum* leaves likely to have the potential phytomedicinal value for its considerable antioxidant, anxiolytic and antidepressant effects.

Keywords: *Clerodendrum indicum*, Antioxidant, Anxiolytic, CNS-depressant, IC50, Phytochemical screening.

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INTRODUCTION

Oxidative stress is commonly known to be a key factor in developing Central Nervous System (CNS) disorders. Hence, the underlying cause of these disorders is often connected to the excess production of oxygen-derived free radicals, which are considered to be greatly harmful to the CNS. Likewise, the reduced antioxidant defense ability of neurons and nervous tissues leads to a number of neurological and psychiatric disorders[1].

Numerous biological investigations have emphasized on the use of plant-derived drugs in modern medicine considering their folkloric or traditional medicinal uses. Isolation, identification and evaluation of active phytochemical compounds by different bioassays guide to find out potent biological activity of plant-derived drugs. It has been established that many medicinal plants have played a vital role in the development of potent therapeutic agents and they are used to cure mental illness, diabetes, hypertension, tuberculosis and even cancer[2]. Therefore, the study was designed to investigate a certain medicinal plant namely *Clerodendrum indicum* Linn, locally known as Bamanhatti or Vamot (family Verbenaceae), which

has considerable reputation for its medicinal values as traditional medicine.

C. indicum is widely distributed throughout Southeast Asia, India, Nepal, Bhutan, Sri Lanka and southern China[3]. The plant has traditional uses in serofulous infection, buboes problem, venereal infections and skin diseases. In addition to this, it has been employed as a vermifuge and febrifuge[4]. Pharmacological studies carried out by *C. indicum* showed that rheumatism, asthma and other inflammatory diseases can be treated with the root and leaf extracts of *C. indicum*, the root extract also possess cytotoxic activity and the juice of the leaf has wider application in hepatic eruption and pemphigus[5,6,7]. The methanolic extract of the plant has been shown to inhibit lipid peroxidation in bovine brain[8]. Moreover, the methanolic extract and its different fractions of leaves of *C. indicum* possess significant anti-nociceptive, antimicrobial and antidiarrheal activities[9].

The aim of the present study was to determine the antioxidant activity of *Clerodendrum indicum* ethanolic leaf extract *in vitro* and also neurobehavioral effect of it *in vivo* by using experimental animal models of anxiety and depression.

METHODS AND MATERIALS

Collection of plant material: The fresh green leaves of *Clerodendrum indicum* (Linn.) were collected from Dhaka district of Bangladesh, in the month of April 2018. The plant was identified and authenticated by the expert taxonomist from Bangladesh National Herbarium, Mirpur, Dhaka, Bangladesh. (Accession number of *C.indicum*-45999)

Preparation of plant extract: The leaves were dried by shade drying for ten days and then grounded into a coarse powder with the help of a suitable blender. Two hundred grams of powdered material was extracted with 1.5 liter ethanol for 7 days with occasional shaking upto 2 inch height above the sample surface as it could sufficiently cover the sample surface. Then it was filtered through whatman filter paper. The liquid extract was then concentrated with a rotary evaporator under reduced pressure at 50°C temperature, and then it was evaporated under ceiling fan and in a water bath to give a greenish black type residue of 6 gm.

Experimental animals: Young Swiss-albino mice of either sex aged 5-6 weeks, average weight 22-30gm were used for the experiment. The mice were purchased from the animal Research Branch of the International Centre for Diarrheal Disease and Research, Bangladesh (ICDDR, B). They were kept in standard environmental condition and fed ICDDR, B formulated rodent food and water. After randomization in to various groups and before initiation of experiment, the mice were to the animal house under experimental conditions at the Department of Pharmacy, Southeast University.

Acute toxicity test: Oral administration of crude ethanolic leaf extract of *C. indicum* at the doses of 500–1500 mg/kg did not produce any mortality or noticeable behavioral changes in mice within 72 h observation period. Therefore, it can be suggested that the plant extract has low toxicity profile with LD_{50} greater than 1500 mg/kg.

Preliminary phytochemical screening: Preliminary phytochemical analysis of the ethanolic leaf extract of *Clerodendrum indicum* was carried out based on the standard methods to identify the presence phytochemical constituents[10].

Tests for antioxidant activity:

DPPH free radical scavenging activity: The free radical scavenging activity of the extract, based on the scavenging activity of the stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical, was determined by the method described[11]. Plant extract (0.1 mL) was added to 3 mL of a 0.004 % methanol solution of DPPH. Absorbance at 517 nm was determined after 30 min and the percentage inhibition activity was calculated from [(Ao-A1)/ Ao] ×100, where Ao is the absorbance of the control (DPPH solution) and A1 is the absorbance of the extract/standard. The inhibition curves were prepared and IC_{50} values were calculated.

Ferric reducing antioxidant power (FRAP): The ferric reducing antioxidant power was determined according to the method described[12]. According to this method, the reduction of Fe3+ to Fe2+ is determined by measuring the absorbance of Perl's Prussian blue complex. Briefly, different concentrations of extracts in 1 mL of distilled water were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide [K3Fe(CN)6] (2.5 mL, 1 %). The mixture was incubated at 50°C for 20 min. An aliquot (2.5 mL) of trichloroacetic acid (10 %) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The supernatant (2.5 mL) was mixed with distilled water (2.5 mL) and FeCl3 (0.5 mL, 0.1 %) and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Ascorbic acid was used as the reference.

Determination of total antioxidant activity: The antioxidant activity of the extract was evaluated by the phosphomolybdenum method according to the procedure described[13]. A 0.3 mL extract was combined with 3 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95ºC for 90 min. Then the absorbance of the solution was measured at 695 nm using a spectrophotometer against blank after cooling at room temperature. Methanol (0.3 mL) in the place of extract is used as the blank. The

antioxidant activity was expressed as the number of equivalents of ascorbic acid.

Determination of total phenolic content: The total phenolic content of plant extract was determined employing the method as described involving Folin-Ciocalteu reagent as oxidizing agent and gallic acid as standard[14]. Firstly, 0.5 ml of plant extract or standard of different concentration solution was taken in a test tube and 2.5 ml of Folin – ciocalteu (diluted 10 times with water) reagent solution was added into the test tube. Then 2.5 ml of sodium carbonate (7.5%) solution was added and incubated for 20 minutes at 25°c to complete the reaction. Then the absorbance of the solution was measured at 760 nm using a spectrophotometer against blank.

Determination of total flavonoid content: The content of flavonoids compounds in the extract was determined by the method described[15]. Catechin was used as standard and the flavonoid content of the extract was expressed as mg of catechin equivalent/gm of dried extract. Firstly, 1ml of extract was placed in a volumetric flask, then 5ml of distilled water added followed by 0.3ml of 5% $NaNO_2$. After 5 minutes, 0.6 ml of 10% $AlCl_3$ was added and volume made up with distilled water. The solution was mixed and absorbance was measured at 510 nm.

Tests for neurobehavioral activity:

Anxiety models:

Open field test: Open field behavioral test is routinely used with slight modification to evaluate both locomotor activity and emotionality in rodents. The open field apparatus consisted of a wooden field of half square meter, with a series of squares alternatively painted in black and white. It had a 50 cm high wall and was placed in a dimly lit room. Mice were treated with normal saline, extract and diazepam and were place in the middle of the open field. Then the number of squares visited by the mice was counted for 5 min at 0, 30, 60, 90 and 120 min after the treatments[16].

Hole cross test: The method was adopted with slight modification[17]. A steel partition was fixed in the middle of a cage having a size of 30 × 20 × 14 cm. A hole of 3 cm diameter was made at a height of 7.5 cm in the center of the cage. Mice were treated with normal saline, extract and diazepam, and were place in one side of the cage. The number of passage of a mouse through the hole from one chamber to the other was counted for a period of 5 min at 0, 30, 60, 90 and 120 min after the treatments.

Depression models:

Forced swimming test: The Force swimming test was carried out on mice according to the described method with slight modification[18, 20]. The test was consisted of two parts; an initial training period of 15 min and an actual test for 5 min after 24 h. Swimming sessions were conducted by placing the mice in individual Plexiglas's cylinders (40 cm high, 24 cm diameter) containing 20 cm of water. The mice were treated with the normal saline, extract and nortriptyline, 45 min before the test. All mice were forced to swim for 6 min, and the time spent in immobility during the last 5 min of a 6 min observation period was recorded as immobile when floating motionless or making only those movements necessary to keep the head above water. A decrease in the duration of immobility during the forced swimming test taken as a measure of antidepressant activity.

Tail suspension test: Tail suspension induced the immobility was measured as per the established method[19]. This is a simple, rapid and reliable method to screen antidepressants. The mice were treated with the normal saline, extract and diazepam, 45 min before the test. In this method mice were suspended above the floor by adhesive tape placed approximately 1-2 cm from the tip of the tail and shows alternate agitation and immobility which is indicative of a state of depression. The remained immobile time of tail suspension test was quantified for 6 min. Mice were considered immobile only when they hung passively and completely motionless. A decrease in the duration of immobility during tail suspension test taken as a measure of antidepressant activity.

Wire hanging test: A standard linear wire hang apparatus was constructed, comprising a large plastic box 55cm long by 40cm wide by 35cm deep with a 2.5mm wire suspended in the top centre of the longest dimension. For linear wire hang tests, the mice after treatment with normal saline and extract and diazepam, were placed in the centre of the wire with all four paws and a timer set running for 6 min. The timer is stopped when the mouse falls off the wire, or if it crawls along the wire and reaches the end. In either case, the mouse is then repositioned in the centre of the wire and the timer restarted, and repeated as many times as necessary up to 6 min. In all cases, mice fell and/or reached the end of the wire within 6 min and were repositioned at least 4 times. The individual times and number of reaches and falls were recorded[21].

STATISTICAL ANALYSIS

The results were presented as mean ± SD. The statistical analysis was performed using one way analysis of variance (ANOVA) followed by Dunnett's post hoc test as using SPSS 20.00 software. Differences between groups were considered significant at a level of *p< 0.05.

RESULTS AND DISCUSSION

Phytochemical screening:

Preliminary phytochemical screening revealed the presence of various bioactive components like alkaloid, carbohydrate, glycoside, flavonoid, steroid and tannin in plant extract shown in Table 1.

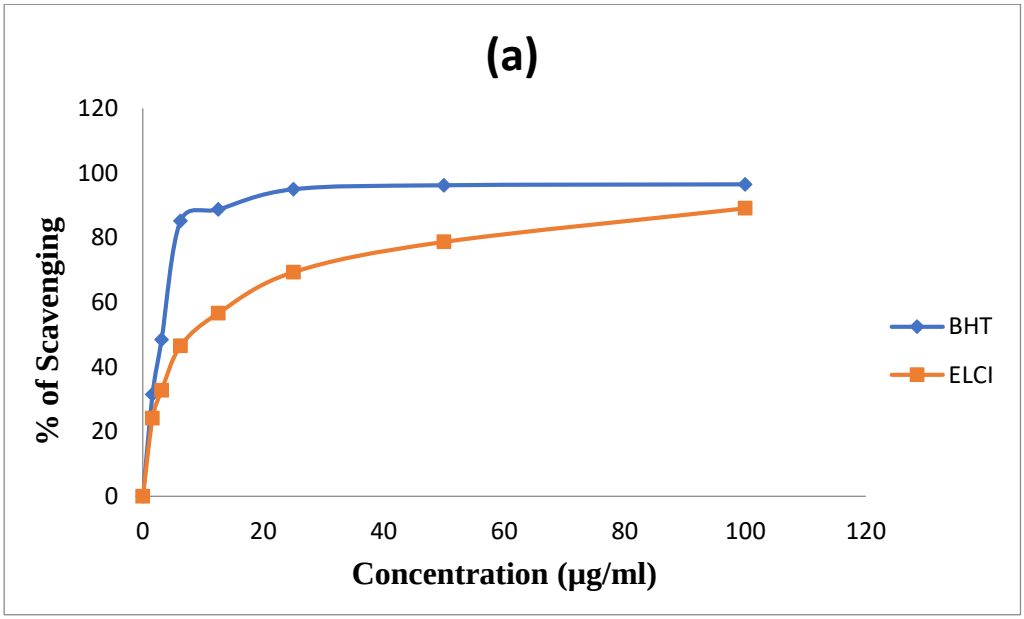
Table 1. Results of phytochemical screening of ethanolic extract of *C. indicum* leaf.

Extract	Alkaloid	Glycoside	Tannin	Flavonoid	Steroid	Phenol	Saponin
ELCI	+	+	+	+	+	+	-

ELCI denotes for ethanolic leaf extract of C. indicum; (+): Present; (-): Absent

Antioxidant activity evaluation:

DPPH free radical scavenging assay: In DPPH free radical scavenging assay, Fig 1(a) showed plant extract exhibited a concentration dependent antiradical activity by inhibiting $DPPH^{\cdot}$ radical. Butylated hydroxytoluene (BHT), which is a well-known antioxidant, showed higher degree of free radical-scavenging activity than that of the extract at each concentration points. The IC_{50} value of the extract shown in Fig 3b was 7.89 µg/mL , whereas IC_{50} value for the reference BHT was 3.25 µg/mL. DPPH antioxidant assay is based on the ability of 2, 2-diphenyl-1-picryl-hydrazyl (DPPH), a stable free radical, to decolorize in the presence of antioxidants. The DPPH radical contains an odd electron, which is responsible for the absorbance at 517 nm and also for a visible deep purple color. When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolorized, which can be quantitatively measured from the change in absorbance and percentage of scavenging activity is calculated. The extract was able to reduce DPPH radical (visible deep purple color) to the yellow coloured diphenylpicrylhydrazine[22]. Additionally, it has been determined that the antioxidant effect of plant products is mainly due to radical scavenging activity of phenolic compounds such as flavonoids, polyphenols, tannins, and phenolic terpenes[23]. Therefore, one of the possible mechanisms of the good antioxidant activity of the extract might be the resultant of containing good amount of phenolic compounds, which shows antioxidant activity due to their redox properties.



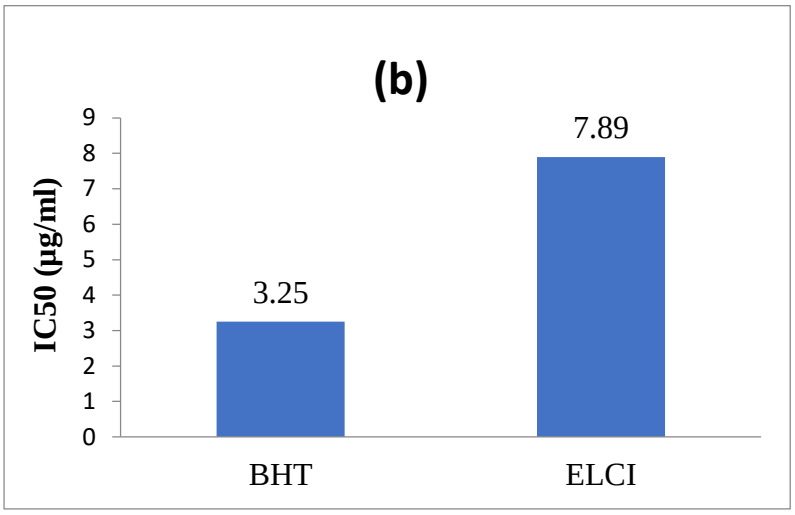


Figure 1. (a) DPPH radical scavenging activity of ethanolic leaf extract of *C. indicum* (ELCI) along with BHT (Standard) and (b) IC50 (µg/ml) values of ELCI of *C. indicum* and BHT (Standard)

Ferric reducing antioxidant power (FRAP): Fig. 2. showed the reducing power capabilities of the plant extract compared to ascorbic acid. The extract displayed good reducing power which was found to rise with increasing concentrations of the extract. In reducing power assays, the presence of antioxidants in the extract can reduce the oxidized form of iron (Fe3+) to its reduced form (Fe2+) by donating an electron. Thus, it can be assumed that the presence of reductants (i.e. antioxidants) in *C. indicum* extract causes the reduction of the Fe3+/ferricyanide complex to the ferrous form. Therefore, the Fe2+complex can be monitored by measuring the formation of Perl's Prussian blue at 700 nm. A higher absorbance indicates greater reducing power ability[24].

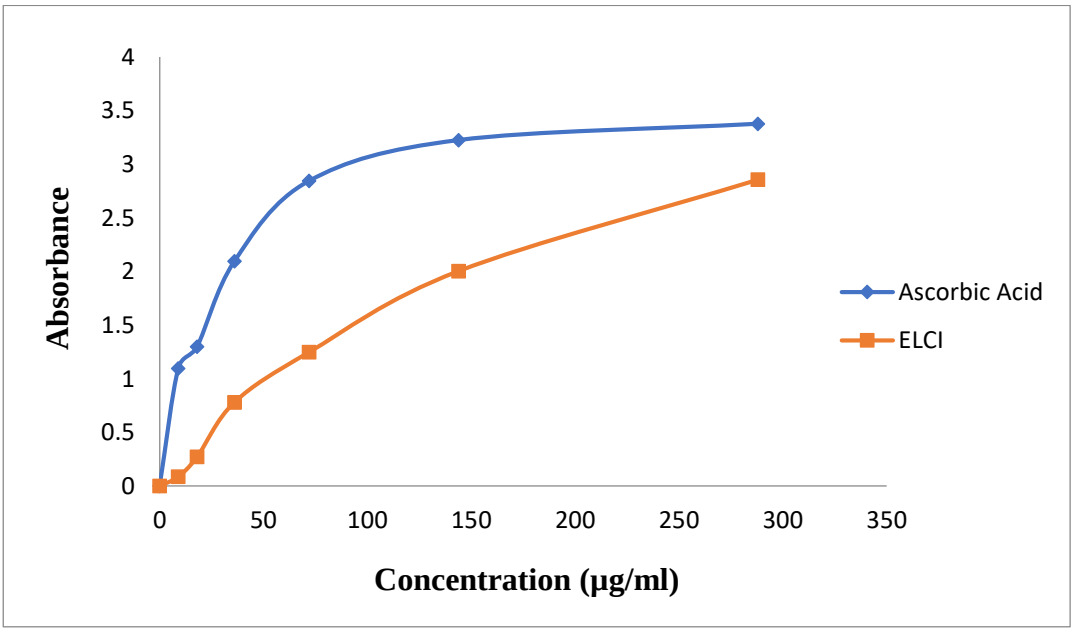


Figure 2. Ferric reducing antioxidant power of ethanolic leaf extract of *C. indicum* (ELCI) along with Ascorbic acid (Standard) at different concentrations.

Determination of total antioxidant activity: The assay was based on the reduction of Mo (VI) to Mo (V) by the antioxidant compound and subsequent formation of a green phosphate/Mo (V) complex at acidic pH with a maximal absorption at 695 nm [13]. The total antioxidant activity of the plant was measured and compared with the reference standard catechin. The high absorbance values indicated that the extract possessed significant antioxidant activity. The result shown in Fig. 3 revealed that the extract had good antioxidant activities and the effects increased with increasing concentration.

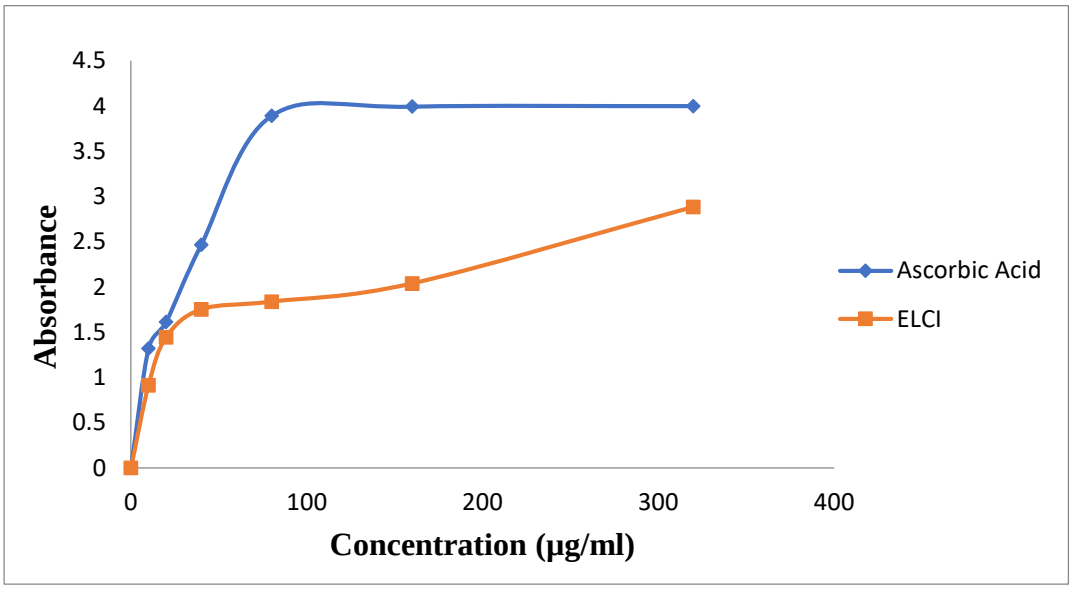


Figure 3. Determination of total antioxidant activtiy of ethanolic leaf extract of *C. indicum* (ELCI) along with Ascorbic acid (Standard) at different concentrations.

Determination of total phenolic content: Phenolic content of the plant extract was determined by using Folin-Ciocalteu reagent. Phenolic content of the extract was calculated on the basis of the standard curve for gallic acid in Fig. 4. The result was expressed as mg of gallic acid equivalent (GAE)/gm of dried plant extract shown in Table 2. The values represented the mean of triplicates ± SD of crude ethanolic extract respectively. Several reports have conclusively shown close relationship between total phenolic content and antioxidant activity of the fruits and vegetables. Moreover, the antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in adsorbing and neutralising free radicals, quenching singlet and triplet oxygen, or decomposing peroxides[25].

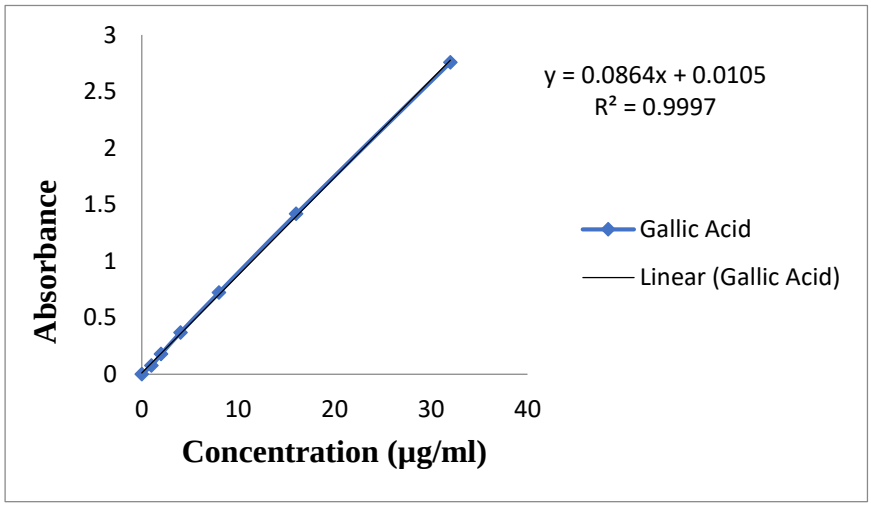


Figure 4. Standard curve of gallic acid for the determination of total phenolic content

Table 2. Determination of total phenolic content of ethanolic leaf extract of *C. indicum* (ELCI).

Sample	No. of sample	Concentration (µg/ml)	Absorbance	GAE/gm of dried sample	GAE/gm of dried sample Mean ± SD
ELCI	1.	31.25	1.014	92.83	99.28 ± 0.945
	2.	31.25	1.179	108.18	
	3.	31.25	1.057	96.83	

Determination of total flavonoid content: Different studies suggest that different types of polyphenolic compounds (flavonoids, phenolic acids) found in plants have multiple biological effects, including antioxidant activity[25] and present studies indicate the presence of flavonoid in the ethanolic leaf extract of *C. indicum*. Flavonoid content of the extract was calculated on the basis of the standard curve for catechin in Fig. 5. The result was expressed as mg of catechin equivalent (CE)/gm of dried extract shown in Table 3. The values represented the mean of triplicates ± SD of crude ethanolic extract respectively.

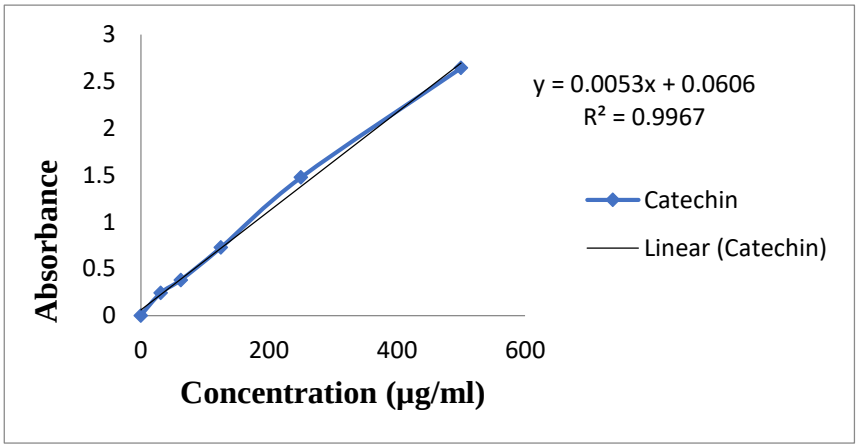


Figure 5. Standard curve of catechin for the determination of total flavonoid content

Table 3. Determination of total flavonoid content of ethanolic leaf extract of *C. indicum* (ELCI)

Sample	No. of sample	Concentration (µg/ml)	Absorbance	CE/gm of dried sample	CE/gm of dried sample Mean ± SD
ELCI	1.	125	0.826	75.34	75.13±0.863
	2.	125	0.843	76.93	
	3.	125	0.802	73.12	

Anxiolytic-like activity evaluation:
In the present study, anxiolytic activity of the ethanolic extract of *C. indicum* was studied in mice models by means of the open field and hole cross tests.
The decrease in locomotion was evident from the results of open field test. Both the doses of extract showed CNS depressant effect (Fig. 6) in a dose dependent manner. The statistically significant ($p< 0.05$) effect was observed for ELCI 400 from 3rd observation (60 min) to 5th observation (120 min) and for ELCI 200 at 5th obsercation (120 min) respectively.

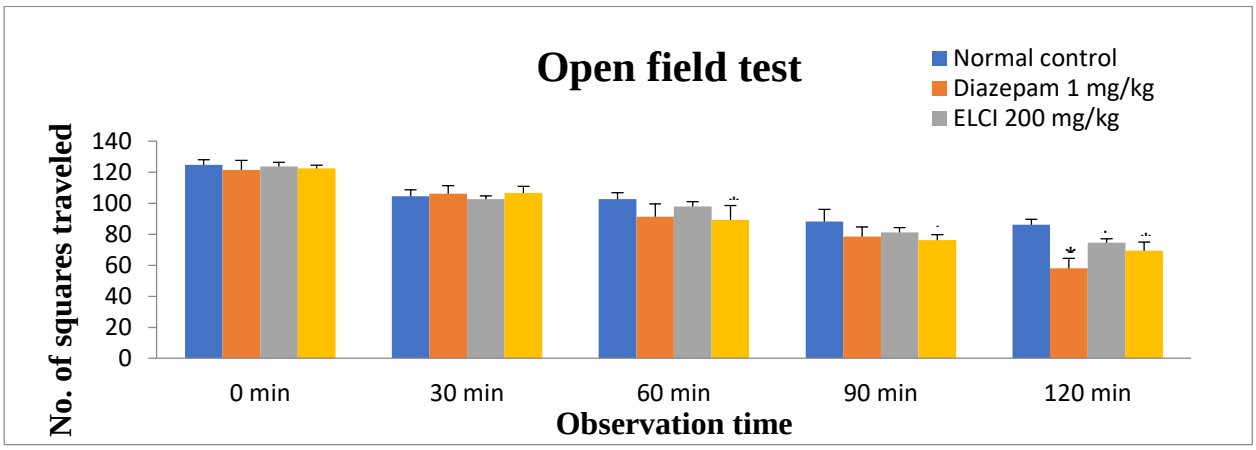


Figure 6. The anxiolytic effect of *C. indicum* extract in open field test. Each value is presented as the mean ± SD. *p< 0.05, Dunnett's test compared with control group. ELCI = Ethanolic leaf extract of *C. indicum*.

In hole cross test, the extract also showed marked decrease in the locomotor activity which represent the CNS depressant effect of the extract (Fig. 7). The significant depressant effect ($p< 0.05$) was observed for ELCI 400 from 3rd (60 min) to 5th (120 min) and for ELCI 200 from 4th (90 min) to 5th (120 min) observation period respectively. The effect was dose-dependent and statistically significant compared to control.

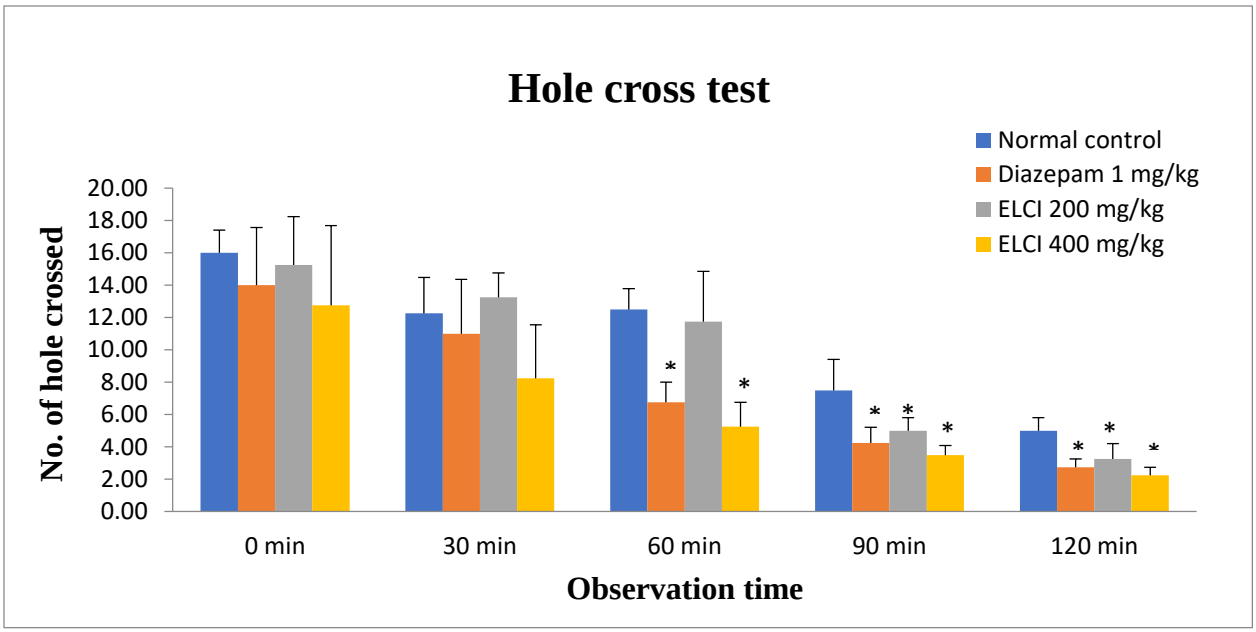


Figure 7. The anxiolytic effect of *C. indicum* extract in hole cross test. Each value is presented as the mean ± SD. *$p< 0.05$, Dunnett's test compared with control group. ELCI = Ethanolic leaf extract of *C. indicum*.

The decrease in locomotion in open field and hole cross tests therefore confirms the CNS depressant activity of ELCI 400 and ELCI 200. The depressant activity may be due to the presence of alkaloids in the extracts[27]. This general depressant and sedative effect of the extracts may be due to the action of alkaloids on the cerebral mechanism involved in the regulation of sleep[28]. Flavonoids with anxiolytic activities have also been described in numerous plant species used in folk medicine to depress the CNS. This effect has been ascribed to their affinity for the central benzodiazepine receptors[29]. It could be suggested that flavonoids of the *C. indicum* contribute to the hypnotic effect of this plant through central benzodiazepine receptors. Furthermore, tannins have been reported to show nonspecific CNS depression in mice[30]. So the reported central depressant effect of the ethanolic extracts of *C. indicum* may be due to the presence of alkaloid, flavonoid or tannin-like constituents in the plant.

Antidepressant-like activity evaluation:

The present study was also designed to investigate the antidepressant activity of ethanolic extract of *C. indicum* in mice using well-tried-out and standardized behavioral tests of depression.

As it is seen in Fig. 8, nortriptyline significantly ($p < 0.05$) decreased immobility time compared to the control group. Although the antidepressant effect of the *C. indicum* extract increased with time but none of the doses of the extract could significantly reduce immobility time in comparison with control group. However, the best result was obtained from the dose 400 mg/kg of the extract.

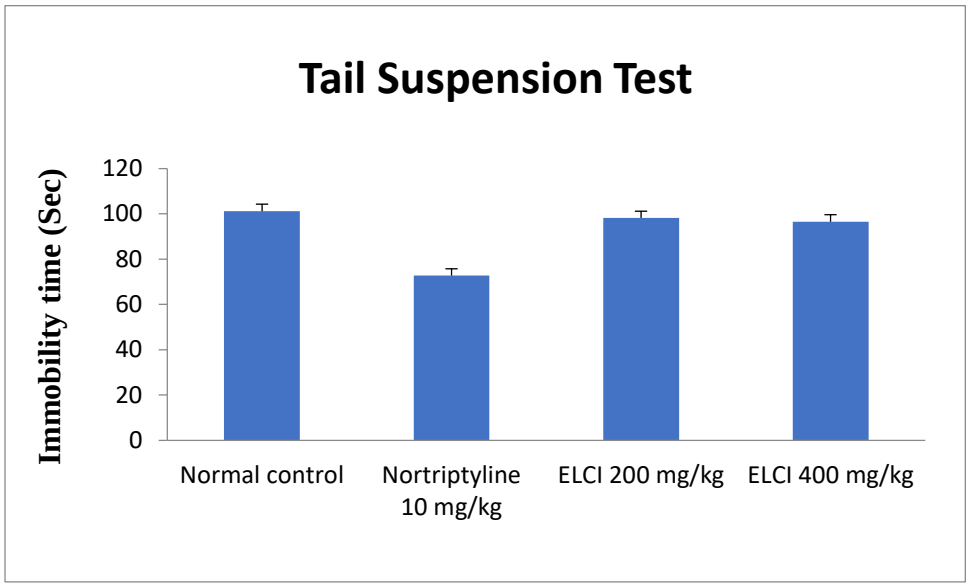


Figure 8. The antidepressant effect of *C. indicum* extract in tail suspension test. Each value is presented as the mean ± SD. *p< 0.05, Dunnett's test compared with control group. ELCI = Ethanolic leaf extract of *C. indicum*.

As it is seen in Fig. 9, diazepam significantly ($p < 0.05$) increased immobility time compared to the control group. None of the doses of the ELCI could significantly affect the immobility time in comparison with control group. However, lower antidepressant activity was found in both doses of the extract in a dose dependent manner, but the best result was obtained from the dose 400 mg/kg of the extract.

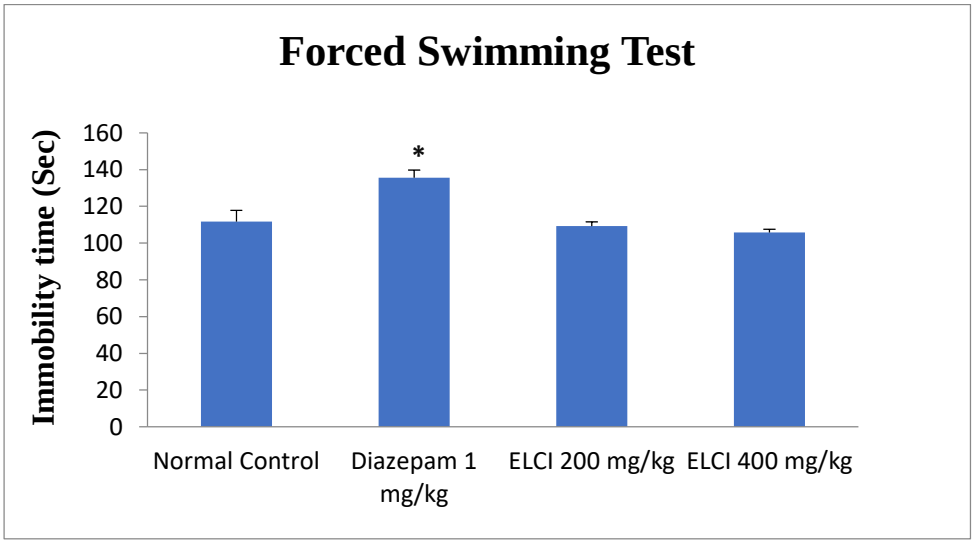


Figure 9. The antidepressant effect of *C. indicum* extract in forced swimming test. Each value is presented as the mean ± SD. *p< 0.05, Dunnett's test compared with control group. ELCI = Ethanolic leaf extract of *C. indicum*.

As it is seen in Fig. 10, None of the doses of the ELCI could significantly affect the immobility time in comparison with control group. However, a dose dependent antidepressant activity was found with the highest result obtained from the dose 400 mg/kg of the extract.

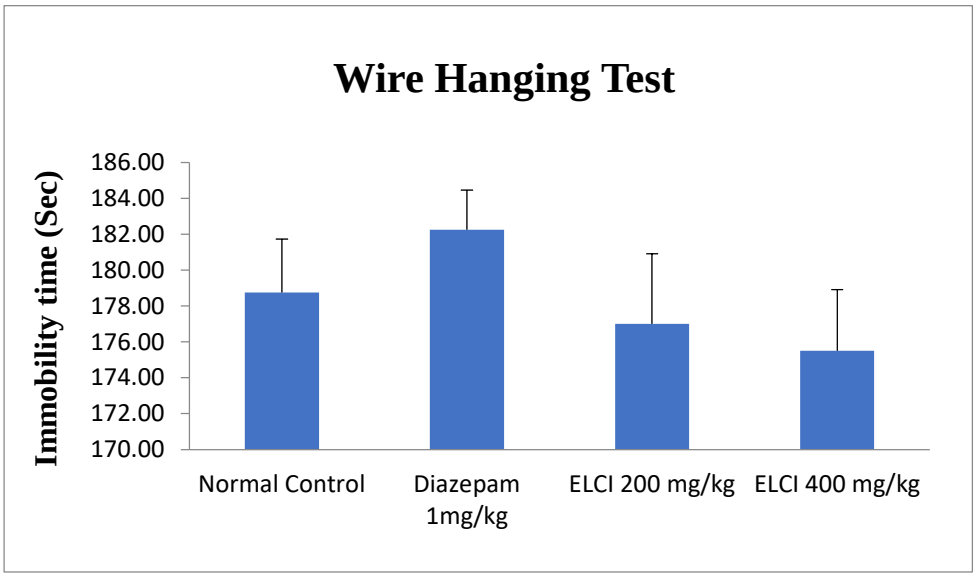


Figure 10. The antidepressant effect of *C. indicum* extract in forced swimming test. Each value is presented as the mean ± SD.

The immobility displayed by rodents when subjected to unavoidable stress is thought to reflect a state of despair or lowered mood, which are thought to reflect depressive disorders in humans. Hence, any agent having capability to significantly reduce the immobility time is evident to be an antidepressant drug[18, 19]. Even so, in all aforementioned models, results indicated that none of the doses of the extract could reduce the immobility time significantly compared with the control. However, in the present study, the experiments were performed acutely. Since the duration of administration may affect the pharmacokinetics of the active components, the therapeutic doses and the extent of effects of preparations obtained here cannot be extrapolated to human and oral and chronic administration of the extract may have better results[31].

CONCLUSION

The finding of the present investigation suggests that the ethanolic leaf extract of *C. indicum* possesses low antidepressant activity in acute animal models of depression, but significant anxiolytic effect in a dose dependent manner. The extract also exhibited good but different levels of antioxidant activity in some models studied. However, further studies are warranted to clearly understand the underlying mechanism of the observed bioactivities and to isolate the active phytochemical constituent (s) responsible for such activities in animal models.

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