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

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ABSTRACT: The aim of the study was to investigate the antioxidant and neurobehavioral activities of the ethanolic leaf extract of *Clerodendrum indicum*. Antioxidant potential of the extract was investigated by multiple *in vitro* assays at different concentrations. *In vivo* neurobehavioral activities were assessed in mice models at two test doses (200,400 mg/kg BW). A preliminary phytochemical screening of the crude extract was also done. The IC_{50} value of the extract for scavenging the DPPH radicals was 7.89 µg/ml, whereas the IC_{50} value of the standard BHT was 3.25 µg/ml. Moreover, the extract showed remarkable ferric reducing power and total antioxidant activity in a dose-dependent manner. The extract also exhibited moderate phenolic and flavonoid content, which were expressed as gallic acid and catechin equivalent respectively. The results of neurobehavioral activities demonstrated moderate antidepressant, but significant ($p<0.05$) CNS-depressant activity of the extract compared to the control group. Phytochemical analyses were found to be positive for alkaloid, phenol, flavonoid, tannin, glycoside and steroid. The present results support that the ethanolic leaf extract of *C. indicum* is likely to have the potential phytomedicinal value for its considerable antioxidant and CNS-depressant activities.

Keywords: *Clerodendrum indicum*, Antioxidant, IC_{50}, CNS-depressant, Phytochemical screening.

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INTRODUCTION

Oxidative stress is commonly known to be a key factor in developing Central Nervous System (CNS) disorders. The underlying causes of these disorders are 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 activities of plant-derived drugs. It has been established that many medicinal plants have played vital roles in the development of potent therapeutic agents and they are also used to cure mental illness, diabetes, hypertension, tuberculosis and even cancer[2]. Therefore, the study was designed to investigate a plant namely *Clerodendrum indicum*, locally known as Bamanhati or Vamot (family Verbenaceae), which has considerable reputation for its medicinal values and has usage in folkloric medicine.

C. indicum is widely distributed throughout Southeast Asia, India, Nepal, Bhutan, Sri Lanka and Southern China[3]. The plant has traditional uses in serofulousinfection, buboes problem, venereal infections and skin diseases. In addition to this, it has been employed as a vermifuge and febrifuge[4]. Pharmacological studies showed that rheumatism,

asthma and other inflammatory diseases can be treated with the root and leaf extracts of *C. indicum*, the root extract also possesses 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 leaf extract of *C. indicum* and its different fractions possess significant anti-nociceptive, antimicrobial and antidiarrheal activities[9].

The aim of the present study was to determine the antioxidant activity of *C. indicum* ethanolic leaf extract *in vitro* and also neurobehavioral activity 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 *C. indicum* 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 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. Then it was filtered through whatman filter paper and the liquid extract was concentrated with a rotary evaporator under reduced pressure at 50°C temperature. After that, 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 22-30 gm were used for the experiment. They were purchased from the Animal Research Branch of the International Centre for Diarrheal Disease and Research, Bangladesh (ICDDR, B). The mice 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 accommodated into 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 BW did not produce any mortality or noticeable behavioral changes in mice within 72 h observation period.

Preliminary phytochemical screening: Preliminary phytochemical analysis of the ethanolic leaf extract of *C. 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 plant extract/standard. The IC_{50} value was calculated and inhibition curve was also prepared considering Butylated hydroxytoluene (BHT) as standard.

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 Fe^{3+} to Fe^{2+} is determined by measuring the absorbance of Perl's Prussian blue complex. Briefly, different concentrations of extract in 1 ml of distilled water were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide $[K_3Fe(CN)_6]$ (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 $FeCl_3$ (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. Ascorbic acid was used as the reference.

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, and 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:

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 placed 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^3. 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.

Forced swimming test: The Force swimming test was carried out on mice according to the described method with slight modification [18, 19]. 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 was taken as a measure of antidepressant activity.

Tail suspension test: Tail suspension test to measure the magnitude of the immobility in depressive- like state of mice model, was carried out as per the established method [20]. 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 was 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 center of the longest dimension. For linear wire hang tests, the mice after treatment with normal saline, extract and diazepam, were placed in the center of the wire with all four paws and a timer set running for 6 min. The timer was stopped when the mouse fell off the wire, or if it crawled along the wire and reached the end. In either case, the mouse was then repositioned in the center 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, glycoside, flavonoid, phenol, steroid and tannin in plant extract shown in Table 1.

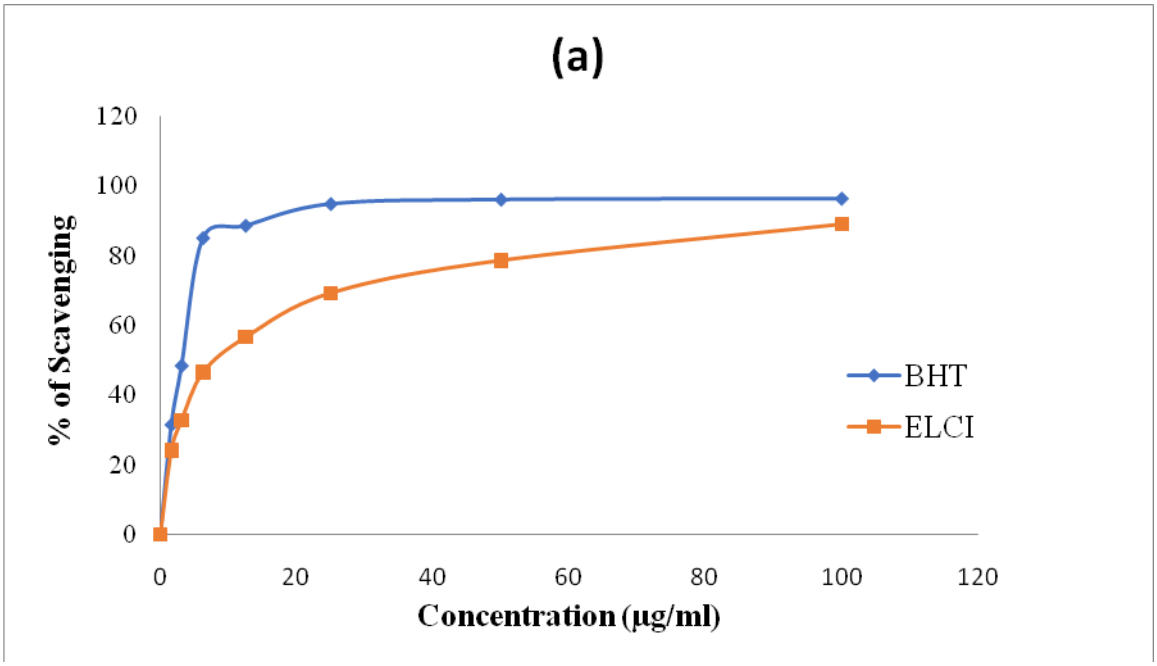
Table 1. Results of phytochemical screening of ethanolic leaf extract of *C. indicum* (ELCI)

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

'+' and '–' denotes for present and absentrespectively.

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 $DPPH^{-}$ radical. Butylatedhydroxytoluene (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 1(b) 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 scavenging DPPH (stable free radical) by antioxidants through reduction reaction. 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 colored 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.



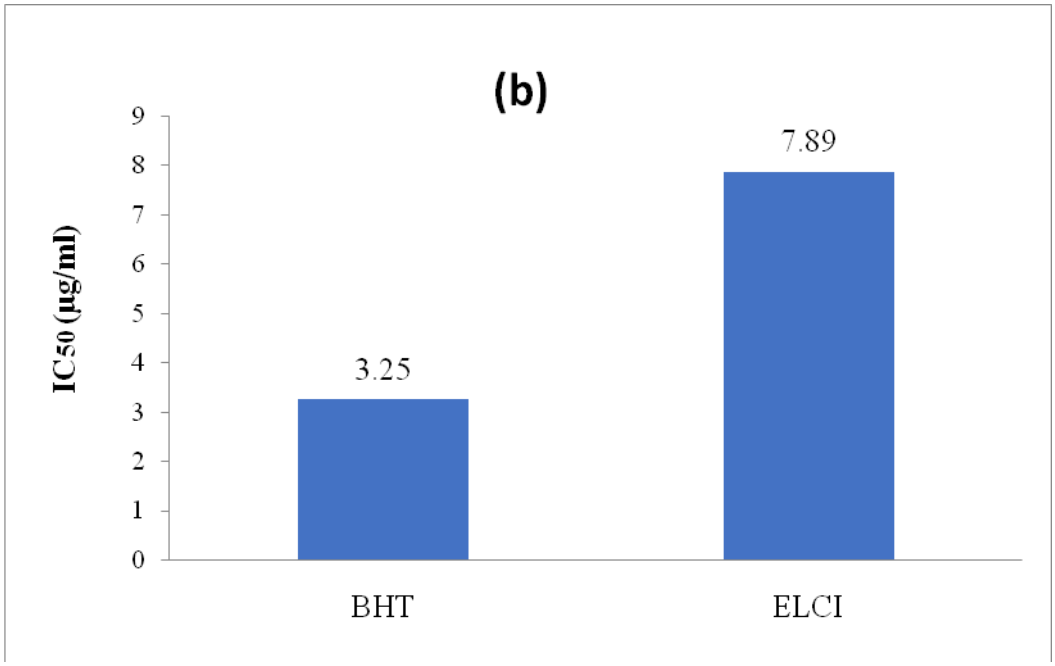


Figure 1. (a) DPPH radical scavenging activity of ethanolic leaf extract of *C. indicum* (ELCI) along with BHT (Standard) and (b) IC_{50} (µg/ml) values of ELCI and BHT (Standard)

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 (Fe^{3+}) to its reduced form (Fe^{2+}) 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 Fe^{3+}/ferricyanide complex to the ferrous form. Therefore, the Fe^{2+}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].

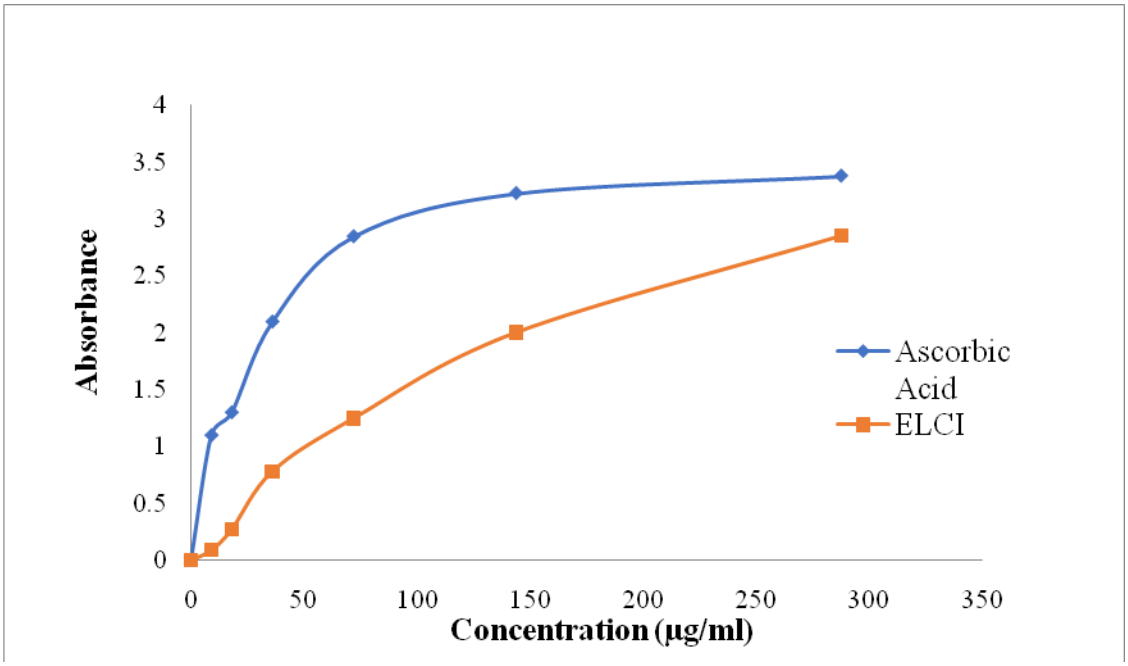


Figure 2. Ferric reducing antioxidant power of ethanolic leaf extract of *C. indicum* (ELCI) along with Ascorbic acid (Standard) at different concentrations

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.

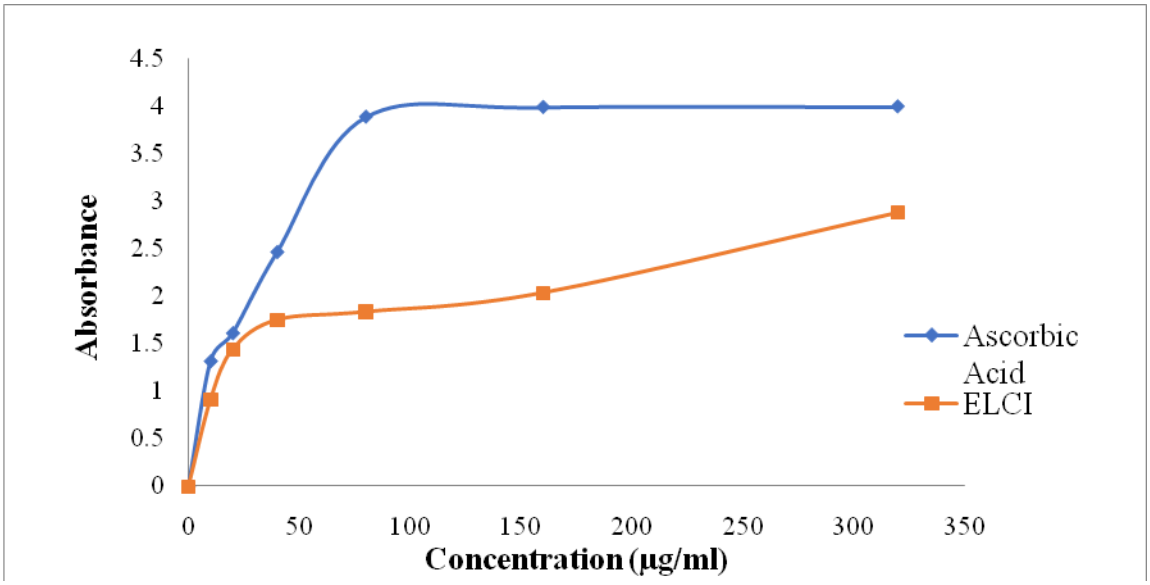


Figure 3. Determination of total antioxidant activity of ethanolic leaf extract of *C. indicum* (ELCI) along with Ascorbic acid (Standard) at different concentrations

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 neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides[25].

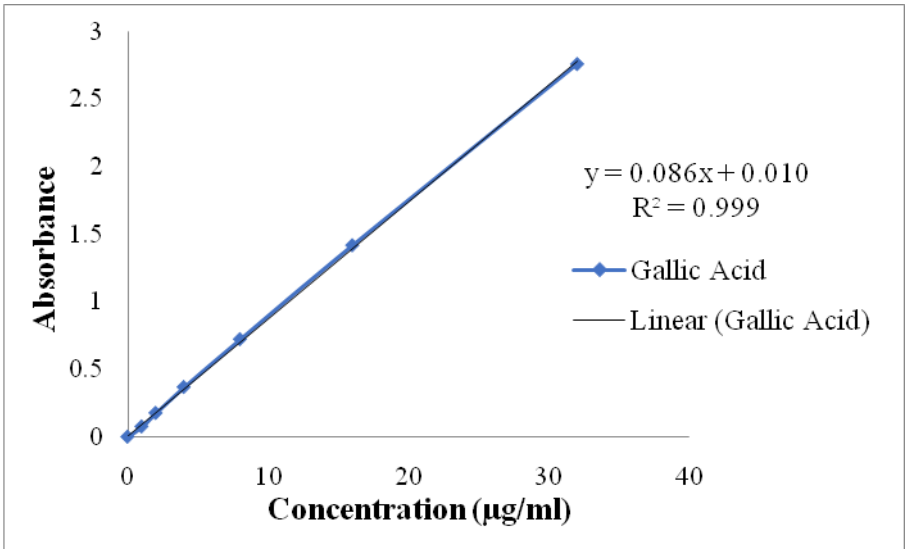


Figure 4. Standard curve of gallic acid for the determination of total phenolic content

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 diversified 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 ethanolic leaf extract respectively.

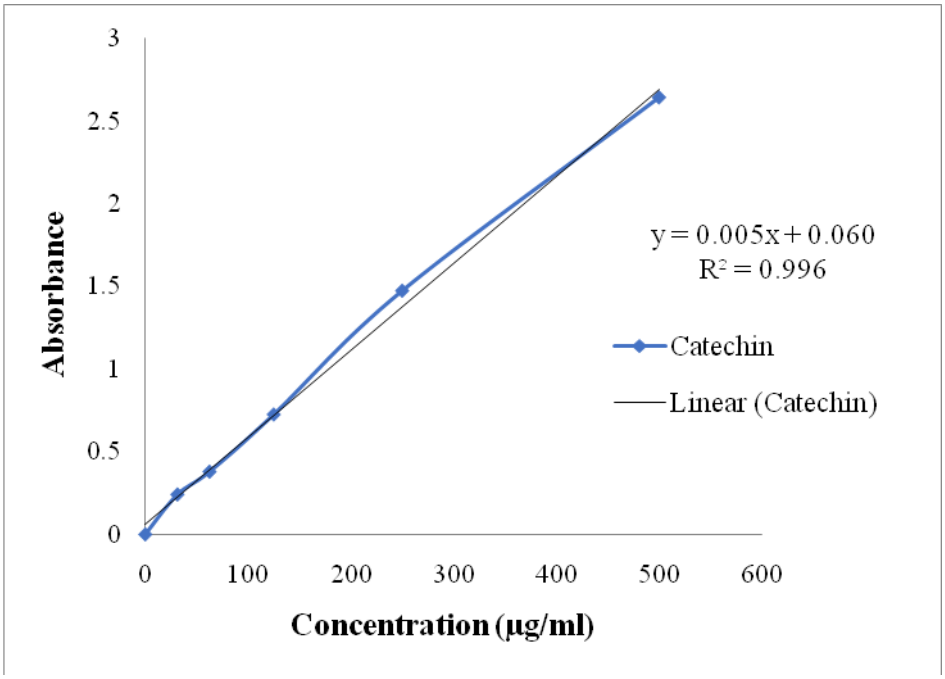


Figure 5. Standard curve of catechin for the determination of total flavonoid content

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	

CNS-depressant activity evaluation:

In the present study, CNS-depressant activity of the ethanolic leaf 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 anxiety reducing CNS-depressant effect (Fig. 6) in a dose dependent manner. The statistically significant ($p < 0.05$) effect was observed for ELCI 400 mg/kg from 3rd observation (60 min) to 5th observation (120 min) and for ELCI 200 mg/kg at 5th observation (120 min) respectively.

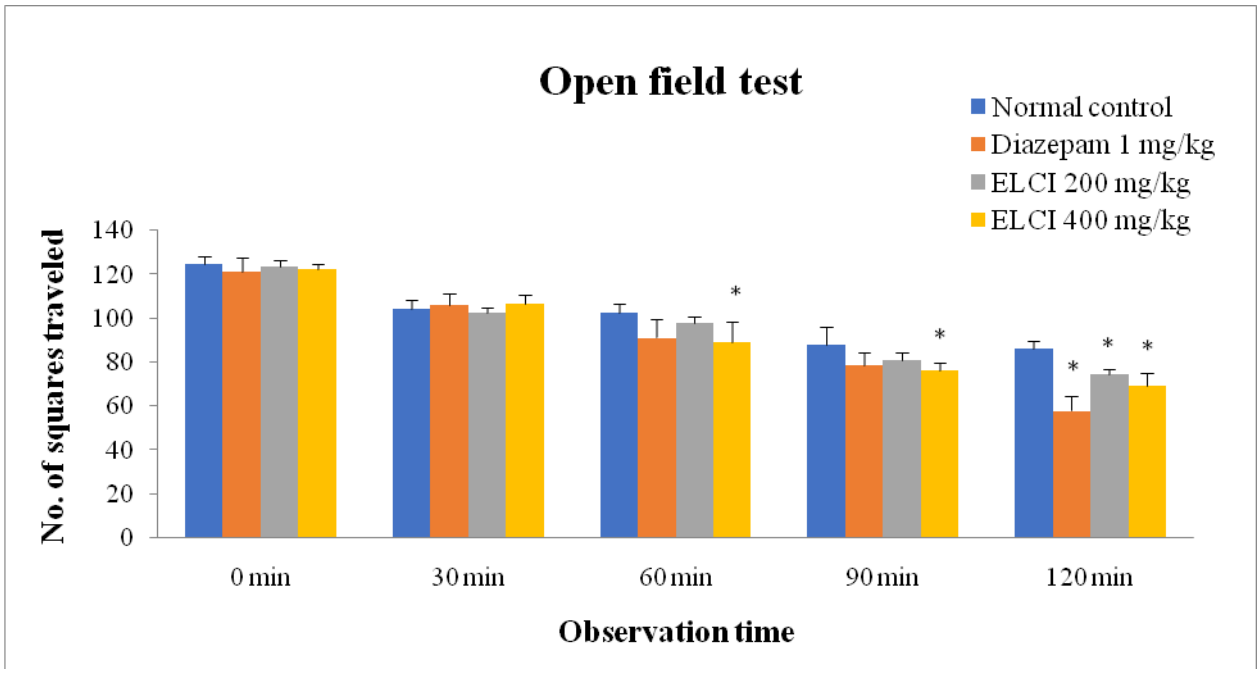


Figure 6. The CNS-depressant effect of ethanolic leaf extract of *C. indicum* (ELCI) in open field test. Each value is presented as the mean ± SD. *p< 0.05, Dunnett's test compared with control group

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 (p< 0.05) effect was observed for ELCI 400 mg/kg from 3rd (60 min) to 5th (120 min) and for ELCI 200 mg/kg from 4th (90 min) to 5th (120 min) observation period respectively. The effect was dose-dependent and statistically significant compared to control.

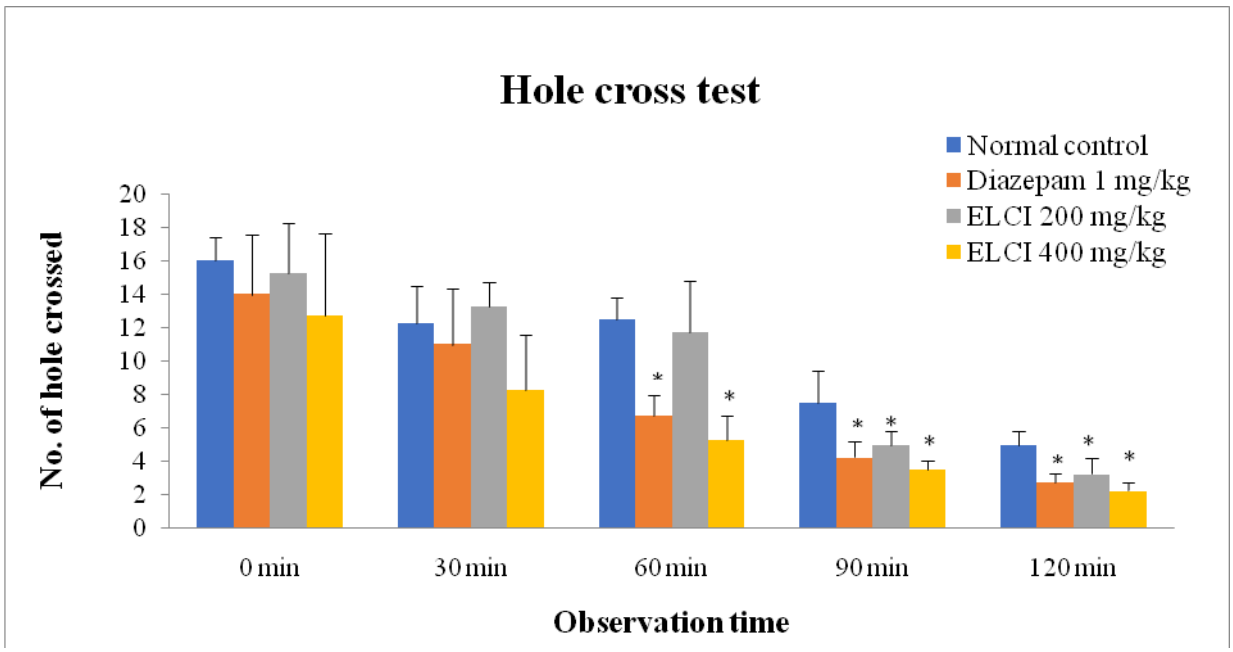


Figure 7. The CNS-depressant effect of ethanolic leaf extract of *C. indicum C. indicum* (ELCI) in hole cross test. Each value is presented as the mean ± SD. *p< 0.05, Dunnett's test compared with control group

The decrease in locomotion in open field and hole cross tests therefore demonstrates the anxiety reducing depressant activity of both the doses of ELCI. The CNS-depressant activity may be found due to the presence of alkaloids in the extract [26,27]. The general depressant and sedative effect of the extract could also be correlated to the action of alkaloids on the cerebral mechanism involved in the regulation of sleep [28]. Moreover, 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 CNS-depressant effect of this plant through same aforementioned mechanism. Furthermore, tannins have been reported to show nonspecific CNS-depression in mice[30]. So the reported central depressant effect of the ethanolic extract of *C. indicum* may be due to the presence of alkaloid, flavonoid or tannin-like constituents in the plant.

Antidepressant activity evaluation:

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

As it is seen in tail suspension test (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 ELCI 400 mg/kg.

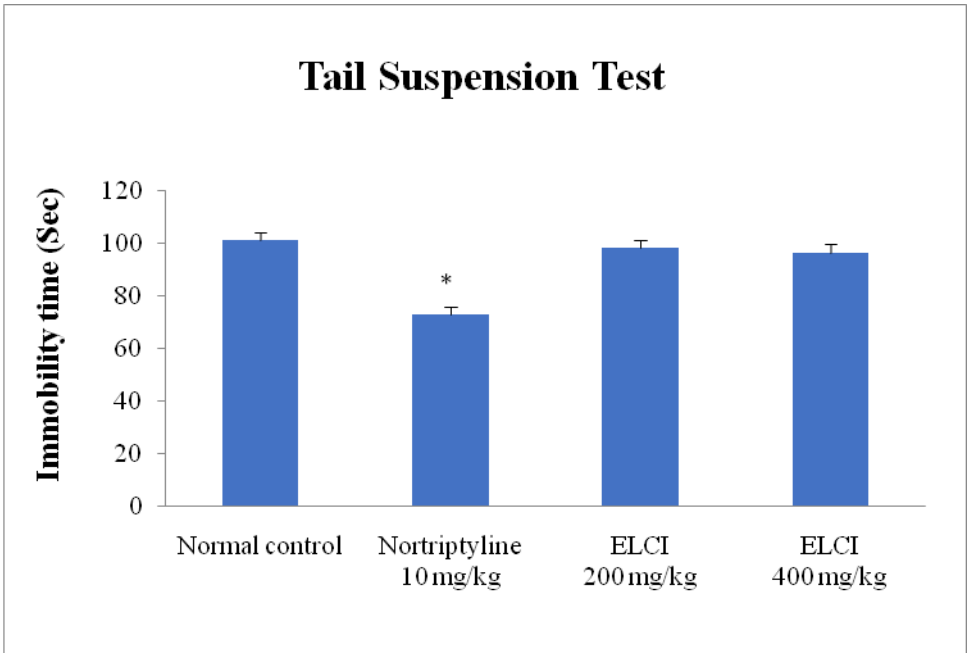


Figure 8. The antidepressant effect of ethanolic leaf extract *C. indicum* (ELCI) in tail suspension test. Each value is presented as the mean ± SD. *p< 0.05, Dunnett's test compared with control group

In forced swimming test (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, moderate antidepressant activity was found in both doses of the extract in a dose dependent manner, but the best result was obtained from the ELCI 400 mg/kg.

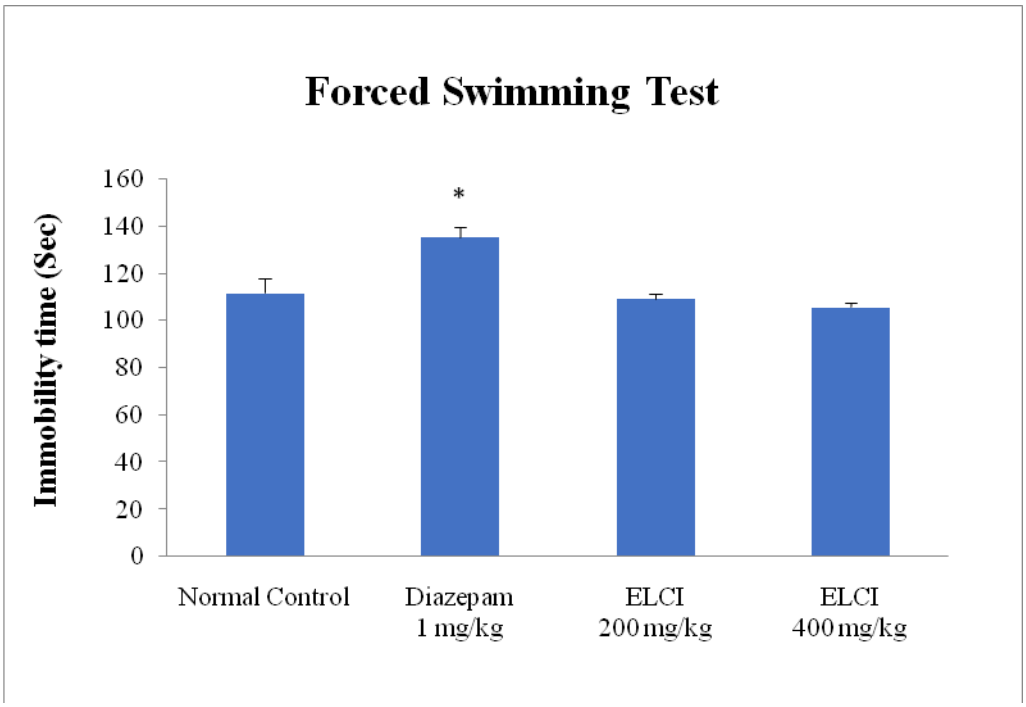


Figure 9. The antidepressant effect of ethanolic leaf extract *C. indicum* (ELCI) in forced swimming test. Each value is presented as the mean ± SD. *p< 0.05, Dunnett's test compared with control group

In wire hanging test (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 better result obtained from the ELCI 400 mg/kg.

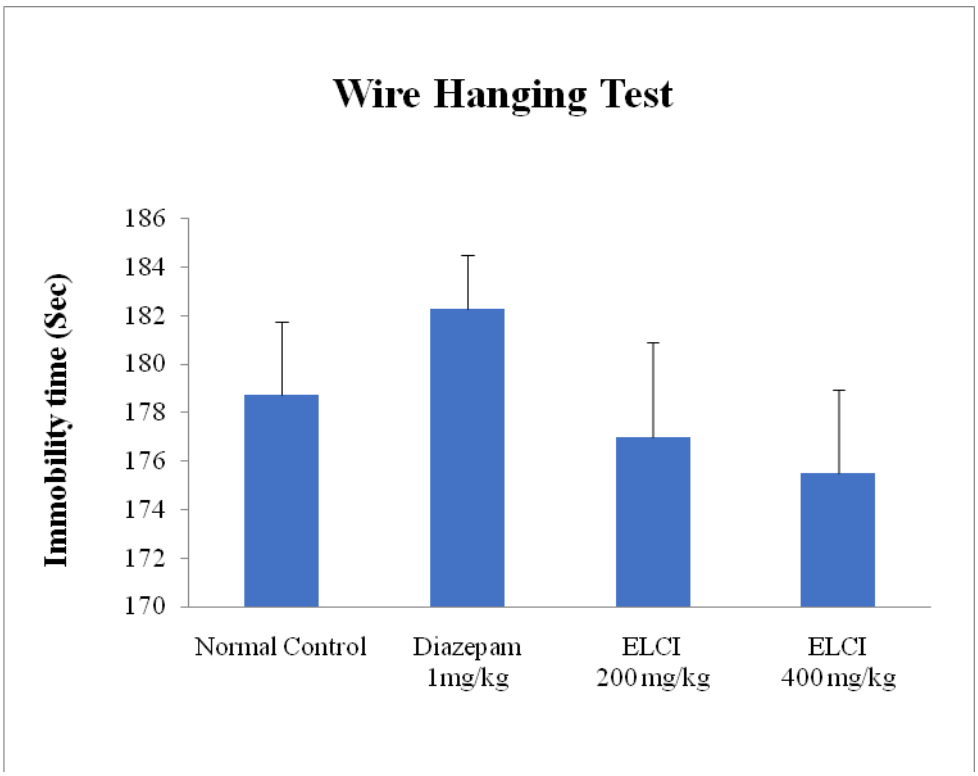


Figure 10. The antidepressant effect of ethanolic leaf extract *C. indicum* (ELCI) in wire hanging test. Each value is presented as the mean ± SD

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]. In all aforementioned experimental models, results indicated that none of the doses of the extract could reduce the immobility time significantly compared to the control. However, in the present study, the experiments were performed acutely. Since the duration of administration and therapeutic doses may affect the pharmacokinetics of the active components, chronic administration of the extract with a wide range of doses may exhibit better results[31].

CONCLUSION

The findings of the present investigation suggest that the ethanolic leaf extract of *C. indicum* possesses moderate antidepressant activity in acute animal models of depression, but significant CNS-depressant activity in a dose dependent manner. The extract also exhibited good but different levels of antioxidant activity in some studied models . However, further investigations 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 different experimental models.

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CONFLICTS OF INTEREST

Authors declare that they have no conflicts of interest.

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