Evaluation of antioxidant and neuropharmacological properties of *Leea aequata* leaves

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Abstract

The Bangladeshi medicinal plant *Leea aequata* is utilized for many health ailments due to its phenolics and flavonoids; however, its bioactive qualities are unknown. An Ethanolic Extract of *Leea aequata* (EELA) was tested for antioxidant activity in a controlled lab study. It was also tested on rodents for feelings of depression and anxiety. Hole Board Test (HBT) and Elevated Plus Maze (EPM) assessed anxiolytic activity in intact mice. The Forced Swim Test (FST) and Tail Suspension Test (TST) assessed antidepressant action through immobility. DPPH scavenging, total phenolic, and total flavonoid assays assessed *in vitro* antioxidant capabilities. In the *in vitro* DPPH scavenging activity model, the half-Inhibition Concentration (IC_{50}) of the plant sample for free radicals is 323.88 µg/mL, which is significant compared to that of ascorbic acid (759.03 µg/mL). The Total Phenol Content (TPC) of 25.78±3.75 Gallic Acid Equivalent (GAE) mg/g of dry extract and the Total Flavonoid Content (TFC) of 20.19 mg Catechin Equivalent (CAE) per gram of dry extract in the *Leea aequata* extract were found to be substantial. In the *in vivo* anxiolytic activity model, EELA showed substantial ($p<0.01$) anxiolytic efficacy at 400 mg/kg in the EPM test. The test extract's anxiolytic action is shown by the open arm's decreased entry at 400 mg/kg (81.33±13.96). Increased head dipping with strong anxiolytic effects at 400 mg/kg (27±4.04) ($p<0.0001$) was observed in HBT. In TST, EELA showed greater antidepressant effectiveness at 200 mg/kg (64.33±6.58). In the FST, EELA at 200 mg/kg had the strongest anti-depressant effect ($p<0.0001$) due to its short immobility period. These results suggest that *L. aequata* has antioxidant and neuropharmacological properties and is a major antioxidant source. According to considerable research, *Leea aequata* may reduce oxidative stress, anxiety, and depression.

Introduction

The relationship between life and illness has persisted since prehistoric times. Modern medicine encounters a plethora of novel ailments every year, many of which defy treatment with established pharmaceuticals. Thus, it is a desire to discover an effective yet safer fresh option.[1] The potential for herbal plants to elicit a therapeutic response and their status as a possible option for the development of pharmaceutical products have piqued a great deal of interest in their use for many years.[2] Approximately 80% of the global population utilizes them for the treatment of several ailments.[3] Herbal products are often the primary source of medicine in impoverished countries and even in affluent societies due to economic or geographical constraints. In contrast, more affluent neighborhoods employ them as a choice rather than need.[4] Plants have a vital role in medicine, with around 25% of pharmaceutical

prescriptions in the US including plant-derived ingredients.[5] In recent decades, the investigation of the therapeutic characteristics of several plants has acquired significant momentum due to their potent pharmacological effects, low toxicity, user-friendly nature, affordability, and economic feasibility.[6] There has been a renewed effort to identify plant-based antioxidants due to the safety concerns around synthetic antioxidants.[7] The latter are notoriously dangerous, and the health management community is worried about the toxic effects of these substances.[8] Several diseases, such as cancer,[9] coronary artery disease,[10] cataracts,[11] diabetes,[12] arthritis,[13] immune deficiency diseases,[14] and aging[15] are associated with oxidative damage. However, these diseases can be prevented by increasing the consumption of green vegetables and seeds, which are rich in naturally occurring antioxidants such as ascorbic acid, vitamin E, and phenolic compounds.[16] Vegetables and fruits constitute the principal dietary sources of natural antioxidants; however, fast food is progressively supplanting them in contemporary societies as a means to accommodate hectic schedules. Consequently, individuals are becoming increasingly vulnerable to health complications linked to oxidative stress.[17] Antioxidants have a vital function in the prevention of diseases. Antioxidant compounds have the ability to function as scavengers of harmful free radicals, binders of metals that promote oxidation, substances that alleviate oxidative stress, and inhibitors of singlet oxygen production.[16] To retard the process of oxidation, antioxidants are commonly included in oils and fatty meals. Regulations are in place to control the utilization of synthetic antioxidants in food due to their potential carcinogenic effects, namely those of Butylated Hydroxytoluene (BHT) and Butylated Hydroxyanisole (BHA). Consequently, the study of natural antioxidants has gained significant importance, and various unprocessed extracts and entirely natural constituents derived from plants have already demonstrated antioxidant properties.[18] Neuropsychiatric diseases, including depression, are prevalent and significant mood disorders that manifest in profound symptoms such as sorrow, feelings of guilt, and loss of interest, which adversely impact sleep, appetite, and other routine activities. Untreated depression, in conjunction with anxiety, can exacerbate symptoms, intensify problems, and eventually increase the likelihood of suicidal thoughts.[19] Depression and anxiety are the two most prevalent mental disorders.[20] Anxiety and depression are presently the most widespread manifestations of mental disorders, impacting around 10 to 20% of the general population.[21] Effective contemporary psychological interventions for depression and anxiety encompass the following: tricyclic and tetracyclic antidepressants, like amitriptyline, benzodiazepines, serotonin reuptake inhibitors, such as paroxetine, serotonin-norepinephrine reuptake inhibitors, like venlafaxine, monoamine oxidase inhibitors, such as phenalgine, and others.[22] Although there are many different types of antidepressants and antianxiety drugs available, the decrease in clinical symptoms has been small. Unfortunately, some of the currently accessible anxiolytics and antidepressants also exhibit specific undesirable effects, therefore leading to less-than-ideal compliance among patients.[23] It is crucial to discover new pharmaceutical leads, and researchers must explore other sources, such as medicinal plants. The *Leea aequata*, a small tree, is indigenous to Asia and may be found in countries such as Bangladesh, Bhutan, Cambodia, China, India, Malaysia, Myanmar, Nepal, the Philippines, Thailand, and Vietnam (*Supplementary Figure 1*).[24] The peripheral woods of Bangladesh, particularly those in Chittagong, Cox's Bazar, Moulvibazar, and Sylhet, harbor a flourishing population of *Leea aequata*. Previous studies have demonstrated the presence of antibacterial action in the pods, stems, and tubers of *L. aequata*.[25] The seeds, stems, tubers, and roots of *L. aequata* are employed in traditional medicine for their anesthetic, anthelmintic, and antibacterial properties.[26] Nevertheless, there have been few investigations carried out about the antioxidant capabilities and neuropharmacological activities of this particular species. Hence, the main aim of this work is to investigate the antioxidant characteristics of an ethanolic extract obtained from the leaves of *Leea aequata* through in *vitro* experiments. In addition, the study aims to evaluate the anxiolytic and depressive properties of the extract using *in vivo* analysis.

Materials and Methods

Preparation of plant extract

The *Leea aequata* leaves were verified by Professor Dr. Sheikh Bokhtear Uddin from the Department of Botany at the University of Chittagong, Bangladesh. The leaves were collected from the Hazarikhil Wildlife Sanctuary, which is located in the Ramgarh-Sitakunda forests, approximately 45 km north of the Chittagong port in southeast Bangladesh. The specimen was assigned the number MS-020422-9174. Our investigations exclusively utilized freshly collected leaves of *L. aequata.* Subsequently, they were fragmented into smaller segments in order to facilitate grinding. Next, the raw sections were vigorously rubbed for a period of 10 days and subsequently pulverized using a mechanical grinder. The powder (180 g) was immersed in 500 mL of 96% ethanol at room temperature for a duration of 14 days, with regular agitation using a shaker machine. Subsequently, it passed through a cotton plug and a Whatman filter paper. The Ethanolic Extract of *L. aequata* (EELA) was prepared by filtering the filtrate and evaporating it at 50°C under reduced pressure using a water bath machine (Labline; London, UK). The resulting extract weighed 6.7 g. The extract was refrigerated at a temperature of 4°C until it was required.

Experimental animals

Swiss Albino rodents of both sexes from the International Center for Diarrheal Diseases Research (ICDDRB) in Dhaka, Bangladesh, were obtained at six to seven weeks of age. Seven days prior to the investigation, the animals were acclimated to the laboratory environment (25±2°C, 12 hours of light and darkness). The Institutional Animal Ethical Committee of the School of Pharmacy at the International Islamic University Chittagong in Bangladesh granted approval for the research (reference number [P&D-147/13-18]).

Antioxidant activity

DPPH scavenging assay

The antioxidant capacity of the plant extract was assessed using the DPPH assay, which follows the methodology described by Brand-Williams *et al.* (1995).[27] Subsequently, 3 mL of a DPPH ethanol solution (20 µg/mL) was combined with each test sample containing 2 mL of various concentrations serially diluted from 500 µg/mL to 0.977 µg/mL. After a reaction period of 30 minutes at 25°C in a dark environment, the absorbance at 517 nm was determined using a UV spectrophotometer. Fifty percent Inhibitory Concentration (IC_{50}) values were subsequently determined by graphing the concentration of the sample against the percentage of free radical inhibition. Ascorbic acid and butylated hydroxyl toluene were employed as positive controls.

$$(\%)\ Radical\ scavenging = \{(A_0-A_1)/A_0\} \times 100$$

where, A_0=absorbance of the control; A_1=absorbance of the

extract. The lower the absorbance values, the higher the free radical scavenging activity.[28] The IC_{50} was calculated as it indicated the effective concentration of the extract needed to scavenge 50% of the free radicals of DPPH.

Total phenolic component analysis

The total phenolic content of *L. aequata* leaves was assessed by using the previously described method of Skerget *et al.* (2005).[29] As an oxidizing agent, Folin-Ciocalteu reagent was utilized, while gallic acid served as a reference. Distilled water was used to dissolve 2 mg of the *L. aequata* leaf extract in order to obtain a sample concentration of 2 mg/mL. Following that, a solution was prepared by combining 0.5 mL of extract solution (containing 2 mg/mL), 2.5 mL of Folin-Ciocalteu reagent (diluted tenfold with water), and 2 mL of Na2CO3 (7.5% w/v). The solution was then incubated at ambient temperature for 20 minutes. The absorbance of the mixture at 760 nm was subsequently measured using an ultraviolet spectrophotometer, and the absorbance was utilized to calculate the total phenolic content of the sample. A standard curve was likewise generated by combining solutions of gallic acid at various concentrations. Phenolic content is quantified in grams of Gallic Acid Equivalent (GAE) per gram of the extract.

Total flavonoid content analysis

The total flavonoid content of the extract was carried out by using a standard colorimetric method of using quercetin as standard.[30] The solution consisted of 500 µg/mL extract, 1.5 µL ethanol, and 100 µL aluminum chloride (AlCl3) (10%). The solution was supplemented with 2.8 mL of distilled water and 100 µL potassium acetate (1 M). For thirty minutes, the mixture was incubated at ambient temperature to complete the reaction. The absorbance at 415 nm against a baseline solution containing all the reagents, with the exception of the extract, was then measured. The total flavonoid content was calculated using a standard quercetin graph, denoted in milligrams of quercetin equivalent concentration.

Experimental design

Three male and female Swiss albino mice were allocated to each of the following groups: control, standard, and test (200 and 400 mg/kg, b.w.). EELA was administered at these concentrations. The control group was given a solution containing 1% Tween-80 in water (10 mL/kg, b.w), while the experimental groups were orally gavaged with EELA at concentrations of 200 and 400 mg/kg, b.w, respectively. Intraperitoneal (i.p.) administration of the standard medication diazepam (1 mg/kg, b.w.) was utilized in the Elevated Plus Maze (EPM) test and Hole Board Test (HBT).

In vivo anxiolytic activity

Elevated Plus Maze

The EPM test was employed to assess anti-anxiety behavior (*Supplementary Figure 2*). The test apparatus consisted of two open arms measuring 35×5 cm^2, two closed arms measuring 35×20 cm^2, and a center square measuring 5×5 cm^2. The EPM was raised 25 cm above the floor. The mice, which were randomly divided, were given the extract and control solution. After 60 minutes of treatment, the mice were individually put in the middle square part of the EPM. The observation was documented for duration of 5 minutes. The proportion of time spent in the open arm was computed:[31]

$$\%\ of\ time\ spent\ into\ open\ arm = \frac{time\ spent\ in\ open\ arm}{time\ spent\ in\ open\ arm + time\ spent\ in\ close\ arm} \times 100$$

Hole Board Test

The HBT was conducted using a wooden board, measuring an area of 20×40 cm^2 with 16 evenly spaced holes which was erected and raised on four-cornered wooden staffs (15 cm in height) (*Supplementary Figure 3*).[32] The mice in the study were divided into several groups. Some animals were given oral EELA at doses of 200 and 400 mg/kg b.w/day, while others were given diazepam at a dose of 1 mg/kg b.w i.p/day. The mice in the negative control group were just given normal saline water at a dose of 1 mL/day, administered twice daily at 12-hour intervals. Sixty minutes after administering the medication or extract, one participant from each group was placed on the board individually. The number of times the subject dipped their head into the holes at eye level was tallied using a tally counter during a 5-minute trial session. A single trial cycle for all three groups (n=5×3) required around 90-95 minutes to complete. Specifically, the groups consisted of normal saline-treated (normal control), diazepam-treated, and EELA-treated groups. The therapy and trial cycle were repeated every 24 hours for a total of 3 consecutive days, starting with the first trial day.

Antidepressant activity

Forced Swim Test

The antidepressant activity was assessed by Forced Swim Test (FST) according to the previously explained method of David *et al.* with some slight modifications (*Supplementary Figure 4*).[32] The groups were treated according to the specifications outlined in the experimental design section. 60 minutes after being given the extract and reference medication, the mice were put individually in a plastic apparatus that measured 25×15×25 cm^3. The apparatus was filled with water (15 cm) at a constant temperature of 25±2 °C. The positioning of individual mice within the device was documented for a duration of 7 minutes, where the first 2 minutes were designated as the adaptation period and the subsequent 5 minutes were quantified as the duration of immobility. Upon completion of the experiment, the mice were euthanized by the administration of diethyl ether anesthesia. The equation employed for calculating the percentage of inhibition of immobility was as follows:

$$Inhibition\ (\%) = \frac{A - B}{A} \times 100$$

where, A is the mean immobility time of the control and B is the mean immobility time of the test sample, respectively.

Tail Suspension Test

The Tail Suspension Test (TST) was used to evaluate the antidepressant activity of *L. aequata* extracts according to the explained method with some slight modifications (*Supplementary Figure 5*).[33] The groups were treated according to the specifications outlined in the experimental design section. After a time span of sixty minutes following the therapy, the mice were individually suspended by their tails using adhesive tape, which was placed approximately 1 cm away from the tail's tip. The calculation of immobility duration and its proportion were determined as the TST outcomes. Upon completion of the experiment, the mice were euthanized by diethyl ether anesthesia.

Statistical analysis

The study's data were shown as mean ± standard error of the mean. A value of $p<0.05$ was judged statistically significant. GraphPad Prism version 8.4 (GraphPad Software Inc.; San Diego,

CA, USA) was used for the statistical analysis, which included Dunnett's test and one-way Analysis of Variance (ANOVA). The test groups were compared to the negative control (1% Tween-80).

Results

In vitro antioxidant activity

DPPH free radical scavenging assay

Supplementary Figure 6 illustrates the DPPH free radical scavenging activity of the ethanolic extract derived from the leaves of *Leea aequata*. The radical scavenging effect of the extract was observed to be dose-dependent in the DPPH assay. As shown in *Supplementary Figure 6*, the IC_{50} of the extract for free radicals is 323.88 µg/mL, which is significantly higher than the IC_{50} of ascorbic acid (759.03 µg/mL). When compared to ascorbic acid, the antiradical activity of plant extracts is evident.

Total Flavonoid Content

The colorimetric technique using aluminum chloride was utilized to evaluate the Total Flavonoid Concentration. The quantification of total flavonoids was represented in terms of Catechin Equivalent (CAE). Using a linear regression equation y=0.0013x+1.7213(R^2=0.8288) derived from a standard quercetin calibration curve (*Supplementary Figure 2)*, the crude extract's total phenolic content was computed to be 20.199 mg QE/g dried extract. *Supplementary Figure 7* and Table 1 show the outcome.

Total Phenol Content

The antioxidant properties exhibited by phenolic compounds are outstanding. As the hydroxyl groups of phenol compounds function as a free radical terminator, the total phenol content in plant extract was expressed as GAE (mg/g of dried extract). The total phenolic content of the crude extract was determined using a linear regression equation, y=0.0019x+1.4261(R^2=0.5677), derived from a calibration curve using standard gallic acid. The total phenol content of the extract (EELA) was ascertained to be 25.775±3.75 mg/g of dried extract (GAE). *Supplementary Figure 8* and Table 2 present the results.

Anxiolytic activity

Elevated Plus Maze

The results of the EPM test are displayed in *Supplementary Figure 9*. The results demonstrated that the anxiolytic properties of the widely used medication diazepam (210±2.51) were substantially (p<0.001) superior to those of the experimental plant (EELA). The study found that the administration of EELA at a dosage of 400 mg/kg resulted in a significant increase in the time spent by mice in the open arms (81.33±13.97), compared to the dosage of 200 mg/kg. This increase was observed in a dose-dependent manner, with higher doses of EELA leading to greater increases in time spent in the open arms. The extract (EELA) showed a mentionable reduction in anxiety at a dosage of 400 mg/kg, as indicated by increased admission into the open arm, which is indicative of anxiolytic effects. The reduction in the duration of time spent in the open arm at a dosage of 200 mg/kg (52.67±17.02) indicates a mild-to-moderate anxiolytic effect of the test extract.

Hole Board Test

The findings of the hole board test are illustrated in *Supplementary Figure 10*. The study revealed a notable reduction in the head-dipping reaction, which was directly influenced by the dosage and induced by the application of EELA. The decrease was statistically significant (p<0.001) when compared to the control group (23.33±1.76). The findings indicate that the plant extract, EELA, had a notable anxiolytic effect. During the hole board test, the application of the EELA led to a decrease in the frequency of head dips, with the extent of the decrease varying based on the dose. The experimental plant showed a highly substantial reduction (p<0.001) in the occurrence of heads falling into the hole when administered at a high dosage of 400 mg/kg (27±4.04). When administered at a lower dose of 200 mg/kg (20.67±5.81), there was a significant fall in head dipping, suggesting a considerable reduction in anxiety (p<0.0001). The observed impact was comparable to that of the reference medication diazepam (64.33±0.33).

Table 1. Data for determination of Total Flavonoid Content (TFC) of Ethanolic Extract of *Leea aequata* (EELA).

Serial No.	Sample solution concentration (µg/mL)	Weight of dry extract per mL, m (gm)	Sample solution absorbance at 765 nm	Quercetin conc c (µg/mL)	Quercetin conc. c (mg/mL)	TFC as CAE, A=(cXv)/m (mg/gm)	TFC Mean±SEM (mg/gm)
1	1000	0.0004	1.822	77.461	0.0775	20.712	20.199±0.513
2	1000	0.0004	1.821	76.692	0.0767	19.173	20.199±0.513
3	1000	0.0004	1.829	82.846	0.083	20.712	20.199±0.513

TFC, Total Flavonoid Content; CAE, Catechin Equivalent; SEM, Standard Error of the Means.

Table 2. Data for determination of Total Phenol Content (TPC) of Ethanolic Extract of L*eea aequata* (EELA).

Serial No.	Sample solution concentration (µg/mL)	Weight of dry extract per mL, m (gm)	Sample solution absorbance at 765 nm	GAE conc. c (µg/mL)	GAE conc. c (mg/mL)	TPC as GAE A=(cXv)/m (mg/gm)	TPC Mean±SEM (mg/gm)
1	1000	0.0004	1.65 1	118.36	0.118	29.59	25.775±3.75
2	1000	0.0004	1.565	73.105	0.073	18.276	25.775±3.75
3	1000	0.0004	1.65	117.84	0.117	29.46	25.775±3.75

TPC, Total Phenol Content; GAE, Gallic Acid Equivalent, SEM, Standard Error of the Means.

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Antidepressant activity

Forced Swim Test

The current study sought to examine the possible antidepressant effects of EELA when administered orally, using the FST as the experimental model. The current investigation utilized mice as experimental subjects, which were given different samples at two distinct doses (200 and 400 mg/kg) as seen in *Supplementary Figure 11*. The findings demonstrated a substantial decrease in times of immobility in the mice administered with these dosages, as compared to the positive control group (mean immobility time: 153.23±2.89 sec). The administration of EELA at a dosage of 200mg/kg had the strongest antidepressant effect, leading to a substantial decrease in immobility time (108.67±10.72 s). Similarly, it was noted that mice given fluoxetine at a dose of 10 mg/kg showed a significant decrease in the amount of time they remained motionless, with an average length of 83±7.08 s.

Tail Suspension Test

The current study involved an experimental group of mice that were exposed to two different doses (200 and 400 mg/kg) of *Leea aequata* extracts. The mice in the experimental group showed a statistically significant decrease in the amount of time they remained immobile compared to the negative control group. The aforementioned observation is recorded in *Supplementary Figure 12*. Out of the several treatments analyzed, it was noted that the 200 mg ethanolic extract had the most significant antidepressant effect, as shown by the lowest recorded immobility time (64.33±6.58). Similarly, it was demonstrated that mice treated with fluoxetin at a dose of 10 mg/kg had a significant decrease in the amount of time they remained immobile (105.3667±3.64), as expected.

Discussion

We began our investigation by thoroughly investigating the antioxidant capabilities of the ethanolic leaf extract of *Leea aequata*. Free radicals, which possess one or more unpaired electrons, are recognized as the causative agents for numerous life-threatening pathological conditions in the human body, including cancers, cardiovascular diseases, inflammatory diseases, respiratory diseases, diabetes mellitus, cataracts, male infertility, and the aging process.[34] Antioxidants have a crucial role in neutralizing free radicals, which are responsible for oxidative stress and linked to several illnesses. Plants are a very beneficial reservoir of natural antioxidants, as they contain several phytochemicals with antioxidant capabilities. Their main function is to safeguard against oxidative damage caused by free radicals.[35-37] In order to assess the antioxidant capacity of the extract, we performed a series of experiments, which encompassed the DPPH scavenging assay, determination of TPC, and quantification of TFC. The evaluation of the antioxidant activities of *Leea aequata*'s ethanolic leaf extract revealed a remarkable ability to eliminate free radicals, as evidenced by a notable result in the DPPH Scavenging Assay (IC_{50}) of (323.88 µg/mL). The concentration and efficacy of the antioxidants are directly correlated with the degree of color change. Significant free radical scavenging activity of the substance under test is indicated by a considerable drop in the absorbance of the reaction mixture.[38] The study's findings imply that the plant extract contains phytochemical components that have the ability to give free radical hydrogen in order to scavenge any possible harm. This indicates that the extract includes bioactive chemicals with the ability to counteract oxidative damage. This discovery is consistent with other studies on plant extracts that include high levels of phenolic and flavonoid chemicals, known for their well-established antioxidant capabilities.[39] The *Leea aequata* extract exhibited a significant TPC of 25.78±3.75 GAE mg/g of dry extract and a TFC of 20.19 mg CAE per gram of dry extract. These readings suggest the existence of substantial quantities of phenolic and flavonoid chemicals. The extract's significant phenolic and flavonoid concentration substantiates its antioxidant capacity and may enhance its health-enhancing characteristics.[39]

Anxiety and stress-related disorders are debilitating psychiatric conditions that hinder daily occupational functioning. Charles Darwin posited that animals, including rats, have the same mechanism as humans for generating emotions. Advancements in animal models of anxiety and stress have provided insights into the pharmacological activity and potential therapeutic consequences of a limited number of medications.[40] The EPM is a highly important behavioral test used to evaluate the anxiolytic-like effects in animals, and it is considered to be among the most significant animal tests. This test has a great sensitivity to the impact of anti-anxiety and anxiogenic medications that work on the Gamma-Aminobutyric Acid (GABA) benzodiazepine complex.[41] Both the EPM and the hole board methods demonstrated encouraging anxiolytic effects for the ethanolic leaf extract of *Leea aequata*. In the EPM test, 400 mg/kg EELA resulted in a significant increase in the time spent by mice in the open arms (81.33±13.97), demonstrating considerable anxiolytic activity. Based on the raised plus maze paradigm, the anxiolytic power of 400 mg/kg of EELA was practically significant compared to that of diazepam. Indicating less anxiety, EELA demonstrated a substantial decrease in the amount of time spent with the arm closed and a rise in the amount of time spent with the arm open. The HBT, which assesses exploratory behavior apart from locomotor activity, revealed a noteworthy rise in head-dippings in EELA-treated mice, indicating a reduction in anxiety.[42] When comparing the amplitude of movements to the standard medication diazepam, the mice that got a dosage of 400 mg/kg body weight had the greatest result (27±4.04) in comparison to the positive control (64.33±0.33). A reduction in locomotor activity, which is a measure of CNS excitability, might be a sign of the extract's sedative or depressant effects. Consistent with research demonstrating the anxiolytic effects of several natural chemicals and plant extracts, these experiments yielded good results.[43]

Symptoms similar to depression are attributed to the GABA-A receptor, as shown in the study. Individuals with low levels of GABA-A receptors or a GABA deficiency may experience depression. This suggests that GABAergic receptor agonists may be effective in reducing depressive symptoms.[33] The plant *L. aequata* possesses a significant quantity of flavonoids. Therefore, our objective was to assess the antidepressant properties of *L. aequata* leaves. The current study demonstrates that the extracts derived from *L. aequata* leaves significantly decreased the duration of immobility in both the TST and swimming tests. During the FST test, it was seen that administering EELA at a dose of 200 mg/kg resulted in the most potent antidepressant effect. This led to a significant reduction in immobility time (108.67±10.72 s) compared to the group that received EELA at a dosage of 400 mg/kg. The TST test revealed that the ethanolic extract with a dosage of 200 mg/kg had the most pronounced antidepressant effect, as evidenced by the lowest observed immobility time of 64.33±6.58. The findings indicated that, in comparison to the reference medication, fluoxetine, an extract of EELA at a dosage of 200 mg/kg reduced the length of immobility time. The results of FST and TST confirm Leea aequata's significant antidepressant properties. Promising

pathways for the antidepressant effect of crude plant extracts may be attributed to inhibition of monoamine oxidase A. Fluoxetine demonstrated significant antidepressant activity due to the specific suppression of serotonin reuptake. Based on the results of the FST and TST, which are indicators of substances with antidepressant-like effects, these results show mild-to-moderate antidepressant effect.[44] The findings of this study indicate that the ethanolic leaf extract of *Leea aequata* has notable antioxidant and mild-to-moderate anxiolytic and depressive characteristics. These discoveries might have significant ramifications for the advancement of natural treatments for anxiety and depression. Nevertheless, this investigation serves as the first stage in comprehending the entire range of skills possessed by the extract. Additional inquiries, such as conducting clinical studies and doing toxicological assessments, are essential in order to verify the safety and effectiveness of this substance for human utilization. Moreover, the determination of distinct bioactive components accountable for the reported impacts might augment its practicality and facilitate the progress of pharmaceutical development.

Future implication and limitations of the study

Our findings show that the ethanolic leaf extract of *Leea aequata* has strong antioxidant activity as well as mild-to-moderate anxiolytic and antidepressant properties. The study offers possible future applications in pharmaceutical development, mental health therapies, nutraceuticals, and environmental protection. However, limitations such as animal model specificity, a single dosage and administration route, short-term effects, and a limited scope of neuropharmacological effects are highlighted, emphasizing the need for dose-response studies, clinical trials, mechanistic studies, long-term investigations, and bioactive compound exploration to enhance the translational potential of research findings and facilitate their practical applications in medicine and healthcare.

Conclusions

Finally, the moderate levels of TPC and TFC and notable effectiveness in the DPPH scavenging experiment show that *Leea aequata*'s ethanolic leaf extract has impressive antioxidant capabilities. In addition, our studies of the effects of drugs on the nervous system in living organisms demonstrate promising effectiveness in reducing anxiety and depression. The EPM, HBT, FST, and TST all produced positive results, which support these findings. These data indicate that the extract has diverse medicinal potential in reducing oxidative stress and treating anxiety and depression. However, conducting more research, such as clinical trials, is crucial to solidify its effectiveness and safety.

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Online supplementary material.
Supplementary Figure 1. Leea aequata in its natural habitat.
Supplementary Figure 2. Elevated Plus Maze (EPM) apparatus used in anxiolytic activity.
Supplementary Figure 3. Hole Board Test (HBT) apparatus used in anxiolytic activity.
Supplementary Figure 4. Apparatus used in Forced Swimming Test (FST), which measures the antidepressant activity of crude extract.
Supplementary Figure 5. Apparatus used in Tail Suspension Test (TST), which measures the antidepressant activity of crude extract.
Supplementary Figure 6. DPPH radical scavenging activity of ascorbic acid and Ethanolic Extract of L. aequata (EELA) at different concentrations.
Supplementary Figure 7. Determination of Total Flavonoid Content (TFC) using quercetin calibration curve.
Supplementary Figure 8. Determination of Total Phenolic Content (TPC) from gallic acid calibration curve.
Supplementary Figure 9. Anxiolytic activity of Ethanolic Extract of L. aequata (EELA) on the Elevated Plus Maze (EPM) test in mice. All values are presented as mean ± Standard Error of the Means (SEM). To compare the test samples with the control, a one-way Analysis of Variance (ANOVA) was used, followed by Dunnett's test (n=3, per group).
*NS, Not Significant; **p<0.01; ***p<0.001, significantly different from control.*
Supplementary Figure 10. Anxiolytic activity of Ethanolic Extract of L. aequata (EELA) on the Hole Board Test (HBT) in mice. All values are presented as mean ± Standard Error of the Means (SEM). To compare the test samples with the control, a one-way Analysis of Variance (ANOVA) was used, followed by a Dunnett's test (n=3, per group).
*NS, Not Significant; **p<0.01; ***p<0.001; ****p<0.0001, significantly different from control.*
Supplementary Figure 11. Anti-depressant activity of Ethanolic Extract of L. aequata (EELA) on the Forced Swim Test (FST) in mice. All values are presented as mean ± Standard Error of the Means (SEM). To compare the test samples with the control, a one-way Analysis of Variance (ANOVA) was used, followed by a Dunnett's test (n=3, per group).
*NS, Not Significant; *p<0.1; **p<0.01; ***p<0.001; ****p<0.0001, significantly different from control.*
Supplementary Figure 12. Anti-depressant activity of Ethanolic Extract of L. aequata (EELA) on the Tail Suspension Test (TST) in mice. All values are presented as mean ± Standard Error of the Means (SEM). To compare the test samples with the control, a one-way Analysis of Variance (ANOVA) was used, followed by a Dunnett's test (n=3, per group).
*NS, Not Significant; *p<0.1; **p<0.01; ***p<0.001; ****p<0.0001, significantly different from control.*