

Acute Toxicity Study and Evaluation of the Anxiolytic Activity of the Ethanol Leaf Extract of *Bryophyllum Pinnatum* (Kurz) in Mice

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ABSTRACT

Introduction: Anxiety is a psychiatric disorder and identified as the most common stress-related mood disorders causing disability and premature death. Due to the several adverse effects of conventional anxiolytics that have reduced the compliance tendencies of patients, alternative therapies are being sought. Although, studies have shown relative central nervous system effects of different fractions of *Bryophyllum pinnatum*, no study has specifically evaluated the anxiolytic activity of the ethanol leaf extract of the plant (EEBP) hence, this study.

Materials and Methods: Mice (22–25 g) were randomly distributed into six groups ($n = 5$) and administered thus: Group I and II received intraperitoneally 1 mL/kg saline and 1 mg/kg diazepam as negative and positive controls respectively whereas Groups III, IV, V and VI received oral doses of 250 mg/kg, 500 mg/kg, 1000 mg/kg, and 2000 mg/kg of *B. pinnatum* extract respectively followed by open field (OF) paradigm procedure. Similarly, the pattern of EEBP administration was repeated for the mice and then subjected to Elevated Plus Maze (EPM) test. Data were expressed as Mean \pm Standard Error of Mean (SEM) using one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test. Results were regarded as significant at values of $P < 0.05$.

Result: LD₅₀ of EEBP is greater than 2000 mg/kg. EEBP exhibited a significant decrease in locomotion and rearing of mice at 500 mg/kg and 2000 mg/kg respectively. Contrarily, a significant increase in the duration of time spent by the mice in the open arm was observed at 1000 mg/kg whereas, none of the treated doses showed a significant reduction in the frequencies of entries in the EPM paradigm. However, EEBP showed a reduction in the index of open arm avoidance compared to the saline group.

Conclusion: EEBP exhibited dose-dependent inhibitory central effects and may possess potential anxiolytic effect. However, further studies are required to determine its molecular mechanism of action

Keywords: *Bryophyllum pinnatum*, anxiolytics, elevated plus maze, Open Field test, Open Arm Avoidance Index

INTRODUCTION

Anxiety disorders are the most common of mental disorders and affect nearly 30 percent of adults at some point in their lives and are the most pervasive mental issue (with a current overall predominance of 2.5 – 7.3%.^{1,2} They differ from ordinary sentiments of apprehension or nervousness, and include excessive fear and uneasiness³. Anxiety has been identified as the most common stress-related mood disorders causing disability and premature death.⁴

Anxiety disorders are treatable and various effective medicines are readily available including anxiolytic agent. Management

with an anxiolytic (sedative) agent leads to decreased activity, moderation of excitement and calming of the recipient. Treatment enables a great many people to lead ordinary profitable lives.⁵ Benzodiazepines are the most common class of anxiolytics and act via central GABA_A benzodiazepine receptors. However, in addition to fast relief of anxiety, they possess unwanted side effects such as sedation, muscle relaxation, synergism with alcohol, and development of tolerance and dependence.^{6,7}

Due to the several adverse effects of conventional anxiolytics that have reduced the compliance tendencies of patients,

alternative therapies with fewer adverse effects are being sought.⁸ *Bryophyllum pinnatum* (Lam.) Kurz (Crassulaceae) (synonym: *Kalanchoe pinnata*, [Lam.] common names include Life plant, air plant [Mexican], love plant, Canterbury bells, Cathedral bells, e.t.c.) is a perennial herb usually classified as a weed that grows widely and is used in folkloric medicine in tropical regions of the world including Nigeria.⁹

A number of active compounds, including flavonoids, glycosides, steroids, bufadienolides and organic acids, have been identified in *Bryophyllum pinnatum*.¹⁰ Although, studies have shown relative central nervous system effects of different fractions of *Bryophyllum pinnatum*,¹¹⁻¹³ no study has evaluated the anxiolytic activity of the ethanol leaf extract of the plant (EEBP), hence, this study

MATERIALS AND METHOD

Animals

Male Swiss albino mice (22–25 g) were used in the study obtained from the Animal House of the Department of Pharmacology and Toxicology, University of Ilorin, Ilorin. Ethical clearance was

obtained from the University of Ilorin Ethics Review Committee (ethical approval number; UERC/ASN/2019/1604). Animals were housed in plastic cages at room temperature with a 12 h light-dark cycle. They were fed with standard rodent pellet diet and water *ad libitum*. The animals were acclimatized for at least one week before being used for the experimental procedures. All experimental procedures were carried out in accordance with the guidelines for laboratory procedures laid down by the University of Ilorin Ethics Committee on Research as well as the International Animal Care and Use Committee (IACUC) in Nigeria.

Collection and identification of plant material

The leaves and stem of *Bryophyllum pinnatum* were collected from Olunlade area in Ilorin. Identification and authentication was done in Department of Plant biology/Botany, Faculty of Life Sciences, University of Ilorin, Ilorin, Kwara State, Nigeria. A voucher specimen number UILH/001/909 was allocated to the plant.

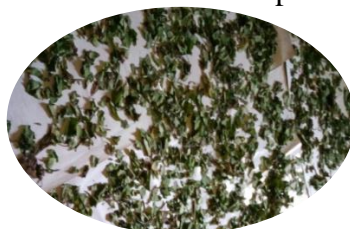


Figure 1: Image showing the leaves of *B. pinnatum* dried in the laboratory after collection

Preparation of ethanol extract of *Bryophyllum pinnatum*

The leaves were dried under shade condition and coarsely powdered. A total of 200 g of the powdered leaves were weighed into a clean jar which was then extracted with ethanol by maceration for a period of 48 hour. The mixture was filtered using cotton wool and the resultant filtrate was then evaporated to dryness on a water bath at a temperature of 45° C. The percentage yield of the ethanol extract of *B. pinnatum* was determined. The extract was stored in a refrigerator and used for the pharmacological investigations.

Acute toxicity test

Swiss albino mice of single sex, weighing between 20 and 25 g were put in a single group consisting of six animals. They were maintained under standard conditions (room temperature at 22 ± 3 °C, 12 hr light/dark) and allowed free access to water along with standard pelleted diet for one week before the experiment. The method described by Organization for Economic Co-operation and Development (OECD) was used to determine the acute toxicity of the extract. Mice were fasted overnight and weighed. The extract was dosed in a stepwise procedure, with the

initial dose being selected as the dose expected to produce some signs of toxicity and animals observed for a period of two weeks.

The animals were subjected to acute toxicity study using the extract at a dose of 2000 mg/kg orally and observed at regular intervals of 1, 2, 4, 8, 12 and 24 hours for skin/fur changes, mucus secretion, sleep, coma, eyes, lethargy and mortality.¹⁴

Behavioural assessment of anxiolytic activities

The anxiolytic activity was assessed using the open field test (OFT) and elevated plus maze (EPM) test. The animals were divided into six groups ($n = 5$). Group I received normal saline (1 mL/Kg as negative control); group II received Diazepam (1 mg/kg as positive control); while groups III, IV, V and VI received different doses of 250 mg/kg, 500 mg/kg, 1000 mg/kg, 2000 mg/kg of EEBP respectively.

Open field test

The floor of a half square meter open field is divided into a series of squares. The structure consisted of a rectangular box

composed of a hardboard floor surrounded by a wall both made with white painted wood. Mice were distributed into negative control, positive control and test groups ($n = 5$) each. Different groups were treated with normal saline (1 mL/kg) intra-peritoneally (i.p.), Diazepam (1 mg/kg) intra-peritoneally, and the extract at doses of 250, 500, 1000, and 2000 mg/kg body weight orally (orally.). After the administration of the doses each animal was introduced into the centre of the field. The following behavioural parameters: locomotion, rearing and grooming was observed in each of the mouse. The total locomotion (characterized by movement from one square to another with all the four paws), the frequency of rearing (characterized by standing upright on the two hind limbs, leaning or otherwise to the edge of the cage with the two forelimbs), frequency of grooming (characterized by mouth washing, face washing, body picking, body cleaning with paws). The observation was made in five (5) minutes session.¹⁵ Before each mouse was introduced, the cage was cleaned with methylated spirit to remove olfactory cue.¹⁶



Figure 2: Image showing the open field behavioural assessment box

Elevated plus maze (EPM) test

The elevated plus maze consists of two closed arms and two open arms emanating from a central platform. Each of the arms is identical and opposite to one another. The close arms are bordered by high walls. The preference of an animal to closed arm is a reflection of aversion to the open arms characterized by fear and anxiety induced by height and exposure.¹⁶ Thirty (30) mice were used and distributed into six groups ($n = 5$). They were treated as follows: Group I: Treatment with standard drug (Diazepam 1 mg/kg intra-peritoneally.); Group II: Treatment with normal saline (1

mL intra-peritoneally.); Group III-VI: Treatment with different doses ethanol leaf extract of *B. pinnatum* (250, 500, 1000, 2000 mg/kg oral) of ethanolic leaf extract (determined from preliminary toxicity test).

After 30 minutes (intraperitoneal administration) or 1 hour (oral administration) post-treatment, each of the animals was placed at the centre of the EPM in a position that faces the open arm. The observation lasted for 5 minutes during which the number of times (frequency) a mouse entered each of the arm, time spent (duration) in any of the

arms, and total arm entries were recorded. An arm entry is calculated as when all the four paws of the animal enters any of the arm entry. Subsequently, the percentage of time spent (duration) in the open arms [$100 \times \text{open}/(\text{open} + \text{enclosed})$] and percentage of the number of open arm entries (frequency, $100 \times \text{open}/\text{total entries}$) was calculated for each animal. The apparatus was thoroughly cleaned after each trial with methylated spirit to eliminate all olfactory cues.¹⁶

$$[100 - \frac{(\% \text{ time on open arm} + \% \text{ entries into open arm})}{2}]$$

The results were expressed as mean ratio of time spent in open arms to total time spent in both open and closed arms, (percentage of time spent in open arms); mean ratio of entries into open arms to total entries into both open and closed arms (percentage of number of entries) and number of entries of open arms. The index of open arm avoidance, interpreted as level of anxiety (Trullas and Skolnick, 1993) was calculated as:



Figure 3: Image of the elevated plus maze

Statistical analysis

The data will be expressed as Mean \pm Standard Error of Mean (S.E.M.) and analyzed using Students-t test and one – way analysis of variance (ANOVA) followed by the Student-Newmann Keuls test as specified in figures or table legends. The results will be regarded as significant at values of $p \leq 0.05$.

RESULTS

Determination of the percentage yield of EEBP

The percentage yield of ethanol leaf extract of *Bryophyllum pinnatum* was 4.75% w/w.

Determination of the acute toxicity of EEBP

The lethality range of EEBP in mice was found to be > 2000 mg/kg according to the OECD guidelines.

Assessment of behavioural parameters using the open field and elevated plus maze models

Behavioural assessment of locomotion, rearing and grooming in mice

The ethanol leaf extract of *Bryophyllum pinnatum* exhibited an effect on the novelty induced behaviour (NIB) of mice by causing a significant decrease ($p < 0.05$) in locomotion, rearing, and grooming of mice at 500 mg/kg, 2000 mg/kg, and 2000 mg/kg respectively when compared to the saline group as shown in Figures 4, 5 and 6 respectively. The effects observed at the three doses were statistically comparable to that observed with diazepam ($p < 0.05$) as respectively shown in Figures 4, 5 and 6.

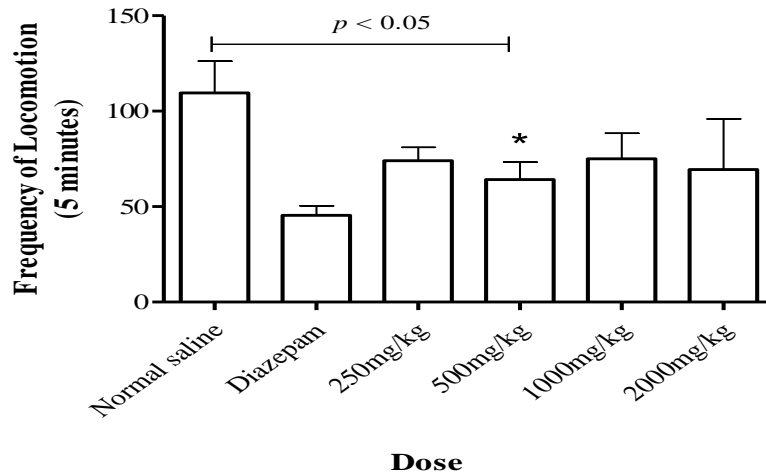


Figure 4: Frequency of locomotion induced by the ethanol leaf extract of *B. pinnatum*, Normal saline (1 mL/kg) and Diazepam (1 mg/kg) in mice. Locomotion of animals were assessed in the open field box using 250 mg/kg, 500 mg/kg, 1000 mg/kg, 2000 mg/kg of the ethanolic extract of *Bryophyllum pinnatum*. A significant decrease ($p < 0.05$) in locomotion of animals at 500 mg/kg was recorded when compared to the normal control and was statistically comparable to diazepam. Data is expressed as \pm Standard Error of Mean (S.E.M.) and analyzed using one-way ANOVA with $p < 0.05$ considered as significant.

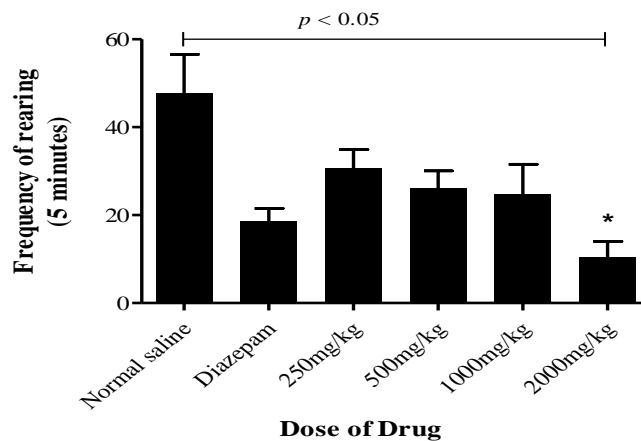


Figure 5: Frequency of rearing produced by ethanol leaf extract of *B. pinnatum*, Normal saline (1 mL) and Diazepam (1 mg/kg) in mice. Rearing of animals were assessed in the open field box using 250 mg/kg, 500 mg/kg, 1000 mg/kg, 2000 mg/kg of the ethanol extract of *Bryophyllum pinnatum*. A significant decrease ($*p < 0.05$) in rearing of animals was observed, at 2000 mg/kg dose when compared to the normal control. Data is expressed as \pm Standard Error of Mean (S.E.M.) and analyzed using one-way ANOVA with $p < 0.05$ considered as significant.

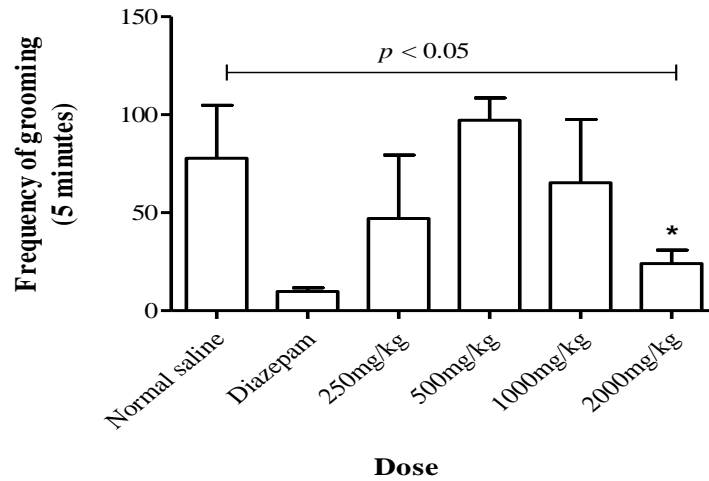


Figure 6: Frequency of grooming produced by ethanol extract of *B. pinnatum*, in comparison to Normal saline (1 mL/kg) and Diazepam (1 mg/kg) as observed in mice. Grooming of animals were assessed in the open field box using 250 mg/kg, 500 mg/kg, 1000 mg/kg, 2 000mg/kg of the ethanol extract of *Bryophyllum pinnatum*. A significant decrease (* $p < 0.05$) in grooming of animals was observed, at 2000 mg/kg dose when compared to the normal control and was statistically comparable to diazepam. Data is expressed as \pm Standard Error of Mean (S.E.M.) and analyzed using one-way ANOVA with $p < 0.05$ considered as significant.

Assessment of anxiolytic activity using the elevated plus maze (EPM) model
Assessment of the frequency and duration of entries into the open arm

The frequency and duration spent by animals in the open arms of the EPM were determined. Only 1000 mg/kg of EEBP showed a significant increase in the duration of time spent by the mice

compared to the saline group in the open arm but none of the treated groups showed significant reduction in the frequencies of entries in the EPM paradigm as depicted in Figure 7. Meanwhile, there were no significant differences in either the frequency or duration of entry into the closed arms at all the treated groups.

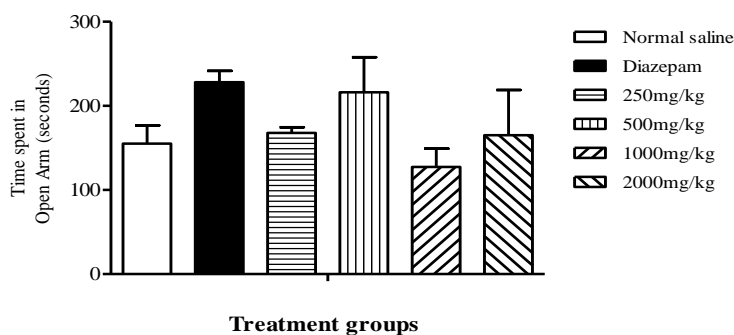


Figure 7: Duration of open arm entries produced by ethanol extract of *B. pinnatum*, in comparison to Normal saline (1 mL/kg) and Diazepam (1 mg/kg) as observed in mice. Duration of open arm entries of animals were assessed in the elevated plus maze 250 mg/kg, 500 mg/kg, 1000 mg/kg, 2000 mg/kg of the ethanol extract of *Bryophyllum pinnatum*. An increase in duration of OAE of animals was observed at 500 mg/kg dose when compared to the normal control but was not statistically significant. Data is expressed as \pm Standard Error of Mean (S.E.M.) and analyzed using one-way ANOVA with $p < 0.05$ considered as significant.

Assessment of the index of open arm avoidance

The effect of the administration of the ethanol extract of *Bryophyllum pinnatum* on the index of open arm avoidance was

also assessed. The extract produced a reduction in the index of open arm avoidance compared with the normal saline control group.

Table 1: Index of open arm avoidance values of controls and ethanol extracts of *Bryophyllum pinnatum*

	Open Arm Entry Parameters				Index of Open Arm Avoidance
	Frequency	Time	% Frequency	% Time	
N/Saline	11.2 ± 4.9	155.0 ± 43.9	57.2 ± 5.0	45.6 ± 12.9	48.6
Diazepam	27.0 ± 7.1	228.0 ± 27.4	81.2 ± 5.7	69.5 ± 6.3	24.7
2000 mg/kg	6.7 ± 6.5	180.7 ± 107.5	47.6 ± 24.7	48.5 ± 33.9	52.0
1000 mg/kg	9.8 ± 5.9	127.4 ± 43.2	55.7 ± 29.6	37.2 ± 12.7	53.6
500 mg/kg	11.2 ± 6.9	216.2 ± 83.1	56.3 ± 9.6	59.9 ± 21.9	41.9
250 mg/kg	9.4 ± 3.4	167.8 ± 13.8	50.9 ± 4.1	47.1 ± 6.9	51.0

DISCUSSION

Anxiety is an emotional state which occurs naturally, and may be negative when it becomes a disorder. It could be produced internally or externally manifesting as either real or imagined having both cognitive and behavioural pattern.¹⁶ The anxiolytic activities of the ethanol leaf extract of *B. pinnatum* using different animal models at four different concentrations was evaluated.

From the toxicity result obtained, it could be inferred that the ethanol leaf extract of *B. pinnatum* is non-toxic at a relatively high dose of 2000 mg/kg. Since the result of the acute toxicity showed neither mortality nor any significant toxic effect in the mice (even after 14 days of observation for delayed lethality or mortality), it can therefore be conveniently stated that the dose at which EEBP could cause lethality or mortality is far greater than 2000 mg/kg. Hence, EEBP could be said to belong to Category 5 of the Globally Harmonized System (GHS) for the classification of chemicals.¹⁴

Considering the results of open field test, the behavioural assessment study showed that the ethanol leaf extract of *Bryophyllum pinnatum* possesses some dose-dependent central effects. These novelty induced behaviours (NIB) were assessed with respect to locomotion, rearing and grooming in mice where

EEPM clearly showed significant decrease at 500 and 2000 mg/kg doses when compared to the normal control groups. Both locomotor activity and rearing effects of mice indicates/ suggests their exploratory behaviour and are considered to be central excitatory activity.¹⁷ This alteration of behaviour produced at different doses of the extract reinforces the hypothesis that a central effect most likely depressant in nature exists. The reduction caused in the rearing and locomotor activity by the administration of the extract could be as a result of induction of inhibitory effect on excitatory neurotransmitters such as glutamate and dopamine or potentiation of inhibitory neurotransmitters such as: aminobutyric acid (GABA).¹⁵

It is commonly known that rodents, when confronted with a novel environment, either explore it or try to escape; many behavioural procedures therefore use unconditioned responses to measure anxiety. The elevated plus-maze was used to measure "state anxiety". The time spent in the open arms in the elevated plus-maze is usually considered as a measurement of anxiety-related behaviour: the more time an animal spends in the open arms, the less anxious it is.^{18,19,20} In the present study, the positive control (diazepam 1 mg/kg) produced a clear anxiolytic profile which showed an increase in the duration spent in

the open arms. This agrees with earlier studies,^{19,21} and validates our EPM experimental conditions. Therefore, the significant increase in the duration of time spent by the mice in the open arm at 1000 mg/kg of EEBP when compared to the saline group is an indication of anxiolysis. Similarly, the effect observed with the EEBP at 500 mg/kg reveals that the extract possesses anxiolytic activity since an increase in the open arm entries is one of the most significant indices for determining anxiolytic property of an agent.

CONCLUSION

The result of the acute toxicity showed neither mortality nor significant toxic effects at the highest dose used, and an

increase in the time spent in the open arms. Thus, it can be concluded that the ethanolic leaf extract of *Bryophyllum pinnatum* is relatively safe and may possess potential anxiolytic effect.

RECOMMENDATION

However, further studies will be required to elucidate the specific neurotransmitters and receptors implicated in these effects thereby determining its molecular mechanism(s) of action.

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