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EVALUATION OF ANXIOLYTIC POTENTIAL OF ETHANOLIC EXTRACT OF *MANILKARA ZAPOTA* L. LEAVES IN RAT MODELS

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ABSTRACT

Background: Anxiety disorders, marked by persistent fear and excessive worry, significantly affect daily life. While conventional anxiolytics are effective, their long-term use often leads to side effects, prompting the search for safer, plant-based alternatives. Manilkara zapota L is a traditional medicinal plant used for various ailments. **Objective:** This study explored the anxiolytic effects of ethanolic extract of Manilkara zapota L. leaves in rats, focusing on its impact on anxiety through behavioral and biochemical assessments Methodology: Powdered leaves *of Manilkara zapota* L. were extracted using Soxhlet extraction with the 70% ethanol at a 1:10 w:v ratio and 60°C. Phytochemical screening and an acute oral toxicity study were conducted per OECD guidelines 423 over 28 days on four groups of rats: control, standard (diazepam), low dose (100 mg/kg), and high dose (200 mg/kg). Behavioral assessments, including the Elevaed Plus Maze (EPM), Open Field Test (OFT), Hole Board Test (HBT), and rotarod, were conducted weekly to evaluate anxiety and motor function. On day 29, biochemical assays on rat brain homogenates measured monoamine oxidase-A (MAO) activity and lipid peroxidation to assess oxidative stress and neurochemical effects **Result:** The EEMZ achieved a yield of 15.16% and was confirmed to contain various Phytochemicals such as alkaloids, glycosides, flavonoids, and others. Acute oral toxicity testing, following OECD guidelines 423, showed no mortality at a dose of 2000 mg/kg, leading to the selection of 100 mg/kg and 200 mg/kg for further studies. The study found that a high dose of the extract (200 mg/kg) showed potential anxiolytic effects, comparable to diazepam. Behavioural tests indicated reduced

anxiety, evidenced by increased time in open arms of the EPM and central zone of the OFT, enhanced exploratory behaviour, and HBT and Rota-rod performance, indicating no sedation. Biochemical analysis revealed significant MAO activity and lipid peroxidation reductions, suggesting protective effects against oxidative stress, especially in the high-dose group. **Conclusion:** The ethanolic extract of Manilkara zapota L showed significant anxiolytic effects, improving behavioural and biochemical parameters. This suggests its potential as a natural anxiolytic effect, requiring further exploration to understand its active constituents and mechanisms of action.

KEYWORDS: *Manilkara zapota* L, OECD, Elevated Plus Maze, Open Field Test, Hole Broad Test, anxiolytic effects, oxidative stress markers, Ethanolic extract, MOA-A, MAD.

INTRODUCTION

Anxiety is derived from the Latin word “anxietas” (to choke, throttle, trouble, and upset). In common words, anxiety can be said a feeling of fear. Here, the reason for fear may be true or just an imagination. Some general causes of anxiety are tension of examination, fear of standing in front of a crowd, etc.[1]

Few terms in psychopathology and clinical psychiatry give rise to more ambiguity of interpretation than the terms “anxiety,” “phobia,” and “panic.” However, the symptoms and conditions these words denote are neither rarities nor phenomena too refractory to clinical analysis.[2]

Some major symptoms of anxiety include fear, nervousness, dizziness, sweating, uncontrolled urination, headache, sleeplessness, bradycardia, hypertension, isolation feelings, feeling of imminent danger, chest pain, nausea, and abdominal discomfort.[3]

Fear and anxiety are nature’s first line of defence. The ability to sense potential danger before it strikes is an evolutionary key to survival. Unfortunately, this form of protection comes at a price. It is also the cornerstone of human malaise and unease. The anxious person is miserable because he or she expects doom and danger at every turn, instead of enjoying any moments of peace that do occur.[4]

By gender, age, culture, and other characteristics, anxiety disorders are more common in some people than others. The prevalence of anxiety disorders is currently estimated to be

7.3% worldwide. 3.6% of people globally, or over 264 million people, have an anxiety disorder, according to the World Health Organization. Females are more likely than males to experience anxiety disorders.[5]

Anxiety disorders are the most common mental disorder in India, affecting 40 million persons aged 18 and over, or 18.1% of the population, based on the National Institute of Mental Health. Anxiety disorders are also frequent in India, with an estimated 20% of the population suffering from them.[6]

Manilkara zaopta L. (Sapotaceae) is a spiny, evergreen tree of small to medium size known for its diverse array of chemical constituents, including alkaloids, glycosides, flavonoids, steroids, tannins, phenols, and saponins. The leaves of this plant have found their place in folk remedies, traditionally used to address various health concerns such as leprosy, intestinal disorders, peptic ulcers, toothaches, and earaches.[7]

The common constituents present in leaves of the plant are cyclitol, dulcitol, octacosanol, α-spinasterol, kaempferol-3- rhamnoside, quercetin and afzelin. The leaves also reported to show antifungal and antibacterial activity.[8-11]

The leaves are used as a remedy for diarrhoea, colds, and coughs. Due to its antimicrobial and antioxidant properties, diarrhoea and dysentery are treated with bark. The seeds have febrifuge, diuretic, and aperient properties. The bark is febrifuge, astringent, and antibacterial. Dental surgery makes use of bark chicle. The bark is employed as a tonic, and diarrhoea and paludism are treated with a decoction of the bark.[12]

Modern allopathic anxiety medications have a history of adverse effects and poor results. As a result, there is a need to seek safe herbal sources that might aid with anxiety. In light of the foregoing, the current study will use animal models to evaluate the anti-anxiety properties of an ethanolic extract of *Manilkara zapota* L. leaves.

MATERIALS AND METHODS

Chemicals: Diazepam, Monobasic phosphate buffer, Trisodium phosphate buffer (PH 7.0), Normal saline, L tyramine plus, L pargyline, 4-aminoantipyrine, and Horseradish peroxidase purchased from **Sigma-Aldrich®** products, India. The remaining reagents were of analytical grade.

Collection and Authentication of plant

Leaves of *Manilkara zapota L.* were collected from a local garden in Chitradurga, Karnataka. The fresh leaves were inspected for damage and then dried in the shade. Once dried, the leaf material was ground into a coarse powder using an electric grinder. Ms. Niveditha B.T., Assistant Professor at Jyanagangothri PG Centre, GR Halli, Davanagere University, Chitradurga, Karnataka. 577502, identified and authenticated the leaf material.

Preparation of Plant Extract[13]

Manilkara zapota L. leaves were collected, cleaned, shade-dried at room temperature for seven days, and pulverized. The 300 grams of powdered leaves were subjected to Soxhlet extraction using 70% ethanol solvent in a 1:10 w/v ratio at 60^0C. Concentrated under reduced pressure and stored in an airtight container for further use. The percentage yield of the corresponding extract was calculated.

Preliminary phytochemical screening[14,15]

Preliminary phytochemical investigations were carried out on the ethanolic extract of *Manilkara zapota* L. leaves for the detection of various phytochemicals by using standard methods prescribed in practical pharmacognosy by C K Kokate and R K Khandelwal.

Experimental Animals

Animal ethical clearance was obtained from the Institution Animal Ethics Committee (IAEC) for experimental purposes **(Ref No: 3E/SJMCP/IAEC/Sept. 2023/2022-23)**. Healthy Adult Wistar Albino rats weighing about 150-200g of either sex was used for this study. The animals were obtained from Biogen Laboratory Animal Facility, Bangalore – 562107. Before the initiation of the experiment, the animals were acclimatized for 10 days and randomized under standard environmental conditions such as temperature (26 ± 2^0C), relative humidity (45-55%), and 12hrs light/dark cycle maintained as per Committee for Control and Supervision of Experiments on Animal (CCSEA) guidelines. All the animals were allowed free access to standard laboratory pellets and drinking water ad libitum under strict hygiene conditions.

Acute oral toxicity of *Manilkara zapota* L. ethanolic leaves[16]

Acute toxicity OECD (TG 423) test guideline for testing of chemicals. Albino mice (females) fasted overnight but were allowed access to water *ad. libitum*. Animals were randomly divided into four groups (n=3). The control received water. Group I-IV were orally treated

with test material at doses of 5, 50, 300 and 2000 mg/kg respectively the animals were observed at 15, 30, 60, 120 and 240 minutes with no intake of food and water and thereafter for 24 h. The mice were further observed for 14 days with food and water intake.

Table No. 01: Experimental design.[17]

Sl. No	Groups	Treatment
1	Control	Standard diet and water *ad libitum.*
2	Standard	Diazepam 1mg/kg p.o for 28days.
3	Test group I	Low dose of EEMZ p.o for 28 days. (1/20 LD50 value of Acute oral toxicity)
4	Test group II	High dose of EEMZ. p.o for 28 days. (1/10 LD50 value of Acute oral toxicity)

The animals were divided into five groups with six rats each: (n=6).

Behavioral parameters assessment

1. Elevated plus maze[18]

Elevated plus maze is a commonly used behavioural assay to determine anti-amnesic activity. An elevated plus maze test was performed according to Handley and Mithani. The elevated plus maze consists of 4 arms (2 open arms and 2 closed arms) attached at a junction (central platform). The height of the plus maze was raised to 50 cm above the ground. Animals of each group control, Positive control, standard, and test were treated with normal saline, standard drug (Diazepam 1 mg/kg), and test extracts of EEMZ at 100mg/kg and 200mg/kg respectively. Rats were placed at the junction of four arms, facing toward an open arm. The number of entries and time spent in each arm were recorded for 5mins. An increase in open-arm activity reflects anti-amnesic behaviour. The apparatus was cleaned with alcohol in between the trials. Precautions are taken to maintain a noise-free environment.

2. Open field model[17]

An open-field model/ test was performed according to Hall. The apparatus consisted of a wooden box (60 × 60 × 60 cm). The floor of the box was divided into 16 squares (15 × 15 cm). Animals of each group control, Positive control, standard, and test were treated with normal saline, (Diazepam 1 mg/kg), and test extracts of EEMZ at 100mg/kg and 200mg/kg respectively. After 30mins, animals were placed individually in one corner square. The number of rearing, assisted rearing (forepaws touching the walls of the apparatus), and number of squares crossed were counted for 5mins. Increasing in square crossing indicates locomotory activity.

1. Hole board model.

The hole board was a wooden box measuring 60 x 60 x 30 cm, featuring four 2 cm diameter holes evenly spaced on the floor. After treatment, each animal was placed at the center of the field, and locomotion was recorded for 20 minutes. The number of head dips and the time spent head-dipping were measured for 10 minutes. To eliminate odour cues, the hole board was cleaned with a 10% alcohol solution after each trial. Head dipping was defined as the animal's head entering the holes up to eye level. Animals of each group control, Positive control, standard, and test were treated with normal saline, (Diazepam 1 mg/kg), and test extracts of EEMZ at 100mg/kg and 200mg/kg respectively. An increase in head dips and duration indicates greater exploratory activity, while a decrease suggests sedative behavior. An anxiolytic-like state is shown by increased head-dipping.

2. Rota rod model[19]

The assessment of motor coordination was conducted using the rota-rod model. This model consists of a stable base platform and a non-slip rotating rod with a diameter of 3 cm, which is divided into five equal sections. This design effectively evaluates motor coordination skills. During a training session, animals were pre-selected based on their ability to remain on the rod (rotating at 12 rpm) for 2 minutes. Animals from each group—Control, Positive Control, Standard, and Test—were treated with either normal saline, Diazepam (1 mg/kg), and test extracts of EEMZ at doses of 100 mg/kg and 200 mg/kg, respectively. The animals were placed on the rod, which was set to rotate at 20 rpm, and the time until they fell off was automatically recorded. The total time spent on the apparatus was observed for 5 minutes (300 seconds). The apparatus was thoroughly cleaned with alcohol between trials.

Biochemical assessment of brain homogenate includes the following

1. Monoamine oxidase-A assay[20]

Animals were anesthetized with i.p. injection of pentobarbital sodium at a dose of 50 mg/kg body weight after the completion of the behavioural tests. Brain was dissected and homogenized in ice-cold 100 mmol/L potassium phosphate buffer, pH 7.4 with a glass Potter-Elvehjem homogenizer. The homogenates were incubated with 500 mmol/L tyramine plus 500 nmol/L pargyline to inhibit MAO-B activity. The chromogenic solution prepared in the assay contained vanillic acid (1 mmol/L), 4-aminoantipyrine (500 mmol/L), and horse radish peroxidase (4 IU/mL) in potassium phosphate buffer (0.2 mol/L, pH 7.6). The absorbance was measured spectrophotometrically at 490 nm. The MAO-A activity was then determined

and expressedas mmol/min g tissue.

1. Lipid peroxidation[20]

Malondialdehyde (MDA) level was determined following the method outlined by Satoh. 75 mg of Thiobarbituric acid (TBA) is dissolved in 15% trichloroacetic acid (TCA). To this, 2.08 ml of 0.2 N HCl was added. Using 15% TCA, the final volume was increased to 100 ml. Then, 0.75 ml of brain homogenate was mixed with 3.0 ml of this reagent. The test tubes were kept in a boiling water bath for 15 min. Then it was cooled and centrifuged for 10 min at 10,000 rpm. The absorbance of the supernatant is read against the blank at 535 nm. The results were expressed in mol/mg of protein.

CALCULATION

$$Conc.of\ MDA = \frac{Abs\ 532 \times 100 \times VT}{(1.56 \times 105) \times WT \times VU}$$

- Abs_{532} is absorbance
- V_T is total volume of mixture (4ml)
- 1.56×10^5 is molar extinction co-efficient
- W_T is weight of dissected brain
- V_U is aliquot volume (1 ml).

STATISTICAL ANALYSIS

The data obtained from the above findings was subjected to statistical analysis using one-way ANOVA followed by Tukey's Kramer Multiple Comparison Test to assess the statistical significance of the result.

RESULTS

The percentage yield of ethanolic extract: **15.16%.** Colour and consistency: Dark green and Crystal.

Preliminary phytochemical screening

Preliminary phytochemical screening of EEMZ leaves confirms the presence of alkaloids, glycosides, flavonoids, tannins, steroids, saponins, triterpenes, resins, phenols, and carbohydrates.

Acute Oral Toxicity Studies (LD50)

The acute oral toxicity studies were conducted following OECD Guidelines 423. The

ethanolic extract of *Manilkara zapota* L (EEMZ) did not cause any deaths in animals when administered at a dose of 2000 mg/kg body weight. As a result, 1/20th of 2000 mg/kg (100 mg/kg) is considered a low dose, and 1/10th of 2000 mg/kg (200 mg/kg) is considered a high dose.

Behavioral Results Elevated plus maze model

The ethanolic extract of *Manilkara zapota* L. leaves was Evaluated for anxiety activity in an elevated plus maze model. The result indicated that, as evidenced by increased entries and time spent in closed arms, along with reduced time in open arms. In contrast, the Diazepam-treated group (2 mg/kg) and EEMZ (100 mg/kg & 200mg/kg) showed a significant increase in the number of entries & duration of time spent in open arm as well as it showed a reduction in the number of entries & duration of time spent in the closed arm when compared to a control group.

The administration of Diazepam led to a significant increase (****P < 0.0001) in the number of entries and time spent in the open arms of the maze. This suggests that Diazepam has anxiolytic properties. Additionally, the high dose of EEMZ (200 mg/kg) resulted in a notable improvement (****P < 0.0001) compared to the low dose (100 mg/kg) against the control group. Importantly, the effects increased each week, demonstrating a dose-dependent response and a consistent improvement in anxiolytic-like behaviour throughout the trial. The effect of EEMZ on cognitive behaviour was evaluated by using an elevated plus maze model. The results are presented in **Tables 01, 02, 03, and 04,** and in **Figures 1, 2, 3 &** 4.

Table No. 1: Effect of EEMZ on the Number of entries in the Open arm by EPM.

Group	Treatment	Number of entries in Open-arm (Counts/5min)			
		1st Week	2nd Week	3rd Week	4th Week
Group I	Control	3.00 ± 0.36	2.83 ± 0.30	2.66 ± 0.49	3.00 ± 0.57
Group II	Standard (Diazepam)	7.00 ± 0.73 ***	9.83 ± 1.10 ***	11.00 ± 1.03 ****	12.5 ± 1.17 ****
Group III	Low Dose of EEMZ (100mg/kg)	4.16 ± 0.60^{ns}	5.33 ± 0.84^{ns}	4.66 ± 0.55^{ns}	5.5 ± 0.42^{ns}
Group IV	High Dose of EEMZ (200mg/kg)	5.83 ± 0.47 **	7.66 ± 1.45 **	8.83 ± 1.40 ***	10.5 ± 1.83 ***

Values were expressed as Mean ± SEM (n=6); Significance values are: ****P < 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05 and ns P > 0.05. Control group vs all groups. (By one-way ANOVA followed by Tukey-Kramer Multiple comparison tests).

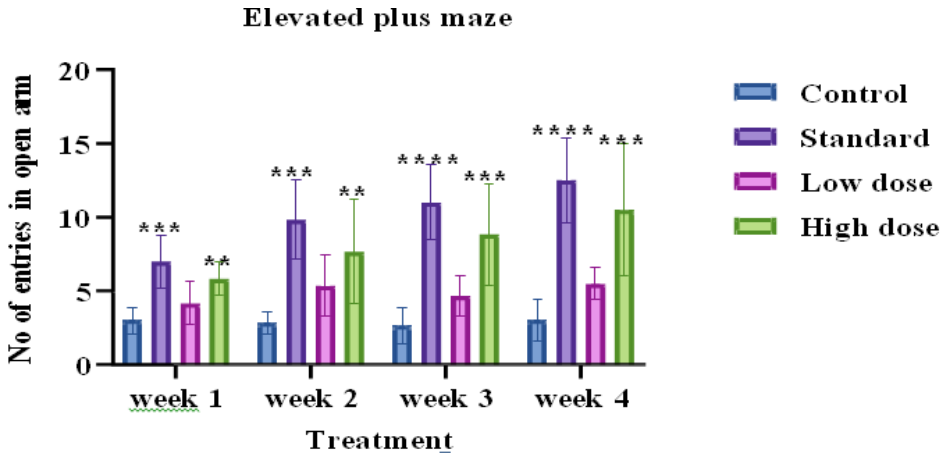

Figure No. 1: Effect of EEMZ on the Number of entries in the Open arm by EPM.

Table No. 2: Effect of EEMZ on the Time spent in the Open arm by EPM.

Group	Treatment	Time spent in Open arm (Counts/5min)			
		1st Week	2nd Week	3rd Week	4th Week
Group I	Control	17.83 ± 2.08	44.33 ±3.49	41.16 ± 4.82	48.66 ± 4.08
Group II	Standard (Diazepam)	117.33 ± 10.97****	122.17 ± 8.83****	156.83 ± 7.79****	164.67 ± 6.55****
Group III	Low Dose of EEMZ (100mg/kg)	39.33 ± 5.37ns	71.83 ± 9.05*	74.33 ± 5.17**	81.83 ± 4.85**
Group IV	High Dose of EEMZ (200mg/kg)	66.83 ± 6.17***	89.16 ± 6.82**	95.33 ± 3.69****	108.33 ± 6.10****

Values were expressed as Mean ± SEM (n=6); Significance values are: ****P < 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05 and ns P > 0.05. Control group vs all groups. (By one-way ANOVA followed by Tukey-Kramer Multiple comparison tests).

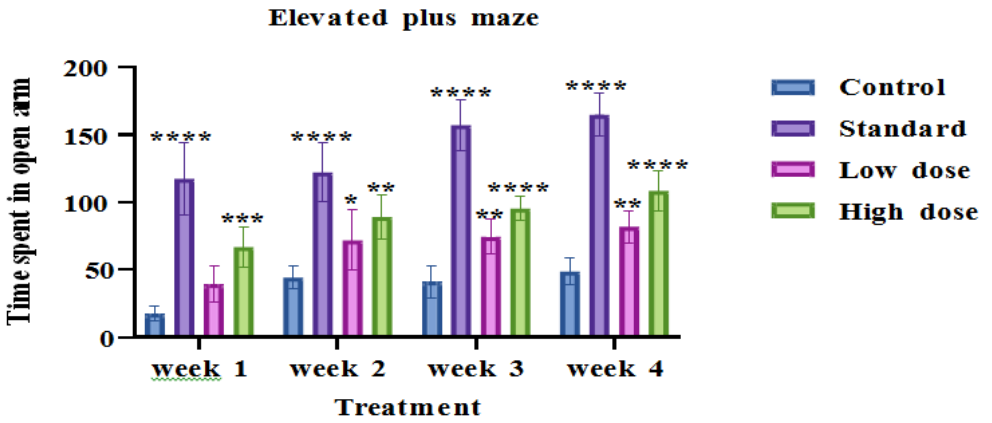

Figure No. 2: Effect of EEMZ on the Time spent in the Open arm by EPM.

Table No. 3: Effect of EEMZ on the Number of entries in the Closed arm by EPM.

Group	Treatment	Number of entries in Closed arm (Counts/5min)			
		1st Week	2nd Week	3rd Week	4th Week
Group I	Control	11.00 ± 1.31	10.33 ± 2.04	9.16 ± 1.85	9.16 ± 1.66
Group II	Standard (Diazepam)	5.16 ± 0.90**	2.83 ± 0.60***	2.66 ± 0.49***	2.66 ± 0.33***
Group III	Low Dose of EEMZ (100mg/kg)	9.00 ± 0.93^{ns}	6.66 ± 0.95^{ns}	5.5 ± 0.71^{ns}	6.00 ± 0.57^{ns}
Group IV	High Dose of EEMZ (200mg/kg)	7.33 ± 1.60	4.66 ± 0.42**	4.83 ± 0.47*	4.33 ± 0.76**

Values were expressed as Mean ± SEM (n=6); Significance values are: ****P < 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05 and ns P > 0.05. Control group vs all groups. (By one-way ANOVA followed by Tukey-Kramer Multiple comparison tests).

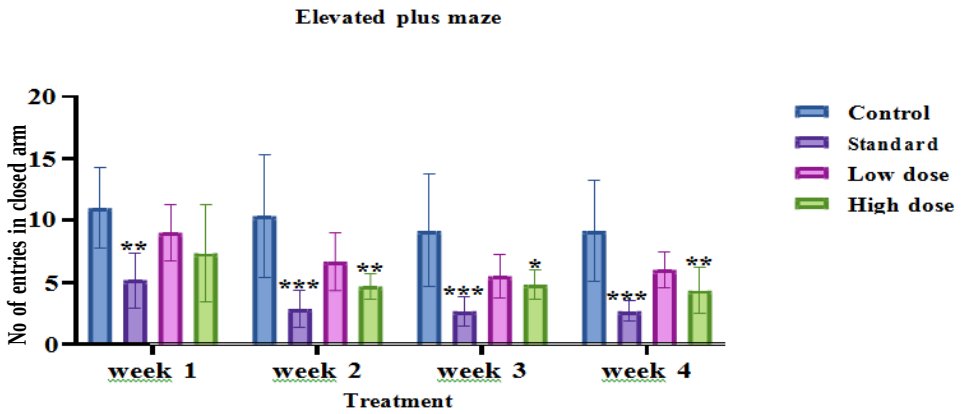

Figure No. 3: Effect of EEMZ on the Number of entries in the Closed arm by EPM.

Table No. 4: Effect of EEMZ on the Time spent in the Closed arm by EPM.

Group	Treatment	Time spent in Closed arm (Counts/5min)			
		1st Week	2nd Week	3rd Week	4th Week
Group I	Control	200.83 ± 14.83	196.2 ± 18.27	204.16 ± 14.41	205.33 ± 13.363
Group II	Standard (Diazepam)	113.00 ± 8.14****	77.16 ± 6.45****	73.33 ± 7.05****	101.16 ± 8.25****
Group III	Low Dose of EEMZ (100mg/kg)	168.00 ± 8.65^{ns}	162.17 ± 8..91^{ns}	160.83 ± 10.53*	165.83 ± 10.31*
Group IV	High Dose of EEMZ (200mg/kg)	144.17 ± 9.86**	143.00 ± 8.83*	136.50 ± 4.13***	128.16 ± 5.33****

Values were expressed as Mean ± SEM (n=6); Significance values are: ****P < 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05 and ns P > 0.05. Control group vs all groups. (By one-way ANOVA followed by Tukey-Kramer Multiple comparison tests).

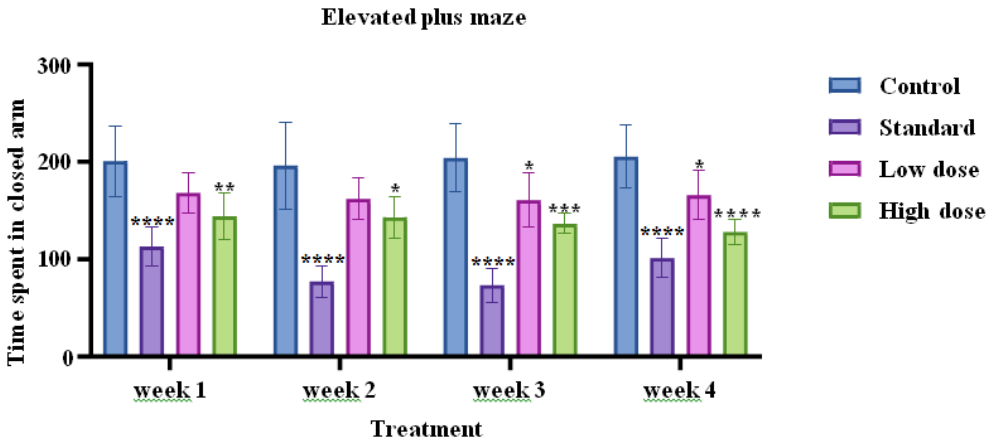

Figure No. 4: Effect of EEMZ on the Time spent in the Closed arm by EPM. Open field model.

In open field apparatus, locomotion (rearing, assisted rearing) and exploratory (number of squares traversed i.e., central, and peripheral). Locomotion and exploratory activities of Positive control group were compared with standard group (Donepezil 2.5 mg/kg), low dose of EEPD (200 mg/kg) and high dose of EEPD (400 mg/kg). Donepezil treated group shows highly significance value (***P<0.001) increase in locomotion and exploratory activity compared to remaining four group of animals. In a low dose of EEPD (200 mg/kg) it shows less significance value (*P<0.05) when compare with control group. In a high dose of EEPD (400 mg/kg) it shows moderately increase in significance value (**P<0.01) when compare with Positive control group. Dose dependent activity was showed by animals in open field model. Results shown in Fig no:04, Table no:05.

The group administered with Diazepam demonstrated a highly significant increase in locomotor and exploratory activities (***P < 0.001) compared to the other groups, indicating robust anxiolytic effects. The high dose of EEMZ (200 mg/kg) resulted in a gradual increase in locomotor and exploratory activity, albeit less pronounced than the Diazepam group and displaying diminishing effects over time. In contrast, the high dose of EEMZ (200 mg/kg) elicited a gradually significant increase in activity (**P < 0.01) compared to the control and low dose of EEMZ (100 mg/kg) group. These findings merit further consideration and exploration for comprehensive understanding. Throughout the experimental period, significance levels progressively increased over the four weeks in the high dose of EEMZ (200 mg/kg) and the gradual development of anxiolytic responses compared to control and low dose of EEMZ (100 mg/kg). The results are presented in **Tables 05, 06, 7, and 8,** and **Figures 5, 6, 7 & 8.**

Table No 5: Effect of EEMZ leaves in Open field model in 1st Week.

Group	Treatment	Rearing	Assisted rearing	Number of squares traversed in 5 mins
Group I	**Control**	4.5 ± 0.76	3.66 ± 0.88	81.83 ± 3.21
Group II	**Standard group (Diazepam 1 mg/kg)**	12.66±1.02 ****	12.5 ± 1.76 ***	110 ± 1.46 ****
Group III	**Low dose of EEMZ (100 mg/kg)**	4.33 ± 0.95ns	4.5 ± 1.33ns	78.5 ± 4.72ns
Group IV	**High dose of EEMZ (200 mg/kg)**	8.16 ± 2.56*	8.33 ± 0.88*	94.66± 3.95*

Values were expressed as Mean ± SEM (n=6); Significance values are: ****P < 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05 and ns P > 0.05. Control group vs all groups. (By one-way ANOVA followed by Tukey-Kramer Multiple comparison tests).

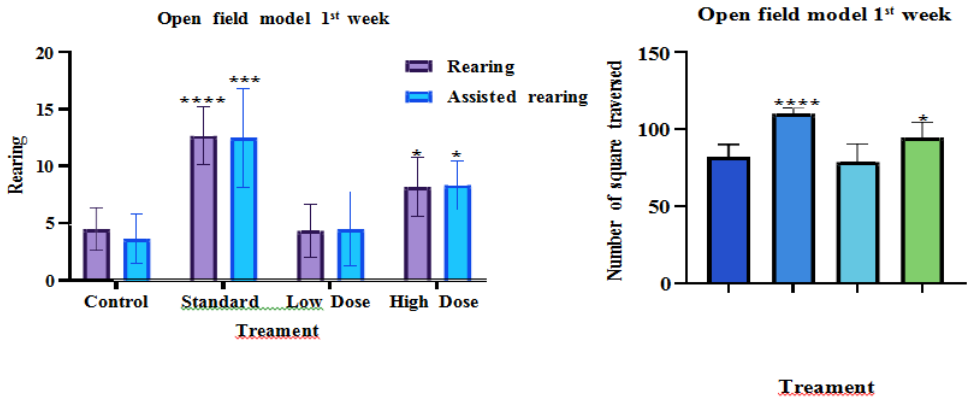

Figure No. 5: Effect of EEMZ leaves in Open field model in 1st Week.

Table No 6: Effect of EEMZ leaves in Open field model in 2nd Week.

Group	Treatment	Rearing	Assisted Rearing	Number of squares traversed in 5 mins
Group I	**Control**	**4.83 ± 0.60**	**4.66 ± 0.55**	**81.83 ± 83**
Group II	**Standard group (Diazepam 1 mg/kg)**	**10.5 ± 0.99*** **	**11.66± 1.38 **** **	**110.00 ± 1.46**** **
Group III	**Low dose of EEMZ (100 mg/kg)**	**4.66 ± 0.76ns**	**5.5 ± 0.61ns**	**79.00± 3.41ns**
Group IV	**High dose of EEMZ (200 mg/kg)**	**8.5 ± 0.88***	**8.66 ± 0.88***	**100.83 ± 3.41*** **

Values were expressed as Mean ± SEM (n=6); Significance values are: ****P < 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05 and ns P > 0.05. Control group vs all groups. (By one-way ANOVA followed by Tukey-Kramer Multiple comparison tests).

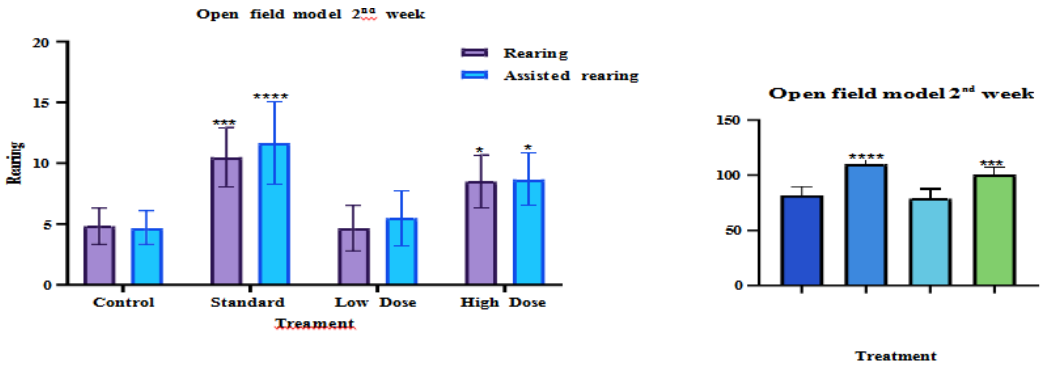

Figure No. 6: Effect of EEMZ leaves in Open field model in 2nd Week.

Table No 7: Effect of EEMZ leaves in Open field model in 3rd Week.

Group	Treatment	Rearing	Assisted Rearing	Number of squares traversed in 5 mins
Group I	**Control**	4.83 ± 0.60	4.66 ± 0.55	81.83 ± 83
Group II	**Standard group (Diazepam 1 mg/kg)**	10.5 ± 0.99***	11.66± 1.38 ****	110.00 ± 1.46****
Group III	**Low dose of EEMZ (100 mg/kg)**	4.66 ± 0.76^{ns}	5.5 ± 0.61^{ns}	79.00± 3.41^{ns}
Group IV	**High dose of EEMZ (200 mg/kg)**	8.5 ± 0.88*	8.66 ± 0.88*	100.83 ± 3.41***

Values were expressed as Mean ± SEM (n=6); Significance values are: ****P < 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05 and ns P > 0.05. Control group vs all groups. (By one-way ANOVA followed by Tukey-Kramer Multiple comparison tests).

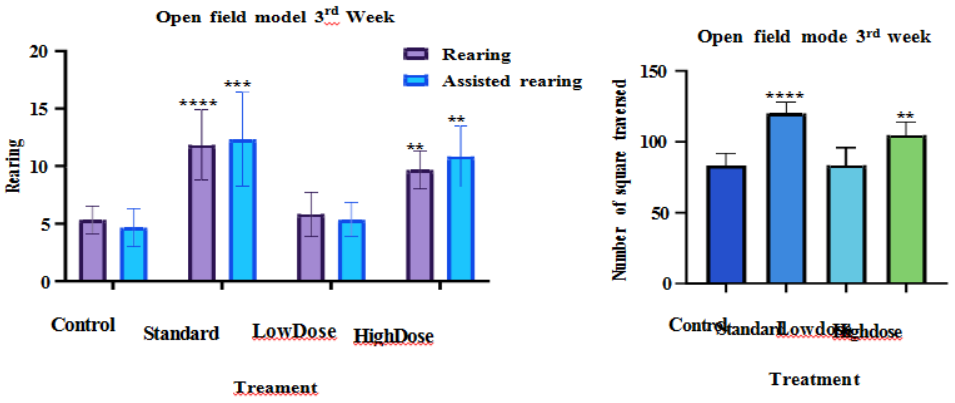

Figure No 7: Effect of EEMZ leaves in Open field model in 3rd Week

Table No 8: Effect of EEMZ leaves in Open field model in 4th Week.

Group	Treatment	Rearing	Assisted rearing	Number of squares traversed in 5 mins
Group I	**Control**	5.16 ± 0.79	2.5 ± 0.42	76.16 ± 2.21
Group II	**Standard group (Diazepam 1 mg/kg)**	13 ± 1.06****	15.5 ± 1.38****	126.67 ± 3.44****
Group III	**Low dose of EEMZ (100 mg/kg)**	7.16 ± 0.74^{ns}	6.16 ± 0.60*	82.5 ± 5.13^{ns}
Group IV	**High dose of EEMZ (200 mg/kg)**	10.16 ± 0.70**	12.16 ± 1.07****	106.17 ± 5.8**

Values were expressed as Mean ± SEM (n=6); Significance values are: ****P < 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05 and ns P > 0.05. Control group vs all groups. (By one-way ANOVA followed by Tukey-Kramer Multiple comparison tests).

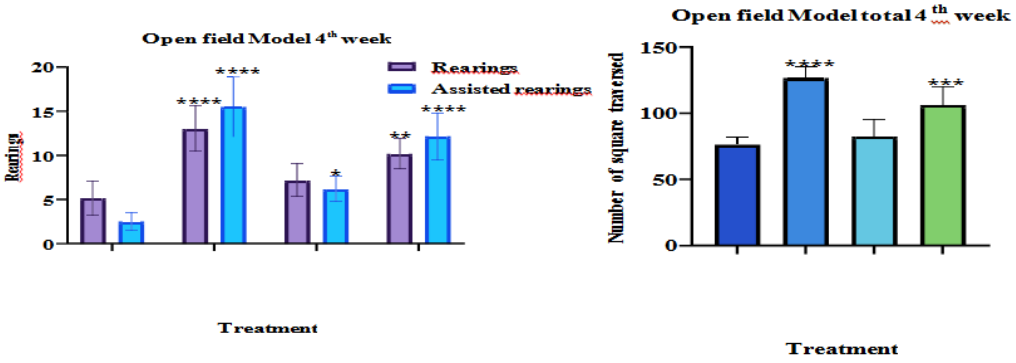

Figure No 8: Effect of EEMZ leaves in Open field model in 4th Week.

1. **Evaluation of the anxiolytic potential of *Manilkara zapota* L leaves in Hole board apparatus**

The anxiolytic activity of the ethanolic extract of Manilkara zapota L Leaves (EEMZ) was tested using the Hole Board model in Wistar albino rats of either sex, weighing between 150-200g. The experiment lasted for 28 days, and behavioural assessments were conducted weekly, starting from the 7th day. The rats' exploratory behaviour was evaluated by measuring their head-dipping activity.

In the experiment, the rats were categorised into four groups, namely a control group. This standard group received Diazepam at 1 mg/kg, a low-dose EEMZ group at 100 mg/kg, and a high-dose EEMZ group at 200 mg/kg. It was observed that in the standard group, Diazepam led to a significant increase in the number of head dips (***P < 0.001), indicating a substantial anxiolytic effect in comparison to the other groups.

In the group that received a high dose of EEMZ (200 mg/kg), there was a slight but statistically significant increase in head dips (**P < 0.01) compared to the low dose of EEMZ (100 mg/kg) against the control group.

The anxiolytic effect in the high-dose EEMZ group improved progressively throughout the four weeks, with the significance level increasing gradually each week. By the final assessment, the results from the high-dose group demonstrated a more pronounced anxiolytic effect, indicating an increase in efficacy over time. The results are presented in **Table No. 9** and **Figure No. 9.**

Table No 9: Effect of EEMZ leaves in Hole Board Modal.

Group	Treatment	Number of head dipping in 5 mins			
		1st Week	2nd Week	3rd Week	4th Week
Group I	Control	4.16 ± 0.65	3.83 ± 0.70	4.16 ± 1.04	2.66 ± 0.42
Group I	Standard (Diazepam 1mg/kg)	10.16 ± 0.70***	12.83 ± 1.55****	14.66 ± 1.66****	18.16 ± 3.04****
Group III	Low Dose of EEMZ (100mg/kg)	6.33 ± 0.98^{ns}	6.00 ± 0.77^{ns}	6.5 ± 0.76^{ns}	8.5 ± 0.88^{ns}
Group IV	High Dose of EEMZ (200mg/kg)	7.66 ± 0.88*	7.66 ± 0.76*	8.5 ± 0.99*	12.66 ± 1.66**

Values were expressed as Mean ± SEM (n=6); Significance values are: ****P < 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05 and ns P > 0.05. Control group vs all groups. (By one-way ANOVA followed by Tukey-Kramer Multiple comparison tests).

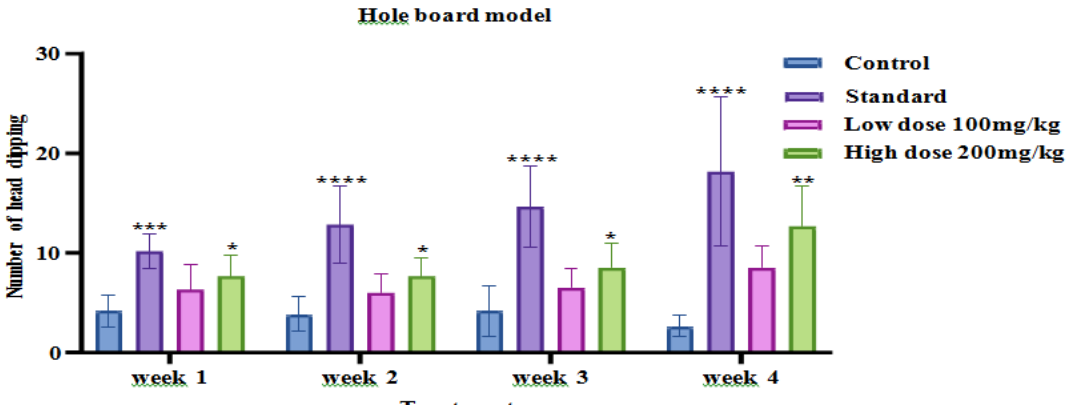

Figure No 9: Effect of EEMZ leaves in Hole Board Modal Rota rod model.

Muscle grip strength was assessed using a rota-rod apparatus, with mean fall-off time as the measure of muscular rigidity. The control group exhibited reduced fall-off time, indicating

muscle incoordination. However, standard group, treated with Diazepam (1 mg/kg), exhibited a highly significant reduction in locomotor activity and motor coordination (****P < 0.0001) compared to the other groups, indicating a pronounced anxiolytic effect. In the low-dose EEMZ group (100 mg/kg), a slight but significant reduction in locomotor activity was observed (*P < 0.05) compared to the control group. The high-dose EEMZ group (200 mg/kg) showed a moderately significant reduction in motor coordination (***P < 0.001) compared to the control group.

Importantly, the anxiolytic effect in the high-dose EEMZ group increased progressively over the 28 days, with the significance of the results improving gradually each week. By the final week, the high-dose group demonstrated a more substantial anxiolytic effect, indicating a time-dependent enhancement of motor coordination improvement. The results are presented in **Table No. 10** and **Figure No. 10**.

Table No 10: Effect of EEMZ on muscle grip strength by Rota-rod model.

Group	Treatment	Fall off time in 5 mins (Sec)			
		1st Week	2nd Week	3rd Week	4th Week
Group I	Control	140.00 ± 8.26	138.83 ± 3.44	136.16 ± 5.06	126.33 ± 2.48
Group II	Standard (Diazepam 1mg/kg)	93.00 ± 3.51****	83.83 ± 4.12****	77.00± 5.79****	75.00 ± 2.95****
Group III	Low Dose of EEMZ (100mg/kg)	122.5 ± 7.37ns	118.66 ± 5.66*	117.00 ± 4.64*	113.16 ± 3.53*
Group IV	High Dose of EEMZ (200mg/kg)	112.33 ± 3.70*	114.66 ± 5.55**	111.33 ± 3.85**	104.66 ± 4.70***

Values were expressed as Mean ± SEM (n=6); Significance values are: ****P < 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05 and ns P>0.05. Control group vs all groups. (By one-way ANOVA followed by Tukey-Kramer Multiple comparison tests).

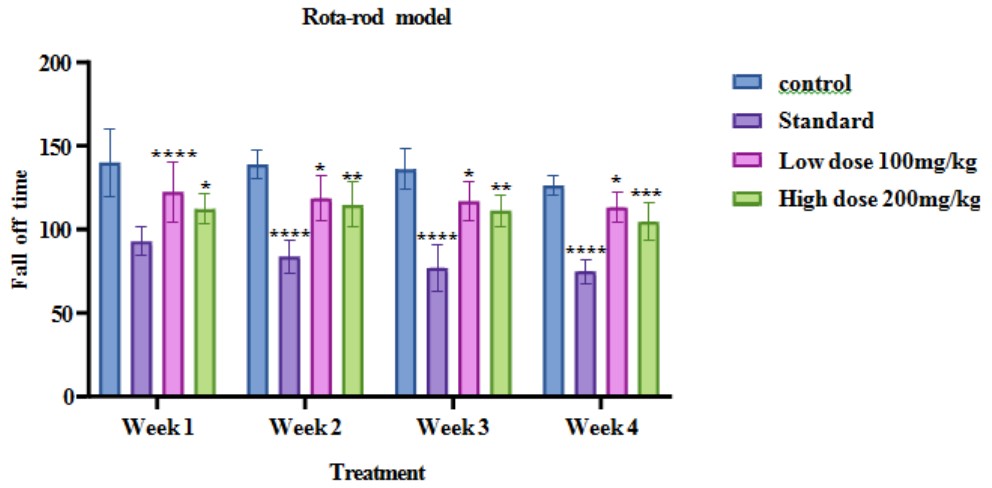

Figure No. 10: Effect of EEMZ on muscle grip strength by Rota-rod apparatus.

Biochemical assessment of brain homogenate

1. Monoamine oxidase-A assays

The evaluation of the anxiolytic potential of the ethanolic extract *Manilkara zapota* L. leaves was conducted over 28 days, during which the animals were divided into four groups: a control group, a standard group treated with Diazepam (1 mg/kg), a low-dose group (100 mg/kg of extract), and a high-dose group (200 mg/kg of extract). On the 29th day, a monoamine oxidase (MAO) assay was performed as a key biochemical parameter to assess the anxiolytic effects of the extract.

Monoamine oxidase is an enzyme responsible for the breakdown of neurotransmitters such as serotonin, dopamine, and norepinephrine, which are directly related to mood regulation and anxiety. Elevated levels of MAO activity have been linked to increased anxiety and depression, as these neurotransmitters are metabolized more rapidly, reducing their availability in the brain. Therefore, inhibiting MAO activity can lead to higher levels of these neurotransmitters, promoting anxiolytic (anxiety-reducing) effects.

The results of the MAO assay revealed a slightly anxiolytic effect of the ethanolic extract of *Manilkara zapota* L. leaves. The standard group, treated with Diazepam, exhibited the highest level of significance in reducing MAO activity (***$P < 0.001$), confirming its potent anxiolytic action. This was followed by the high-dose group (200 mg/kg), which showed a mild significant reduction (*$P < 0.05$) in MAO activity compared to the control and low-dose group (100 mg/kg).

The results suggest that the ethanolic extract of *Manilkara zapota* L. leaves exhibits mild

anxiolytic properties, particularly at higher doses. These high dose of EEMZ are linked to a slight inhibition of monoamine oxidase (MAO) activity, which may result in increased levels of neurotransmitters that help reduce anxiety in the brain. The results are presented in **Table No. 11** and **Figure No. 11.**

Table No. 11: Effect of EEMZ on Monoamine oxidase-A assays.

Group	Treatment	µmol/min/mg tissue
Group I	Control	2.223±0.049
Group II	Standard Diazepam (1mg/Kg)	1.215±0.082***
Group III	Low dose of EEMZ (100mg/Kg)	2.029 ± 0.240^{ns}
Group IV	High dose of EEMZ (200mg/Kg)	1.673±0.084*

Values were expressed as Mean ± SEM (n=6); Significance values are: *P < 0.05, **P< 0.01 and ***P < 0.001, ^{ns}P>0.05. Control group vs all groups. (By one-way ANOVA followed by Tukey-Kramer Multiple comparison tests).

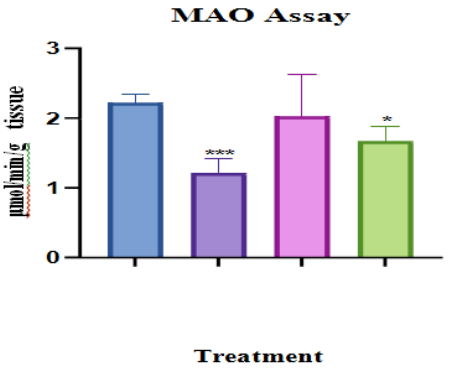

Figure No. 11: Effect of EEMZ on Monoamine oxidase-A assays.

Lipid peroxidation assay

Malondialdehyde (MDA) is a reliable indicator of peroxidation. Increase in free radical causes over production of MDA this was determined by reactive brain homogenate sample with Thiobarbituric acid (TBA) in lipid peroxidation assay. Scopolamine-induced cognitive impairment may lead to increased ROS production or decreased antioxidant defenses in the brain, resulting in oxidative stress. This oxidative stress can damage lipids, leading to the production of malondialdehyde (MDA), which is a marker of lipid peroxidation.

The results of the lipid peroxidation assay showed a moderate reduction in the high dose of EEMZ (200 mg/kg) in oxidative stress. The standard group (Diazepam) exhibited the greatest reduction in lipid peroxidation levels (**P < 0.01), indicating its potent anxiolytic and antioxidant effects. This was followed by the high-dose group (200 mg/kg), which showed a

moderately significant reduction (**P < 0.01), and the low-dose group (100 mg/kg), which displayed a slight but notable reduction (*P < 0.05) when compared to the control group.

These findings suggest that the ethanolic extract of *Manilkara zapota* L. leaves not only exerts anxiolytic effects but also plays a role in reducing oxidative stress, as evidenced by decreased lipid peroxidation. This effect indicates that higher doses of the extract are mildly effective in combating oxidative stress, which may contribute to its anxiolytic potential. The results are presented in **Table No. 12** and **Figure No. 12.**

Table No. 12: Effect of EEMZ on Lipid Peroxidation Assay.

Group	Treatment	MDA (nmol /mg of protein)
Group I	Control	6.582±0.0.926
Group II	Standard Diazepam (1mg/Kg)	3.358±0.121**
Group III	Low dose of EEMZ (100mg/Kg)	4.088±0.281*
Group IV	High dose of EEMZ (200mg/Kg)	3.712±0.692**

Values were expressed as Mean ± SEM (n=6); Significance values are: *P < 0.05, **P< 0.01 and ***P < 0.001. Control group vs all groups. (By one-way ANOVA followed by Tukey-Kramer Multiple comparison tests).

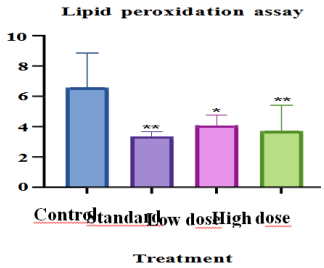

Figure No. 12: Effect of EEMZ on Lipid peroxidation.

DISCUSSION

The 300 gm of shade-dried powdered leaves underwent Soxhlet extraction with 70% ethanol (1:10 w/v) at 60^0C, yielding 45.5 grams of extract (15.16% yield). Phytochemical tests confirmed the presence of alkaloids, glycosides, flavonoids, tannins, steroids, saponins, phenols, and carbohydrates. The EEMZ extract was evaluated for acute oral toxicity following OECD guidelines 423. Notably, no mortality was observed at a 2000 mg/kg dose. As a result, 100 mg/kg and 200 mg/kg were selected as screening doses for further studies. The current research aims to evaluate the anxiolytic effects of the ethanolic extract of *Manilkara zapota* L. (EEMZ) leaves on Wistar albino rats against behaviour animal models

for a 28-day treatment period. On the 29th day, biochemical parameters were assessed to further explore the potential mechanisms underlying the anxiolytic properties of *Manilkara zapota* L. The study focused on measuring the activity of brain homogenates for monoamine oxidase (MAO) and lipid peroxidation levels.

Behavioural assessment

Behavioural assessment was carried out on the 7^{th}, 14^{th}, 21^{st} and 28^{th} day using the Elevated plus maze (EPM), Open field, hole board, and Rota rod model. In all these models, the rats treated with different doses of EEMZ (100mg/kg & 200mg/kg) exhibited significant improvements, indicating notable anxiolytic activity.

Elevated plus maze

The elevated plus maze is a well-established model for evaluating anxiety in both male and female rats. This study focused on the anxiolytic properties of the ethanolic extract of *Manilkara zapota* L. by comparing the number of entries and the time spent in the open and closed arms of the maze.

The findings revealed that the extract produced a significant anxiolytic effect, particularly at 200 mg/kg dosage during the fourth week (****P<0.0001), as indicated by an increased duration spent in the open arms. The lower dose of 100 mg/kg also showed some effect (**P<0.01), while the diazepam group exhibited the most pronounced anxiolytic response. The extract demonstrated significant anxiolytic effects, especially at a higher dosage than the control group.

Open field model

The open-field apparatus is a model used to investigate anxiolytic activity. In the open field apparatus, locomotion (number of squares traversed i.e., central and peripheral) and exploratory behaviour (rearing and assisted rearing) were observed. In the open field test, it was observed that the standard group (Diazepam 1mg/kg) spent significantly more time exploring and moving around when compared to the control group, with a statistical significance of ****P<0.0001. Additionally, the high dose of EEMZ (200 mg/kg) resulted in a notable increase in exploration and locomotion over four weeks, with a significance of **P<0.01, while the low dose of EEMZ (100 mg/kg) did not show any significant effects.

Hole board model

The hole board model is commonly used to evaluate the anxiolytic behaviour of animals. The number of head dipping by the animals in the standard and extract groups was compared with the control group. There was a highly significant increase in the number of head dipping in the standard group ****P<0.0001 (Diazepam 1 mg/kg) compared to the control group. A low dose of EEMZ (100 mg/kg) also did not show any significance in the number of head dipping. However, a high dose of EEMZ (200 mg/kg) showed mild significance in increasing the number of head dipping compared to the low dose of EEMZ (100 mg/kg) in contrast to the control group.

Rota rod model

The Rota rod test is used to assess peripheral neuromuscular blockade and motor coordination in animals. Performance deficits are likely at 7, 14, 21, and 28 days. Diazepam (1 mg/kg) demonstrated a highly significant effect (****p < 0.0001). The low dose of EEMZ (100 mg/kg) showed no significant effect, while the high dose (200 mg/kg) was moderately significant (***p < 0.001). The low dose also displayed mild significance (*p < 0.05) compared to the control. This suggests that the reduction in spontaneous activity may be due to the muscle-relaxant effect of EEMZ, while its anxiolytic effect was moderate at the high dose.

Furthermore, the results from the previously mentioned models suggest that the moderate calming effects of the extract may be attributed to the presence of various plant compounds. These compounds could potentially act on different receptor types or exhibit varying affinities for the relevant receptors.

The study's results indicate that the high dose of EEMZ (*Manilkara zapota* L. leaf extract) possesses moderate anxiolytic effects. Future studies will need to isolate and identify the phytoconstituents responsible for these observed effects from *Manilkara zapota* L. leaves.

Biochemical assessment

On the 29th day, biochemical parameters were evaluated to further investigate the potential mechanisms underlying the anxiolytic effects of *Manilkara zapota* L. The two main biochemical markers measured were monoamine oxidase (MAO) activity and lipid peroxidation levels.

Monoamine oxidase-A assay

The monoaminergic system, which includes serotonin, norepinephrine, and dopamine, is important for regulating anxiety. Monoamine oxidase (MAO) is an enzyme that breaks down these neurotransmitters. High MAO-A activity can reduce levels of these chemicals, contributing to anxiety and depression. Conversely, lower MAO activity can increase their levels, potentially alleviating anxiety symptoms.[21]

Our study findings indicate that ethanolic extracts of Manilkara zapota L. leaves have the slightly to reduce MAO-A activity after 28 days of dosing, suggesting a possible anxiolytic effect based on the results we observed.

The MAO-A assay showed that the ethanolic extract had a mild anxiolytic effect. Diazepam significantly reduced MAO-A activity (***P < 0.001), confirming its strong anxiolytic properties. The high-dose group (200 mg/kg) also showed a slight reduction in MAO-A activity (*P < 0.05), while the low dose (100 mg/kg) had a non-significant effect, resulting in a slight increase in anxiety-reducing neurotransmitters.

In our study, we found that *Manilkara zapota* L reduces the activity of MAO-A, which is an enzyme that oxidizes serotonin. This reduction in MAO-A activity could potentially enhance the treatment of anxiety.

Lipid peroxidation assay

There were high levels of phospholipids in the cell membrane of the brain which act as substrates for lipid peroxidation which is related to oxidative damage to the tissue. The increase in free radicals damages the cell membrane due to instability in the phospholipids, resulting in cellular toxicity and accumulation of MDA known as lipid peroxidation chain reaction.

The polyunsaturated fatty acids indicate MDA levels, which serve as markers for oxidative cell membrane damage. Elevated MDA levels reflect cellular toxicity and increased lipid peroxidation, often linked to oxidative stress that can adversely affect brain function and contribute to neuroinflammation and neurodegeneration associated with anxiety disorders.23 The standard group treated with Diazepam showed a significant decrease in MDA levels (**P < 0.01), while the high dose of EEMZ (200 mg/kg) showed a moderate reduction (**P < 0.01), and the low dose (100 mg/kg) showed lesser significance (*P < 0.05) compared to the

control group. These findings suggest reduced brain tissue damage in the treated groups. Recent evidence indicates a strong link between anxiety disorders and oxidative stress in both humans and animals, impacting anxiety-related behavior. Brain tissue is particularly susceptible to oxidative damage due to high oxygen consumption, leading to excessive production of free radicals. The presence of vulnerable polyunsaturated fatty acids in neuronal membranes and neurotoxic metabolites further exacerbates this issue. An imbalance between reactive oxygen species and antioxidative protection results in cellular dysfunction and neuronal death. Our study shows that treatment with Manilkara zapota L. effectively reduces MAO-A levels and lipid peroxidation (MDA) in brain tissue, promoting neuronal survival.[22,23]

Flavonoids, major bioactive compounds present in *Manilkara zapota* L, have been reported on the antioxidation function via radical scavenging capacity on MAO-A and MAD. Therefore, it could be suggested the anxiolytic potential of *Manilkara zapota* L. Leaves possibly acts via the antioxidant defence mechanism and preferentially modulates the brain's serotoninergic system specifically in the brain.

We have shown that the extract from *Manilkara zapota* L. leaves has a maximal effect at a dosage of 200 mg/kg body weight in reducing anxiety. This suggests that *Manilkara zapota* L. could be beneficial for treating patients with anxiety disorder. However, additional research is required to uncover its active constituents and precise mechanisms of action.

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