



# Anxiolytic activity of hydroalcoholic *Manilkara zapota* (Linn.) leaf extract in Swiss albino mice

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## ABSTRACT

The current research assessed the anxiolytic activity of the methanol extract of leaves of *Manilkara zapota* (MEMZ) using behavioural models and neurotransmitter estimation in mice. The preliminary phytochemical screening showed the presence of alkaloids, flavonoids, saponins, triterpenoids, glycosides, and tannins in the extract. The results obtained in the elevated plus maze model showed that MEMZ (100 and 200 mg/kg) significantly ( $p < 0.01$ ) increased the number of entry and time spent in open arms and decreased in the closed arms, indicating dose- and time-dependent anxiolytic effects. MEMZ (200 mg/kg) also produced a significant decrease in the anxiety index (AI), with AI decreasing from 0.45 to 0.10 by the end of the 21-day experimental duration in comparison to the standard anxiolytic Diazepam (2 mg/kg,  $p < 0.05$  vs. control). In the light-dark transition model, the MEMZ-treated group spent a greater time in the light chamber, implying the treatment reduced anxiety, similar to the Diazepam group. Neurochemical analysis of brain homogenates showed a significant and dose-dependent increase in  $\gamma$ -aminobutyric acid (GABA) and a corresponding decrease in serotonin levels in mice receiving MEMZ treatment. The dose of 200 mg/kg MEMZ increased GABA to 34.42  $\mu\text{g/ml}$  and reduced serotonin to 44.52  $\mu\text{g/ml}$  levels, values not significantly different from Diazepam ( $p > 0.05$ ). These results offer support for the potential use of MEMZ leaf extract as a natural anxiolytic. Additional studies are necessary to ascertain its mechanism of action and clinical utilization.

## 1. INTRODUCTION

Anxiety is defined as a state of intense uneasiness, uncertainty, and fear resulting from the prediction of a threatening event or situation, often to a degree that normal physical and psychological functioning is disrupted. Anxiety disorder is the feeling of apprehension, uncertainty, or tension of an imagined or unreal threat [1]. Anxiety disorder is increasingly recognized as a highly prevalent and chronic disorder with onset in teenage years, with an incidence of 18.1% and a lifetime prevalence of 28.8% [2].

Benzodiazepines are currently the most often prescribed medications for anxiety disorders that act as positive allosteric modulators of  $\gamma$ -aminobutyric acid (GABA)-A receptors, which enhance chloride ion influx and neuronal inhibition, producing anxiolytic, sedative, and muscle relaxant effects. However, the clinical usage of these drugs is constrained by their negative effects [1]. Despite its relative safety, benzodiazepines can have side effects like forgetting, dependency, sedation, tolerance, misuse potential, psychomotor impairment, and potentiation of other central nervous system (CNS) depressants, which are quite worrying [3].

Nature has given us abundant fauna and flora with various biological activities. The Sapotaceae family's methanol extract of leaves of *Manilkara zapota* (MEMZ) is one of the most important tropical fruit crops grown in India. Originally from tropical South America, this crop has now naturalized in

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tropical areas around the Indian coast and is frequently regarded as indigenous to the area. In addition, the Caribbean, Thailand, the Maldives, Vietnam, Sri Lanka, Bangladesh, Pakistan, Indonesia, and other locations cultivate MEMZ. It is a well-known commercial crop that is widely grown in Malaysia, Sri Lanka, India, and Indonesia. The world's largest producer of sapodilla, known locally as sapota or chikoo, is India. Sapota is a fruit that is known for its exceptional qualities with diverse biological activities, including antioxidant, anti-inflammatory, antimicrobial, antidiabetic, anticancer, hepatoprotective, and cardioprotective effects [4].

The use of traditional natural products as anxiolytics for the management of neurological disorders is gaining a lot of interest, which can show fewer side effects and a fast onset of action [2]. MEMZ, commonly known as sapodilla, chickoo, or sapota, belongs to the family Sapotaceae [5]. It is an evergreen, glabrous tree, 8–15 m in height [6]. Lupeol acetate, oleanolic acid, apigenin-7-O-L-rhamnoside, myricetin-3-O-L-rhamnoside, and caffeic acid are the primary components found in sapodilla leaves [7]. Phytochemicals found in MEMZ make it one of the most significant plant species used to cure a variety of diseases [8].

Triterpenoids and flavonoids were shown to exhibit antioxidant, anti-inflammatory, antipyretic, antidiabetic, anti-aging, antilipidemic, and acaricidal activities in the bark and seeds of this plant [5]. The polyphenolic chemicals found in MEMZ fruit have substantial antioxidant, antihyperglycemic, and hypercholesterolemic properties [6]. The bark and fruit of MEMZ were used to isolate several triterpenoids that had anticancer properties [7]. Both the seed coverings and the seeds themselves had tyrosinase inhibitory and antibacterial properties [9]. Although the phytochemical profile of MEMZ leaves is promising, their anxiolytic potential has yet to be evaluated scientifically. Previous literature has primarily described the antioxidant, antimicrobial, and anticancer activities of the fruit, bark, and seeds, but has not explored potential effects in any neuropsychiatric disorder. *Manilkara zapota* leaves are rich in phytoconstituents such as quercetin, myricetin, apigenin, and triterpenoids like lupeol and ursolic acid, many of which have been reported to modulate the GABA-A receptor and exert anxiolytic effects [10,11].

The objectives of this research were to conduct a phytochemical investigation, to determine the anxiolytic activity of the methanolic extract of MEMZ leaves by using an elevated plus maze (EPM) and light-dark model. It also aimed to assess GABA and serotonin levels in the brain through biochemical estimation. Addressing these key objectives has yielded a study that is the first to provide evidence to support the anxiolytic role of MEMZ leaves and support that MEMZ leaves may be an important novel source of plant-based anxiolytic agents.

## 2. MATERIALS AND METHODS

### 2.1. Extraction method

The leaves of MEMZ were collected from the local area of Sangli. Authentication of the plant was carried out at Kasturbai Walchand College, Sangli, and deposited with voucher specimen number KWC/Herb/243/24.

The leaves of MEMZ were cleaned, shade-dried, and ground into a coarse powder. Twenty grams of the powdered leaves were extracted using 100 ml of methanol as the solvent at a controlled temperature of 60°C–65°C for 6 hours, completing 14 cycles using a Soxhlet apparatus. The obtained methanolic extract was then filtered, dried, and concentrated at room temperature using a rotary evaporator. Prior to use in animal studies, the dried extract was reconstituted in appropriate concentrations using distilled water.

### 2.2. Experimental design

Swiss albino mice are used in anxiety studies because they are easy to handle and exhibit stable, well-characterized anxiety-like behaviours in established models [12]. Swiss albino mice of both sexes, weighing between 18 and 24 g, were obtained for the study. The animals were maintained in standard laboratory conditions, in a room maintained at a temperature of 22°C ± 2°C, and at a relative humidity of 55% ± 5% with a 12 hours light/dark cycle and maintained with ad libitum food and water. The study protocol was approved by the Institutional Animal Ethics Committee (IAEC) of Appasaheb Birnale College of Pharmacy, Sangli, Maharashtra, India, constituted in accordance with the Control and Supervision of Experiments on Animals (CSEA), Government of India (Protocol No. IAEC/ABCP/01/2022–23).

As per OECD guidelines, the mice were randomly assigned to one of four groups of six animals (3 males and 3 females) and dosed orally for 21 consecutive days as presented in Table 1. Since the methanolic extract of MEMZ was determined to be safe at a dose of 2,000 mg/kg, hence the doses of 100 and 200 mg/kg were taken for evaluation. The animals underwent behavioural assessments on days 1st, 7th, 14th, and 21st, to assess anxiolytic activity, and to ensure the absence of sedative effects.

### 2.3. Experimental models

The anxiolytic effects of a MEMZ leaf extract were assessed utilizing two standard exteroceptive behavioural assessments: the EPM and the Light-Dark Transition (LDT) tests.

#### 2.3.1. EPM model

The EPM apparatus (Fig. 1) consisted of two open arms (16 × 5 cm) and two closed arms (16 × 5 × 12 cm), with the four arms arranged in a plus shape and elevated 25 cm above the platform. Each mouse was placed in the centre of the maze, facing one of the two open arms, to be observed individually; thus, animals were only tested once, and were tested at the same time on each testing day. Behavioural parameters recorded during a 5 minutes session included the number of entries and time spent in open and closed arms, along with the calculated anxiety index (AI). AI is calculated as follows:

$$\text{AI} = \text{Time spent in closed arms} / \text{Total time spent in all arms}$$

Testing was carried out on 1, 7, 14, and 21 days of the treatment application, under low-light conditions [13–15]. The EPM underwent thorough cleaning after each test session in order to eliminate possible olfactory cues.

### 2.3.2. LDT model

The cage measuring  $21 \times 42 \times 25$  cm was used in the study. The length of the compartment was divided equally in order to create the light and dark compartments with a small connecting hole (3 cm high  $\times$  5 cm wide). The light compartment had a high-intensity light placed at an angle, while the dark compartment received dim lighting to create a contrast. The number of transitions between the two compartments, as well as the time spent in each compartment, were recorded during a 10 minutes assessment of anxiety-related behaviour [16].

All groups of animals were assigned both the EPM and LDT on the same respective day, with exactly 1 hour in between the two tasks. First, the EPM, followed by the LDT after 1 hour.

### 2.4. Biochemical estimation of neurotransmitters from the brain

All of the neurochemical measurements shown represent values to a brain region (hippocampus, amygdala, stratum), obtained from  $n = 6$  euthanized animals per group, pooled per animal, and processed as a single averaged sample; results are expressed as mean  $\pm$  standard error of the mean (SEM).

**Table 1.** Experimental design for evaluation of the anxiolytic activity of methanolic extract of *MEMZ* leaves.

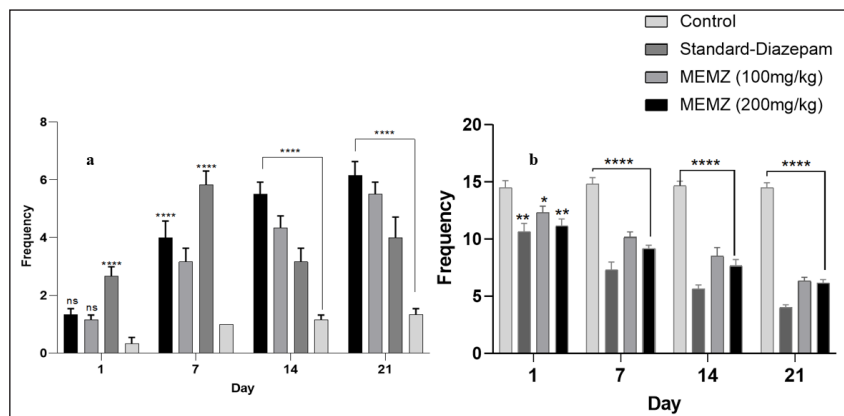
Sr. No.	Groups	Treatment	Route of administration
1	Group 1	Control- 1% Gum Acacia P. O for 21 days	Orally
2	Group 2	Standard drug- Diazepam (1 mg/kg P.O) for 21 days	Orally
3	Group 3	Methanolic extract of <i>MEMZ</i> leaves (100 mg/kg P.O) for 21 days	Orally
4	Group 4	Methanolic extract of <i>MEMZ</i> leaves (200 mg/kg P.O) for 21 days	Orally

### 2.4.1. Estimation of GABA level

The level of GABA was estimated by multiple development paper chromatography methods. A GABA calibration curve was created by paper chromatography followed by UV-Visible spectrophotometry. A series of standard GABA solutions (10–80  $\mu\text{g/ml}$ ) were prepared in 0.1N 80% ethanol, derivatized, and read using a UV-Visible spectrophotometer at 515 nm. Further, 1 ml of the brain homogenate supernatant was evaporated to dryness in an oven at 70°C, and the residue was then reconstituted in 100 ml of distilled water. A standard solution of GABA at a concentration of 2  $\mu\text{M}$ , along with the sample, is spotted on Whatman no. 1 chromatography paper using a micropipette. It was placed in a chamber containing butanol: acetic acid: and water (12:3:5 v/v) as solvent. The solvent front was withdrawn and dried when it reached the top of the paper. Similar procedures are followed for a second run, following which the papers are dried, sprayed with ninhydrin reagent, and placed in an oven at 100°C for 4 minutes. The portions, which carry GABA corresponding with the standard, are cut and eluted with 0.005%  $\text{CuSO}_4$  in 75% ethanol. Their absorbance is read against a blank at 515 nm in a spectrophotometer and using statistical graph was plotted with corresponding values [17,18].

### 2.4.2. Estimation of serotonin

A calibration curve for serotonin was constructed using standards (10–80  $\mu\text{g/ml}$ ). The fluorescence intensity was quantified after derivatization, with spectrophotometric analysis, and plotted against concentration for the regression equation and  $R^2$  value. Further, the freshly isolated brain tissues were weighed and homogenized (5 ml HCl-butanol (50–50) together for 1 minute, and the homogenates were then centrifuged for 10 minutes at 2,000 rpm. For the supernatant, 1 ml was pipetted into a centrifuge tube that contained 2.5 ml of heptane and 0.31 ml of 0.1 M HCl. The tube was shaken vigorously for 10 minutes and centrifuged at 2,000 rpm, and the contents were separated into upper and lower phases. The upper organic layer was discarded, and 0.2 ml was taken from the bottom aqueous



**Figure 1.** Effect of methanolic extract of *MEMZ* leaves on frequency of (a) open arm (b) close arm entries in EPM. Values are expressed as (Mean  $\pm$  SEM) and  $n = 6$ . The significance level for the data was \*\*\*\*  $p < 0.0001$ , \*\*\*  $p < 0.001$ , \*\*  $p < 0.01$ , ns (non-significant)  $p > 0.0$  when compared with control group by two-way ANOVA followed by Dunnett's test.

phase for the estimation of 5-hydroxytryptamine. All operations were conducted at 0°C (to preserve the sample) [17,18].

To 0.2 ml of the aqueous extract, 0.25 ml of O-phthalaldehyde (OPT) reagent (the OPT reagent was prepared by dissolving 20 mg OPT in 100 ml of concentrated HCl) was added. The reaction mixture was heated at 100°C for 10 minutes to form the fluorescent product. After cooling the reaction mixture until thermally equilibrated with the surrounding environment, fluorescence readings were taken using a photo fluorimeter with an excitation wavelength of 360 nm and an emission wavelength of 470 nm. Tissue blanks were prepared by adding 0.25 ml of concentrated HCl with no OPT reagent. An internal standard was prepared by mixing HCl and butanol in a 1:2 volume 500 µg/ml concentration of serotonin dissolved in distilled water.

### 2.5. Statistical analysis

The data were expressed as mean ± SEM. Biochemical parameters and behavioural data obtained from the study were statistically analysed using two-way analysis of variance (ANOVA) followed by Dunnett's *post hoc* test using GraphPad Prism. The significance level for the data was \*\*\*\*  $p < 0.0001$ , \*\*\*  $p < 0.001$ , \*\*  $p < 0.01$ , ns (non-significant)  $p > 0.0$  when compared with the control group.

## 3. RESULTS

### 3.1. Qualitative analysis

The methanolic extract of MEMZ leaves tested positive for various phytochemical constituents such as alkaloids, flavonoids, saponins, glycosides, and so on [19–22]. Considering the phytochemical richness, the extract may have significant therapeutic potential.

### 3.2. EPM model

#### 3.2.1. Frequency of entries

Figure 1a summarizes the effects of the methanolic extract of MEMZ leaves on the number of entries into the open arm in the EPM). Both doses of MEMZ (100 and 200 mg/kg) produced a progressive and significantly greater number of entries than the control starting on Day 7. On Day 1, there were no significant differences between any of the treatments. By Day 7, there was a significant increase in open arm entries in the 100 and 200 mg/kg treatment with frequency of showed  $3.16 \pm 0.47$  and  $4.00 \pm 0.57$ , respectively (\*\*\*\*  $p < 0.0001$ ). By Day

14 and 21, the 100 and 200 mg/kg treatment open arm entries were similar and significantly greater than all other groups ( $5.5 \pm 0.42$  and  $6.16 \pm 0.47$  entries, respectively, \*\*\*\*  $p < 0.0001$ ). From the responses observed so far, MEMZ has potential anxiolytic activity that appears to be dose- and time-dependent.

Figure 1b represents that the methanolic extract of MEMZ leaves exhibited a progressive decline in closed-arm entries over time and increasing dose level, suggesting decreasing anxiety-related behaviour. MEMZ at doses of 100 and 200 mg/kg caused a decrease of 15% and 23% in entries on Day 1, respectively. The standard diazepam group exhibited a larger decrease with a total of  $10.66 \pm 0.71$  entries (\*\*  $p < 0.01$ ). On Day 7, the MEMZ at the 100 and 200 mg/kg decreased to  $10.16 \pm 0.471$  (a decrease of 32%) and  $9.16 \pm 0.30$  (a decrease of 38%). Day 14 showed further decline in 100 and 200 mg/kg group entries to  $8.5 \pm 0.76$  and  $7.66 \pm 0.55$ , respectively, while the diazepam-treated mice exhibited an average of only  $5.66 \pm 0.33$  (61 percent decrease) closed-arm entries. By Day 21, the behaviour of the MEMZ 100 and 200 mg/kg groups was almost stable, whereas the diazepam group amounted to the least with an average of  $4.0 \pm 0.25$ . From these results, it is demonstrated that MEMZ produces an anxiolytic effect that is similar to the active control in a progressively administered dose-dependent paradigm, through the reductions in closed-arm entries over time.

#### 3.2.2. Duration of time spent in open and closed arms

The EPM test results show that the methanolic extract MEMZ leaves produces anxiolytic effects that are dose- and time-dependent (Table 2). On Day 1, both MEMZ doses (100 and 200 mg/kg) slightly increased the time spent in the open arms compared to the control group, which indicates some initial mild anxiolytic behaviour. The time spent in open arms increased steadily over the 21-day treatment period. On Day 21, the control group spent on average 8.66 seconds in the open arm as opposed to the standard drug diazepam, which had a significantly greater time, reaching more than 83 seconds. The MEMZ doses at 100 and 200 mg/kg also showed a gradual increase over the study time to equal 49 and 60.5 seconds in the open arms accordingly. When compared to the control group, that represent 6.5 times greater or approximately 7 times greater for MEMZ treatments. It clearly illustrates that the extract is anxiolytic in a dose-response and time-response manner.

In addition, MEMZ treatment for both doses showed a decrease in closed arm time. The 100 mg/kg dose reduced closed arm time to  $97.16 \pm 3.26$  seconds by Day 21, and the

**Table 2.** Effect of methanolic extract of MEMZ leaves extract on time spent (seconds) in open and close arm in EPM.

Group	Day 1		Day 7		Day 14		Day 21	
	Open	Close	Open	Close	Open	Close	Open	Close
Control	$2.33 \pm 1.49$	$214.16 \pm 6.24$	$4.33 \pm 0.33$	$204.16 \pm 2.85$	$6.5 \pm 0.84$	$200.16 \pm 3.16$	$8.66 \pm 1.25$	$186 \pm 2.14$
Standard	$25.66 \pm 1.05$	$175.66 \pm 7.02$	$60.16 \pm 2.10$	$111.66 \pm 3.06$	$73.83 \pm 2.08$	$90.66 \pm 1.56$	$83 \pm 2.12$	$78.33 \pm 4.46$
MEMZ (100 mg/kg)	$7.16 \pm 0.87$	$183.16 \pm 2.85$	$30.83 \pm 2.05$	$133.5 \pm 1.60$	$40.16 \pm 1.49$	$119.16 \pm 1.49$	$49 \pm 3.75$	$97.16 \pm 3.26$
MEMZ (200 mg/kg)	$10.16 \pm 1.30$	$153.33 \pm 4.79$	$38.33 \pm 2.41$	$123.16 \pm 3.54$	$54.83 \pm 1.30$	$106.83 \pm 3.61$	$60.5 \pm 1.91$	$90.83 \pm 4.64$

Values are expressed as (Mean ± SEM) and  $n = 6$ . All values are  $p < 0.0001$  statistically significant when compared with control group by Two-way ANOVA followed by Dunnett's test. ns is considered as non-significant.

200 mg/kg dose resulted in  $90.83 \pm 4.64$  seconds of closed arm time. The decreased time spent in the closed arms, along with the increased time spent in the open arms, indicates that the extract produced an attenuation of anxiety-like behaviour and the potential use of the MAN extract as an anxiolytic treatment. As indicated, the 200 mg/kg dose has greater efficacy as an anxiolytic treatment with a longer duration of treatment.

### 3.2.3. Anxiety index

The data in Table 3 indicate that the methanolic extract of MEMZ leaves produced a dose-dependent reduction in AI over 21 days. Although the control group exhibited little to no increase in AI over the 21-day duration of the study, the standard treatment group (Diazepam) experienced a significant and gradual decline in AI scores over time reaching extremely low AI score of 0.02 on day 21. Both doses of MEMZ (100 and 200 mg/kg) significantly reduced the AI compared to the control group throughout the study period; however, the 200 mg/kg exhibited a greater decline in AI from 0.45 on day 1 to 0.10 on day 21. These results of MEMZ suggest similar anxiolytic activity, particularly at higher doses, but to be slightly less potent compared to the standard treatment, Diazepam.

## 3.3. LDT model

### 3.3.1. Time spent in light chamber

The methanolic extract of MEMZ significantly increased the amount of time spent in the light chamber, and this was both dose- and time-dependent (Fig. 2a). Overall, the animals that were treated with MEMZ, particularly at a dose of 200 mg/kg, increased exploration of the light chamber from Day 1 to 21, which indicates that MEMZ reduced anxiety-like behaviours.

**Table 3.** Effect of methanolic extract of MEMZ leaves on AI.

Group	Day 1	Day 7	Day 14	Day 21
Control	0.98	0.98	0.95	0.93
Standard- Diazepam	0.30	0.10	0.07	0.02
MEMZ (100 mg/kg)	0.54	0.30	0.26	0.20
MEMZ (200 mg/kg)	0.45	0.22	0.13	0.10

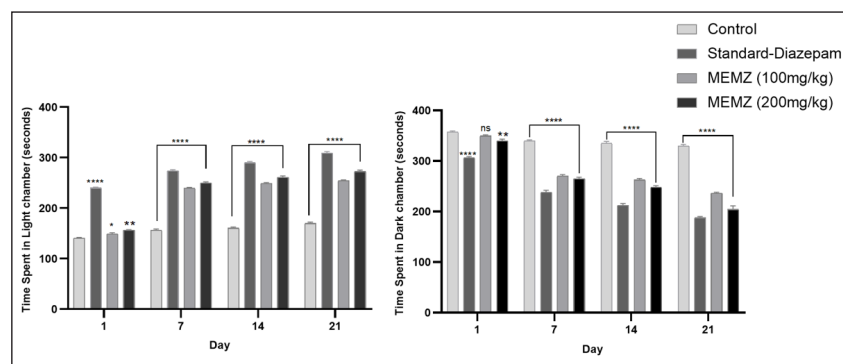
The only condition that showed an increase in time spent in the light chamber that exceeded control (the reference standard was Diazepam) was with MEMZ at 100 and 200 mg/kg. The time spent in the light chamber demonstrated to be statistically different from the control groups ( $****p < 0.0001$ ). The increase in time spent in the light chamber is indicative of increased exploratory behaviour and reduced aversion to bright locations, which suggests further evidence for anxiolytic properties.

### 3.3.2. Time spent in dark chamber

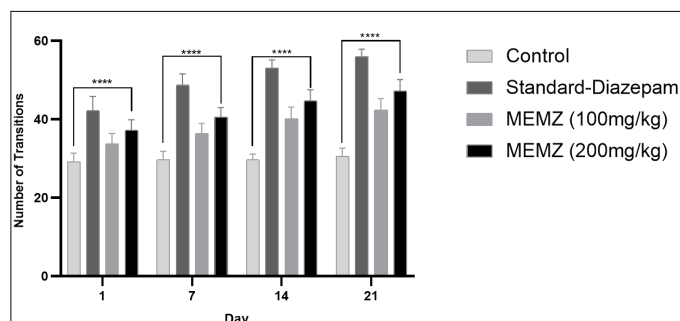
In contrast to the light chamber, MEMZ treatment resulted in a significant decrease in the amount of time spent in the dark chamber and further embeds support for the anxiolytic action of the treatment ( $*p < 0.05$  to  $****p < 0.0001$ ). The control conditions remained the same overall and always needed to spend more time in the dark chamber, which demonstrates the natural preference they felt to remain in the dark enclosed spot due to anxiety. However, the MEMZ treatment groups, particularly the MEMZ 200 mg/kg treatment, significantly decreased ( $****p < 0.0001$ ) the occupancy of the dark chamber over the 21-day period, with similar patterns and degree of reduction demonstrated as with Diazepam. The decrease in the amount of time spent in the dark chamber supports the extract's ability to decrease anxiety-like behaviours in animals (Fig. 2b).

### 3.3.3. Number of transitions

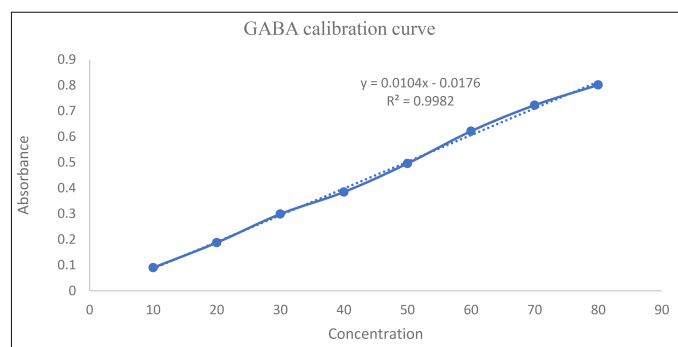
Figure 3 shows that the methanolic extract of MEMZ leaves increased the number of transitions between the light and dark chambers in both dose- and time-dependent manners, indicating increased exploration and decreased anxiety. In the control, the number of transitions was nearly constant throughout the experiment, while Diazepam resulted in significantly greater transitions from Day 1 to 21 ( $****p < 0.0001$ ). MEMZ at 100 mg/kg resulted in a statistically significant increase by Day 14 and 21; however, the MEMZ 200 mg/kg dose resulted in more robust transitions and increases that were highly statistically significant from Day 7 onward ( $****p < 0.0001$ ). These results demonstrate that MEMZ improves locomotor activity and decreases anxiety-like behaviour in this animal model at 100 and 200 mg/kg doses, with the greatest improvements at the higher MEMZ dose.



**Figure 2.** Effect of methanolic extract of MEMZ leaves on time spent (Seconds) in (a) light chamber (b) Dark chamber in LDT model. Values are expressed as (Mean  $\pm$  SEM) and  $n = 6$ . The significance level for the data was  $****p < 0.0001$ ,  $***p < 0.001$ ,  $**p < 0.01$ , ns (non-significant)  $p > 0.0$  when compared with control group by Two-way ANOVA followed by Dunnett's test.



**Figure 3.** Effect of methanolic extract of *MEMZ* leaves on number of transitions between light and dark chamber in LDT model.



**Figure 4.** Standard calibration curve of GABA.

### 3.4. Neurotransmitter level estimation

#### 3.4.1. Level of GABA in brain tissue homogenate in mice

The calibration curve of GABA (in terms of Absorbance) with good linearity ( $R^2 = 0.9982$ ) across the concentration range of 10–80  $\mu\text{g/ml}$  is shown in Figure 4, indicating a strong linear relationship between absorbance and concentration. Table 4 illustrates that the methanolic extract of MEMZ significantly elevated the GABA levels present in the brain homogenate from the mice. The low GABA concentration (14.50  $\mu\text{g/ml}$ ) in the control group treated with gum acacia (1% w/v) supports the potential anxiolytic activity of MEMZ. The standard drug, Diazepam (1 mg/kg), showed the highest concentration of GABA (46.47  $\mu\text{g/ml}$ ) investigated, and validated its previously established site of action by indicating GABA enhancement. Notably, the GABA levels at the lower (100 mg/kg) dose of MEMZ (29.55  $\mu\text{g/ml}$ ) and higher (200 mg/kg) dose (34.42  $\mu\text{g/ml}$ ) increased significantly in comparison to the control ( $p < 0.0001$ ). The dose-dependent increase of GABA levels further supports the potential mechanism of action of MEMZ, which may provide anxiolytic activity through the facilitation of GABAergic neurotransmission by the brain, in the same way Diazepam produces its actions.

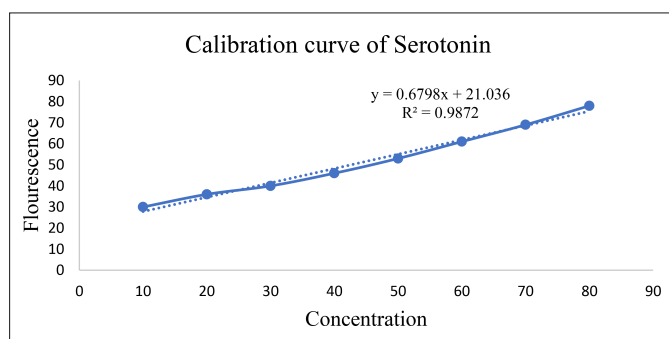
#### 3.4.2. Results of serotonin estimation

The calibration curve of serotonin (in terms of Fluorescence) across the range of 10–80  $\mu\text{g/ml}$  confirms the linear relationship with  $R^2 = 0.9872$  (Fig. 5). This curve was used to estimate the amount of serotonin in brain homogenates.

**Table 4.** Effect of methanolic extract of *MEMZ* leaves on level of GABA in brain homogenate in mice.

Groups	Dose, route of administration	GABA ( $\mu\text{g/ml}$ ) (mean $\pm$ SEM)
Control	1% Gum Acacia P. O	14.5008 $\pm$ 0.1447
Standard-Diazepam	1 mg/kg P. O	46.46721 $\pm$ 0.2951****
MEMZ (100 mg/kg)	100 mg/kg P. O	29.55229 $\pm$ 0.3609****
MEMZ (200 mg/kg)	200 mg/kg P. O	34.42217 $\pm$ 0.4869****

Values are expressed as (Mean  $\pm$  SEM) and  $n = 6$ . The significance level for the data was \*\*\*\*  $p < 0.0001$ , when compared with control group by Two-way ANOVA followed by Dunnett's test.



**Figure 5.** Standard calibration curve of serotonin.

**Table 5.** Effect of methanolic extract of *MEMZ* leaves on level of serotonin in brain homogenate in mice.

Groups	Dose, route of administration	Serotonin ( $\mu\text{g/ml}$ ) (mean $\pm$ SEM)
Control	1% Gum Acacia P. O	68.08 $\pm$ 1.41
Standard-diazepam	1 mg/kg P. O	33.47 $\pm$ 0.61
MEMZ (100 mg/kg)	100 mg/kg P. O	53.49 $\pm$ 1.43
MEMZ (200 mg/kg)	200 mg/kg P. O	44.52 $\pm$ 0.98

Values are expressed as (Mean  $\pm$  SEM) and  $n = 6$ .

As observed in Table 5, administering the methanolic extract of MEMZ leaves showed a dose-dependent reduction in serotonin in a homogenate taken from the brain of mice. The control group had the most serotonin (68.08  $\mu\text{g/ml}$ ), while administering Diazepam, the standard anxiolytic, established a consistent connection to lowered serotonin (33.47  $\mu\text{g/ml}$ ). Administering MEMZ at 100 mg/kg and MEMZ at 200 mg/kg induced a reduction in serotonin to 53.49 and 44.52  $\mu\text{g/ml}$ , respectively. The results show potential anxiolytic properties based on the reduction in serotonin levels in the brain homogenation and supports the conclusion that MEMZ may be contributing to its anxiolytic action by changing levels of serotonin, like Diazepam.

## 4. DISCUSSION

Leaves from *M. zapota* have shown significant potential pharmacological utility as a result of their substantial antioxidant and anti-inflammatory activities exhibited in many

studies [23]. In one study, the acetonetic extract of MEMZ leaves demonstrated potent DPPH and superoxide radical scavenging activities, surpassing the standard antioxidants [24]. In another study, the ethanolic extract not only showed potent antioxidant effects in the *in vitro* and *in vivo* models (isoprenaline-induced myocardial damage) but also exhibited hepatoprotective effects against liver injury induced by CCl<sub>4</sub> in rats [25]. Further, in another study, the ethyl acetate extract inhibited phospholipase A2 (PLA2) and the 5-lipoxygenase (5-LOX) enzymes, but the authors did not claim to show inhibition of 5-LOX, and the high concentrations of MEMZ and bold claim of PLA2 inhibition, noted that it reduced carrageenan-induced paw edema in rats [26]. It was reported that the ethanolic extract of MEMZ demonstrated a dose-dependent inhibition in a protein denaturation model with anti-arthritis activity [27]. This presents an opportunity to explore the therapeutic abilities of M leaf extracts and support investigations on its bioactive component(s).

Much literature supports that phytoconstituents in plants contribute significantly to the treatment of disorders within the CNS, which in this plant includes alkaloids, flavonoids, phenolic compounds, tannins, and saponins. MEMZ leaves include phytochemicals that have the potential to execute an anxiolytic action. Their anxiolytic activity had not been documented earlier; therefore, the present study was designed to investigate the anxiolytic potential of the methanolic extract of MEMZ leaves.

In the present study, the EPM results showed that MEMZ-treated rats demonstrated substantial increases in the number of entries and time spent in the open arms, while showing reductions in time and percent time spent in the closed arms, signifying a reduction in anxiety-like behaviours. In the Light-Dark Box test, MEMZ treatment resulted in a significant increase in time spent in the light compartment compared to the control group, providing further evidence for its anxiolytic effectiveness. Biochemical evaluation of neurotransmitters showed that MEMZ (200 mg/kg) and the standard treatment diazepam (1 mg/kg) did significantly raise GABA in the brain while lowering serotonin compared to control animals. These neurochemical changes are consistent with the mechanisms for anxiolytic activity since increased GABAergic activity while decreasing activity of serotonin is a common description for anxiolytics.

Phytochemical analysis of MEMZ leaves showed that several flavonoids and phenolics were detected, including apigenin, quercetin, myricetin, apocynin, caffeic acid, and rutin—all of which have previously documented anxiolytic activity. Flavonoids like apigenin are known to be structural analogues to benzodiazepines and have been suggested to exert partial agonism at benzodiazepine-site binder on GABA receptors, thus providing a plausible mechanism for the observed effects [28].

Therefore, the presence of these constituents provides further supporting pharmacology of the MEMZ anxiolytic activity.

## 5. CONCLUSION

The present study demonstrated that MEMZ extract possesses anxiolytic activity. The pattern of change in neurotransmitters was observed to be the same as standard Diazepam. Thus, the results indicate that the methanolic extract of MEMZ leaves exhibits anxiolytic activities, which may be

due to the presence of flavonoids and the possible mechanism through GABAergic and serotonergic pathways. In this study, classical neurotransmitter measurement methods (including paper chromatography and spectrophotometry) were a convenient and easy option, but future scope includes the use of advanced methods (HPLC or LC-MS/MS) for better sensitivity and specificity. Further, a study must be needed in the future for the isolation of phytoconstituent from the MEMZ leaves to find out the exact mechanism of action as an anxiolytic activity.

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## 7. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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## 9. CONFLICT OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

## 10. ETHICAL APPROVALS

Ethical approval details are given in the 'Materials and Methods' section.

## 11. DATA AVAILABILITY

The authors confirm that the data supporting the findings of this study are available within the article.

## 12. PUBLISHER'S NOTE

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## 13. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declare that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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