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Evaluation of the anxiolytic activity of ethyl acetate fraction of *Calotropis gigantea* leaves in rats

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Abstract
Anxiety disorders are a serious threat to public health, affecting a major section of the world's population. Although effective treatments exist, only a fraction of people with anxiety disorders receive treatment due to barriers such as lack of awareness, insufficient mental health services, and social stigma. In this study, we evaluated the anxiolytic activity of the ethyl acetate fraction of *Calotropis gigantea* leaves in rats. The study involved behavioral tests, such as the elevated plus maze and open field test, to evaluate the anxiolytic potential of the extract. The results showed that the ethyl acetate fraction of *Calotropis gigantea* leaves significantly increased the time spent in the open arms of the elevated plus maze and the central zone of the open field test, indicating its anxiolytic activity. Therefore, the results of this study suggest that the ethyl acetate fraction of *Calotropis gigantea* leaves has potential as an anxiolytic agent.

Keywords: Anxiolytic, *Calotropis gigantea*, ethyl acetate fraction

1. Introduction
Currently, herbal drugs are widely used as green medicine for their safe and dependable health care paradigms. Traditional herbal medicines have drawn uprising attention for a couple of decades due to their incredible pharmacological activities, economic viability, and fewer side effects in different healthcare management [1].

Drugs that affect the central nervous system (CNS) are widely used as pharmacological agents [2]. CNS depressants such as barbiturates, benzodiazepines, and ethanol interact with the postsynaptic gamma-aminobutyric acid receptor (GABAA receptor) to produce their effects [3]. However, the use of barbiturates as a CNS depressant is limited due to their narrow margin of safety, and just 10 times their therapeutic dose can be lethal [4]. Barbiturates can also lead to both psychological and physiological dependence. Benzodiazepines are commonly used but can lead to tolerance and physical dependence [5, 6]. Ethanol is another CNS depressant that changes membrane fluidity and interacts with the GABA system, leading to tolerance and physical dependence [4, 8]. Alcohol addiction affects 5% to 10% of men and 3% to 5% of women in American society [9]. It is important to find a natural CNS depressant with reduced or no toxicity. Medical plants have been used for thousands of years for a variety of purposes, including food preservation, pharmaceuticals, alternative medicine, and natural therapies. Naturally produced compounds are considered more environmentally friendly as they can be biodegraded more easily than synthetic compounds.

Calotropis gigantea (*C. gigantea*) and R. Br. (Asclepiadaceae) is a wild weed commonly known as Boro Akanda. It can grow up to 4 meters in height and has sessile leaves that are 10 cm in length and 8 cm in width. The plant has oval, light green leaves, milky stems, and clusters of waxy flowers that are either white or lavender. The flowers are 14-15 mm long and 3-4.5 cm in diameter. Several medicinal properties of *C. gigantea* have been scientifically reported. The flowers of the plant are said to possess analgesic and antimicrobial properties [10], as well as cytotoxic activity [11]. The leaves and other parts of the plant are reported to have anti-Candida [12] and antibacterial properties [13], as well as antioxidant activity [14]. The roots are known to contain antimicrobial [16] and insecticidal activity [17], as well as wound healing [18] and CNS Activity [10]. Moreover, the latex of the plant is reported to have wound healing [19] and antimicrobial properties [20].

2. Materials and Methods

2.1. Drugs and chemicals

Diazepam was given as a gift sample from Square Pharmaceuticals Ltd., Bangalore. Thiopental sodium and Tween 80 were purchased from the local chemical market.

2.2. Collection and extraction *of C. gigantea* leaves

Healthy and mature *Calotropis gigantea* plants were verified by ABS Medicinal Garden, Salem, Tamil Nadu. After the leaves were gathered in May from the surrounding areas of Dharmapuri. When leaves from the highest portion of the plant were chosen and collected in the summer, a high phytochemical content was anticipated. During collection, care was taken to prevent contamination from dirt, dust, or other extraneous objects. To get rid of any contaminants, the gathered leaves were thoroughly cleaned in distilled water. After washing, the leaves were either spread out in a well-ventilated area to dry naturally or dried completely in a drying oven until they attained a consistent weight. To preserve consistency in particle size, the dried leaves were crushed into a coarse powder using a mortar and pestle or grinder [24, 32].

The dried and powdered plant material was weighed in a predetermined amount. Because it extracts a variety of phytochemicals with a moderate degree of polarity, ethyl acetate was selected as the extraction solvent. The Soxhlet extraction procedure, also known as maceration, was used to extract the material [23]. Throughout the Soxhlet extraction, ethyl acetate was constantly pumped through the dried and powdered material for several hours while it was contained in a thimble inside the Soxhlet extractor.

2.3 Animal

For this investigation, 180±20 g of male Sprague Dawley rats, six weeks of age, were employed. The mice were kept in groups of six animals per cage in a temperature-controlled environment (21-22°C) with a reversible light-dark cycle (12 h/12 h) had normally to fed and water *ad libitum* [37].

3. Preliminary phytochemical Screening

Preliminary phytochemical studies of the ethyl acetate fraction of *Calotropis gigantea* leaves aim to identify and characterize the chemical constituents present in this fraction [24-31]. Phytochemical analysis provides valuable insights into the potential bioactive compounds responsible for the medicinal properties of the plant [32-36]. Here are some common phytochemical groups that are often investigated:

4. *In vivo* Neuropharmacological activity studies

The neuropharmacological activities of ethyl acetate fraction extract of C. gigantea leaves were estimated by hole cross test, open field test, and elevated plus-maze (EPM) test. During each experiment, male Sprague Dawley rats were divided into three groups, namely, control, positive control, and test samples. Each group containing 5 mice was treated as the following arrangement: control, 1% v/v Tween-80 in water, 0.5 mL/mice; positive control, diazepam, 1 mg/kg body weight; test sample, ethyl acetate fraction extract at the dose of 400 mg/kg body weight.

4.1. Elevated Plus Maze Test (EPM)

The anxiolytic activity of plant extracts was evaluated using the EPM test. The apparatus was situated 40 cm above the floor, consisting of two open arms (5×10 cm) and two closed arms (5×10×15 cm) radiating from a platform (5×5 cm) to form a plus-sign figure. The open-arm edges were 50 cm in height to keep the mice from falling and the closed-arm edges were 15 cm in height. Sixty minutes after the administration of the test drug, each animal was placed at the center of the maze facing one of the enclosed arms. During the 5-minute test period, the number of open and enclosed arms entries, plus the time spent in open and enclosed arms, was recorded *via* the method of *Pillow and File*. Entry into an arm was defined as the point when the animal places all four paws onto the arm. The procedure was conducted in a sound-attenuated room; observations were made from an adjacent corner [38].

4.2 Open field test (OFT)

This test is one of the most frequently used methods to evaluate the loco motor activity and emotionality of rodents. The apparatus is a square box consisting of a 50 cm high wall and a wooden floor with a series of squares alternatively painted in black and white. Animals were administered with the vehicle, EAFCG, or diazepam and placed in the middle of the open field allowing free exploration. The animals were then scored with the number of squares they visited for 3 min before and at 30, 60, 90, and 120 min post treatments. The percentage of inhibition was calculated for each time point as described in the hole cross-test [39].

4.3 Marble burying test (MBT)

A normal glass cage with bedding materials was used in this experiment. Before testing, the individual animal was acclimatized in one cage for 30 min. After removal of the animal, 25 glass marbles were uniformly distributed on top of the 4 cm layer of bedding materials. Following EAFCG**,** vehicle, or diazepam treatment, each animal was placed in the cage for 30 min. The number of buried marbles was then counted as a score of anxiety [40]. The percentage of inhibitions was calculated as follows.

% Inhibition N = [(Control-Treatment) / Control] *100

4.4 Acute toxicity test

The animals were divided into five consecutive groups containing five animals in each [41-45]. Animals were kept in close observation for 72 h and for a total of seven days following the oral treatments with vehicle or EAFCG at 500, 1000, 2000, and 3000 mg/kg doses (adjusted volume 0.1 ml/animal) to check any allergic reaction, swelling, vomiting, diarrhea, and mortality induced by EAFCG. In the meantime, they were allowed to have access to food and water *ad libitum*.

4.5 Statistical analysis

The results are presented as Mean ± SEM. The statistical analysis was performed using a one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test, for these tests, One-way, two-way ANOVA followed by Bonferroni's post hoc tests was adopted. In all the cases $p<0.05$ was considered significant. All statistical analysis was performed using SPSS software. Moreover, graphical presentations are made by using Graph Pad Prism software version 10.2.0 (392).

5. Results

5.1 Ash value

The results of the usual process were used to determine the total ash value, water soluble ash value, and acid soluble ash value. Table 1 displays the results.

Table 1: Ash values of *Calotropis gigantea* leaves in % w/w

S. No.	Name of the plant and part used	Type of ash	% Ash in w/w
1.	*Calotropis gigantea* leaves	Total ash	9.53
2.		Acid soluble ash	4.51
3.		Water soluble ash	3.15

5.2 Extractive value

Table 2 provides the extractive values of *Calotropis gigantea* leaves.

Table 2: Extractive value of *Calotropis gigantea* leaves in % w/w

S. No.	Name of the plant and part used	Type of extract	Extractive value w/w
1.	*Calotropis gigantea* leaves	Alcohol soluble extract	09.23
2.		Water soluble extract	23.34
3.		Hydro-alcohol soluble extract	31.22

5.3 Heavy metal analysis

Table 3 demonstrates that iron (Fe) is the only heavy metal (of the five) that has been determined to be present in significant amounts, with the remaining elements falling within the acceptable range percentages.

Table 3: Results of the metal contents from EAFCG (ppm)

S. No	Fractions	Cd (ppm)	Cu (ppm)	PB (ppm)	Zn (ppm)	Fe (ppm)
1	EAFCG	0.0016	0.0012	0.2599	0.0087	0.559

EAFCG-Ethyl acetate fraction of Calotropis gigantea leaves. PPM-Parts Per Million

5.4 Preliminary phytochemical analysis

Using established protocols, various qualitative chemical tests were performed on the ethyl acetate and n-hexane fractions to identify the components, as reported by Harborne (1973) [33]. Table 6 displays the findings of the initial phytochemical group test of the *Calotropis gigantea* fractions. Glycosides, flavonoids, triterpenoids, alkaloids, phenolic compounds, and tannins (trace) were detected by the phytochemical tests (Table 4).

Table 4: Qualitative analysis of the various phytoconstituents on the ethyl acetate fraction of EAFCG

S. No	Tests	EAFCG
1.	Alkaloids	+
2.	Flavonoids	++
3.	Tannins	+
4.	Terpenoids	++
5.	Steroids and Sterols	-
6.	Anthraquinones	-
7.	Glycosides	++
8.	Phenolic Compounds	+
9	Resins	-

EAF-Ethyl Acetate Fraction; ++ -Abundantly present; + - Moderately present; -Absent

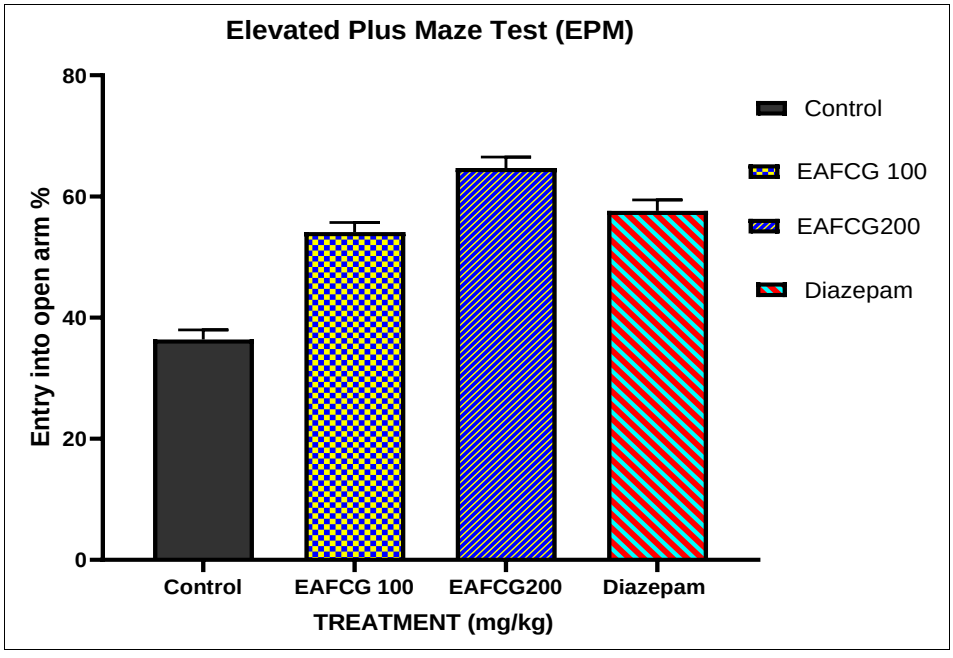
The anxiolytic effect of EAFCG in the elevated plus maze using rats

The anxiolytic activity was indicated by the dose-dependent increase in the percentage of entries into the open arm (p<0.0001) R2 Values found to be 0.9802 and the amount of time spent in the open arm (P<0.0001) R2 Values found to be 0.9971 and, following acute administration of EAFCG in different doses (Table 5).

Table 5: Effects of the ethanolic extract of *Calotropis gigantea* leaves on the percentage of entries and the time spent in open arms of the elevated plus-maze during the 5-min test session

Treatment	Dose (mg/kg)%	Entry into open arm%	Time spent in open arm%
Control	10 ml/kg, I.P.	36.4±1.5	35.8±0.87
EAFCG10	100 mg/kg, P.O.	54.13±1.49*	56.12±0.63*
EAFCG20	200 mg/kg, P.O	64.69±1.74**	67.18±0.64**
Diazepam	1 mg/kg, I.P.	57.61±1.74*	61.16±0.64**

Data expressed as mean±SEM; N=*6* ***p*<0.05, **p*<0.0001 compared to vehicle-treated group One-way ANOVA followed by post hoc Bartlett's test was performed.



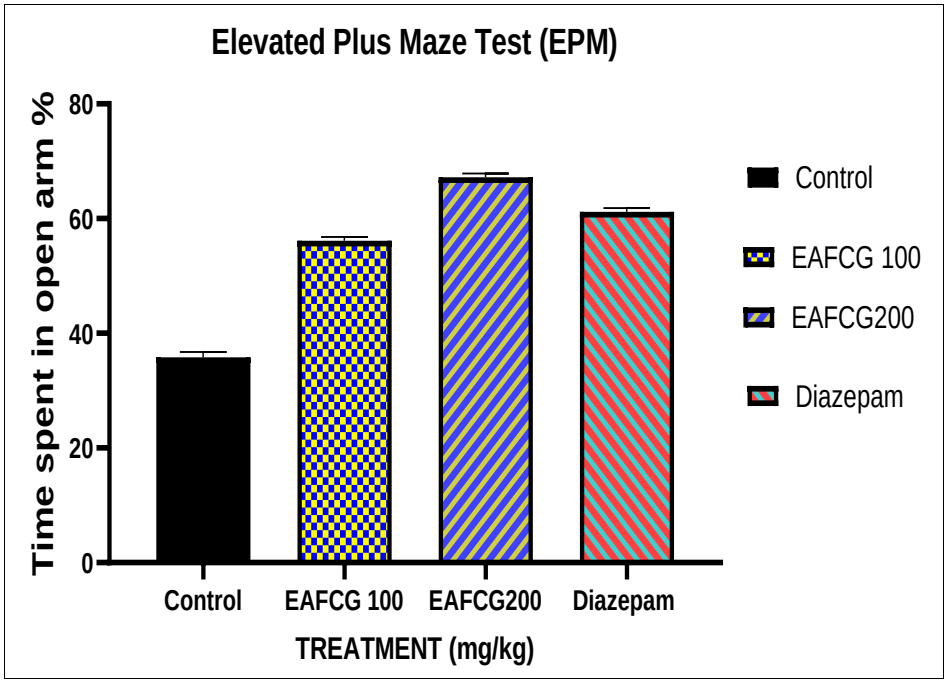


Fig 1: Effects of the ethanolic extract of *Calotropis gigantea* leaves on the percentage of entries and the time spent in open arms of the elevated plus-maze during the 5-min test session

5.5 The EAFCG decreased the frequency and amplitude of movements in the open field Test (OFT)

Rats that were given EAFCG (100 and 200 mg/kg) showed a substantial increase (p<0.0001) in the amount of time spent in the central compartment and the number of crossings of the open field. Table 3 shows that there were no significant (p>0.05) differences in the total number of ambulation or rearing.

Table 6: Effect of EAFCG on open field test

Treatment	Dose (mg/kg) (PO)* (ip)**	Number of squares crossed (% of inhibition)				
		Pre-treatment	30 min	60 min	90 min	120 min
Control	10 ml /kg**	96.80±3.21	96.81±3.11	95.88±3.41	95.80±3.91	94.23±3.28
EAFCG100	100 mg/kg*	101.40±3.74	67.52±2.34	52.45±2.54	17.18±3.0	17.18±2.98
EAFCG200	200 mg/kg*	99.60±3.19	58.28±2.54*	42.14±2.89**	15.78±0.58***	15.59±2.51***
DIAZEPAM	2 mg/kg**	98.66±3.39	52.35±2.95	45.24±3.01	33.78±2.19**	16.78±2.25***

Effect of EAFCG on open field test. Values are presented as the Mean ± SEM (N=6). EAFCG = Ethanolic extract of *Calotropis gigantea*; **p<0.001, ***p < 0.0001 compared with the control group (two-way ANOVA followed by Bonferroni's test)

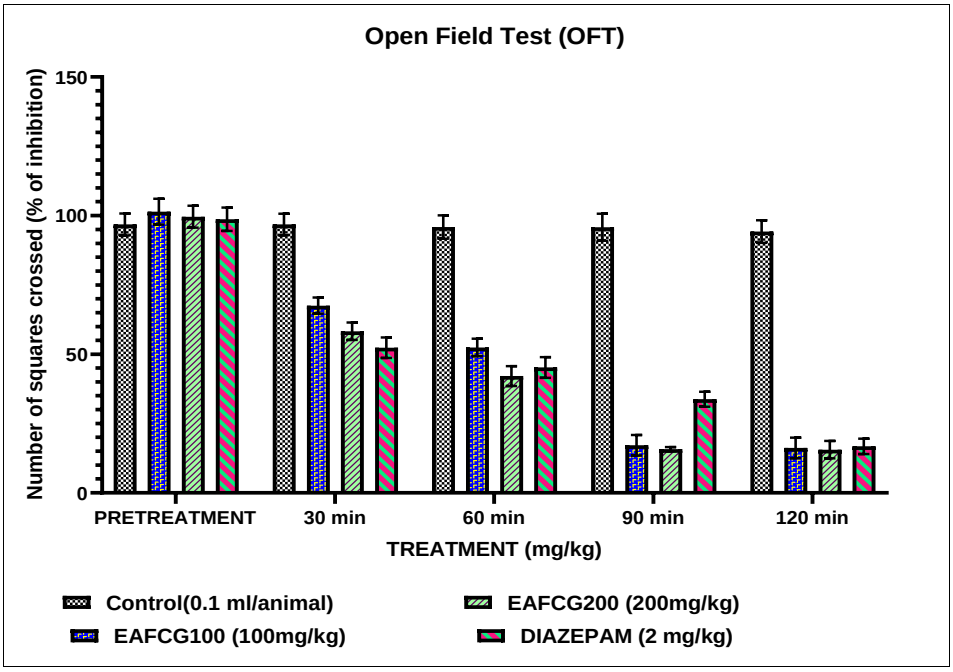


Fig 2: Effect of EAFCG on open field test

Table 7: Effect of EAFCG on marble burying test (MBT)

Treatment	Dose (mg/kg)	Responses	
		Numbers of marbles buried	% Inhibition
Control	0.1 ml/animal	9.88±2.320	0
Diazepam	1	1.20±0.240	87.341
EAFCG100	100	3.52±0.470	62.869
EAFCG200	200	2.04±0.500	78.481

Values are presented as the Mean ± SEM (N=5). EAFCG = Methanolic extract of *Calotropis gigantea;* **p*<0.05; ***p*<0.01; compared with the control group (one-way ANOVA followed by Barlett's test)

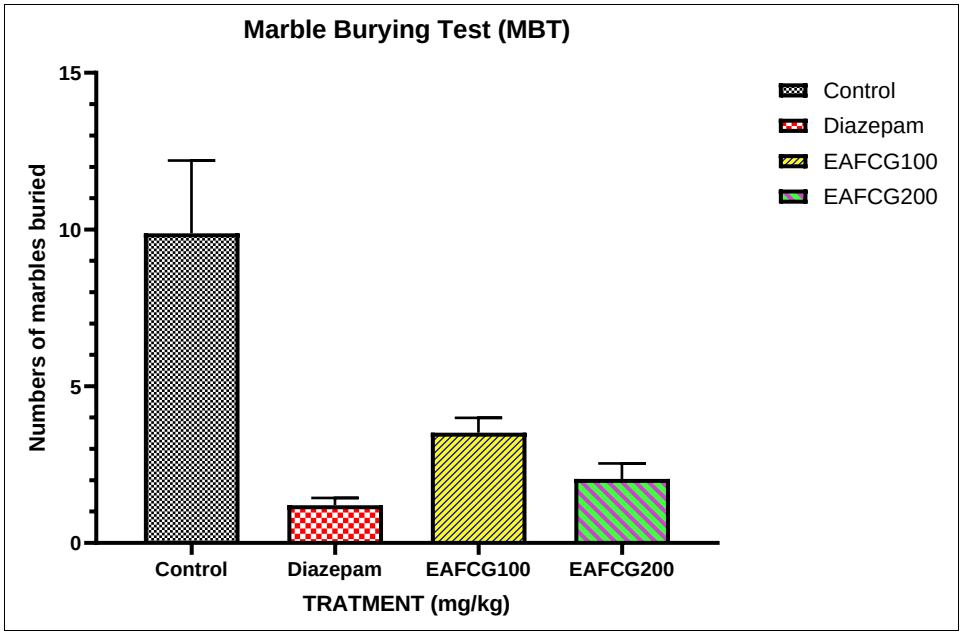


Fig 3: Marble burying test-no of marbles buried

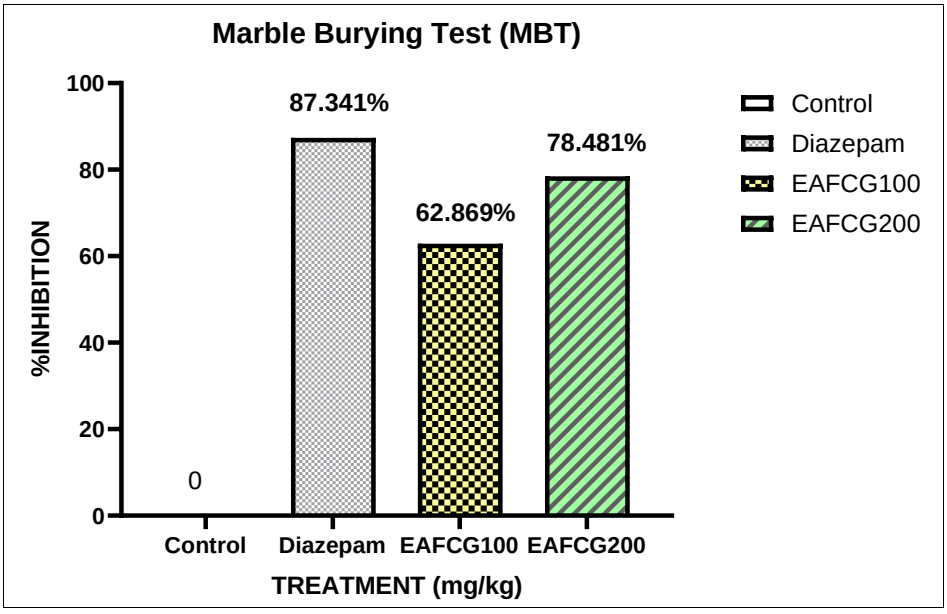


Fig 4: Marble burying test% of inhibition

6. Discussion

Anxiety is a psychological illness that causes unreasonable fear and affects mood. Medications like antidepressants, buspirone, and benzodiazepines are used to treat anxiety, but they have drawbacks like drowsiness, forgetfulness, and dependence. Medicinal plants are being explored as a potential source of new drugs to treat anxiety.

Numerous traditionally used plants have pharmacological qualities that make them highly promising for use in therapeutic applications to treat problems of the central nervous system. *Calotropis gigantea*, a member of the Apocynaceae family, was chosen for this study due to its rich flavonoid content and historical use. Flavonoids have been shown to have potential effects on the central nervous system.

The plant was collected from the Dharmapuri district of Tamil Nadu, and authenticated by a Botanical Survey of India, Coimbatore. The total ash, alcohol-soluble ash, and

water-soluble ash values were calculated to identify the inorganic component contained in the leaf powder as shown in Table 1. These identified ash values can be used to compare the values for their pharmacognostical standards and as standard parameters for the original plant.

The extractive values of *Calotropis gigantea* leaves were determined to reveal the aqueous and organic soluble fractions. A hydroalcoholic extract was made and further fractionated for this investigation. The percentage yield obtained from different solvent fractions was calculated. Extractive values are shown in Table no-2.

The results of the heavy metal analysis indicated that the levels of metals such as iron, zinc, copper, lead, and cadmium in the EAFCG were below permitted ranges as shown in Table no-3.

Based on an initial analysis, it was found that the ethyl acetate fraction of *Calotropis gigantea* (EAFCG) included flavonoids and phenolic chemicals. Glycosides, flavonoids, triterpenoids, alkaloids, phenolic compounds, and traces of tannins were detected by the Preliminary phytochemical analysis, and the various phytoconstituents on the ethyl acetate fraction of EAFCG were shown in Table no-4

Pharmacological assays to identify anxiolytic behavior in animals typically involve various behavioral tests designed to assess anxiety-like responses. We employed the following assays, Elevated Plus Maze Test (EPM), Open field test (OFT), Marble burying Test (MBT), and Acute toxicity study also included in study.

The EPM study measures the anxiety-like behavior of animals by recording their time spent in open versus enclosed arms and the number of entries into each arm. EAFCG is an anxiolytic compound that reduces anxiety-like behavior, while anxiogenic compounds have the opposite effect. The study found that the percentage of entries into the open arm and time spent in the open arm increased with the different doses of EAFCG, compared to the standard dose of Diazepam 2mg/kg. EAFCG had a dose-dependent effect and worked similarly to Diazepam by enhancing the activity of gamma-aminobutyric acid (GABA), the major inhibitory neurotransmitter in the central nervous system. Diazepam can modulate the activity of other neurotransmitter systems, including serotonin and dopamine, contributing to its diverse pharmacological actions.

The Open Field Test (OFT) is a widely used behavioral assay to assess general locomotor activity and anxiety-like behavior in rodents. OFT is valuable for studying the effects of various interventions, including pharmacological compounds, on anxiety-related behaviors and general locomotor activity. OFT test was found to be highly significant where $p<0.0001$ was given by the two-way ANOVA test. Fig no-1 showed significant values in 30,60,90 and 120 min when compared with standard diazepam 2mg/kg versus EAFCG in two different doses 100 and 200mg/kg, it showed that EAFCG200 was highly significant when compared with the standard dose, the result responses were shown in the figure no-9. The OFT is valuable for studying the effects of various interventions, including pharmacological compounds, on anxiety-related behaviors and general locomotor activity.

The Marble Burying Test (MBT) is a behavioral assay commonly used to assess anxiety-like and compulsive behaviors in rats. In this test, rats are placed in a cage containing a layer of marbles evenly spaced across the bedding. The natural digging and burying behavior of rodents leads them to interact with the marbles. An increase in the number of marbles buried is considered indicative of higher anxiety or compulsive-like behavior. The response of EAFCG was studied with 100 and 200 mg/kg doses and compared with the standard drug of Diazepam 1mg/kg, the observed responses were shown in Table 8, the one-way ANOVA employed for the statistical analysis where $p<0.0001$ and R^2 was found to be 0.9075 which was highly significant. The percentage of inhibition was shown in fig no-2 it was noted that the EAFCG extracts effectively control anxiety or compulsive-like behaviour. Also, it was found that EAFCG 200 mg/kg showed 78.41% inhibition when compared to the standard drug Diazepam 1mg/kg which was nearly the same at 87.341%. The above responses might be the same mechanism as diazepam which was through enhancing the activity of gamma-aminobutyric acid (GABA), which leads to an increase in the frequency of chloride channel opening, resulting in hyperpolarization of the neuronal membrane and inhibition of neuronal excitability. This hyperpolarization reduces the likelihood of action potential generation and transmission, leading to an anxiolytic effect.

The Acute toxicity test revealed that the oral administration of EAFCG at the doses of 500, 1000, 2000, and 3000 mg/kg did not show any mortality or allergic manifestations during 7 days of the observation period. Therefore, it can be assumed that EAFCG possesses a low toxicity profile and is safe within our experimental doses up to 3000 mg/kg.

Hence, it is predictable that the extract may act by potentiating GABAergic inhibition in the CNS via membrane hyperpolarization leading to a reduction in the firing rate of critical neurons in the brain or it may be due to direct activation of GABA receptors. It may also be due to enhanced affinity for GABA or an increase in the duration of the GABA-gated channel opening.

It has also been reported that some flavonoids exhibit high affinity binding to the benzodiazepine site of $GABA_A$ receptors. Therefore, the CNS depressant activity may be due to the phytoconstituents present in the EAFCG extract.

7. Summary and Conclusion

The study isolated the ethyl acetate fraction from *Calotropis gigantea* leaves and assessed its anxiolytic effects using established EPM, OFT, and MBT. The study also explored the neuropharmacological mechanisms underlying the observed anxiolytic effects. The effects of drugs or other interventions on anxiety-related responses and studied the underlying neurobiology of anxiety disorders and obsessive-compulsive behaviors in rats with the EPM, OFT, and MBT tests.

In conclusion, the selected animal models exhibited anxiolytic efficacy in response to the ethyl acetate fraction from the hydroalcoholic extract of *Calotropis gigantea* leaves. Since flavonoids are known to promote anxiolytic activity, the presence of flavonoids in these plant extract fractions may be the cause of the anxiolytic activity.

8. Conflict of interest statement

We declare that we have no conflict of interest.

9. Acknowledgments

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