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Research Article

Multimodal Evaluation of the Anxiolytic Activity of Phyllanthus niruri Extract in Mice: A Comparative Behavioral and Neuropharmacological Approach

Kuldeep Prajapati *, Mangesh Tote, Subhendu Mathur, Juverya Kazi, Abhishek Prajapati, Sahil Patil

Department of Pharmacology, Oriental College of Pharmacy, Navi Mumbai, Maharashtra, India, Pin- 400705

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*For Correspondence:

Mr. Kuldeep Prajapati, Department of Pharmacology, Oriental College of Pharmacy, Navi Mumbai, Maharashtra, India, Pin- 400705.

Abstract

Anxiety is a widespread mental health issue that impacts millions of individuals globally, often interfering with daily functioning and overall well-being. While conventional anti-anxiety medications like benzodiazepines are widely used, their prolonged use is often limited due to side effects such as sedation, dependence, and drug tolerance. As a result, there is increasing interest in exploring herbal remedies with better safety profiles. *Phyllanthus niruri* Linn., a medicinal herb widely recognized in Ayurvedic medicine for treating liver and urinary problems, is known to contain neuroactive constituents such as flavonoids, tannins, phenolics, and alkaloids. This study aimed to assess the anti-anxiety potential of the hydroalcoholic extract of *Phyllanthus niruri* in Swiss albino mice using multiple validated behavioral models. The plant was collected and authenticated, then extracted using a 70:30 ethanol-water mixture and screened for key phytochemical constituents. Mice were randomly assigned into four groups: a control group, a standard group receiving diazepam (2 mg/kg), and two test groups treated orally with the extract at 100 mg/kg and 200 mg/kg. Behavioral tests including the Elevated Plus Maze (EPM), Open Field Test (OFT), Actophotometer, and Rotarod were conducted to evaluate anxiety levels, movement activity, and motor coordination. The extract demonstrated a dose-related reduction in anxiety-like behaviors, with effects comparable to the standard drug, and did not cause significant motor impairment. Phytochemical screening confirmed the presence of active compounds likely responsible for the observed anxiolytic action. These findings support the traditional claims of *Phyllanthus niruri* in stress relief and highlight its potential as a safe, natural alternative for managing anxiety disorders.

Keywords: *Phyllanthus niruri*, anxiolytic activity, hydroalcoholic extract, behavioral models

INTRODUCTION

Anxiety is one of the most common psychological disorders affecting people across all age groups worldwide. It goes beyond temporary worry or fear, and when persistent, it can interfere with daily life, productivity, and emotional well-being. Symptoms like restlessness, irritability, muscle tension, rapid heartbeat, and trouble sleeping often reduce an individual's quality of life. According to global estimates, anxiety-related disorders affect more than 300 million individuals and represent a major cause of disability and psychological distress[1–5]. Anxiety can show up in different forms, affecting people in various ways.

Pharmacological treatment for anxiety generally includes benzodiazepines, selective serotonin reuptake inhibitors (SSRIs), and other central nervous system (CNS) depressants. Although these medications are clinically effective, their prolonged use is often associated with adverse effects such as drowsiness, cognitive dulling, dependence, tolerance, and withdrawal symptoms. These limitations have generated interest in natural alternatives that can provide anxiolytic benefits without causing significant side effects or dependency[18].

Herbal medicines have been used for centuries in traditional systems such as Ayurveda, Siddha, and Traditional Chinese Medicine (TCM) to manage emotional imbalances and nervous system disorders. Many plants used in these systems are believed to act on the nervous system in a gentle but effective manner. Recent research has shown that several plant-based compounds can influence neurotransmitters like GABA, serotonin, and dopamine, all of which play key roles in anxiety regulation.

Table 1: Types of Anxiety Disorders

S. No.	Type of Anxiety Disorder	Description
1	Generalized Anxiety Disorder (GAD)	Constant, uncontrollable worry about daily matters such as health, work, or family, often with fatigue and sleep trouble[6,7].
2	Panic Disorder	Sudden episodes of intense fear (panic attacks) with symptoms like rapid heartbeat, breathlessness, and dizziness[6–8].
3	Social Anxiety Disorder	Strong fear of social situations due to worry about being judged or embarrassed, leading to avoidance of people or events[9].
4	Specific Phobias	Extreme and irrational fear of particular things (e.g., animals, heights), causing strong anxiety or panic in their presence[10].
5	Separation Anxiety Disorder	Deep fear or distress when away from close people or familiar places; may affect both children and adults[11,12].
6	Obsessive-Compulsive Disorder (OCD)	Involves recurring unwanted thoughts (obsessions) and repetitive behaviors (compulsions) to reduce distress[13].
7	Post-Traumatic Stress Disorder (PTSD)	Triggered by past trauma, includes flashbacks, emotional numbness, and hyper-alertness[14,15].
8	Health Anxiety (Hypochondria)	Persistent fear of having a serious illness, even after medical reassurance; leads to repeated checking or doctor visits[16,17].

One such herb is *Phyllanthus niruri*, commonly known as Bhumi Amla or Stonebreaker. This small annual plant belongs to the family Phyllanthaceae and is distributed widely across India, South America, and Southeast Asia. Traditionally, it has been used to treat liver conditions, urinary disorders, infections, and inflammatory diseases. Modern phytochemical studies have identified a wide range of active constituents in *Phyllanthus niruri*, including flavonoids (like quercetin and rutin), alkaloids, tannins, lignans (such as phyllanthin and hypophyllanthin), saponins, and polyphenols.

Many of these bioactive compounds are known to possess neuroactive properties. For instance, flavonoids and lignans may interact with GABAergic receptors, which are involved in regulating anxiety and CNS excitability. Some of these constituents also have antioxidant and anti-inflammatory properties, which may provide indirect neuroprotection. Despite this promising phytochemical profile, the anxiolytic potential of *Phyllanthus niruri* has not been fully explored in well-structured scientific studies[19,20].

A limited number of experimental studies have been conducted to evaluate the calming effects, but most of these have used only one or two behavioral model, Relying on a limited behavioral test may not provide a complete understanding of a substance's effect, as many herbal extracts can also produce muscle relaxation or sedation, which may mimic an anxiolytic response. Therefore, to draw reliable conclusions about its effect on anxiety, it is essential to use multiple validated behavioral models that can assess different aspects of behavior, motor activity, and neuromuscular coordination.

In this context, the present study was designed to evaluate the anxiolytic effect of hydroalcoholic extract of *Phyllanthus niruri* using a multimodel behavioral approach in mice. This included four different experimental models Elevated Plus Maze (EPM), a classic test for anxiety based on natural aversion to open spaces. Open Field Test (OFT), used to assess exploratory behavior and emotionality. Actophotometer, to measure spontaneous locomotor activity and rule out central depressant effects. Rotarod test, to evaluate motor coordination and neuromuscular integrity.

By using this combination of tests, the study aims to distinguish true anxiolytic effects from general sedation or impaired motor function, which is a crucial aspect in validating CNS-active herbal drugs. The outcomes of this study are expected to contribute valuable scientific evidence regarding the anxiolytic potential of *Phyllanthus niruri*. By adopting a multimodal testing strategy and correlating behavioral outcomes with the plant's phytochemical profile, this work may help to position *Phyllanthus niruri* as a promising natural alternative for anxiety management, encouraging further research into its mechanism of action and possible clinical applications.

MATERIALS AND METHODS

Plant materials

The plant material used in this study was collected from the Palghar region of Maharashtra, India. The selection was done based on common physical traits and identification features of *Phyllanthus niruri* observed in the field. To ensure proper botanical identification, the collected specimen was authenticated by experts at the Department of Botany, Maharashtra College of Science, Arts, and Commerce, located at 246-A, Jahangir Boman Behram Marg, opposite Alexandra Cinema, Nagpada, Mumbai, Maharashtra – 400008. The authentication confirmed that the collected sample belonged to the species *Phyllanthus niruri* (Family: Phyllanthaceae), thereby ensuring the authenticity and reliability of the plant material used in this research.

Chemicals

Diazepam of analytical grade purity was obtained from a certified pharmaceutical supplier and used as the standard anxiolytic reference drug in the study. All other chemicals and reagents employed, including solvents and reagents for phytochemical screening, were of analytical grade and procured from reputable local suppliers.

Sample Preparation and Extraction

Fresh aerial parts of *Phyllanthus niruri* Linn. were carefully collected from their natural growing site, ensuring that the selected plants were healthy and free from any visible signs of disease or contamination. Immediately after collection, the plant material was thoroughly rinsed with distilled water to remove dust, soil particles, and other external impurities. After washing, the clean plant parts were evenly spread and dried in a hot air oven maintained at a controlled temperature between 40°C and 45°C. This temperature range was chosen to effectively remove moisture without damaging any heat-sensitive or volatile phytochemicals. Drying was continued until the plant material achieved a consistent weight, indicating complete removal of water content. Once dried, the plant material was ground into a fine powder using a mechanical grinder. The powdered sample was then passed through a sieve to ensure uniform particle size, which is important for efficient solvent penetration during extraction.

The final powder was stored in a clean, air-tight, and labeled glass container placed in a dry, dark environment until further use. For the extraction process, a precise amount of the powdered material was weighed using a digital balance and placed into a wide-mouthed glass jar with a tight-sealing lid. A hydroalcoholic solvent mixture consisting of ethanol and distilled water in a 70:30 ratio was freshly prepared and added to the container in a quantity sufficient to completely immerse the plant material, maintaining a solvent-to-material ratio of approximately 10:1 (w/v).

The mixture was left to undergo cold maceration at room temperature (25–30°C) for 48 to 72 hours, with occasional shaking to enhance the contact between the plant powder and solvent. After the initial extraction period, the mixture was filtered using Whatman No.1 filter paper to separate the extract from the residual plant matter. To improve the overall yield, the remaining plant residue (marc) was subjected to a second round of maceration using fresh solvent for an additional 24 hours. Filtrates from both rounds were combined and concentrated using a rotary evaporator under reduced pressure at a temperature between 40°C and 50°C. This low-temperature vacuum evaporation method was used to preserve sensitive phytoconstituents like flavonoids and phenolic compounds. The resulting semi-solid mass was further air-dried to yield the final dry crude extract. The dried extract was transferred to an air-tight container and stored in a desiccator over silica gel to protect it from moisture. It was kept refrigerated at a temperature of 4–8°C until further use in phytochemical analysis, toxicity testing, and pharmacological evaluations[21].

Phytochemical Screening

The hydroalcoholic extract of *Phyllanthus niruri* was subjected to preliminary qualitative phytochemical analysis to detect the presence of major secondary metabolites. Standard procedures were followed to test for flavonoids, phenolic compounds, tannins, and alkaloids, which are commonly associated with neuropharmacological activity[22].

Table 2: Phytochemical Screening

Phytochemical Group	Test Name	Observation	Inference
Flavonoids	Ammonia Test	Yellow color appeared which faded after some time.	Flavonoids likely present.
	Lead Acetate Test	Yellow solid (precipitate) formed after adding lead acetate solution.	Confirms presence of flavonoids.
	Shinoda Test	Pink or red color developed after adding magnesium and HCl.	Indicates flavonoid content.
Phenolic Compounds	Dilute Iodine Test	Dark green or blue color observed after iodine addition.	Suggests phenolic compounds.
	Ferric Chloride Test	Blue, green, or black color appeared after ferric chloride treatment.	Confirms phenolic group presence.
Tannins	Salkowski Reaction	Reddish-brown layer formed between chloroform and sulfuric acid.	Possible presence of tannins/terpenes.
	Ferric Chloride Test	Blue-black or green-black color appeared.	Confirms tannins.
Alkaloids	Hager's Test	Yellow solid formed after adding picric acid solution (Hager's reagent).	Indicates alkaloid presence.
	Dragendorff's Test	Orange to reddish-brown solid formed after adding Dragendorff's reagent.	Strong indication of alkaloids.

Experimental animals

For this study, Swiss albino mice (*Mus musculus*) were selected due to their genetic uniformity, well-established physiological profile, and wide acceptance in pharmacological and behavioral research. These animals are commonly used in toxicological and CNS-related studies, making them a suitable model for evaluating the anxiolytic activity of the plant extract. A statistical power analysis was performed prior to experimentation to determine the minimum number of animals required for obtaining significant results while adhering to the ethical principles of the 3Rs-Replacement, Reduction, and Refinement. The animals were procured from a certified breeder, the National Institute of Biosciences, located at Gat No. 69, Dhangawadi, Taluka Bhor, Pune, Maharashtra. The breeder complies with national regulatory guidelines and maintains high standards of animal care, transport, and breeding. Upon arrival at the animal house, the mice were first placed under a 14-day quarantine period to screen for infections and general health conditions. Animals were closely monitored during this time, and only those deemed healthy were selected for inclusion in the study. Each animal was marked using non-toxic identifiers (e.g., markers or ear tags) for individual tracking. After quarantine, the mice were acclimatized to laboratory conditions for seven days in the experimental animal facility. During this period, they were handled gently each day by trained personnel to reduce handling-induced stress and help them adapt to the test environment. Animals were housed in standard polypropylene cages with bedding, under controlled environmental conditions (temperature: 22–26°C; relative humidity: 45–65%) and a 12-hour light/dark cycle, in line with CCSEA and OECD guidelines. Throughout the acclimatization period, the animals were given unrestricted access to autoclaved standard pellet diet and filtered drinking water to ensure optimal physiological stabilization before the start of experimental procedures.

ACUTE ORAL TOXICITY

To assess the safety profile of the hydroalcoholic extract of *Phyllanthus niruri*, an acute oral toxicity study was conducted using healthy adult male Swiss albino mice, each weighing between 27–30 grams. The animals were randomly grouped and housed in standard polycarbonate cages, with three mice per cage, under well-maintained laboratory conditions. The room temperature was kept at 25 ± 2°C, with relative humidity between 50–60%, and a 14-hour light / 10-hour dark cycle. Prior to dosing, the animals were allowed to acclimatize for 7 days. During this period, they had free access to a balanced standard pellet diet and filtered drinking water, which ensured stabilization of physiological parameters before experimentation. All procedures involving animal care and handling were conducted in accordance with ethical standards approved by the Institutional Animal Ethics Committee (IAEC), University of Mumbai, and in compliance with CCSEA guidelines (Committee for the Purpose of Control and Supervision of Experiments on Animals). The toxicity study was designed according to the OECD Guideline 423 (Acute Toxic Class Method). A single oral dose of 2000 mg/kg body weight of the hydroalcoholic extract was administered using an oral gavage and a calibrated gastric feeding needle. After dosing, animals were closely monitored for signs of toxicity or abnormal behavior. Observations were carried out at frequent intervals during the first 30 minutes, followed by periodic checks during the first 24 hours, and then daily for 14 days. Parameters recorded included changes in behavior, appearance, and physical activity such as piloerection, tremors, salivation, convulsions, lethargy, respiratory irregularities, and alterations in movement. Animals were also observed for mortality or signs of distress throughout the study duration. In addition, body weights were recorded on Day 0, Day 7, and Day 14 to detect any significant weight loss or gain, which might indicate systemic toxicity. The absence of adverse effects at this limit dose was considered an indicator of the extract's safety for further pharmacological evaluation[23,24].

Preparation of Stock Solution of the Extract for Dosing

The required quantity of the hydroalcoholic extract of *Phyllanthus niruri* was accurately weighed and freshly dissolved in distilled water prior to each dosing session. Fresh solutions were prepared daily to maintain stability and consistency of the extract. The prepared solution was administered orally (p.o.) to the animals at two dose levels: 100 mg/kg and 200 mg/kg body weight, maintaining a constant volume for each administration.

Experimental Design

A total of 24 healthy Swiss albino mice were randomly divided into four groups, with six animals in each group (n = 6). The grouping was done as follows:

- Group I – Control: Received distilled water (vehicle) orally.
- Group II – Standard: Received Diazepam (2 mg/kg, i.p) as the standard anxiolytic drug.
- Group III – Test Low Dose: Received *Phyllanthus niruri* extract at a dose of 100 mg/kg orally.
- Group IV – Test High Dose: Received *Phyllanthus niruri* extract at a dose of 200 mg/kg orally.

SELECTION OF DOSE

The doses used for both the standard drug and the hydroalcoholic extract of *Phyllanthus niruri* were selected carefully, based on existing pharmacological literature, previous experimental studies, and toxicity data. For diazepam (used as the standard anxiolytic), the dose was chosen by referring to earlier validated studies in mice, ensuring it was effective and aligned with standard pharmacopoeial guidelines. This helped ensure that the dose was therapeutically relevant without causing adverse effects.

For the *Phyllanthus niruri* extract, dosage selection was guided by results from an acute oral toxicity study conducted as per OECD Guideline 423. In that study, a high dose of 2000 mg/kg caused no toxicity or mortality in mice, confirming the extract's safety profile. Based on

these findings, two lower doses—100 mg/kg and 200 mg/kg—were chosen for further pharmacological evaluation. These doses were selected to assess potential anxiolytic activity while ensuring a wide safety margin.

Past ethnomedicinal records and preclinical research on *Phyllanthus niruri* and other herbs with similar phytochemical profiles were also reviewed to predict how the extract might behave inside the body. This helped support the rationale for the chosen dose range.

- High Dose (200 mg/kg): Chosen to evaluate maximum potential effects, while staying within the safe range established by toxicity studies.
- Low Dose (100 mg/kg): Included to observe possible dose-dependent effects and to help define the effective therapeutic window.

All doses were administered orally and adjusted according to each mouse's body weight. The timing and route of administration were optimized to ensure consistency and accuracy across all experimental groups. This approach enabled a reliable comparison between the extract and the standard drug, and also allowed investigation into the extract's possible mechanism of action[23–27].

BEHAVIORAL MODELS

Elevated plus maze test

The Elevated Plus Maze (EPM) is a well-established behavioral model used to assess anxiety levels in rodents. It takes advantage of the animal's natural tendency to avoid open and elevated spaces due to fear of heights and potential threats. A reduction in anxiety-like behavior is reflected when the animal shows greater willingness to explore these open arms. In the present study, the maze consisted of four arms: two open arms (16 cm × 5 cm) and two closed arms (16 cm × 5 cm × 15 cm), connected by a central platform measuring 5 cm × 5 cm. The maze was elevated 25 cm above ground level to create a mild anxiety-inducing environment. Each mouse was placed at the center of the maze, facing an open arm, and observed for a duration of 5 minutes. The parameters recorded included:

- Number of entries into open and closed arms
- Time spent in each type of arm

An entry was considered valid when all four paws of the mouse entered an arm. To avoid scent-based bias, the apparatus was thoroughly cleaned with ethanol after each trial. Increased time spent in the open arms and a higher number of open arm entries are taken as signs of reduced anxiety. The performance of mice treated with the test extract of *Phyllanthus niruri* was compared with both control and standard (diazepam-treated) groups. If treated mice showed enhanced open arm exploration without signs of sedation or motor impairment, it was considered indicative of an anxiolytic-like effect of the extract[28–30].

Open field test

The Open Field Test is a widely recognized method for studying anxiety-related behavior and general locomotor activity in rodents. The test is based on the natural tendency of mice to avoid open and brightly lit spaces and stay close to walls (a behavior called thigmotaxis). When a mouse feels anxious, it avoids the center and limits its movement. However, if it feels less anxious, it explores the center more freely and shows more vertical (rearing) activity. In this study, the apparatus consisted of a square open box with high walls to prevent escape. The floor was marked with lines to divide it into multiple equal squares, helping to track movement across the field. The environment was kept quiet and consistent to avoid external stress. Each mouse was placed gently in the center of the field and observed for 5 minutes. The chamber was cleaned with 70% ethanol between each trial to eliminate scent cues from previous animals. The following behavioral parameters were recorded:

- Number of squares crossed: indicating horizontal exploratory activity
- Number of rearings: standing upright on hind legs (free rearing)

These measures reflect the animal's level of curiosity, anxiety, and overall activity. A more relaxed and less anxious mouse is expected to explore more (cross more squares), rear more frequently, and use less wall support[29,31,32].

Actophotometer – Locomotor Activity

The Actophotometer test is a commonly used method to measure how active a mouse is, and to check how certain drugs or plant extracts affect the brain and nervous system. It helps researchers understand whether a substance works as a stimulant, a sedative, or has anti-anxiety (anxiolytic) effects. The overall activity level of the animal reflects how alert or calm it is, which makes this test a good tool for assessing changes in behavior. In this test, a mouse is placed inside a clear box that contains infrared light beams around its edges. Every time the mouse moves and crosses a beam, the beam is broken, and this is automatically recorded as one movement. The total number of movements is counted digitally over a period of 5 minutes. The more the mouse moves, the higher its activity score. In our study, each mouse was tested one at a time, and the chamber was cleaned with 70% ethanol after every trial to remove any smells that could affect behavior. The main thing we measured is the Total locomotor activity (number of movements recorded)This test was particularly useful in checking whether the *Phyllanthus niruri* extract made the mice sleepy or less active (which could mean sedation), or whether it reduced anxiety without affecting movement. If the mice treated with the extract moved less like the mice treated with diazepam, a known sedative it could suggest a sedative effect. But if their movement stayed normal, and they still showed anti-anxiety behavior in other tests (like EPM or OFT), then it would mean that the extract is working as an anxiolytic without causing sedation , which is ideal. This test helped confirm whether the plant extract had any unwanted sedative effects or if it safely reduced anxiety while keeping the mice alert and active[32,33].

Rotarod test (Motor Coordination Activity)

The Rotarod test is a standard method used to check how well mice can maintain their balance and coordination. It helps researchers find out if a drug or plant extract affects the animal's physical abilities like causing drowsiness, muscle relaxation, or other changes in movement. This is especially important when testing substances that act on the brain, such as those used for reducing anxiety, to ensure they don't also cause unwanted side effects like poor coordination. In this study, the setup included a metal rod that rotated at a steady speed of 25 rotations per minute (rpm). The rod is raised above the ground, so the mice have to balance on it as it spins. If a mouse is sedated or its muscle control is weakened, it will fall off the rod sooner. After giving the treatment, each mouse was placed on the rotating rod one at a time, and the time it took to fall off was measured in seconds. Each test lasted for 5 minutes, and the equipment was cleaned with alcohol after every trial to remove any scent or dirt left by the previous mouse.

This test was used to check if the Phyllanthus niruri extract caused any problems with motor skills. If the treated mice fell off quickly—similar to the ones given diazepam—it would suggest reduced coordination or sedation. But if the mice stayed on the rod for about the same time as the untreated (control) group, it would show that the extract didn't interfere with their physical abilities. This is an important check because a good anti-anxiety treatment should calm the animal without making it sleepy or uncoordinated. So, the Rotarod test helped confirm whether the calming effects of Phyllanthus niruri observed in other tests were free from side effects like sedation or muscle weakness[33–37].

Corticosterone Analysis

To evaluate the effect of *Phyllanthus niruri* extract on stress-induced hormonal changes, plasma corticosterone levels were measured following three weeks of treatment and behavioral assessment. After the final behavioral test, mice were subjected to 60 minutes of restraint stress to induce acute physiological stress response.

Approximately 0.1 mL of blood was collected from each mouse via the facial (submandibular) vein using a sterile lancet. Blood samples were transferred into pre-chilled EDTA-coated microcentrifuge tubes and immediately placed on ice. Plasma was separated by centrifuging the samples at 2200 × g for 10 minutes at 4°C. The collected plasma was stored at −20°C until analysis. Plasma corticosterone levels were determined using a commercial radioimmunoassay (RIA) kit (125I RIA, MP Biomedicals, Orangeburg, NY), as per the manufacturer's instructions[38–40].

STATISTICAL ANALYSIS

All the data were expressed as mean ± SEM. the data were analyzed using (ANOVA) one-way analysis of variance followed by Dunnett's test. A P value of <0.05 was considered as the level of significance.

RESULTS

PHYTOCHEMICAL ANALYSIS

The confirmatory qualitative phytochemical screening of phyllantus niruri was performed to identify the main class of compounds present in the phyllantus niruri.

Table 3: Results of Qualitative phytochemical Analysis of *Phyllanthus niruri*

Phytochemical Group	Test Performed	Observation	Results
Flavonoids	Ammonia Test	Yellow color appeared	Present
	Lead Acetate Test	Yellow precipitate formed	Present
	Shinoda Test	Pink to red color developed	Present
Phenolic Compounds	Dilute Iodine Test	Blue-green coloration observed	Present
	Ferric Chloride Test	Blue-black/green-black color appeared	Present
Tannins	Terpenoid Test	Reddish-brown layer at interface	Present
	Ferric Chloride Test	Green-black color appeared	Present
Alkaloids	Hager's Test	Yellow precipitate formed	Present
	Dragendorff's Test	Reddish-brown/orange precipitate formed	Present

DPPH ANTIOXIDANT ASSAY

Table 4: DPPH radical scavenging assay of HEPN

DPPH ANTIOXIDANT SCAVENGING ASSAY			
CONCENTRATION (µG/ML)		**ABSORBANCE**	**% SCAVENGING ACTIVITY**
200	***PHYLLANTHUS NIRURI* EXTRACT**	0.350	29.5774648
400		0.248	50.1006036
500		0.183	63.1790744
600		0.140	71.8309859
10	**ASCORBICACID**	0.321	35.41247485
15		0.212	57.34406439
20		0.157	68.41046278
25		0.105	78.87323944

Control absorbance – 0.497

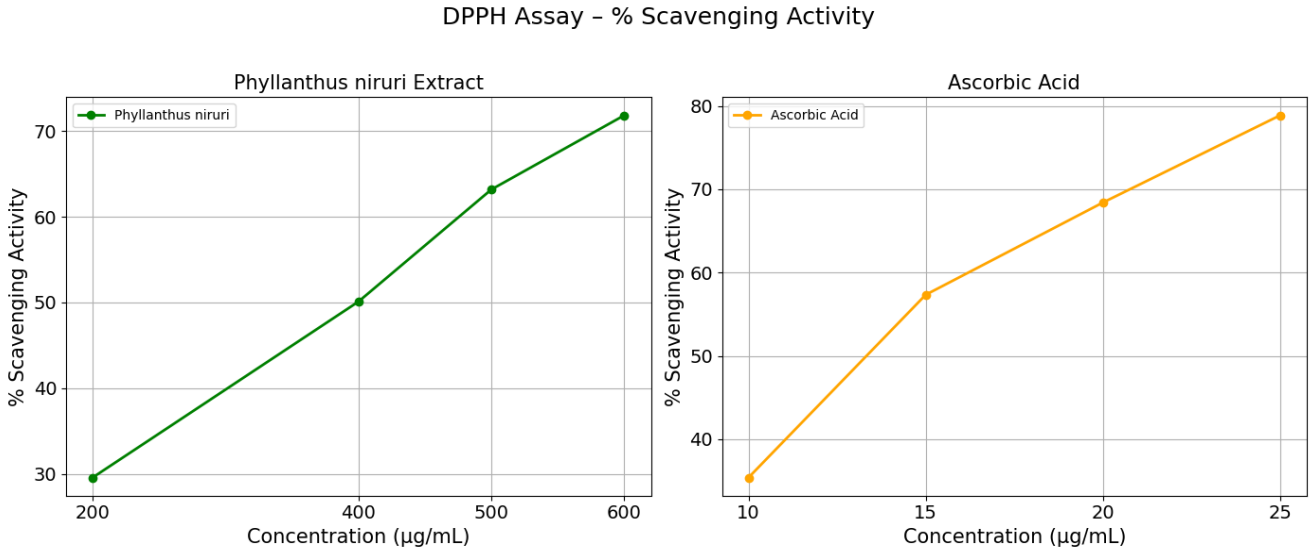

Figure 1: DPPH Radical scavenging assay

IC_{50} value of herbal extract and ascorbic acid was found to be 399.02 µg/ml and 13.96 µg/ml form the graph.

ACUTE ORAL TOXICITY

The acute oral toxicity study conducted according to OECD Guideline 423 (Limit Test) revealed that the Ethanolic extract of P.niruri did not produce any signs of toxicity or mortality at a limit dose of 2000 mg/kg body weight. All animals remained normal in terms of behaviour, clinical appearance, and physiological parameters throughout the 14-day observation period. No abnormalities were observed in central nervous system activity, motor coordination, autonomic responses, or vital signs. These findings indicate that the extract is well-tolerated at the tested dose and can be considered non-toxic under the conditions of this study. Based on these results, the LD_{50} of the extract is estimated to be greater than 2000 mg/kg, classifying it as relatively safe for oral administration.

BEHAVIOURAL TEST:

ELEVATED PLUS MAZE (EPM)

Table 5: Elevated plus Maze results

Group	Open Arm Entries	Closed Arm Entries	Time spent in Open Arms (s)	Time spent in Closed Arms (s)
Control	3.83 ± 0.31	11.17 ± 0.60	22.17 ± 1.30	173.67 ± 4.74
Diazepam	9.50 ± 0.22 **	5.50 ± 0.34 **	75.33 ± 1.78 **	72.00 ± 1.48 **
HEPN (100mg/kg)	7.17 ± 0.31 *	6.33 ± 0.33	33.83 ± 1.08 *	109.00 ± 6.17
HEPN (200mg/kg)	9.17 ± 0.31 **	6.33 ± 0.21	49.67 ± 0.49 **	76.33 ± 7.05

Values are Mean ± SEM, n = 6. Significance *p < 0.05, **p < 0.01 vs. Control (one-way ANOVA followed by Dunnett's test)

In the Elevated Plus Maze test, the control group showed low open arm entries (3.83 ± 0.31) and minimal time in open arms (22.17 ± 1.30 s), indicating a high anxiety level. Treatment with diazepam (2 mg/kg) significantly increased both the number of open arm entries (9.50 ± 0.22) and time spent in open arms (75.33 ± 1.78 s), confirming its strong anxiolytic activity ($p < 0.01$).

The group treated with *Phyllanthus niruri* extract at 100 mg/kg showed a moderate but significant increase in open arm entries (7.17 ± 0.31) and time spent (33.83 ± 1.08 s) compared to control ($p < 0.05$), indicating a mild anxiolytic effect.

At 200 mg/kg, the extract produced results (9.17 ± 0.31 entries; 49.67 ± 0.49 s) much closer to diazepam, with statistically significant improvement ($p < 0.01$) in exploratory behavior, suggesting a dose-dependent anxiolytic effect. Meanwhile, closed arm entries and time in closed arms were proportionally reduced in extract and diazepam-treated groups, supporting the anxiolytic response.

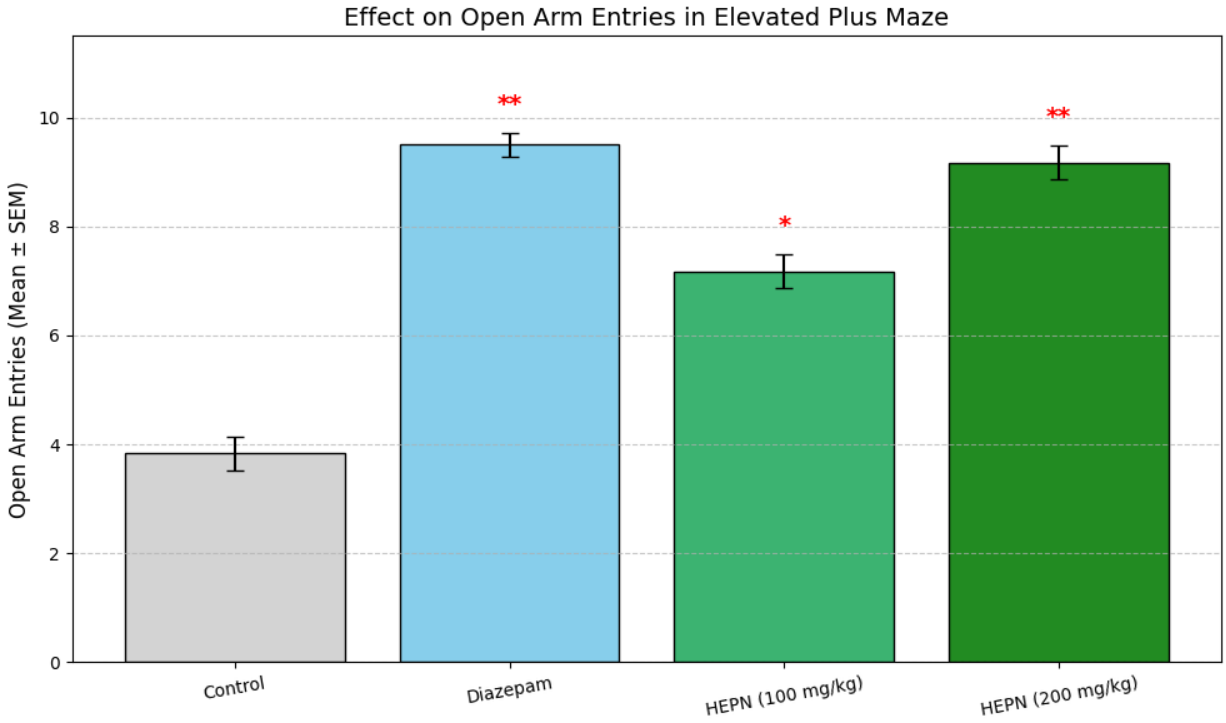

Figure 2: Effect of hydroalcoholic extract of *Phyllanthus niruri* on Open Arm Entries in Elevated Plus Maze

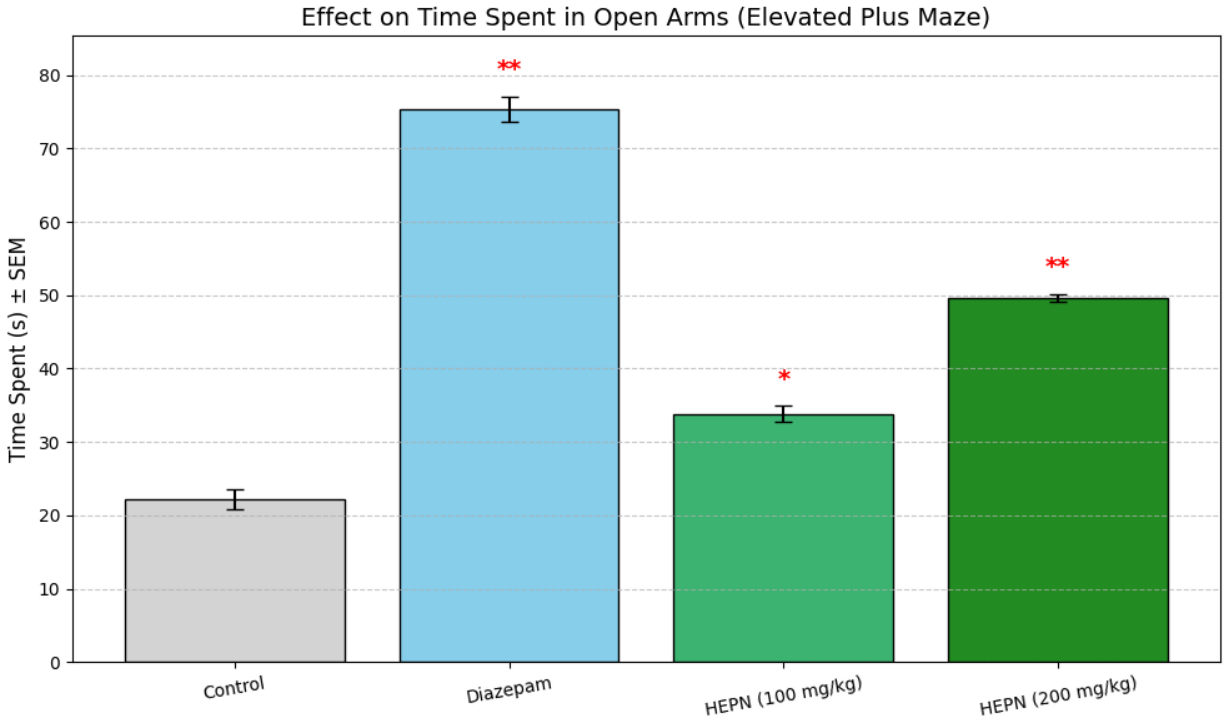

Figure 3: Effect of hydroalcoholic extract of *Phyllanthus niruri* on Time spent in Open Arm in Elevated Plus Maze

OPEN FIELD TEST

Table 6: Open field test results

Group	Squares Crossed	Rearings
Control	48.67 ± 1.28	9.83 ± 0.31
Diazepam (2 mg/kg)	85.17 ± 1.33 **	18.83 ± 0.40 **
HEPN (100 mg/kg)	65.83 ± 1.47 *	13.67 ± 0.49 *
HEPN (200 mg/kg)	78.33 ± 1.60 **	16.67 ± 0.42 **

Values are expressed as Mean ± SEM (n = 6). Significance *p < 0.05, **p < 0.01 vs. Control group. (one-way ANOVA followed by Dunnett's test)

In the Open Field Test, the behavior of mice was assessed to determine the effect of *Phyllanthus niruri* extract on locomotion and exploratory activity—two important indicators of anxiety-like behavior. The control group showed limited activity, with fewer square crossings (48.67 ± 1.28) and a lower number of rearings (9.83 ± 0.31), indicating heightened anxiety. Mice treated with Diazepam (2 mg/kg) exhibited a statistically significant increase in both parameters ($p < 0.01$), showing 85.17 ± 1.33 squares crossed and 18.83 ± 0.40 rearings.

This confirms the anxiolytic action of the standard drug. The group administered *Phyllanthus niruri* extract at 100 mg/kg demonstrated moderate enhancement in activity, with 65.83 ± 1.47 squares crossed and 13.67 ± 0.49 rearings ($p < 0.05$), indicating a mild anxiolytic effect. The 200 mg/kg extract group showed a stronger behavioral response, with 78.33 ± 1.60 squares crossed and 16.67 ± 0.42 rearings ($p < 0.01$), which closely approached the standard group's performance.

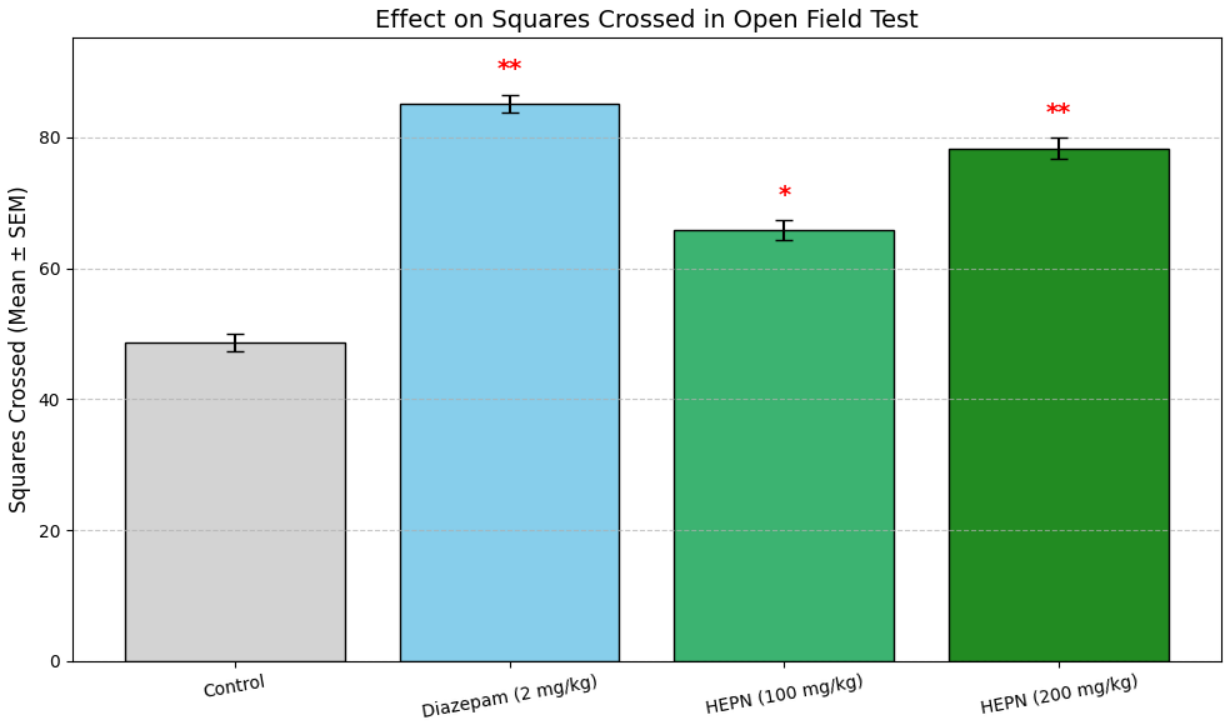

Figure 4: Effect of hydroalcoholic extract of *Phyllanthus niruri* on Squares Crossed in Open Field Test

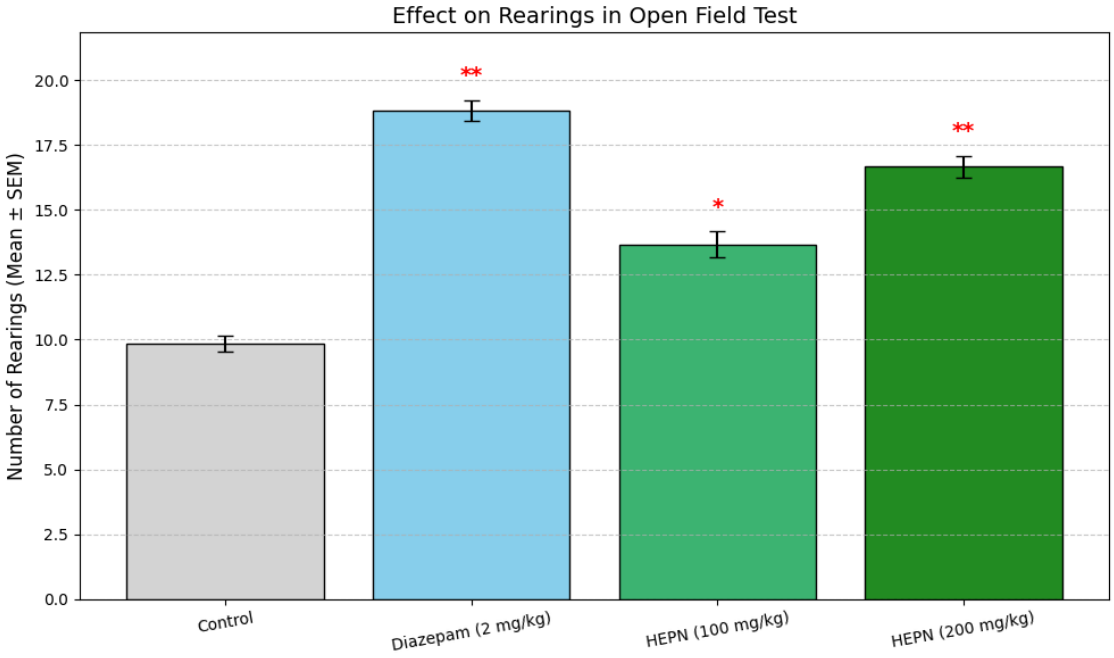

Figure 5: Effect of hydroalcoholic extract of *Phyllanthus niruri* on Rearings in Open Field Test

ACTOPHOTOMETER (LOCOMOTOR ACTIVITY)

Table 7: Actophotometer Test Results

Group	Locomotor activity (Mean ± SEM)
Control	300 ± 4
Diazepam 2 mg/kg	147 ± 3 **
HEPN (100 mg/kg)	206 ± 3 *
HEPN (200 mg/kg)	168 ± 2 **

Values are expressed as Mean ± SEM (n = 6). Significance * p < 0.05, ** p < 0.01 vs control (One-way ANOVA + Dunnett's)

The extract at 100 mg/kg and 200 mg/kg produced a dose-dependent reduction in locomotor activity: 206 ± 3 and 168 ± 2 counts versus 300 ± 4 in control ($p < 0.05$ and $p < 0.01$, respectively). Diazepam (2 mg/kg) led to a stronger suppression (147 ± 3 counts, $p < 0.01$), confirming CNS depressant effects. This indicates that Phyllanthus niruri extract exhibits anxiolytic activity without the marked sedation seen with diazepam, as supported by similar literature findings in other plant extracts.

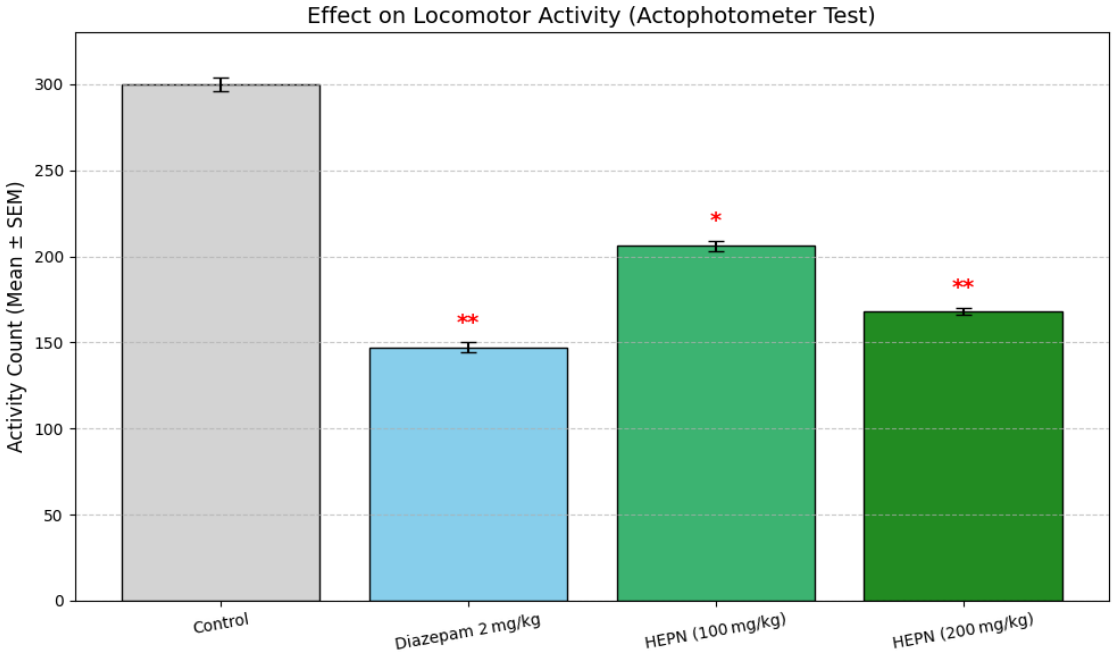

Figure 6: Effect of hydroalcoholic extract of *Phyllanthus niruri* on Locomotor Activity

ROTAROD (MOTOR COORDINATION ACTIVITY)

Table 8: Motor Coordination Activity Results

Group	Mean Fall Off Time Mean ± SEM (s)
Control	293 ± 2
Diazepam 2 mg/kg	131 ± 3 **
HEPN (100 mg/kg)	281 ± 3
HEPN (200 mg/kg)	268 ± 2

Values are expressed as Mean ± SEM (n = 6). Significance **p < 0.01 vs. Control group. (one-way ANOVA followed by Dunnett's test)

The standard reference drug diazepam (2 mg/kg) caused a noticeable drop in the time mice could stay on the rotating rod, with an average fall-off time of 131 ± 3 seconds, which was significantly lower than that of the control group ($p < 0.01$). This result reflects diazepam's known sedative and muscle-relaxing effects, which impair motor coordination. On the other hand, the mice treated with *Phyllanthus niruri* extract at doses of 100 mg/kg and 200 mg/kg were able to stay on the rod for 281 ± 3 seconds and 268 ± 2 seconds, respectively. These values are quite close to the control group's performance (293 ± 2 seconds), indicating that the extract did not interfere with balance or motor ability at either dose.

This outcome suggests that the anti-anxiety effects seen with *Phyllanthus niruri* in earlier behavioral tests (like the Elevated Plus Maze and Open Field Test) are not due to sedation, but rather reflect a genuine anxiolytic action. Therefore, *Phyllanthus niruri* may offer a safe, plant-based alternative for managing anxiety, without causing drowsiness or loss of motor control.

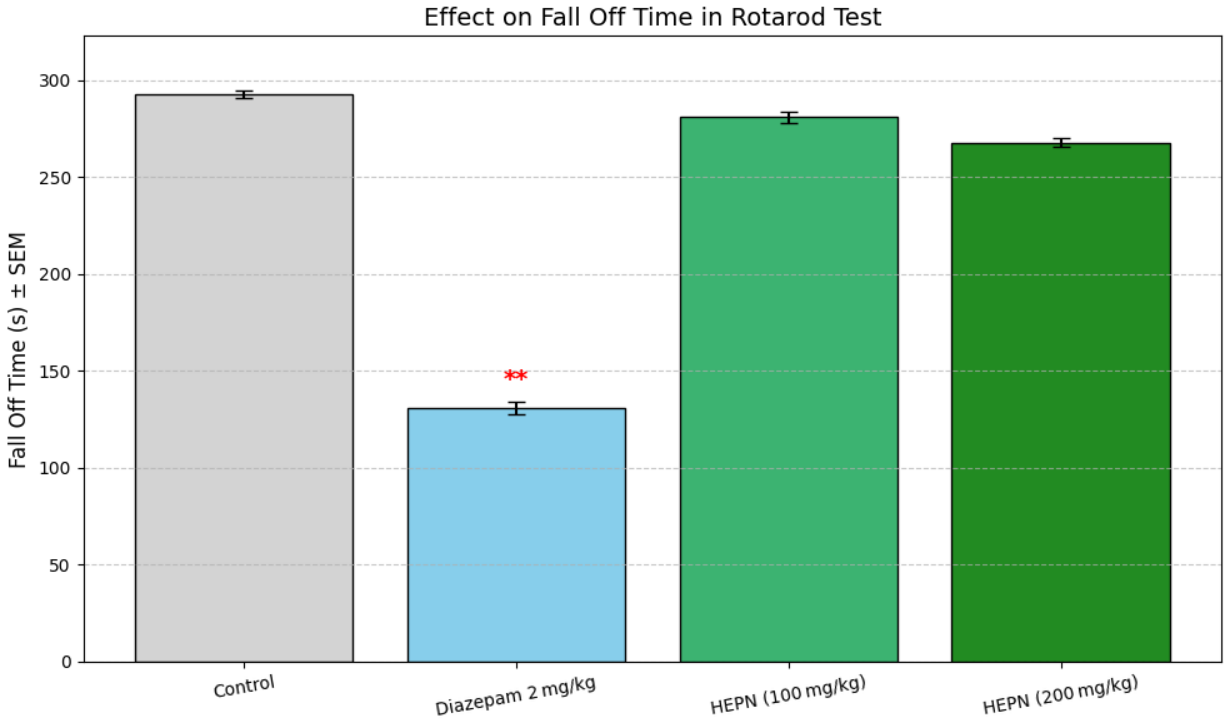

Figure 7: Effect of hydroalcoholic extract of *Phyllanthus niruri* on Motor Coordination

Corticosterone Analysis Results

The control group displayed corticosterone levels of 140.6 ng/mL, which is within the normal biological reference range of 100–200 ng/mL. The standard group treated with diazepam (2mg/kg) showed a significant reduction to 92.1 ng/mL, indicating suppression of the hypothalamic-pituitary-adrenal (HPA) axis. Test groups treated with *Phyllanthus niruri* extract demonstrated a dose-dependent reduction in corticosterone levels. The 100 mg/kg group showed 123.4 ng/mL, while the 200 mg/kg group showed 108.2 ng/mL. both values falling within the normal range. These results indicate that the extract reduces corticosterone levels moderately without excessive suppression, suggesting potential adaptogenic anxiolytic properties.

Table 9: Corticosterone Level

S. No.	Group	Corticosterone Level (ng/mL)	Biological Reference Range (ng/mL)
1	Control	140.6	100–200
2	Diazepam 2 mg/kg	92.1	100–200
3	HEPN (100 mg/kg)	123.4	100–200
4	HEPN (200 mg/kg)	108.2	100–200

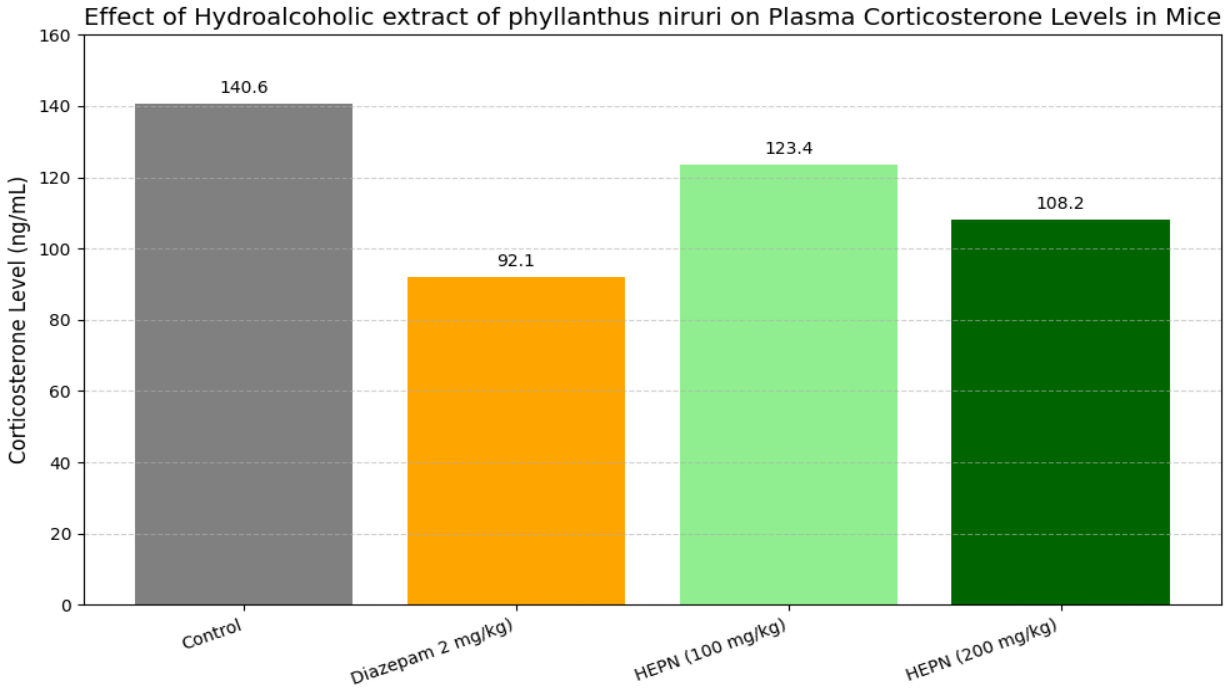

Figure 8: Effect of hydroalcoholic extract of *Phyllanthus niruri* on plasma corticosterone level

DISCUSSION

The current research was undertaken to explore the anxiolytic (anti-anxiety) potential of the hydroalcoholic extract of *Phyllanthus niruri* using various well-established behavioral models in Swiss albino mice. The selection of this plant was influenced by its long-standing use in traditional Ayurvedic medicine for treating a wide range of ailments, including those affecting the nervous system. Although *Phyllanthus niruri* has been widely studied for its hepatoprotective, anti-inflammatory, and antimicrobial properties, its potential role in anxiety disorders remains relatively underexplored. This study aimed to scientifically validate its traditional use and uncover possible neuropharmacological mechanisms of action.

Phytochemical screening confirmed the presence of major neuroactive groups such as flavonoids, alkaloids, tannins, and phenolic compounds. These constituents are known to interact with various neurotransmitter systems, particularly the GABAergic system, which is central to the modulation of anxiety.

To evaluate the behavioral effects, four models were employed: Elevated Plus Maze (EPM), Open Field Test (OFT), Actophotometer, and Rotarod. In the EPM, animals treated with *P. niruri* extract (100 mg/kg and 200 mg/kg) showed a significant increase in open-arm entries and time spent, indicating reduced anxiety levels. Similar anxiolytic responses were observed in the OFT, where extract-treated mice displayed enhanced exploratory behavior and increased center square entries, reflecting decreased anxiety without hyperlocomotion.

The Actophotometer and Rotarod tests ruled out sedative or motor-impairing effects. While diazepam significantly reduced locomotor activity and impaired balance (as expected from a CNS depressant), the extract maintained normal motor function and coordination, emphasizing its non-sedative anxiolytic action.

Importantly, corticosterone analysis provided biochemical support to the behavioral findings. Corticosterone is a primary stress hormone in rodents and an established marker for HPA axis activation. The control group exhibited normal corticosterone levels (140.6 ng/mL), whereas diazepam-treated mice showed significantly lower levels (92.1 ng/mL), suggesting strong suppression of the stress response. Interestingly, the *Phyllanthus niruri* extract groups showed dose-dependent reductions in corticosterone (123.4 ng/mL at 100 mg/kg and 108.2 ng/mL at 200 mg/kg), both within physiological limits. This indicates a moderate yet effective downregulation of the HPA axis, aligning with its adaptogenic properties. The extract appeared to buffer stress without overly suppressing the endocrine response — a feature beneficial in long-term therapeutic use.

Overall, the findings across behavioral and biochemical domains support the anxiolytic efficacy of *Phyllanthus niruri* extract. Its likely mechanisms include enhancement of GABAergic neurotransmission and regulation of stress-induced corticosterone elevation, potentially aided by antioxidant protection against oxidative damage in key brain regions. This dual action enhances its therapeutic promise.

The study provides scientific backing for the use of *Phyllanthus niruri* in anxiety-related conditions. Its favorable safety profile, lack of sedation or motor impairment, and natural origin make it a promising candidate for the development of plant-based anxiolytics.

CONCLUSION

The study successfully demonstrated that the hydroalcoholic extract of *Phyllanthus niruri Linn.* exhibits

significant anxiolytic effects in validated behavioral models in Swiss albino mice. Both tested doses (100 mg/kg and 200 mg/kg) led to notable improvements in anxiety-related parameters, comparable to the standard drug diazepam.

Crucially, the extract maintained normal locomotor activity and motor coordination, indicating it does not cause sedative or muscle-relaxant side effects a major advantage over conventional anxiolytic agents. Preliminary phytochemical analysis confirmed the presence of neuroactive constituents such as flavonoids, phenolics, and alkaloids, which may contribute to its anxiolytic mechanism.

In addition to behavioral benefits, the extract demonstrated a moderate yet significant reduction in plasma corticosterone levels, a key marker of stress response mediated by the hypothalamic-pituitary-adrenal (HPA) axis. This suggests that *Phyllanthus niruri* not only alleviates anxiety symptoms but also helps normalize physiological stress markers, supporting its adaptogenic potential.

Considering its efficacy, safety, non-sedative nature, and stress hormone-modulating effects, *Phyllanthus niruri* emerges as a promising herbal candidate for the management of anxiety disorders. However, further research involving neurochemical assays, receptor-binding studies, and clinical trials is warranted to fully elucidate its mechanism and validate its therapeutic potential in humans.

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