Original Article

EVALUATION OF ANXIOLYTIC ACTIVITY OF ETHANOLIC EXTRACT OF *Phyllanthus niruri* LEAVES IN MICE

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ABSTRACT

Anxiety has become one of the most prevalent and widely experienced mental illnesses, affecting 7-30% of the world's population. It is characterized as a distressing psychological state of mind, which is represented by internal feeling of uneasiness, stress and agony. Various theories, including psychodynamic, psychoanalytic, and genetic perspectives, have been proposed to explain the etiology of anxiety disorders. Certain drugs used for treatment of anxiety have side effects and limitation such as sedation, central complexity, and habituation and during withdrawal of drug. Researchers are actively exploring natural remedies, especially medicinal plants, to address anxiety disorders and minimize potential side effects associated with conventional drugs. Hence in the present study we investigate the anxiolytic activity of the plant *Phyllanthus niruri* leaves in mice using various experimental models such as Elevated Plus Maze and Light And Dark Chamber. In the Elevated Plus Maze (EPM) model, parameters measured included the number of entries in open and closed arms, along with the time spent in each. In the Light and Dark Chamber, measurements encompassed the number of crossings between light and dark sides recorded for 10 minutes, along with the time spent in each side. The administration of the different doses of the plant extract produced significant difference in the locomotor activity of the mice.

Key words: Anxiety, Phyllanthus niruri leaves, EPM, Light and Dark Chamber.

Anxiety is defined as a cognitive emotional response characterized by anatomical activation, typically of the autonomic nervous system.[1] When the manifestation of anxiety significantly disrupts daily life, it is referred to as an anxiety disorder.[2] Some symptoms of anxiety are panic attacks, fear, sweating, elevated blood pressure, stress, tension, tremor and vomiting.[3] According to Freud theory, anxiety is an unpleasant state of mind. He also mentioned the variability between objective anxiety and neurotic anxiety, primarily by considering whether the origin of the danger stemmed from external circumstances or internal impulses. Objective anxiety involves an intricate internal response to the anticipation of injury or harm stemming from an external threat. Neurotic anxiety is characterized by feelings of apprehension and heightened physiological alertness.[4] If anxiety is detected early they can be treated as well as the symptoms can be minimized and will provide quality to one's life.[5]

Anxiety can emerge as a significant concern for individuals of all ages, including both adults and children. Studies have proved that Women tend to experience anxiety more frequently than men.[6] To avoid the occurrence of anxiety, drugs like benzodiazepines, serotonin reuptake inhibitors (SSRI's) serotonin norepinephrine reuptake inhibitors (SNRI) are the first line drugs that are prescribed for treatment of anxiety. Tricyclic antidepressants, Monoamine oxidase inhibitor (phenelzine), antihistamines (hydroxyzine), anti-seizure drugs (gabapentin) are mostly prescribed as a second line treatment. Injecting this drug alters neurotransmitter levels like serotonin or GABA.[7] Prevention of anxiety disorder at early stage is necessarily important.[8] The etiology of anxiety mainly involves psychological factors such as childhood trauma or stressful past events; genetic factors also come into the considerations; physiological factors including alterations in serotonergic and catecholaminergic systems of the body play a role in causing of anxiety.[9]

Due to the trends seen in those years, several authors called the twentieth century as "The age of anxiety". This description suggests that contemporary life has led to increased anxiety levels. In recent years, individuals appear to be experiencing higher levels of anxiety, with concerns about safety, social acceptance, and job security weighing more heavily on their minds than in previous times.[10] According to the World Health Organization (WHO), approximately 450 million people worldwide suffer from anxiety. The current global prevalence rate of anxiety disorders among individuals

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aged 18 years and older is 18.1%.[11] Approximately one-eighth of the total population is affected by anxiety disorders, which is a heterogeneous type of condition.[12] The anxiety disorders are mainly classified as panic disorder, generalized anxiety disorder, post-traumatic stress disorder, obsessive–compulsive disorder, social phobia, and specific phobias.[13] Among anxiety disorders, panic disorder (10.3%), social phobia (2.7%), and generalized anxiety disorder (2.2%) are particularly common.[14]

The symptoms and the disorders following anxiety can be due to the impaired regulation of the central nervous system. These physical and emotional symptoms of this dysfunction are due to increased sympathetic stimulation at varying levels. Anxiety is the result of an imbalance or abnormal functioning of the neurotransmitters in the body. Most commonly, serotonergic and noradrenergic neurotransmitters play a prominent role in the modulatory steps involved. Disruption of the GABA system is also one of the actions in the physiology of the body due to anxiety. It is also seen that the corticosteroid level may increase or decrease the activity of various pathways in the brain.[15]

Plants are said to be rich source of biomolecule which has vast therapeutic uses to cure various illnesses.[16] The synthetic drugs and medications contain multiple side effects; hence the herbal drugs that possess variety of therapeutic values are used in the treatment of anxiety which shows few adverse reactions in the body. For preparation of anxiolytic and antidepressant drugs from plant source requires multidisciplinary approach including ethanopharmacological surveys, careful examination of the folkloric uses of the plant, as well as phytochemical and pharmacological studies.[17]

Phyllanthus niruri belongs to the family *phyllanthaceae*.[18] It is a herb that is seen growing in the tropical and subtropical regions of Asia, America, and China. *Phyllanthus niruri* is an annual herb that thrives in the wild, following the initial monsoon rains in regions such as Jharkhand, Bihar, Chhattisgarh, and other Indian states. There have been reports that this plant also grows habitually in the coastal regions.[19] It is an erect annual herb which grows up to a height of 40-70 cm with ascending herbaceous branch. The leaves are numerous in number; green in colour; sub sessile in structure; they are arranges closely.[20]

In the Ayurvedic system of medicine in India, Chinese traditional medicine, and Indonesian medicine, *Phyllanthus niruri* is utilized.[21] It is rich in bioactive compounds, including lignans (such as phyllanthin, hypophyllanthin, and niranthin), flavonoids, glycosides, tannins, alkaloids, ellagitannins, triterpenes, phenylpropanoids, steroids, ricinolic acid, niruriside, and phyltetralin.Numerous reports have highlighted the anti-inflammatory, anti-viral, anti-cancer, and anxiolytic potential of various Phyllanthus species.[22] Among the various bioactive molecules identified from Phyllanthus sources, Niranthin, a lignan, has shown promise in managing anxiety disorders, primarily through its influence on GABA receptors.[23] Chlorogenic acid obtained from P. niruri, 4-sinapoyl quinic acid not only is a powerful oxidizing agent but also exhibits anti-inflammatory, anticancer and anti-anxiety properties.[22]

MATERIALS

Experimental animals

The experiment employed healthy Swiss albino mice, weighing between 18 to 25 grams, of either sex, which was obtained from the animal facility at Srinivas College of Pharmacy in Mangalore. These mice were kept in a controlled environment with a temperature of 22±2 degrees Celsius, a relative humidity of 60±5%, and a 12-hour light/dark cycle. They were accommodated in clean polypropylene cages filled with sterile paddy husk as bedding material, and they were provided with unrestricted access to a standard pellet diet and water. All the animals were provided with humane care in accordance with the guidelines established in the "Guide for the Care and Use of Laboratory Animals" which was developed by the 'National Academy of Sciences' and published by the 'National Institute of Health'. Prior to their involvement in the study, the animals were allowed a minimum of one week for acclimatization. Furthermore, all procedures were conducted in compliance with the regulations set forth by the Institutional Animal Ethics Committee, as per the directives of the CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), under the Ministry of Animal Welfare Division in the Government of India, located in New Delhi, India.

Plant material

The leaves of *Phyllanthus niruri* belonging to family *Phyllanthaceae* were collected from a local region of Kundapura in Udupi district on July of 2023. It was authenticated by Dr. Siddharaju MN, Assistant Professor and Research Guide, Department of Botany, University College, Mangalore.

Chemicals

Chemical such as Diazepam of pure analytical grade will be procured from E Merck (India) Ltd, Mumbai and all other chemicals of analytical grade were procured from local suppliers.

METHODOLOGY

PREPARATION OF ETHANOLIC EXTRACT OF *Phyllanthus niruri* LEAVES

The fresh leaves of *Phyllanthus niruri* were collected locally during the season of monsoon. The collected material was further shade dried and powdered coarsely for the process of extraction. The powdered material was extracted by subjecting to cold maceration by using ethanol as solvent for 72 hours. In this procedure, 50g of the powder was soaked in 500ml of

ethanol, with continuous shaking using mechanical shaker. The resultant extract was filtered through Whatman No.1 filter paper and the filtrate was subjected to dryness at 50° on water bath.[24]

PREPARATION OF STOCK SOLUTION OF THE EXTRACT FOR DOSING

The ethanolic extract of *Phyllanthus niruri* was weighed and dissolved in required quantity of distilled water.Each time fresh preparation of the extract was prepared before administration. The extract was administered post orally at the constant volume of 100mg/kg and 200mg/kg for each animal.[25]

EXPERIMENTAL DESIGNS

The Swiss albino mice (22-27gms) of either sex were selected. The mice were divided into following groups (n=6) as follows:

Group I: Vehicle control

Group II: Standard group (Diazepam- 1mg/kg) (i.p) [26]

Group III: Ethanolic extract of *Phyllanthus niruri* leaves (100mg/Kg) (p.o.) [25]

Group IV: Ethanolic extract of *Phyllanthus niruri* leaves (200mg/Kg) (p.o.) [25]

The treatment of the plant extract was given through oral route. All animals were pretreated for 20 days except diazepam treated animals. On 21st day, animals was treated, 30 min before the evaluation.

EXPERIMENTAL MODELS

1. Elevated plus maze:

Principle- This model of anxiety has been extensively used for evaluation of novel anxiolytic agents and to investigate psychological and neurochemical basis of anxiety. This test has been proposed for selective identification of anxiolytic and anxiogenic drugs. Anxiolytic compounds, by decreasing anxiety, increase the open arm exploration time; anxiogenic compounds have the opposite effect.

Procedure- Prior to starting the experiment, the mice was handled daily to reduce stress. Two hours after the oral administration of the test drugs and 30 min after the intra-peritoneal administration of diazepam, the animal was placed in the center of the maze, facing one of the open arms. Thereafter, the results were recorded during the next 5 min. An arm entry being defined when all four paws are in the arm.

Following parameters measured:

1. Number of open and closed arm entries.

2. Percentage time spent in open and closed arm.

At the end of each trial the apparatus was wiped clean in order to eliminate any olfactory clues, which might modify the behavior of next animal. The procedure was conducted preferably in a sound attenuated room, with observations made from an adjacent room via web camera attached to the computer system.[27]

2. Light and dark model:

Principle- Crawely and Goodwin (1980) Crawley (1981) described a simple behavior model in mice to detect compounds with anxiolytic effects. In a two chambered system, where the animals can freely move between a brightly-lit open field and a dark corner. Mice tends to explore a novel environment but toretreat from the aversive properties of a brightly-lit open field and a dark corner, they show more crossings between the two chambers and more locomotor activity after treatment with anxiolytic. The number of crossings between the light and dark sites is recorded.

Procedure- Movements through the partition and the time spent in the dark and light chamber were counted. Mice were placed into the cage. The animals were treated 30 min before the experiment with test drugs or vehicle intra-peritoneally and then observed for 10 min, groups of 3 animals are used for each dose. The following behavioral were measured:

1) The number of entries in dark and light chamber.

2) Time spent in minutes in dark and light chambers.

The procedure was conducted preferably in a sound attenuated room, with observations made from an adjacent room via web camera attached to the computer system.[27]

STATISTICAL ANALYSIS

All data were expressed as Mean ± SEM. The statistical significance between groups were compared using One way ANOVA, followed by Dunnett's multiple comparison test. For all tests a "p" value of 0.05 or less was considered for statistical significance.

RESULTS

Phytochemical screening

Phytochemical analysis is a pivotal aspect of a research, delves into the examination of plant-derived compounds. By the phytochemical screening of the Ethanolic Extract of *Phyllanthus niruri* leaves (EEPNL) it was identified that the extract consists of alkaloids, phenolic acids, tannins, flavonoids, saponins, and glycosides which helps understand the pharmacological activities of the plant and helps paving the way for the development of novel drugs, nutritional supplements.

Anxiolytic effects

In EPM (Table 1), animals treated with two doses of EEPNL (100mg/kg and 200 mg/kg) showed increase in the time spent at open arm of the elevated plus maze model which was significant (33.167±1.537; $P<0.05$ and 38.0±0.931; $P<0.01$) when compared with control (22.0±3.512). Similarly, animals

treated with diazepam (1mg/kg), as expected, showed a significantincrease in the time spent at open arm of the elevated plus maze model (75.0±3.651;P<0.001)

Animals treated with two doses (100mg/kg and 200 mg/kg) also showed decrease in the time spent at closed arm of the elevated plus maze model which was significant (109.0±16.73; P<0.01 and 76.0±11.590; P<0.001) when compared with control (173.33±8.33). Similarly, animals treated with diazepam (1 mg/kg), as expected, showed a significant increase in the time spent at open arm of the elevated plus maze model (72.00±3.425; P<0.001).

Animals treated with two doses showed decrease in the number of entries in closed arm of the elevated plus maze model which was significant (6.833±0.60; P<0.01 and 6.167±0.307; P<0.001) when compared with control (11.0±1.211). Similarly, animals treated with diazepam (1mg/kg), as expected, showed a significant decrease in number of entries at open arm of the elevated plus maze model (5.5±0.764; P<0.001).

Animals also showed increase in the number of entries in open arm of the elevated plus maze model which was significant (7.83±0.477; P<0.01 and 8.83±1.249; P<0.001) when compared to control (4.0 ±0.516). Similarly, animals treated with diazepam (1mg/kg), as expected, showed a significant decrease in number of entries at open arm of elevated plus maze model(9.5±0.428; P<0.001).

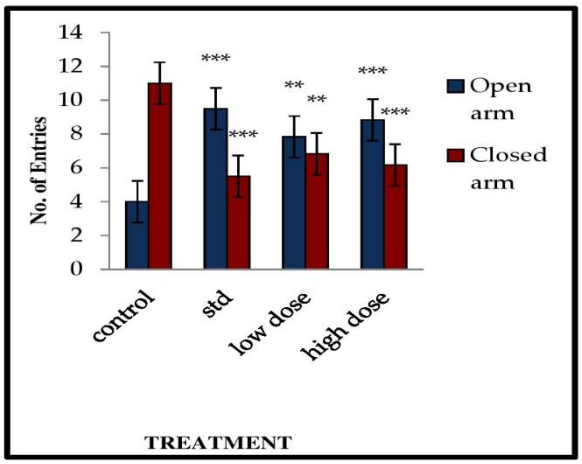

Fig. 1: Comparative profile of number of entries to open and closed arm in EPM after oral administration of 100mg/kg and 200mg/kg of EEPNL.

In LDT (Table 2) animals treated with two doses of EEPNL (100mg/kg and 200mg kg) showed reduced time spent in dark chamber (4.317±0.303 and 2.1±0.230;P<0.01) and with concomitant increase in time spent in light chamber (1.517±0.166; P<0.01 and 1.86±0.051; P<0.001) when compared with controls (4.633±0.704 and 0.650±0.141). Similarly, animals treated with diazepam (1mg/kg) as expected showed reduced the time spent in dark chamber with concomitant increase in time in light chamber (1.2±0.435; P<0.001 and 3.833±0.219; P<0.001) respectively.

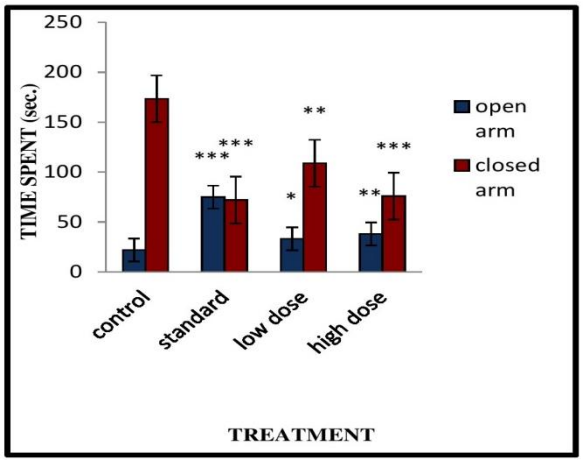

Fig. 2: Comparative profile of time spent in open and closed arm in EPM after oral administration of 100mg/kg and 200mg/kg of EEPNL.

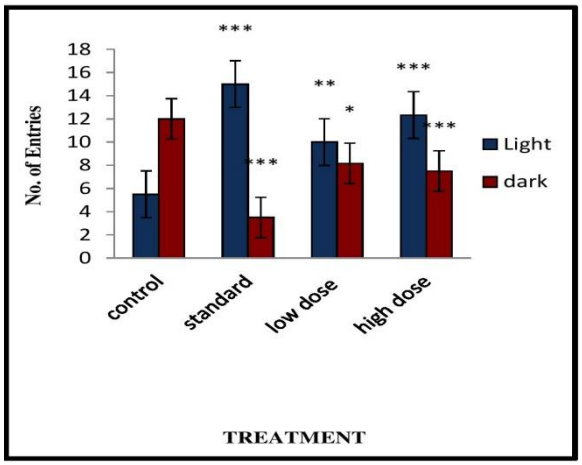

Fig. 3: Comparative profile of number of entries to light and dark chamber in LDC after oral administration of 100mg/kg and 200mg/kg of EEPNL.

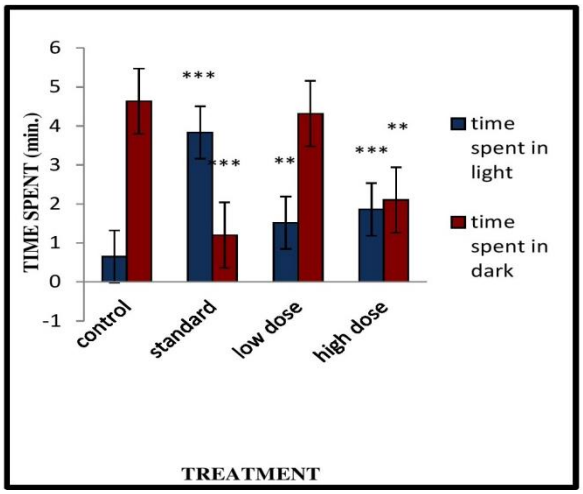

Fig. 4: Comparative profile of time spent in light and dark chamber in LDC after oral administration of 100mg/kg and 200mg/kg of EEPNL.

All animals treated two doses of EEPNL (Table 10) showed increased number of entries in dark chamber (8.167±.792; P<0.05 and 7.5±0.764; P<0.001) and with increase in number of entries in time in light chamber (10.0±1.15;P<0.01 and 12.33±0.843;P<0.001) when compared with controls (12.0±0.577and 5.5±0.764) respectively. Similarly, animals treated with diazepam (1 mg/kg) as expected showed increased number of entries in both dark chamber and light chamber (3.5±0.342; P<0.001 and 15.0±1.1414; P<0.001) respectively.

Table 1: Effect of EEPNL on Elevated Plus Maze in mice

Sl.no	Drug treatment	Dose	No. of entries (mean±SEM)		Time spent in seconds (mean±SEM)	
			Open arm	Closed arm	Open arm	Closed arm
1	Control	Vehicle	4.0 ±0.516	11.0±1.211	22.0±3.512	173.33±8.33
2	Standard	Diazepam	9.5±0.428***	5.5±0.764***	75.0±3.651***	72.00±3.425***
3	Low dose	100mg/kg	7.83±0.477**	6.833±0.601**	33.167±1.537*	109.0±16.73**
4	High dose	200mg/kg	8.83±1.249***	6.167±0.307***	38.0±0.931**	76.0±11.590***

Values aremean±SEM for n=6, expressed as the time (in sec) of 6 animals in each group. Data analysis was performed using Dunnet's test. *P<0.05, **P<0.01, ***P<0.001 v/s control

Table 2: Effect of EEPNL on light dark transition model in mice

Sl.no	Drug treatment	dose	No. of entries (mean±SEM)		Time spent in min (mean±SEM)	
			Light	dark	Light	dark
1	Control	Vehicle	5.5±0.764	12.0±0.577	0.650±0.141	4.633±0.704
2	Standard	Diazepam	15.0±1.1414***	3.5±0.342***	3.833±0.219***	1.2±0.435***
3	Low dose	100mg/kg	10.0±1.15**	8.167±.792*	1.517±0.166**	4.317±0.303 ns
4	High dose	200mg/kg	12.33±0.843***	7.5±0.764***	1.86±0.051***	2.1±0.230**

Values aremean±SEM for n=6, expressed as the time (in min) of 6 animals in each group. Data analysis was performed using Dunnet's test. *P<0.05, **P<0.01, ***P<0.001 v/s control

DISCUSSION

Anxiety encompasses various dimensions, such as cognitive, emotional, physical, and behavioral components. Physiological reactions are typically normal and adaptive, but, they can become exaggerated to the extent that they jeopardize an individual's psychosocial well-being.[28] Benzodiazepines have been extensively used for the last 40 years to treat several forms of anxiety, but due to their unwanted side effects, alternative treatment strategies with favorable side-effect profiles, credible benefits and moderate costs are of interest. Thus, there is a need to investigate alternatives.[29]In the present study, the anxiolytic potential of *Phyllanthus niruri* was determined using various experimental models. The behavioral models employed for this study were elevated plus maze and light and dark test.

The EPM is considered to be an etiologically valid animal model of anxiety - uses natural stimuli, such as a fear of a new, brightly-lit open space and the fear of balancing on a relatively narrow raised platform, moreover it is known that anxiolytic agent increases the frequency of entries and time spent in open arm of the EPM.[29]

Light-dark exploration test titrates a natural tendency of mice to explore a novel environment, against the aversive properties of a brightly lit compartment. Some of the measurable indices of anxiety are number of crossing between chambers, locomotor activity, number of rearing and amount of time spent in the dark area of the apparatus.[30] In the present study it is noted that administration of EEPNL (100mg/kg and 200mg/kg) showed dose dependent increase in the time spent in the open arms and the number of entries into open arms when compared with the control. The present study also showed that EEPNL (100 mg/kg and 200 mg/kg) increased the time spent in the light area and the no. of entries in to the light suggesting again that EEPNL possesses anxiolytic properties.

Earlier reports on the chemical constituents of plants and their pharmacology suggest that plant contains alkaloids, flavonoids, tannins, phenolic compounds and saponins which

are active against anxiety and many CNS disorders in the body.

Phyllanthus niruri shows anxiolytic property due to the presence of phenolic compounds and tannins such as chlorogenic acid and niranthin.[22] The phytochemical gallic acid shows anxiolytic activity primarily mediated by the 5-HT1 A but not BZD receptors.[31]

Flavonoids being structural analogues of benzodiazepines bind to $GABA_A$ receptor and pharmacologically act as a partial agonist.[29]

The effects of EEPNL on EPM and light- dark test were almost equivalent to that of 1mg/kg diazepam. In the present study, the anxiolytic activity of *Phyllanthus niruri* extract was observed at the dose of 200mg/kg in mice. These observations clearly indicate the *Phyllanthus niruri* exerts anxiolytic activity.

It is possible that the mechanism of anxiolytic action of EEPNL could be mediated by synergistic action of these phytochemical. The results obtained in the study suggest that the ethanolic extract of *Phyllanthus niruri* possesses anxiolytic property. Thus, of EEPNL has potential clinic application in the management of anxiety disorder. Future investigations are warranted for elucidating the extract mechanism and bioactive compounds.

CONCLUSION

The present study can be concluded with the fact that ethanolic extract of Phyllanthus niruri reveals significant anxiolytic effect in Swiss albino mice using animal models of anxiety namely Elevated plus maze, light and dark chamber. The data obtained was satisfactory and conclusive so as to achieve our objectives. In the conclusion, present data indicate the administration of EEPNL to mice shown anxiolytic activity supporting the folk information regarding anxiolytic activity of EEPNL.

The extract and preliminary phytochemical studies of EEPNL revealed the presence of chemical and phytochemical constituents such as (chlorogenic acid and niranthin), alkaloid, tannins, flavonoid, phenolic compound and saponnins. The exact mechanism underlying anxiolytic activity is not clear but it may be apparently related to active compounds present in the extract. Hence further studies would be necessary to evaluate the contribution of active chemical constituents for the observed anxiolytic activity.

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