

EVALUATION OF ANXIOLYTIC ACTIVITY OF LEAF EXTRACTS OF *NELUMBO NUCIFERA* IN LABORATORY RODENTS

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ABSTRACT

Nelumbo nucifera Gaertn. leaves (Nymphaeaceae) have been traditionally used for the treatment of various ailments such as ulcer, helminthic, microbial and fungal infections, cancer and diabetes. Despite a long tradition of use, no systematic phytochemical and pharmacological work has been carried out on this potential plant. Thus, *Nelumbo nucifera* (NN) was subjected to preliminary anti-anxiety screening studies, with a view to ascertain the verity of its traditional use as an anxiolytic. In the present investigation, leaves of *Nelumbo nucifera* were extracted using various solvents such as methanol, hydroalcohol and aqueous. All the crude extracts (50, 100 and 200 mg/kg) were evaluated for anxiolytic activity in mice using Elevated Plus Maze (EPM) and Light-Dark apparatus (LD). The mice treated with MENN and HENN extracts at a dose of 200 mg/kg, significantly increased the time spent in the open arm and in the number of entries in the elevated plus maze apparatus, and also increased the time spent in the light area and decreased the time spent in the dark area with respect to control. Furthermore, a diminution in the anxiety response was also observed against elevated plus maze and light dark tests, which signify its anti-anxiety activity when compared with standard anxiolytic drug, diazepam (2 mg/kg). Among all these extracts, the methanol and hydroalcoholic extracts exhibited significant anti-anxiety activity at a dose of 200 mg/kg in mice. Phytochemical screening showed presence of alkaloids and flavonoids in the extract of NN, which might affect certain mediators of brain to reduce anxiety. These results suggest that the extract administration should reduce the aversion fear and produce anxiolytic activity.

KEY WORDS

Nelumbo nucifera leaves, Anxiolytic activity, Elevated Plus Maze and Light-Dark model

1. INTRODUCTION

Human anxiety is a feeling of apprehension, uncertainty or tension stemming from the anticipation of imaginary or unreal threat. Anxiety affects one-eighth population worldwide and has become an important research area in the field of psychopharmacology. Benzodiazepines (BZD's), barbiturates, antidepressants (TCA's) have been used for long time to treat anxiety disorders. The serious side effects associated with these drugs, namely rebound insomnia, sedation, muscle relaxation, withdrawal and tolerance (BZD's, barbiturates and alcohol), sexual dysfunction, anti-cholinergic, antihistaminic effects (TCA's) have limited their use in patients. Due to these adverse effects many researchers are conducting studies to find an alternative medicine to treat anxiety [1]. Several

medicinal plants have been evaluated for their effects on animal models of anxiety. So, Herbal treatments are to be helpful in treating anxiety [2]. The herbal medicines have wide therapeutic actions and safety profile. This makes the herbal therapies to be successful [3]. One of these can be the use of *Nelumbo nucifera* Gaertn. (Family: Nymphaeaceae) also known as Tamara (lotus). *Nelumbo nucifera* is widely distributed in South-East Asia. In India, it occurs from Kashmir in north to Kanyakumari in south [4]. The leaves are large and orbicular; the young leaves are eaten as vegetables and used in traditional medicine. The leaves are rich in a number of alkaloids. The major components are present such as nuciferine, roemerine and benzyl isoquinoline alkaloids [5]. In Ayurveda this plant is used as a diuretic and anthelmintic and in the treatment of

strangury, vomiting, leprosy, skin diseases and nervous exhaustion^[6]. Despite the widespread use of *Nelumbo nucifera* as an anxiolytic, there are no pharmacological data to support such effects. The objective of present study is to investigate the effect of methanolic, hydroalcoholic and aqueous extracts of the leaves of *Nelumbo nucifera* on anxiolytic activity in different experimental models on CNS in mice.

2. MATERIALS AND METHODS

2.1. Collection of Plant material

The *Nelumbo nucifera* Gaertn. (Nymphaeaceae) leaves were collected from the local regions and authenticated by taxonomists. These were made free from the adherent foreign material, air-dried, cut into small pieces and coarsely powdered mechanically.

2.2. Extraction of Herb

The dried and powdered leaves were defatted with petroleum ether (60-80°C). Methanolic extract and Hydroalcoholic extract (70% ethanol) were obtained by Soxhlet extraction method and Aqueous extract by decoction method^[7].

The Methanolic, Hydroalcoholic and Aqueous extracts of *Nelumbo nucifera* are termed as MENN, HENN and AQNN respectively.

2.3. Preliminary Photochemical Investigation

The extracts of *Nelumbo nucifera* were screened for the presence of various Phytochemical constituents like alkaloids, flavonoids, Carbohydrates, Glycosides, Phytosterols, Saponins, phenolic compounds and tannins^{[7]-[10]}.

2.4. Animals

Healthy Swiss albino mice, either sex weighing 20-30gram were procured from the Teena Bio-labs Pvt. Ltd. (Reg. No. 177/1999 CPCSEA), Hyderabad, Telangana. Animals were housed at CPCSEA approved animal house (1553/PO/a/11/CPCSEA). The animals were stabilized for 1 week; they were maintained under standard conditions at temperature of 25 ± 1°C, 60 ± 5 % relative humidity and 12–hours light dark cycle. They had been given standard pellet diet supplied by Hindustan Lever Co., Bombay and water ad libitum throughout the course of study. All the study protocols were reviewed and approved by Institutional Animal Ethical Committee

(IAEC). The studies were strictly followed Ethical norms during all experimental procedures.

2.5. Source of Drugs, chemicals and Equipments

Diazepam (Natco Pharma Ltd), chemicals- Diethyl ether and tween-80 (Merck, Mumbai), Equipment- Elevated Plus maze and Light-dark apparatus.

2.6. Acute Toxicity Study

The Acute Toxicity Studies were performed using Swiss albino mice as per OECD Guideline No.423^[11]. The median lethal dose of the pet-ether alcohol and aqueous were determined by orally administering the extracts in increasing dose levels of 0.1,0.2,0.5, 1, 1.5 and 2 g/kg body weight to healthy mice of either sex. The animals will be observed continuously for 2 h and later 24 hr intervals for a period of 48hrs, at the end of this period, if any mortality in different dose groups were noted.

Nelumbo nucifera (NN) was found to be safe till a dose of 2000 mg/kg since no mortality and abnormal toxicity was observed at this dose. According to OECD guidelines, maximal safe dose was selected for this study. Hence, three doses of NN [MENN, HENN and AQNN] were selected for the study. The doses are 50, 100 and 200 mg/kg. All extracts are given by Oral administration (p.o.).

2.7. Study Design

For the following activities the animals were divided into eleven groups, each group containing six animals.

Group I Served as a control and received 2% Tween 80 (10ml/kg).

Group II, III and IV received Methanolic extracts (ME) of 50, 100 and 200 mg/kg respectively.

Group V, VI and VII received Hydroalcoholic extracts (HE) of 50, 100 and 200 mg/kg respectively.

Group VIII, IX and X received Aqueous extract (AQ) of 50, 100 and 200 mg/kg respectively.

Group XI Served as a standard and received Diazepam (2 mg/kg) a standard Drug.

The test groups were from II to X group. The extracts of *Nelumbo nucifera* (NN) termed as MENN, HENN, and AQNN respectively.

2.8. Evaluation of Anxiolytic Activity (In-vivo methods)

2.8.1. Elevated plus-maze Test

In brief, the apparatus was composed of two open (30 × 5 cm) and two enclosed (30 × 5 × 15 cm) arms

that radiated from a central platform (5 × 5 cm) to form a plus sign. The maze floor and the closed arms were covered with black adhesive tape. The plus-maze was elevated to a height of 20 cm above the floor level by a single central support and four 25-W red fluorescent lights arranged as a cross at 100 cm above the maze were used as the source of illumination. The experiment was conducted during the dark phase of the light cycle (7: 00 –19: 00 pm). The mice in groups from II to X were administered with MENN, HENN and AQNN extracts (50, 100 and 200 mg/kg, p.o. 1 h before test), whereas group I treated with 2% Tween 80 and group XI treated with Diazepam (2 mg/kg i.p 30 min before test). The test was started by placing the mouse on the central platform of the maze facing the closed arm. The number of entries into, and the time spent in each of the two types of arm, were counted during a 5 min test period. A mouse was considered to have entered an arm when all four paws were in the arm. The apparatus was cleaned thoroughly between trials^[1].

2.8.2 Light-dark model transition Test

The light-dark apparatus consists of two-compartment chamber (40×60×20cm/h) comprising of a brightly illuminated area (40×40cm) with a 100 W white light source placed 17 cm above the box and a dark area (40×20 cm) separated by a wall with a round hole (7 cm diameter) was used. Mice were placed individually in the illuminated part of the cage and following parameters were recorded during the test session of 5 min, total no. of crossings between the light and dark area, total time spent in the bright illuminated part of the cage and in the dark part of the cage, total no. of rearing in brightly illuminated and in dark part of the cage, and no. of defecation units. The apparatus was cleaned thoroughly between experiments. All behavioral recordings were carried out with the observer unaware of the treatment the mice had received^[12].

2.8.3 Statistical analysis

Values are expressed in Means ± S.E.M. for six animals in each group and statistically assessed by one-way analysis of variance (ANOVA) and subjected to Dunnett's test. The $p < 0.05$ was considered significant.

3. RESULTS

3.1. Percentage of yield: The percentage yield of extraction of *Nelumbo nucifera* (NN) leaves were showed more in methanolic (MENN) and Hydroalcoholic (HENN) extracts when compared with Aqueous (AQNN) extract. (56.2%, 62.8% and 28.20% respectively)

3.2. Phytochemical Screening: The methanolic, hydroalcoholic and aqueous extracts were showed positive results for Alkaloids, flavonoids, Carbohydrates, Glycosides and tannins but negative result for phytosterols.

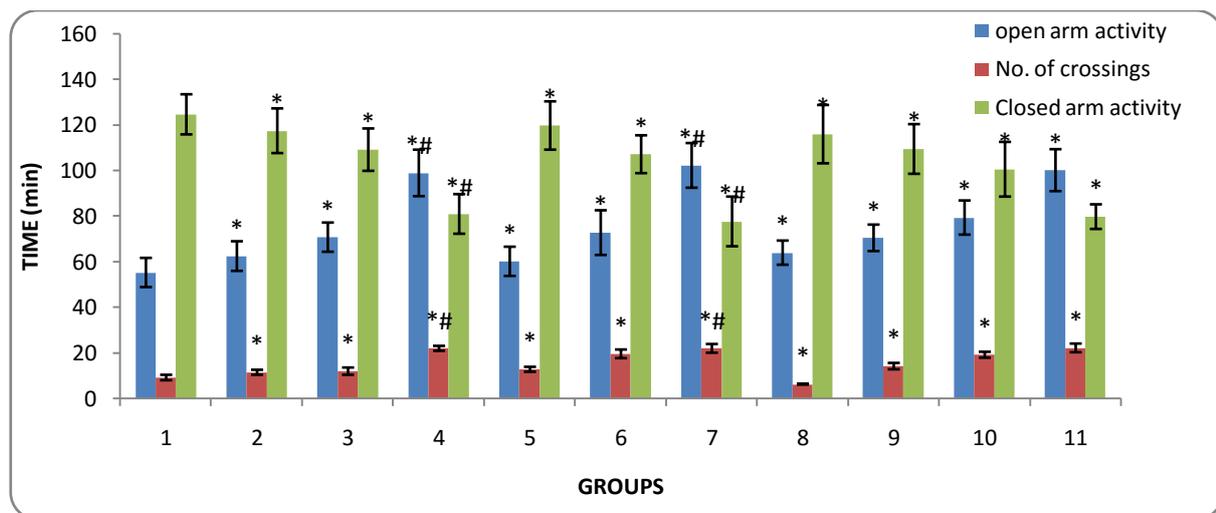
3.3. Effect of *Nelumbo nucifera* extracts on elevated maze method of Anxiety

The total time spent in the two closed quadrants, which have black walls (20 cm high) and two open quadrants, was recorded for 5 min. The total duration of time spent in the open quadrants is thought to negatively reflect anxiety, which indicates anti-anxiety activity. The results are given in Table 1 below and represented in Figure 1. In this study, the extract of MENN and HENN at a dose of 200 mg/kg significantly ($\#p < 0.05$) increased time spent and number of entries into the open arm and decreased time spent and number of entries into the closed arm compared with control which was received 2% tween 80. But the extract MENN and HENN at the 200 mg/kg dose level, when compared with that of diazepam, both extracts were showed similar to that of Diazepam. The extracts MENN (100 mg/kg), HENN (100 mg/kg) and AQNN (100 mg/kg) were showed moderately anxiety activity, in comparison to the control.

Table: 1. Effect of *Nelumbo nucifera* leaves extracts on number of entries and time spent in elevated plus maze method of Anxiety

Group No.	Treatment	Dose	Time spent in open arms (min)	Number of entries	Time spent in close arms (min)
1	2%Tween80	10 ml/kg	55.3± 6.4	9.2± 1.2	124.7±8.8
2		50 mg/kg	62.5±6.5*	11.5±1.1*	117.5±9.8*
3		100 mg/kg	70.8±6.4*	12.0±1.6*	109.2±9.3*
4	MENN	200 mg/kg	99.0±10.2*#	22.0±1.1*#	81.0±8.7*#
5		50 mg/kg	60.2±6.4*	12.8±1.1*	119.8±10.6*
6		100 mg/kg	72.8±9.8*	19.6±1.9*	107.2±8.3*
7	HENN	200 mg/kg	102.3±9.8*#	22.0±1.9*#	77.7±10.9*#
8		50 mg/kg	64.0±5.3*	6.3±0.2*	116.0±12.8*
9		100 mg/kg	70.5±5.8*	14.2±1.4*	109.5±10.9*
10	AQNN	200 mg/kg	79.4±7.5*	19.2±1.3*	100.6±12.0*
11		Diazepam	2 mg/kg	100.2±9.2*	22.2±1.9*

Values are expressed as (Mean ± SEM), n= 6, All groups were compared with Normal control group *p<0.05 and standard group and significance shown by #p<0.05. Statistically analyzed by one- way analysis of variance (ANOVA) followed by Dunnett's test.


Fig: 4.14. Effect of *Nelumbo nucifera* leaves extracts on number of entries and time spent in elevated plus maze method of Anxiety (Values expressed as Mean ± SEM)

3.4. Effect of *Nelumbo nucifera* leaves extracts on Light and Dark model transition test

The animals were placed individually in the illuminated part of the cage and following parameters were recorded during the test session of 5 min, total no. of crossings between the light and dark area, total time spent in the illuminated part of the cage, time spent in the dark part of the cage.

The obtained results are given in Table 2 below and represented in Figure 2. In this study, the mice treated with 2% tween 80 showed less time spent in the light

area and more time spent in the dark area. The mice treated with MENN and HENN extracts at dose of 200 mg/kg and diazepam (2 mg/kg) were showed significantly increased time spent in the light area and decreased time spent in the dark area. All the extracts MENN, HENN and AQNN (50 and 100 mg/kg) significantly reduced time spent in the light area and increased time spent in the dark area when compared with diazepam.

The MENN, HENN extract (200 mg/kg) and diazepam significantly increased the number of entries in the light as well as dark area.

Table: 2. Effect of *Nelumbo nucifera* extracts on number of crossing and time spent in Light box and Dark box in light and dark model transition test

Group No	Treatment	Dose	Time spent in lighted box (min)	Number of crossings	Time spent in Dark box (min)
1	2%Tween 80	10 ml/kg	67.4± 5.1	11.4± 1.0	112.6±8.6
2		50 mg/kg	60.8±6.2*	12.0±1.2*	119.2±5.6*
3	MENN	100 mg/kg	72.8±6.4*	12.5±1.2*	107.2±9.2*
4		200 mg/kg	100.6±9.4*##	20.0±2.1*##	79.4±8.5*##
5		50 mg/kg	70.5±6.8*	10.5±1.0*	109.5±9.8*
6	HENN	100 mg/kg	82.3±8.5*	12.0±1.2*	97.5±9.2*
7		200 mg/kg	120.2±11.0*##	22.2±2.3*##	58.8±8.4*##
8		50 mg/kg	62.5±6.5*	10.3±1.0*	117.5±10.6*
9	AQNN	100 mg/kg	65.7±6.0*	9.6±1.0*	114.4±10.8*
10		200 mg/kg	80.2±7.6*	10.6±1.0*	99.8±12.9*
11	Diazepam	2 mg/kg	121.2±10.6*	20.0±2.01*	59.8±5.4*

Values are expressed as (Mean ± SD), n= 6, All groups were compared with Normal control group *p<0.05 and standard group and significance shown by #p<0.05. Statistically analyzed by one- way analysis of variance (ANOVA) followed by Dunnett's test.

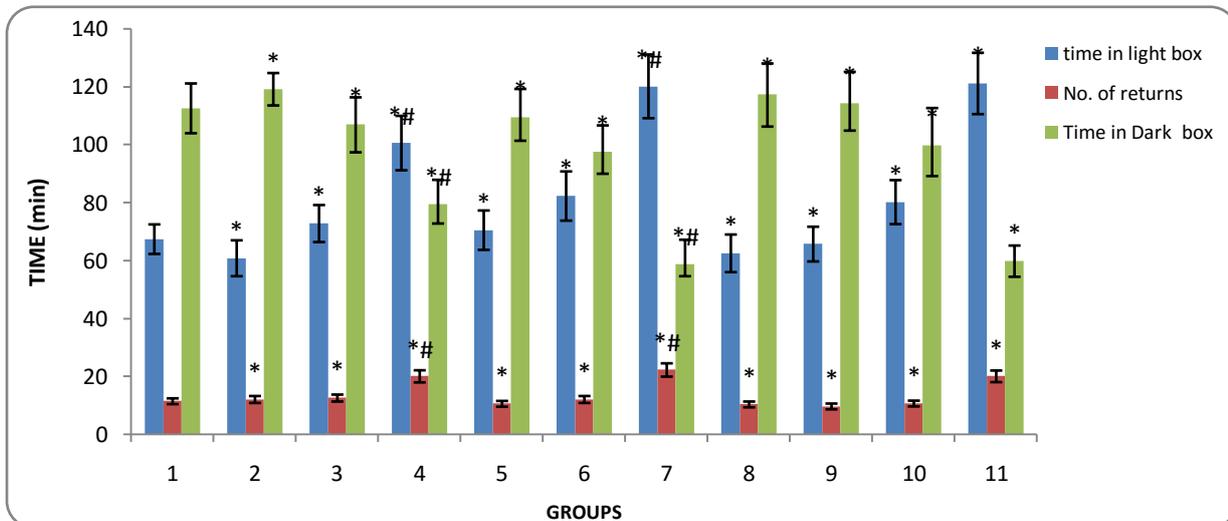


Fig: 2. Effect of *Nelumbo nucifera* extracts on number of crossing and time spent in Light box and Dark box in light and dark model transition test (Values expressed as Mean ± SEM)

4. DISCUSSION

Fear and anxiety are defined as the response of a subject to real or particular threats that may impair its homeostasis, this response may include physiological and/or behavioral. Measuring anxiety like behavior in mice has been mostly undertaken using a few classical animal models of anxiety such

as the elevated plus maze and Light dark model. The test is principally based on the exposure of animal to an elevated maze array, which evokes an approach-avoidance conflict that is considerably stronger than that evoked by exposure to an open maze array. The animals being exposed to the new

environment tend to avoid open entries and prefer to stay in closed arm due to fear^[13].

In the present study, we used the EPM & light dark model of anxiety to evaluate the anxiolytic effects of the methanolic, hydroalcoholic and aqueous extracts of *Nelumbo nucifera* (NN). EPM is a model which uses the natural fear of rodents to avoid open and elevated places. The ratio of open and closed area entries reflects a specific effect on anxiety, provided there is no concomitant change in the total number of entries (open + closed), however, this is not totally true for diazepam which increases preference for the open areas i.e. total entries. As expected, diazepam produced significant increases in both the time spent and in number of entries into the open arms & light chamber.

The methanolic and hydroalcoholic extracts of NN showed significant increases in the time spent in open-sided arms and number of entries into the open arm in the elevated plus maze by the mice at a dose of 200 mg/kg. The ultimate manifestation of anxiety and fear in the animals is exhibited by decrease in the motor activity and preference to remain at safer places. Anxiolytic agents are expected to increase the motor activity, which is measured by the time spent by the animal in the open arms^[14]. Anxiolytics (e.g. diazepam) are known to exert their pharmacological action by increasing the gamma amino butyric acid (GABA) content in the cerebral hemisphere of mice^[15]. Based on the above statements the results were indicating the *Nelumbo nucifera* could reduce the fear and anxiety in mice due to increase in the motor activity and GABA content, hence it produces anxiolytic activity.

In Light dark model the hydroalcoholic extracts of leaves of NN (200 mg/kg) was illustrated the maximum increase in the time spent and number of entries into the light compartment when compared with standard drug diazepam. The anxiolytic activity of NN was showed similar to that of diazepam. Diazepam has shown significant increase in the number of entries in the open arm and produced anxiolytic effects in a variety of anxiolytic screening procedures, including EPM and light-dark model^[1],^[12].

Alkaloids are the most important secondary metabolites in many plants that are held responsible for their anxiolytic actions^[16]. The flavonoids increased the time spent in the open arm of elevated plus maze which clearly indicated its anxiolytic activity^[1]. Flavonoids have a range of activities on GABA_A receptors and have been described as a “new family of benzodiazepine receptor ligands”. They were first linked to GABA_A receptors^[17].

In the present study the phytochemical screening of NN showed the presence of alkaloids, flavonoids and tannins and due to their interaction with the GABA/ benzodiazepine receptor complex in the brain there by showing the anxiolytic effect. These results suggest that extracts of NN administration should reduce the aversion fear and produce anxiolytic activity.

4. CONCLUSION

The effects were dose dependent, the optimum effect was observed at a dose of 200 mg/kg which was significantly higher than vehicle treated control group i.e it was concluded that the *Nelumbo nucifera* possess Anxiolytic activity which was evidenced by all the models as described above. The results have been obtained carefully from the controlled experiment models with laboratory animals. The statistical validity of the findings has been proven and they provide a scientific foundation for the use of the biologically active ingredients of *Nelumbo nucifera* in anxiety, which might affect certain mediators of brain and thus reduce the aversion fear and produce anxiolytic activity.

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