

ANTI-ANXIETY EFFECTS OF LEAVES OF *NELUMBO NUCIFERA* GAERTN. IN MICEMrugaya P. Kulkarni¹, Archana R. Juvekar^{1*}¹Department of Pharmaceutical Sciences and Technology, Mumbai University Institute of Chemical Technology, Nathalal Parikh Marg, Matunga, Mumbai – 400019, India.*Corresponding Author's contact id: arj04@rediffmail.com

Summary

In the present study, the anti-anxiety activity of aqueous extract of leaves of *Nelumbo nucifera* was evaluated in four standard behavioral paradigms in mice. In elevated plus maze model, aqueous extract of *Nelumbo nucifera* at 100 mg/kg and 200 mg/kg increased the frequency and duration of occupancy of mice in open arm, suggesting its anxiolytic potential. Similarly, the extract at both doses, increased the number of dark-light transitions and decreased the latency time required for mice to feed in light-dark model and novelty-induced feeding suppression model respectively. Further, the increased social interaction time displayed by mice treated with *N. nucifera* extract at 200 mg/kg in social interaction test model reinforced its anxiolytic effects. The anxiolytic potential of *N. nucifera* extract observed in all the aforementioned behavioral models was comparable to standard drug, diazepam (1 mg/kg). Hence, combination study of test extract of *N. nucifera* with diazepam (a gamma-aminobutyric acid (GABA) modulator) was performed to explore the putative GABAergic involvement in its anxiolytic mechanism using elevated plus maze model. The extract at 200 mg/kg potentiated the anxiolytic effect of diazepam, inkling its ability to produce anxiolysis by modulating GABAergic transmission and/or action in brain. In conclusion, the results are indicative of the significant anxiolytic effects of aqueous extract of leaves of *Nelumbo nucifera* with GABAergic intervention in its mode of action.

Keywords: *Nelumbo nucifera*, anxiolytic, behavioral models, diazepam.

Introduction

Anxiety disorders are among the most prevalent of all psychological problems worldwide [1]. The hallmark of anxiety disorders is marked, persistent and excessive or unreasonable fear that is experienced to a degree that significantly interferes with everyday life [2]. The etiology of most anxiety disorders, although not fully understood, has come into sharper focus in the recent past. Pharmacological treatment of anxiety is principally based on 1,4-benzodiazepines (BDZs, diazepam and related drugs) or 5-HT_{1A} receptor agonists and selective 5-HT reuptake inhibitors (SSRIs).

However, these approaches have drawbacks because BDZs have a number of unwanted side effects, including tolerance, sedation, cognitive impairments, and alcohol interaction and generally, the onset of action of 5-HT receptor ligands is slow [3].

Considering the limitations of the available conventional pharmacotherapeutic agents for treating the psychiatric conditions, herbal remedies offer an alternative for patients, especially for those with lingering conditions and intolerance to adverse effects [4]. Hence, search for central nervous system (CNS)-active agents, especially from plant sources, as a potential preventive and treatment intervention for psychiatric disorders has already turned into an attractive research field and a very important social issue for improvement of the quality of human life.

The plant *Nelumbo nucifera* (Gaertn.) family: Nymphaeaceae commonly known as Lotus is a fresh water plant that grows in semitropical climates. It originated in India and was brought to other countries ranging from Egypt to China about 2000 yrs ago. Traditionally, decoction of fruits of the plant is used in treatment of poor digestion, enteritis, chronic diarrhoea, insomnia and palpitations, while the root nodes are used in treatment of hemorrhages, excessive menstruation, and nosebleeds [5]. The powdered rhizomes are prescribed for piles as demulcents, beneficial in dysentery and chronic dyspepsia [6].

Preliminary pharmacological screening of *N. nucifera* rhizome has indicated its CNS depressant activity [7]. This was the basic inspiration behind carrying out studies on the entire spectrum of psychopharmacological activities on leaves of this plant. Thereby, recently we have successfully demonstrated the anti-stress and anticonvulsant potential of aqueous extract of leaves of *N. nucifera* [8, 9]. In consideration to aforementioned reported psychopharmacological activities, it was thought prudent to investigate the anxiolytic potential of aqueous extract [AE] of *Nelumbo nucifera* [NN] with the aim of finding agents that are safer and suitable for long-term treatment in anxiety disorders.

Materials and Methods

Experimental animals: Male Swiss Albino mice (20-25 gm body weight) were used for anxiolytic activity study. The animals were housed in groups of five in clean polypropylene cage in standard laboratory conditions of temperature ($25\pm 2^\circ\text{C}$) with 12h/12h light and dark cycle. They had free access to food and water *ad libitum*. The Institutional Animal Ethics Committee (IAEC) approved the experimental protocol.

Preparation of test samples: The leaves of *Nelumbo nucifera* were obtained from local source and were identified and authenticated at the Department of Botany, Ruia College, Mumbai, India. Dried powdered leaves were defatted with Pet ether ($60-80^\circ\text{C}$) and successively extracted with water. The extract so obtained was dried using a vacuum evaporator (40°C) and used for further studies. The aqueous extract for oral administration was prepared in distilled water using 0.2% NaCMC as suspending agent.

Assessment of anxiolytic activity:

Experimental groups: Swiss Albino male mice were divided into four groups of six each. Group I animals served as vehicle control (0.2% NaCMC). Group II animals were treated with diazepam (1 mg/kg *i.p.*), served as positive control. Group III and IV animals were fed orally with NN extract (100 mg/kg and 200 mg/kg) respectively. Drug/vehicle was administered 45 minutes prior to the start of experiment.

(1) Elevated plus maze model (EPM): The apparatus was a slightly modified version of the original plus maze used for mice [10]. The plus maze elevated at 50 cm above a base, had four arms (6 cm wide, 16 cm long) extending from a central platform (8 X 8 cm). Two of the arms had side-walls (12 cm high), while the other two arms did not have side-walls. During the test, a mouse was placed on the central platform facing one of the closed-sided arms. The number of entries and total time spent in the open-sided arms (all four paws entering) during the ensuing 5 min observation period was recorded [11].

The anxiolytic potential of the test extracts was also studied in combination with diazepam (1 mg/kg), a benzodiazepine agonist. Diazepam was administered 30 min before test extract treatment and 45 min later, mice were subjected to anti-anxiety testing in elevated plus maze apparatus [12].

(2) Light–dark model: The mice's light–dark box (30 X 30 X 10 cm) consisted of two parts, the light compartment and the dark-compartment with a volume ratio of 3:1. There was a hole (5 X 5 cm) in the bottom of the clapboard between the two compartments. A 60-W incandescent bulb above provided illumination of 700 lx for the open light-compartment and 0 lx for the enclosed dark compartment [13]. At the start of the experiment, the mouse was placed in the middle of illuminated part of the cage. The number of crossings from dark to light compartment was registered during ten minutes.

(3) Social interaction test model: The general design was essentially as reported by [14]. A plastic arena (30 X 20 X 13.5 cm) located in a wooden soundproof box was used for monitoring the animal's social interaction. The light intensity of the arena floor was 380 lx. The mice used as a test pair were separately housed in a group in non-adjacent holding cages. Experiments were conducted under the high light (380 lx) unfamiliar test conditions. Members of each pair of mice having no prior experience of the test arena were placed in opposite corners of the arena, one pair at a time, and then left undisturbed for 5 min. The behavioral measures were the duration of non-aggressive, active social behaviors, genital investigation, necktail licking, trunk sniffing, facing, and following, and their sum. Passive body contact was not regarded as a social interaction parameter [15].

(4) Novelty-induced feeding suppression model: The test apparatus was a wooden box (30 X 20 X 13.5 cm) with a solid floor placed in a dim lit room. The floor of the wooden box was covered with 2 cm layer of wooden chips/husk, and laboratory chow pellet was placed on the floor. A similar arrangement was made in the home cages of the mice. Food was removed from the home cage 48 hr prior to testing, but water was provided *ad libitum*. In the experiment, mice were placed individually in the test chamber and latency to begin eating (defined as chewing of pellet and not merely sniffing or playing with it), was recorded. If the mice had not eaten within 300 sec, the test was terminated and a latency score of 300 sec was assigned [16].

Statistical analysis: The results obtained in the present study were expressed as the mean \pm SD. The numerical results were evaluated by application of one-way ANOVA with post Tukey or Dunnett's test wherever applicable for statistical significance. $P < 0.05$ was considered as statistical significant.

Results

(1) Elevated plus maze model: Mice pretreated with NN[AE] at (100 and 200 mg/kg) showed significant ($P < 0.01$) increase in the number of entries and prolongation of the cumulative time spent in the open arm relative to vehicle control mice. The effect was comparable to standard anxiolytic, diazepam (1 mg/kg) (**Table 1A**). In mechanism-based combination study with diazepam, NN[AE] at 200 mg/kg showed significantly ($P < 0.05$) increased number of open arm exploration frequency and duration compared to diazepam-treated group alone (**Table 1B**).

Table 1A: Effect of *N.nucifera* aqueous extract on exploratory activity of mice in elevated plus maze model

Treatment	Dose (mg/kg)	No of entries in open arm	Time spent in open arm (sec)
Vehicle control	-	6.75 ± 2.36	26.00 ± 5.22
Positive control (Diazepam)	1 mg/kg	31.50 ± 6.02 **	90.50 ± 11.81 **
NN[AE]	100 mg/kg	17.55 ± 3.05 **	65.69 ± 5.28 **
NN[AE]	200 mg/kg	22.60 ± 1.98 **	74.08 ± 6.66 **

Values are mean ± S.D. (n = 6). Significantly different from vehicle control (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$), by one-way ANOVA followed by Dunnett's Test

Table 1B: Effect of *N.nucifera* aqueous extract in combination with diazepam on exploratory activity of mice in elevated plus maze model

Treatment	Dose (mg/kg)	No of entries in open arm	Time spent in open arm (sec)
Vehicle control	-	6.75 ± 2.36	26.00 ± 5.22
Positive control (Diazepam)	1 mg/kg	31.50 ± 6.02 #	90.50 ± 11.81 #
NN[AE] + Diazepam	100 mg/kg + 1 mg/kg	35.40 ± 4.54	96.40 ± 6.41
NN[AE] + Diazepam	200 mg/kg + 1 mg/kg	39.66 ± 3.14 *	102.83 ± 4.87 *

Values are mean ± S.D. (n = 6). Significantly different as compared to vehicle control ($^{\#}P < 0.05$), Significantly different as compared to positive control (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) by one-way ANOVA followed by Tukey's Test

(2) Light-dark model: Similar to diazepam treated mice, NN[AE] treated mice at both doses (100 and 200 mg/kg) showed significantly ($P < 0.01$) increased number of travellings from dark to light compartment compared to vehicle control mice (**Table 2**).

Table 2: Effect of *N.nucifera* aqueous extract on exploratory behavior of mice in light-dark model

Treatment	Dose (mg/kg)	Total no of travellings from dark to light chamber
Vehicle control	-	4.40 ± 1.34
Positive control (Diazepam)	1 mg/kg	17.60 ± 2.30 **
NN[AE]	100 mg/kg	11.59 ± 2.67 **
NN[AE]	200 mg/kg	15.91 ± 2.07 **

Values are mean ± S.D. (n = 6). Significantly different from vehicle control (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$), by one-way ANOVA followed by Dunnett's Test

(3) Social interaction test model: The significantly ($P < 0.01$) increased social interaction time shown by diazepam-treated mice compared to untreated control was also observed in NN[AE] treated group at higher dose (200 mg/kg) in the social interaction test model (Table 3).

Table 3: Effect of *N.nucifera* aqueous extract on social interactive behavior of mice in social interaction test model in mice

Treatment	Dose (mg/kg)	Total social interaction time (sec)
Vehicle control	-	11.55 ± 1.23
Positive control (Diazepam)	1 mg/kg	18.22 ± 1.40 **
NN[AE]	100 mg/kg	12.33 ± 1.68
NN[AE]	200 mg/kg	15.38 ± 2.22 **

Values are mean ± S.D. (n = 6). Significantly different from vehicle control (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$), by one-way ANOVA followed by Dunnett's Test

(4) Novelty-induced feeding suppression model: The time taken for vehicle-treated mice to feed in a novel environment was found to be significantly ($P < 0.001$) high compared to those kept in their home cages. However, when kept in novel cages, NN extract-treated mice displayed significantly ($P < 0.001$) decreased latency time to feed in a dose-dependent manner compared to untreated mice kept in novel cage (Table 4).

Table 4: Effect of *N.nucifera* aqueous extract on the feeding behavior of mice in novelty-induced feeding suppression model

Treatment	Dose (mg/kg)	Latency time to feed (sec)
Vehicle control + Home cage	-	34.00 ± 5.84
Vehicle control + Novel cage	-	75.00 ± 10.11 #
NN[AE] + Novel cage	100 mg/kg	57.00 ± 5.90 **
NN[AE] + Novel cage	200 mg/kg	49.00 ± 4.48 ***

Values are mean ± S.D. (n = 6). Significantly different as compared to vehicle control in home cage ($^{\#}P < 0.05$), Significantly different as compared to vehicle control in novel cage (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) by one-way ANOVA followed by Tukey's Test

Discussion

Elevated plus maze model was used as the preliminary screening model to selectively identify anxiolytic and anxiogenic compounds. The model involves spontaneous or natural aversive stimuli i.e., height, unprotected opening, and novelty [17]. The elevated plus-maze test is thought to produce unconditioned fear (clinically compatible with panic anxiety) by single exposure to open spaces [18].

Hence, mice normally prefer to spend most of their time in closed arm. The frequency and time spent in the open arms is the major index of the anxiety in the plus maze model, given the fact that an open area is extremely aversive to rodents [19]. In EPM study, mice pretreated with NN extract at both doses (100 and 200 mg/kg) showed less fear and anxiety toward open and elevated area, as evidenced by their increased explorative abilities in open arm compared to untreated mice.

Clinical and preclinical evidence strongly implicates gamma-amino butyric acid (GABA)ergic dysfunction in anxiety [20]. In particular, the ionotropic GABA_A receptors have been a key target for anxiolytic drug development [21], propelled largely by the clinical success of benzodiazepines, the positive modulators of this receptor. Hence, combination study of test extract of *N. nucifera* with diazepam, a classic GABA modulator, was performed to explore the putative GABAergic involvement in its anxiolytic mechanism of action. NN[AE] potentiated the anxiolytic effect of diazepam at highest dose, thereby suggesting that the test extract produces anxiolysis by enhancing GABAergic transmission and/or action in brain, similar to benzodiazepine-like drugs.

The EPM test was supplemented by three other behavioral tests; namely light-dark model, social interaction test model and novelty-induced feeding suppression model to validate the positive findings of the plant extracts. All these behavioral paradigms are useful in modeling different subtypes of human anxiety, thereby aid in giving a sneak preview of effect of test substances on the specific type of anxiety disorder.

Light and dark model based on spontaneous responses reflects a type of anxiety linked with uncontrollable stress (depressive anxiety) because animals are exposed by force to a novel and/or aversive environment from which they cannot escape [22]. Good agreement has been observed between relative potency of drugs clinically used in the treatment of anxiety in humans and their ability to facilitate exploratory activity in the light-dark paradigm in mice [23]. In the light and dark box paradigm, the brightly lit environment is a noxious environment stressor that inhibits the exploratory behaviour of rodents. The test measures natural aversion of mice to brightly lit places [24]. Reduction in the number of entries, in the light chamber is regarded as a marker of anxiety [25]. In the test, NN[AE] treated group at both doses showed promising anxiolytic potential by displaying increased number of travellings from dark chamber to light chamber compared to vehicle treated group.

The social interaction test model is a useful animal model for evaluating anxiolytic compounds, which are prescribed for treating social phobia, social failure impairments, and emotional immaturity [15]. The significantly increased social interaction time shown by diazepam-treated mice in the social interaction test is in agreement with its utility in treating social phobia-type of disorder. Similarly, NN[AE]-treated group at 200 mg/kg, showed increased social interaction time as compared to untreated group, thereby underlying its anxiolytic effect. Finally, feeding latencies in novelty-induced feeding suppression model was used as a measure to underscore the anxiolysis effect of the test plant extract. The decreased latency time to feed required by NN[AE] treated mice at both doses kept in novel cages compared to untreated mice kept in novel cage reinforced its anxiolytic potential.

Thus, the present investigation of test extract of *N. nucifera* in different anxiety models demonstrated its potential anxiolytic effects with putative GABAergic mechanism of action. In concordance, the anticonvulsant effect of aqueous extract of *N. nucifera* reported in our previous study [9] is also mediated by augmenting the GABA levels in brain. Several reports suggest that ligand-binding at the benzodiazepine-binding site on the GABA_A receptor complex is known to exert such pharmacological actions as anxiolysis, anticonvulsion, muscle relaxation, and sedation [26].

Hence, it can be postulated that the plant may have constituents that act as ligands for benzodiazepine receptor binding site, and thereby allosterically modulate the GABA_A binding site to enhance GABA transmission in the brain responsible for its anxiolytic and anticonvulsion effects.

Conclusion

In conclusion, the present study indicates that aqueous extract of *Nelumbo nucifera* possess significant anxiolytic activity, comparable to standard anxiolytic diazepam. Further studies are warranted to isolate the active constituents responsible for its psychopharmacological activities.

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