

Effects of Methanolic Extract of *Nymphaea capensis* Leaves on the Sedation of Mice and Cytotoxicity of Brine Shrimp

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Abstract: Nymphaeaceae family is well known for the flowering water plants which are traditionally used in various neurological diseases. So, the purposes of the examination was to evaluate sedative action of the methanol extract of *Nymphaea capensis* leaves using animal models along with its cytotoxicity assay on brine shrimp. Adult male Swiss albino mice were treated with extract at 200 and 400 mg/kg doses and then subjected to behavioral tests such as open field, hole cross, elevated plus-maze (EPM) and thiopental Na-induced sleeping time test to assess sedative activities. *In-vitro* cytotoxicity assay was performed on shrimps of *Artemia salina*. In hole cross and open field test locomotors activity and exploratory behavior were reduced in the test group compared with the control groups. Percentage time spent in open arm in EPM test was increased for test groups in a dose dependent manner. Reduced onset of sleeping time and increased duration of sleeping time also indicated CNS depressant effect of the extract which was comparable with the standard drug diazepam. The extract shows LC₅₀ value 271.584 µg/ml in the brine shrimp cytotoxic test. The analysis showed that the plant extract possess significant dose dependent sedative effects for the methanol extract of *Nymphaea capensis* with cytotoxicity.

Key words: *Nymphaea capensis* • Sedative • Open field • Hole cross • Thiopental sodium • Elevated plus maze • Brine shrimp and cytotoxicity

INTRODUCTION

Nymphaeaceae is a family of aquatic, flowering and rhizomatous herbs. Members of this family are commonly called water lilies and live in freshwater areas in temperate and tropical climates around the world. Various plants in the Nymphaeaceae family are traditionally used in neurological diseases and some their extracts and isolated compounds have also been scientifically reported. Leaves, dried seeds and fruits of *Nelumbo nucifera* (Padma) are used against headache [1]. The ethanolic extract of seeds and aqueous extract of leaves of *Nelumbo nucifera* possess a significant depression and anxiolytic action in dose dependent manner [2]. Neferine, an alkaloid isolated from chloroform extract of embryos of seeds of *Nelumbo nucifera* has anxiolytic and sedative properties [3]. The flowers and roots of *Nymphaea nouchali* Burm. (also known as *N. stellata* or Red water lily) have mild sedative properties; used for mind altering purposes [4].

The alcohol extract of the defatted fruits of *N. stellata* produced mild sedation and ataxia, potentiated hexobarbitone-induced hypnosis in mice [5]. *Nymphaea caerulea* (Blue Egyptian water lily) contains apomorphine, a dopamine agonist, as well as nuciferine, nupharine and nupharidine. The flowers have also yielded a variety of alkaloids, including kaempferol, which has MAOI properties. In Guinea, an extraction of the flowers is taken as a narcotic and in Tanganyika the root is consumed along with the root sap of *Ipomoea aquatica* to treat mental illness [6]. *Nymphaea lotus* or White Lotus is effectively used to increase memory and create a feeling of euphoria and ecstasy, without the use of narcotics [7]. Root of *Nymphaea odorata* or White water lily is generally used in different types of inflammation [8]. *Nymphaea alba* or White Lotus Water lily produces calming and sedative effects upon the nervous system and is useful in the treatment of insomnia, anxiety and similar disorders [9, 10].

Hence, we have selected *Nymphaea capensis* in the family of Nymphaeaceae to study its sedative and anxiolytic properties. *N. capensis* is an aquatic flowering plant of the water lily family Nymphaeaceae. It is well known to Bangladeshi people as Nil Sapla and Blue water-lily. It is distributed all over the country in Bangladesh especially in lakes and ponds and also throughout different parts of the world as Africa, Australia and Northern America. *N. Capensis* is a clump forming perennial with shiny, glossy leaf-blades that are up to 40 cm in diameter, green, minutely pimples on both sides with prominent veins below, wavy margin, bluntly toothed and cleft almost to the center where the petiole is attached. *Nymphaea capensis* mature leaves are lightly blotched with wavy margins and slightly blunt-toothed. The water lily does not have true stems; the leaves are on long petioles (leaf stalks) that arise directly from the rhizome. The *Capensis* large, elegant blue flowers are held well above the water at the tip of a sturdy green stalk. This plant blooms repeatedly from mid-spring to early fall. The flowers are bisexual, star-like and regular, with 4 sepals, green on the outside and white to blue on the inside, with up to 30 petals, about 5 cm long and 1.5 cm wide. In the center of the flower are numerous blue-tipped bright golden yellow stamens [11].

MATERIALS AND METHODS

Plant Material: Leaves of *N. capensis* were collected from a lake of Anowara, Chittagong, Bangladesh. The plant was previously identified by Dr. Shaikh Bokhtear Uddin, Associate professor, Department of Botany, University of Chittagong. A voucher specimen has been retained in the Chittagong Forestry with accession number CFB-38017.

Preparation of Plant Extract: In the extraction process, the leaves were collected and dried at room temperature by avoiding sunlight to protect thermo-volatile constituents. Thereafter, the dried leaves were pulverized into powder and emerged with methanol in a colored glass container as to confirm no interaction in between sunlight and contained mixture solution. This mixture was shaken for seven days by a shaking machine to ensure rational mixture and to extract constituents with methanol from powder. After seven days, this mixture was filtered and the filtrated solution was kept for drying until getting a semisolid extract. This semisolid extract was used as crude plant extract.

Selection of Animal: The study was conducted on Swiss Albino mice purchased from the International Centre for Diarrhoeal Disease and Research, Bangladesh (ICDDR, B). They were five to six weeks of age, weighing about 20-35 gm, which were housed in colony cages (six rats per cages) at an ambient temperature of 25-27° Celsius with 12 hr light and dark cycles having proper ventilation in the room. The animals were fed with standard diet and water. The set of rules followed for animal experiment were approved by the institutional animal ethics committee, Department of Pharmacy, International Islamic University Chittagong, Bangladesh according to governmental guidelines [12].

Sedative Tests

Open Field Test[13]: The Open Field Test (OFT) is an experiment used to assay general locomotor activity levels and anxiety in rodents in scientific research. In the open field test, the animals were divided into control, positive control and test groups containing five mice each. The test groups received extracts 400 mg/kg and 200 mg/kg body weight orally respectively, whereas the control group received vehicle (1% Tween 80 in water). Animals in the positive control group received diazepam (1 mg/kg B.w.). The floor of an open field of half square meter was divided into a series of squares each alternatively colored black and white. The apparatus had a wall of 40 cm height. The number of squares visited by the animals was counted for 3 min at 0, 30, 60, 90 and 120 min after oral administration of the test drugs and the standard [13].

Hole Cross Test [14]: It is a test in which mice should cross the hole by dipping his head within a certain amount of time. The method was carried out as described by Takagi *et al.* [14]. A steel partition was fixed in the middle of a cage having a size of 30 × 20 × 14 cm. A hole of 3 cm diameter was made at a height of 7.5 cm in the center of the cage. The animals were divided into control, positive control and test groups containing five mice each. The test groups received extracts at the doses of 400 mg/kg and 200 mg/kg body weight orally whereas the vehicle control and positive control groups received vehicle (1% Tween 80 in water) and the standard drug diazepam (1 mg/kg b.w.) respectively. The number of passages of a mouse through the hole from one chamber to another was counted for a period of 3 min at 0, 30, 60, 90 and 120 min after oral administration of the test drugs and the standard.

Thiopental Sodium Induced Sleeping Time [15]: The animals were randomly divided into four groups consisting of five mice each. The test groups received at the doses of 400 mg/kg and 200 mg/kg body weight, respectively while positive control group was treated with diazepam (1 mg/kg) and control groups with vehicle (1% Tween 80 in water). Thirty minutes later, thiopental sodium (at 40 mg/kg b.w. dose) was administered to each mouse to induce sleep. The animals were observed for the latent period (time between thiopental administration to loss of righting reflex) and duration of sleep (time between the loss and recovery of righting reflex) [15].

Elevated Plus-maze (EPM test)[17-19]: The elevated plus maze (EPM) is a rodent model of anxiety that is used as a screening test for putative anxiolytic or anxiogenic compounds and as a general research tool in neurobiological anxiety research. The EPM apparatus consists of two open arms (5×10 cm) and two closed arms (5×10×15 cm) radiating from a platform (5×5 cm) to form a plus sign figure. The apparatus was situated 40 cm above the floor [16]. The open arms edges were 0.5 cm in height to keep the mice from falling and the closed-arms edges were 15 cm in height. Sixty minutes after administration of the test drugs, each animal was placed at the center of the maze facing one of the enclosed arms. During the 5-min test period, the number of open and enclosed arms entries was recorded [17]. Entry into an arm was defined as the point when the animal places all four paws onto the arm. The procedure was conducted in a sound attenuated room; observations made from an adjacent corner [18, 19].

$$\% \text{ of entries in open arm} = \frac{\text{Number of entries in open arm}}{\text{Number of entries in open} + \text{Number of entries in closed arm}}$$

Acute Oral Toxicity Test: An acute oral toxicity test was performed according to the “Organization for Environmental Control Development” guidelines (OECD: Guidelines 420; Fixed Dose Method). Swiss Albino mice (n=5) overnight fasted for 18h were used for the study. Different doses of methanolic plant extract were administered orally into the mice. The maximum given dose was 600 mg/kg body weight. Then the animals were observed for the first three hours of administration and mortality recorded within 48 hours.

Cytotoxic Study [20-22]: Brine shrimp lethality bioassay is widely used in the bioassay for the bioactive compounds. For the preparation of the simulated sea water, 38 grams sea salt was weighted accurately,

dissolved in 1 liter of sterilized distilled water and then filtered to get clear solution. The pH of the sea water was maintained between 8.5 using 1N NaOH solution. For the hatching of brine shrimp eggs, *Artemia salina* Leach (brine shrimp eggs) collected from the pet shop was used as the test organism. Simulated sea water was taken in the small tank and the shrimp eggs (1.5 gm /L) were added to the tank. The shrimp were allowed for 48 hours day/dark cycles to hatch and mature as Nauplii (larvae). Constant oxygen supply was carried out during the hatching time. The stock solution was first prepared by dissolving 3 mg sample in 6 ml sea water (3.8% NaCl in water) plus DMSO (1%) to attain concentrations of 500 µg/ml. Now, seven test tubes were taken and each was filled with 2 ml sea water and then 0, 10, 50, 100, 200, 300 and 500 µl of stock solutions were transferred to marked test tubes to obtain concentrations of 0, 10, 50, 100, 200, 300 and 500 µl µg/ml respectively after final volume adjustment. 1% DMSO was used as a negative control. 10 matured shrimps were applied to each of all experimental test tubes and then volumes were adjusted to 5 ml with sea water. After 24 hrs, the test tubes were inspected using a magnifying glass and the number of survived nauplii in each test tubes were counted. From this data, the percent (%) of mortality of the brine shrimp nauplii was calculated for each concentration using the following formula:

$$\% \text{ Mortality} = \frac{N_t}{N_0} \times 100$$

Where, N_t = Number of death nauplii after 24 hrs of incubation, N_0 =Number of total nauplii initially transferred i.e 10. The LC_{50} (Median lethal concentration) was then determined using regression analysis [20- 22].

Statistical Analysis: The data was expressed as mean ± standard error of mean (S.E.M.). Statistical comparisons were performed using one-way ANOVA followed by Dunnett’s multiple comparison test. The values obtained were compared with the vehicle control group and were considered statistically significant when $P < 0.05$.

RESULTS

Sedative Tests

Open Field Test: Locomotor activity is correlated with the alertness of the CNS and reduction of motor activity could be indicator of sedative effect [23]. Number of squares traveled by the mice was decreased throughout the 120 min study periods in the open field test.

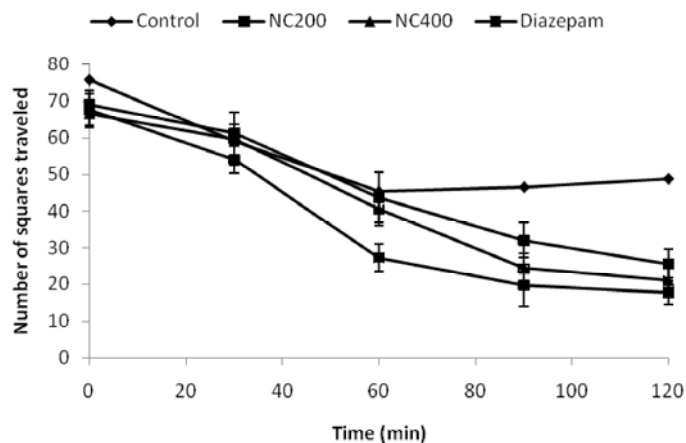


Fig. 1: Number of squares traveled by the mice of different groups in the open field test. All values are expressed as mean \pm SEM (n=5); One way Analysis of Variance (ANOVA) followed by Dunnet's test. * $P < 0.05$, significant compared to control. NC200 = *N. capensis* 200 mg/kg, NC400 = *N. capensis* 400 mg/kg.

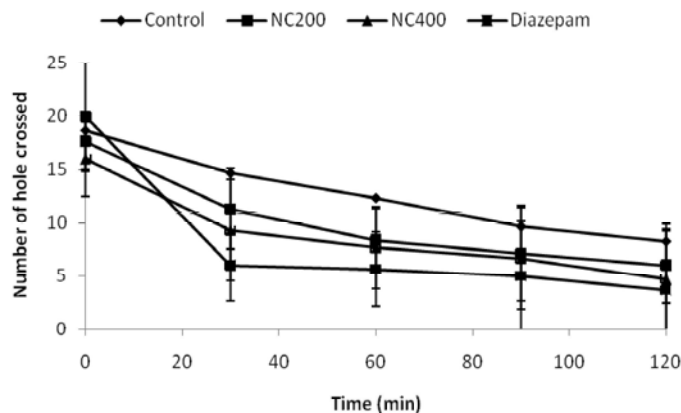


Fig. 2: Number of hole crossed by the mice of different groups in the hole cross test. All values are expressed as mean \pm SEM (n=5); One way Analysis of Variance (ANOVA) followed by Dunnet's test. * $P < 0.05$, significant compared to control. NC200 = *N. capensis* 200 mg/kg, NC400 = *N. capensis* 400 mg/kg.

The numbers of square travelled by the mice for each group were almost equal at the start of experiment (approximately 70 moves at first three minutes). Then the moves decreased gradually for all groups at different magnitude till third period of study. Then the number of squares visited by the animals in the control group was fixed nearly at 46 but those for test groups and standard control group remained decreasing till the end of experiment. From the figure, it is found that the extract decreased locomotor activity in a dose dependent manner at 400 and 200 mg/kg doses. The results for test extract was statistically significant ($p < 0.05$) and comparable to the sedative action of standard drug diazepam.

Hole Cross Test: In the test, any agents with sedative properties will suppress locomotor movements. Our results represented in the figure suggest that the number

of hole crossed from one chamber to another by mice was decreased for all four groups in the study period. However, it is also evident from figure that locomotion movement decreased was steadier in the treatment groups compare to the control group. The result for test extract at both tested doses (400 and 200 mg/kg) were comparable to the reference drug and was statistically significant ($p < 0.05$).

Thiopental Sodium Induced Sleeping Test: Thiopental Sodium, is a rapid-onset short-acting barbiturate general anesthetic, when given appropriate dose, induces sedation in animals by stimulating the inhibitory neurotransmitter gamma-amino butyric acid (GABA) mediated postsynaptic inhibition through allosteric modification of GABA_A receptors (29). In the thiopental sodium induced sleeping test, the mice treated with the

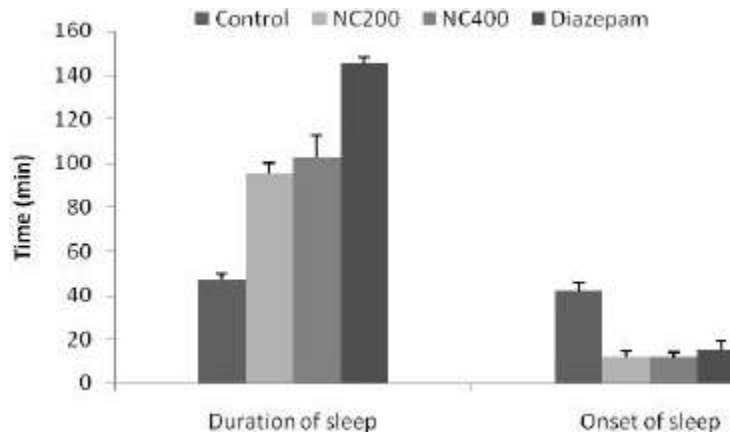


Fig. 3: CNS depressant activity of methanolic extract of leaves of *N. capensis* on thiopental sodium induced sleeping time test in mice. All values are expressed as mean \pm SEM (n=5); One way Analysis of Variance (ANOVA) followed by Dunnet's test. * $P < 0.05$, significant compared to control. NC200 = *N. capensis* 200 mg/kg, NC400 = *N. capensis* 400 mg/kg.

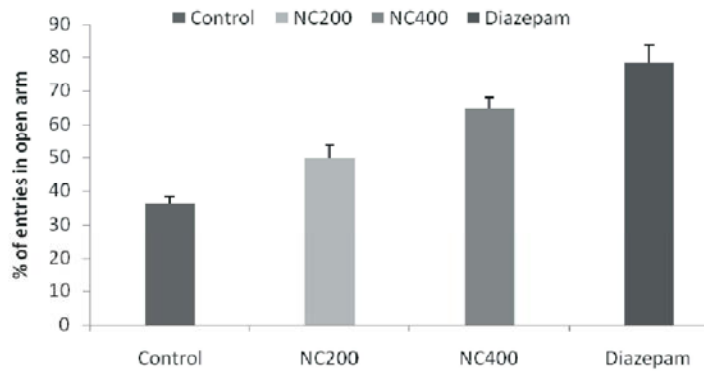


Fig. 4: CNS depressant activity of methanolic extract of leaves of *N. capensis* on elevated plus maze test in mice. All values are expressed as mean \pm SEM (n=5); One way Analysis of Variance (ANOVA) followed by Dunnet's test. * $P < 0.05$, significant compared to control. NC200 = *N. capensis* 200 mg/kg, NC400 = *N. capensis* 400 mg/kg.

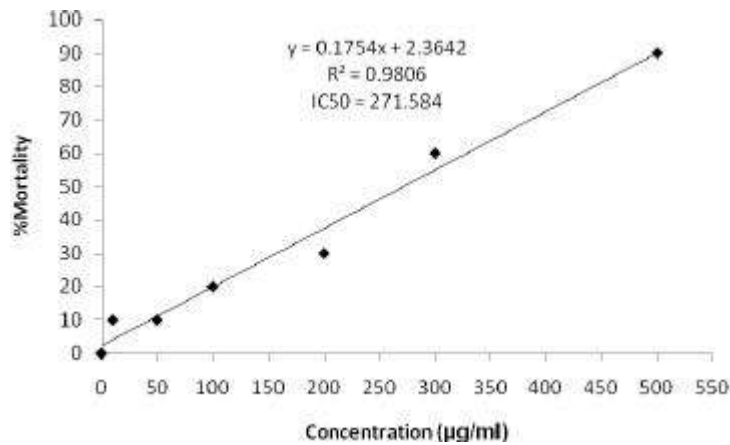


Fig. 5: Brine shrimp lethality bioassay. Determination of LC_{50} values for methanolic extract of *N. capensis* from a linear correlation between log concentrations versus percentage of mortality.

extract induced sleep at an earlier stage compared to the control group. Onset of action for the test groups at 400 and 200 mg/kg doses were 11.67 ± 3.47 and 12 ± 2.85 min respectively while for normal control group, it was 42 min. Diazepam induced sleep at 15 min. On the other hand extract increased duration of sleep significantly at both doses (figure 3) which ultimately justifies that the plant extract might have sedative action on the CNS. Onset of sleep observed for test extract was higher than the diazepam but duration of sleep was highest for standard drug (145.31 ± 2.784 min).

Elevated plus-maze (EPM) Test: The elevated plus-maze test is probably the most widely used model of animal anxiety [24, 25]. A substance which has anxiolytic effect generally increases time and proportion of entrance into the open arms when treated animals are exposed to EPM [26]. Our present results showed that acute treatment with a single IP injection of *N. capensis* extract increased the percentage of entries of mice into the open arms in a dose dependent manner. The extract at dose of 400 mg/kg induced the most significant effects and led to change anxiety-related behavioral parameters. Reference anxiolytic drug diazepam showed similar effects at low dose. At high dose, diazepam affects locomotor activity, so it is not used as positive control in this method.

Cytotoxic Study: In this test, the toxicity of extract was studied by measuring the effect of different concentrations ranges from 0-500 $\mu\text{g/ml}$ on the brine shrimp nauplii. The extract showed concentration dependent cytotoxicity under 24 hrs day-night conditions. LC_{50} value of the extract was 271.584 $\mu\text{g/ml}$ in the brine shrimp cytotoxic test.

DISCUSSION

Medicinal plants have served as sources of readily accessible, inexpensive and effective medication since the earliest times known to man. It is found that various ethnomedicinal plants have neurobehavioral profile and serve as an alternative to modern medicine. Biological evaluation and scientific validation of the ethnomedicinal plants are the need of the hour [27, 28]. The study has examined some neuropharmacological and cytotoxic activities of methanolic extract of *N. capensis*. Comparisons of the data for the four test models exhibit the sedative and anxiolytic-like effects of *N. capensis*.

Depression is a significant contributor to the global burden of disease and affects people in all communities across the world. Today, depression is estimated to affect

350 million people. The World Mental Health Survey conducted in 17 countries found that on average about 1 in 20 people reported having an episode of depression in the previous year [29]. Sedative-hypnotics are drugs that depress or slow down the body's functions. Often these drugs are referred to as tranquilizers and sleeping pills or sometimes just as sedatives. Their effects range from calming down anxious people to promoting sleep. Both tranquilizers and sleeping pills can have either effect, depending on how much is taken. Formal indications for sedation of the critically ill are consists of depression, anxiety, insomnia, fear, irritation, excitement. The most important indication is simply that of anxiolysis, or the lessening of fear. This is most true for patients who are profoundly ill, yet aware enough to realize they are looking directly into the abyss of death. Sedation can mitigate the patient's sense of dyspnea, or suffocation that accompanies ventilatory failure. It is also a little-appreciated fact that sedatives can potentiate the effects of narcotics, thereby insuring better comfort and analgesia for the patient. Additionally, sedation is a mandatory prerequisite prior to and during, co-administration of neuromuscular blockers [30].

The CNS depressant activity obtained for extract was evidenced from the suppression of the number of squares traveled by the mice in the test group throughout the study period. So, reduced movements of mice in the treatment group in open field and hole cross tests indicated sedative action of the test extract. The considerable sedative effect of the extract was also observed by the reduction in sleeping latency and increase of thiopental sodium induced sleeping time. EPM test is widely used for anxiolytic related behavioral assessment. However, the test extract showed anxiolytic action at both 200 and 400 mg/kg doses through the increase of percentage entries into the open arms. Hypnotic and sedative medications (henceforth referred to as hypnotics) work, in general, by increasing the activity of gamma-aminobutyric acid (GABA), a neurotransmitter in the brain. Neurotransmitters are chemicals made and released by nerves that attach to receptors on other nerves and serve as a means of communication between nerves. Increases in GABA activity in the brain produce drowsiness and facilitate or maintain sleep. The sedative activity of methanolic extract of *N. capensis* may be mediated by GABAergic pathway, since GABAergic transmission can produce profound sedation in mice. The inhibitory action of GABA consists in the opening of chloride channels to allow hyperpolarizing the membrane, leading to CNS depression and resulting in sedative and hypnosis activity [31].

On the other hand, the onset and duration of action in thiopental sodium test was also dose dependent and it was found that this plant possessed immense effect. Since the effect of thiopental on the CNS involves the activation of the inhibition GABAergic system [32, 33]. It is suggested by some researcher that the anxiolytic activity of benzodiazepine was direct activation of glycine synapses in the brain [34]. This finding suggests that some constituents in the methanol extract produce facilitation of this inhibitory and activator system.

In the current investigation, the intraperitoneal injection of crude extract of *Nymphaea capensis* leaves showed sedative and anxiolytic effects on mice like other species of the Nymphaeaceae family. During phytopharmacological studies, it is essential to make a safety profile by analyzing its toxicity. Sometimes, cytotoxic assay can lead to finding out active constituents with diverse biological properties such as anti-cancer. In our toxicity assay, the extract showed a moderate cytotoxic effect, as it had seen that the IC₅₀ value of methanol extract of *N. capensis* leaves was 271.584 µg/ml. Sedative and anxiolytic activities of plant extract are likely to be associated with its rich contents of phytochemicals, namely, alkaloids, tannins, flavonoids, glycosides and gums [35-37]. From the present results in the study, it can be inferred that the crude methanolic extract of *N. capensis* leaves possess strong sedative and anxiolytic activity and moderate cytotoxic properties. Therefore, this extract could be considered for the treatment of anxiety and related neuropsychiatric disorders by conducting further pharmacological studies and mechanism of sedative and anxiolytic action, as well as to identify the active compounds responsible for this bioactivity in the animal model.

CONCLUSION

From the above discussion, it can be concluded that the methanol extract of *Nymphaea capensis* possess significant sedative and anxiolytic activities. It is essential for thoroughly investigation to find out the prime constituents of the plant responsible for the actions, either to make lead compound or to make structure activity relationship to avoid the side effect of commonly used benzodiazepine.

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