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In-Vivo Neuropharmacological and Anti-Nutrient Study of Methanolic Extract of *Lemna Minor*

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ABSTRACT

Medicinal plants constitute an important natural wealth of a country. They play a significant role in providing primary health care services to rural people. They serve as therapeutic agents as well as important raw materials for the manufacture of traditional and modern medicine. *Lemna minor*, the common duckweed is an aquatic freshwater aquatic plant of the genus *Lemna*. They belong to the family of Lemnaceae, which is monophyletic to the Araceae family. The study assesses In-vivo Neuropharmacological and Anti Nutrient Study of methanolic extract of *Lemna minor*. The plants were collected from Chandpur were extracted in methanol solvents using soxhlet apparatus. Chemical tests to identify different anti nutrients, total phenolic and flavonoids were carried out using specific reagents. Several test such as open field test, hole cross test, hole board test and phenobarbital induced sleeping time test are performed to check possible neurological activity. The study of anti-nutrient properties showed a very high amount of tannin (5.625gm/kg) but a low amount of phytic acid (0.03gm/kg) & alkaloids (0.526gm/kg) comparison with standard value. Total phenolic (19.22 GAE/g dry weight) and flavonoids (7.68 QE/g dry weight) content determine that *Lemna minor* have a very good amount of phenolic and flavonoids content and which may act as good anti-oxidant. In the study of in-vivo neurological activity the efficacy of *Lemna minor* extract was compared with standard anxiolytic drug diazepam (1 mg/kg b.w.) showed limited sedation as well as anxiolytic properties in mice. The present study of in-vivo & anti-nutrient is biological evaluation of this plant forms a primary platform for further phytochemical, anti-nutrient & pharmacological studies.

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Introduction

Lemna minor as fast growing, nitrogen and phosphorus accumulating aquatic plant with high nutritional value for livestock, finds another application in the nutrient recovery from livestock wastewater. This application is known to be executed in farming systems in south east Asia, where manure and excrement are deposited in small eutrophic ponds. The water of those ponds then fertilizes bigger ponds on which *L. minor* is grown for the further use as fodder for ducks. *L. minor* has been shown to remove heavy metals like Pb, Cu, Zn and As very efficiently from waters with non-lethal concentrations. Medicinal plants, medicinal herbs, or simply herbs have been identified and used from prehistoric times. Plants make many chemical compounds for biological functions, including defense against insects, fungi, and herbivorous mammals. Over 12,000 active compounds are known to science. These chemicals work on the human body in exactly the same way as pharmaceutical drugs, so herbal medicines can be beneficial and have harmful side effects just like conventional drugs. However, since a single plant may contain many substances, the effects of taking a plant medicine can be complex. Medicinal plants are widely used to treat disease in non-industrialized societies, not least because they are far cheaper than modern medicines [1]. World Health Organization (WHO) has defined medicinal plants as plants that contain properties or

compounds that can be used for therapeutic purposes or those that synthesize metabolites to produce useful drugs [2]. The pharmacological evaluation of chemical substances from plants is an established method for the identification of lead compounds which can lead to the development of novel and safe medicinal agents. The importance of medicinal plants and traditional health systems in solving the health care problems of the world is gaining increasing attention. Because of this resurgence of interest, the research on plants of medicinal importance is growing phenomenally at the international level. Most of the developing countries have adopted traditional medical practice as an integral part of their culture. Historically, all medicinal preparations were derived from plants, whether in the simple form of raw plant materials or in the refined form of crude extracts, mixtures, etc. A large proportion of such medicinal compounds have been discovered with the aid of ethnobotanical knowledge of their traditional uses. The rich knowledge base of countries like India and China in medicinal plants and health care has led to the keen interest by pharmaceutical companies to use this knowledge as a resource for research and development programs in the pursuit of discovering novel drugs [3, 4].

Materials and Methods

Plant Material

Lemna minor spreads freely and is usually found growing in colonies that can cover quite large areas of water. The plant is gathered from the wild for local use as a food and medicine. [5]

The plant *Lemna minor* was selected based on its medicinal uses. Using standard taxonomical methods, supplied by the Bangladesh Forest Research Institute (BFRI), Chittagong identified the plant. The plants were collected from Chandpur. It was then separated & cleaned from impurities.

Preparation of Leaf Extract

The plant was dried in room temperature 25-30°C for ten days about 9 hours. About 500 gm of powder of plant species was subjected with 500 ml of methanol in soxhlet apparatus. Extraction was carried out for 10 hours the obtained extract was collected and made to concentrate with the help of air cooler. The obtained extract were collected, filtered and made to evaporate the solvent under air cooler in room temperature for several days. After evaporation of solvent it becomes a gummy concentrate. The gummy concentrate was designated as crude methanol. The above was carried out using methanol and water as solvent [6].

Drug and Reagents

All the chemicals and reagents used in this study were of analytical grade and standards drug diazepam were obtained from Square Pharmaceuticals Ltd.

Experimental Animal

The study was conducted on 30 Swiss Albino mice purchased from BCSIR, Chittagong. They were five to six weeks of age, weighing about 30-40 gm, which were housed in colony cages (six mice per cages) at an ambient temperature of 25-27 Celsius with 12h light and dark cycles having proper ventilation in the room. The mice were fed normal diets purchased commercially from the vendors and water. The animals were allowed to acclimatize to the laboratory environment for one week and then randomly divided into groups for experiments. All protocols for animal experiment were approved by the Institutional Ethics Committee [7].

Determination of Anti-Nutritional Factors

Anti-nutrients are natural or synthetic compounds that interfere with the absorption of nutrients. Nutrition studies focus on those anti-nutrients commonly found in food sources and beverages. These test were carried out in order to detect the presence of anti-nutrients of interest which are tannins, phytic acids and alkaloids. All the test were done using appropriate procedures.

Determination of Tannin

The quantitative analysis of total tannin content in the aqueous extract of *Lemna minor* was determined by Folin-Denis method with minor modifications. A stock solution of *Lemna minor* aqueous extract was prepared in the concentration of 1 mg/ml. For quantification, to 0.1 ml of aqueous extract, 1 ml of distilled water and 0.5ml of Folin-Denis reagent were added and mixed thoroughly. This mixture was alkalized by adding 1 ml of 15% (w/v) sodium carbonate and kept in dark for 30 minutes at room temperature. The absorbance was read at 700 nm. Pure tannic acid (1 mg/ml) was used as standard. Result was expressed as mg tannic acid equivalent per gram of dry weight (mg TE/g) of extract [8].

Determination of Phytic Acid

Phytic acid was determined by the procedure of Lucas and Markakas (1975) 2.0 g of the sample was weighed into a 250 mL conical flask. One hundred mL 2% concentrated HCl was used to soak sample for 3 h and then filtered with a Whatman No. 1 filter paper fifty cm³ of the filtrate and 10 cm³ of distilled water were added in each case to give proper acidity. Ten mL 0.3% ammonium thiocyanate solution was added into the solution as indicated and titrated with standard Iron II Chloride solution containing 0.00195

g Iron/mL, end point observed to be yellow which persisted for 5 min. The percentage phytic acid was calculated thus:

$$\% \text{ Phytic acid} = y \times 1.19 \times 100$$

Where, y = titre value $\times$ 0.00195 g [9].

Determination of Alkaloids

Alkaloid determination was carried out following the procedure of Harborne (Harborne, 1973). 5.0g of the sample was weighed into a 250ml beaker and 200ml of 10% acetic acid in ethanol was added and covered and allowed to stand for 4h. This was filtered and the extract was concentrated on a water bath to one quarter the original volume. Concentrated ammonium hydroxide was added drop wise to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitate was collected and washed with dilute ammonium hydroxide and then filtered. The residue is the alkaloid which was dried and weighed [10].

Determination of Total Phenol Content

Total phenolic content of methanol extract of *Lemna minor* determined employing the method as described by Singleton et al., (1965) involving Folin-Ciocalteu reagent as oxidizing agent and Gallic acid as standard [11]. Briefly, 0.5 ml of plant extract or standard of different concentration solution was taken in a test tube. 2.5 ml of Folin-Ciocalteu (Diluted 10 times with water) reagent solution and 2.5 ml of Sodium carbonate (7.5%) solution was added into the test tube. The test tube was incubated for 20 minutes at 25°C to complete the reaction. Then the absorbance of the solution was measured at 760 nm using a spectrophotometer against blank. A typical blank solution contained all reagents except plant extract or standard solution. The total content of phenolic compounds in methanol extract of plant and in different fractionates in gallic acid equivalents (GAE) was calculated from the calibration curve and the result were expressed as mg of gallic acid equivalent per g dry weight [12].

Determination of Total Flavonoids Content

Total flavonoids content of methanol extract of *Lemna minor* determined employing the aluminium chloride colorimetric method involving quercetin reagent. Quercetin was used as standard and the flavonoid content of the extract was expressed as mg of quercetin equivalent/gm of dried extract. Following, 1.0 ml of plant extract or standard of different concentration solution was taken in a test tube. 3 ml of methanol, 200 μ l of 10% aluminium chloride solution, 200 μ l of 1M potassium acetate solution and 5.6 ml of distilled water was added into the test tube. The test tube was then incubated at room temperature for 30 minutes to complete the reaction. Then the absorbance of the solution was measured at 420 nm using a spectrophotometer against blank. A typical blank solution contained all reagents except plant extract or standard solution. The total content of flavonoids compounds in plant extracts in quercetin equivalents was calculated from a calibration curve and result was expressed as mg of quercetin equivalent per g dry weight [13].

In Vivo Neuropharmacological Study

Neuropharmacology is the study effects of drugs on the nervous system, with the goal of developing compounds that offering therapeutic benefits in humans with psychiatrics and neurological disease. [14] For the neuropharmacology tests it takes four different studies. They are: (1) Open field test, (2) Hole cross test, (3) Hole Board test, (4) Prolongation effect on phenobarbital induced sleeping time in mice.

Open Field Test

The open field test was planned to evaluate behavioral responses such as locomotor activity, hyperactivity and exploratory behaviors [15]. This test was performed because mouse mobility affects their ability to move or climb. In this test, the effects of administration on the animals' mobility were calculated. Each mouse was placed in the center of the open field apparatus, and its behavior was observed for 5 min. The parameters evaluated were the total number of squares crossed, the number of outer squares (those adjacent to the walls) crossed, and the number of inner squares crossed. These three measures are referred to as total (TL), peripheral (PL), and central locomotion (CL), respectively. The numbers of leanings (one or two paws in contact with the wall), rearing (the mouse standing on its two hind paws without touching the walls), grooming (face cleaning, paw licking, fur licking, head scraping, and rubbing), and defecations were also recorded. At the end of each test, the whole area was cleaned with a wet sponge and a dry paper towel [16].

Hole-cross Test

A wood 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 Centre 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 (100,200 mg/kg) body weight orally whereas the vehicle control and positive control groups received vehicle and the standard drug diazepam (1 mg/kg b.w.), respectively. The number of passage of a mouse through the hole from one chamber to other 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 [17].

Hole Board Test

The evaluation of certain components of behavior of mice such as curiosity or exploration has been attempted by Boissier et al. (1964) [18]. Mice of either sex (NMRI strain) with a weight between 18 and 22 g are used. The hole-board has a size of 40 × 40 cm. Sixteen holes with a diameter of 3 cm each are distributed evenly on the floor. The board is elevated so that the mouse poking its nose into the hole does not see the bottom. Nose-poking is thought to indicate curiosity and is measured by visual observation in the earliest description and counted by electronic devices in more recent modifications. Moreover, in the newer modifications motility is measured in addition by counting interruption of light beams. Usually, 6 animals are used for each dose and for controls. Thirty minutes after administration of the test compound the first animal is placed on the hole-board and tested for 5 min. The number of counts for nose-poking of treated animals is calculated as percentage of control animals [19].

Prolongation Effect on Phenobarbital Induced Sleeping Time in Mice

Test samples suspended in normal saline were administered to the animals. All experiments were carried out between 1 pm and 5 pm. Animals were denied food for 24 h prior to the experiment. Thirty minutes after the intraperitoneal administration (i.p.) of test samples, pentobarbital was i.p. administered at a dose of 30 mg/kg body weight to mice placed in a box. The animals in the box that stopped moving within 15 min after pentobarbital injection were immediately transferred to another box, whereas those that remained immobile for more than 3 min were judged to be asleep. The sleeping time was defined as the time from their transfer into the second box to when they resumed spontaneous movements. Mice that failed to fall asleep within 15 min after pentobarbital administration were excluded from the experiments [20, 21].

Results and Discussion

Anti-nutritional Content

The methanolic extract of *Lemna minor* compared to standard Tannin content (16.02g/kg) the plant has 5.62g/kg so it will not alter protein absorption. Compared to standard (phytic acid = 0.280 g/kg ; alkaloid= 2.63 g/kg) the low level of phytic acid & alkaloids will not alter the phytase enzyme as well as absorption of other nutrients. So, we can say that these anti-nutrients will not hamper the normal absorption capacity of the nutrients required for our body development and growth.

Table 1: Showing the concentrations (g/kg) of the Anti- nutrient content of the sample *Lemna minor*

Sample	Tannin content gm/ kg	Phytic acid content gm/kg	Alkaloids content gm/kg
<i>Lemna minor</i>	5.625gm	0.03gm	0.526gm

Figure 1: Showing Anti-nutrient content of *Lemna minor* Phenolic and Flavonoid Content

The total phenolic content of methanolic extract, calculated from calibration curve ($R^2 = 0.998$) was, 19.22 gallic acid equivalents/g and total flavonoid content of methanolic extract. Calculated from calibration curve ($R^2 = 0.972$) was 7.68 quercetin equivalents/g. In Table 2 values are means of three biological replicates. From the results of present study it was concluded that the methanolic extracts of *Lemna minor* has showed significant antioxidant activity. The antioxidant potential of whole plant extracts may be due to presence of good amount of phenolic and flavonoid content. Thus, the methanolic extracts of *Lemna minor* could serve as the potential source of natural antioxidants and may help to develop new natural drugs against various diseases caused by free radicals.

Table 2: Total phenolics and flavonoids content in methanolic extract of *Lemna minor*

Sample	Total phenolic content	Total flavonoid content
<i>Lemna minor</i>	19.22 Gallic acid equivalents/g	7.68 Quercetin equivalents/g

In Vivo Neuropharmacological Analysis

To obtain meaningful results regarding the effect of *Lemna minor* methanolic extracts effect on CNS in mice, a number of methods namely open field, hole cross, hole board & phenobarbital induced sleeping time test were adopted. Test results are follows:

Open Field Test

The number of squares traveled by the mice was not suppressed significantly from the whole observation. The locomotor activity is not found and the results were statistically non-significant.

Hole-cross Test

Lemna minor methanolic extract produced no significant effect of

locomotion from its initial value during the period of experiment which was comparable to the reference drug diazepam.

Hole Board Test

In this hole board test it is significant that, increased in dose shows no evident locomotor activity in mice compare to the reference drug diazepam.

Table 3: Neuropharmacological activity test of methanolic extract of leaves of *Lemna minor* by open field and hole-cross and hole board test

	Minutes	Control	Diazepam	LM 100mg	LM 200mg
Open Field Test	0	104	74.8	75.4	80.4
	30	57.8	62.4	81.4	80.4
	60	35.4	35.6	87.4	80.8
	90	29.4	18.4	88.6	82.2
	120	28.8	10.6	88.8	87.4
Hole-cross Test	0	18.6	7.2	6.6	7
	30	9.2	3.4	10.8	9.6
	60	5.4	1.8	7.2	8.2
	90	4.6	1.4	9.6	8.2
	120	4.6	2	10.6	11.2
Hole-board Test	0	17	19.4	17.4	16.9
	30	18.8	20	14.6	13.4
	60	17.2	19.8	15.2	15.8
	90	17.2	17.8	11.6	15.7
	120	17	14.4	9.6	17.4

Figure 2: Column chart showing the effect of methanolic extract of *L. minor* by open field, hole-cross and hole-board test on wister albino rats

Prolongation Effect on Phenobarbital Induced Sleeping Time in Mice

The result from the prolongation effect on phenobarbital induced sleeping time in mice is presented on Table 4. The study was undertaken to evaluate the sleep potentiating effect of *Lemna minor* methanolic extract in mice and the extract shows no significant activity phenobarbital induced sleeping time in a dose dependent manner.

Table 4: Neuropharmacological activity *Lemna minor* leaves extract on phenobarbital induced sleeping time in mice

Group	Treatment	Dose	Onset of sleep (minute)	Duration of sleep (minute)
1	Phenobarbitone	45	9	88
2	<i>Lemna minor</i>	250	20	39.2
3	<i>Lemna minor</i>	500	19.5	55.2

Figure 3: Showing effect of *Lemna minor* leaves extract on phenobarbital induced sleeping time in mice

The most important step in evaluating drug action on the CNS is to observe the behavior of the test animals. Substances that have CNS depressant activity either decrease the time for onset of sleep or prolong the duration of sleep or both. Another important step in evaluating drug action on CNS is to observe its effect on locomotors activity of the animal. Decrease in locomotors activity of mice may be closely related to sedation resulting from depression of the central nervous system. The rate of locomotors activity of albino mice, after applying *Lemna minor* methanolic extracts, is not decreased. Finally, overall results obtained from this study suggested the methanolic extract of *L. minor* has no CNS depressant activity on experimental animal models.

Conclusions

The present study was designed to investigate anti-nutrient, total phenolic & flavanoids content and neurological effect of methanol extract of *Lemna minor* whole plant. From the study we found a very good amount of phenolic (19.22 GAE/g dry weight) & flavonoids (7.68 QE/g dry weight) content in comparison with black tea & may act as good anti-oxidant. *Lemna minor* have a very good amount of anti-nutrient like tannin content (5.625g/kg), phytic acid content (0.03gm/kg), alkaloids content (0.526gm/kg) which cannot alter the absorption capacity of nutrients required for our body development and growth. In case of in-vivo neurological effect it was observed from the study of methanolic extract of the whole plant of *Lemna minor* show minor sedative and anxiolytic properties in compared to standard drug diazepam (1 mg/kg b.w.). However, the present study of in-vivo & anti-nutrient is biological evaluation of this plant forms a primary platform for further phytochemical, anti-nutrient & pharmacological studies. These promissory extracts open the possibility of finding new clinically effective bioactive compounds.

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