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Evaluation of Bioactivities of *Nymphaea nouchali* (Burm. f) - the National Flower of Bangladesh

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Abstract

Different extractives of petals of *Nymphaea nouchali* were evaluated for membrane stabilizing, antioxidant, cytotoxic and antimicrobial activities. The membrane stabilizing activity was assessed by hypotonic solution and heat induced methods. In the present studies, the aqueous soluble (AQSF) materials of the petals demonstrated strong membrane stabilizing activity, whereas the chloroform (CSF) and petroleum ether soluble fractions (PESF) revealed moderate membrane stabilizing activity in both methods. The total phenolic content was also determined and expressed in gallic acid equivalent. In brine shrimp bioassay, the extractives of *N. nouchali* exhibited no lethality as evident from no death of nauplii after 24 hours of observation. On the other hand, the chloroform (CSF) and aqueous soluble fractions (AQSF) of methanol extract revealed significant antibacterial and antifungal activities against some microorganisms used in the screening.

Key words: *Nymphaea nouchali*, membrane stabilizing, total phenolic content, brine shrimp lethality bioassay, antimicrobial.

Introduction

In most of the traditional systems of treatment, the use of medicinal plant include the fresh or dried part, whole, chopped, powdered or an advanced form of the herb usually made via extraction with water, ethanol or an organic solvent play a major role and constitute the backbone of traditional medicine (Mukherjee, 1986). Botanical medicines or phytomedicines refer to the use of any plant's seeds, berries, roots, leaves, bark, or flowers for medicinal purposes by significant percentage of people (Barrett *et al.,* 1999). The percentage might be higher if over-the-counter (OTC) drugs are included (Duke, 1990). One contributing factor of increased death rate is an increase in antibiotic resistance in nosocomial and community acquired infections (Pinner *et al.*, 1996). The antimicrobial screening, which is the first stage of antimicrobial drug research, is performed to ascertain the susceptibility of various bacteria and fungi to any agent. This test measures the ability of each test sample to inhibit the *in vitro* bacterial and fungal growth (Ayafor *et al*., 1972).

Nymphaea nouchali (Burm. f) belongs to the family Nymphaeceae, which is a family of flowering plants. It is the national flower of Bangladesh and commonly known as "Shapla" in Bengali. *N. nouchali* is a large perennial aquatic herb with short round rhizomes. In the present study, the organic soluble materials of a methanol extract of the petals as well as its different organic soluble partitionates were evaluated for the antioxidant activity in terms of total phenolic content, membrane stabilizing capability, as well as antimicrobial and cytotoxic activities of *N. nouchali* for the first time.

Materials and Methods

Plant materials: The petals of *N. nouchali* were collected from Gazipur in August 2010. A voucher specimen for this plant has been maintained in Bangladesh National Herbarium, Dhaka, Bangladesh (Accession no. 35453) for future reference.

Reagents and chemicals: All chemicals i.e. methanol, petroleum ether (b.p. 60-80^{o}C), carbon tetrachloride, chloroform and other reagents used in these experiments were of the highest analytical grade.

Extraction and fractionation: The petals were sun dried for several days and then oven dried for 24 hours below 40^{o}C to facilitate grinding. The powdered materials

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(500 gm) were macerated in 2.0 L of methanol for 7 days and then filtered through a cotton plug followed by Whatman filter paper number 1. The extract was concentrated with a rotary evaporator at low temperature (40-45ºC) and reduced pressure. The concentrated methanolic extract was partitioned by the modified Kupchan method (Van Wagenen *et al.*, 1993) and the resultant partitionates i.e., petroleum ether (PESF), carbon tetrachloride (CTCSF), chloroform (CSF), and aqueous soluble (AQSF) fractions were used for the experiment.

Membrane stabilizing activity: The erythrocyte membrane resembles to lysosomal membrane and as such, the effect of drugs on the stabilization of erythrocyte could be extrapolated to the stabilization of lysosomal membrane (Omale and Okafor, 2008). Therefore, as membrane stabilizes, it interferes with the release and/or action of mediators like histamine, serotonin, prostaglandins, leukotrienes etc. (Shinde *et al.*, 1999). The membrane stabilizing activity of the extractives was assessed by using hypotonic solution-induced and heat-induced mice erythrocyte haemolysis (Shinde *et al*., 1999). To prepare the erythrocyte suspension, whole blood was obtained using syringes (containing anticoagulant EDTA) from mice through cardiac puncture. The blood was centrifuged and blood cells were washed three times with 154 mM NaCl in 10 mM sodium phosphate buffer (pH 7.4) through centrifugation for 10 min at 3000 g.

Hypotonic solution-induced haemolysis: The test sample consisted of stock erythrocyte (RBC) suspension (0.50 ml) mixed with 5 mL of hypotonic solution (50 mM NaCl) in 10 mM sodium phosphate buffered saline (pH 7.4) containing either the extract (1. 0 mg/ml) or acetyl salicylic acid, ASA (0.1 mg/ml). The control sample consisted of 0.5 ml of RBCs mixed with hypotonic-buffered saline alone. The mixture was incubated for 10 min at room temperature, centrifuged for 10 min at 3000 *g* and the absorbance of the supernatant was measured at 540 nm. The percentage inhibition of either haemolysis or membrane stabilization was calculated using the following equation-

% Inhibition of haemolysis = 100 x (OD_1-OD_2/OD_1), where, OD_1 = optical density of hypotonic-buffered saline solution alone (control) and OD_2 = optical density of test sample in hypotonic solution.

Heat-induced haemolysis: Isotonic buffer containing aliquots (5 ml) of the different extractives were put into two duplicate sets of centrifuge tubes. The vehicle, in the same amount, was added to another tube as control. Erythrocyte suspension (30 µl) was added to each tube and mixed gently by inversion. A pair of the tubes was incubated at 54ºC for 20 min in a water bath. The other pair was maintained at 0-5ºC in an ice bath. The reaction mixture was centrifuged for 3 min at 1300 g and the absorbance of the supernatant was measured at 540 nm. The percentage inhibition or acceleration of hemolysis in tests was calculated according to the equation:

% Inhibition of hemolysis = 100 x [1- (OD_2-OD_1/ OD_3-OD_1)]

where, OD_1 = optical density of unheated test sample, OD_2 = optical density of heated test sample and OD_3 = optical density of heated control sample

Total phenolics analysis: The antioxidant effect of phytochemicals is mainly due to phenolic components, such as flavonoids (Pietta, 1998), phenolic acids, and phenolic diterpenes (Shahidi *et al.*, 1992). Total phenolic content of *N. nouchali* extractives was measured by employing published method (Skerget *et al.*, 2005) involving Folin-Ciocalteu reagent as an oxidizing agent and gallic acid as standard. To 0.5 ml of extract solution (2 mg/ml) in water, 2.5 ml of Folin-Ciocalteu reagent (diluted 10 times with water) and 2.0 ml of sodium carbonate (7.5% w/v) solution were added. After 20 minutes of incubation at room temperature the absorbance was measured at 760 nm using a UV-visible spectrophotometer. The total phenolics were quantified by calibration curve obtained by measuring the absorbance values of known concentration of gallic acid (0-100 µg/ml) and were expressed as mg of GAE (gallic acid equivalent)/gm of the dried extract.

Brine shrimp lethality bioassay: Brine shrimp lethality bioassay (McLaughlin, 1998; Persoone, 1980) is a rapid and comprehensive bioassay for the bioactive compounds of natural and synthetic origins. By this method, natural product extracts, fractions as well as the pure compounds can be tested for their bioactivities. This technique was applied for the determination of general toxic property of the plant extractives (Meyer *et al*., 1982 and McLaughlin *et al*., 1998). DMSO solution of the samples was applied against *Artemia salina* in a 1-day *in vivo* assay. For the experiment, 4 mg of each of the

petroleum ether, carbon tetrachloride, chloroform and aqueous soluble fractions were dissolved in DMSO and solutions of varying concentrations (400, 200, 100, 50, 25, 12.50, 6.25, 3.125, 1.563, 0.781 µg/ml) were obtained by serial dilution technique using DMSO. Vincristine sulphate was used as positive control.

Antimicrobial activity: The antimicrobial screening was performed by the disc diffusion method (Ayafor *et al*., 1972; Bauer *et al*., 1966; Rahman and Rashid, 2008) against thirteen bacteria and three fungi (Table-1) collected as pure cultures from the Institute of Nutrition and Food Science (INFS), University of Dhaka, Bangladesh. Solutions of known concentration (µg/ml) of the test samples were made by dissolving measured amount of the samples in calculated volume of methanol. Dried and sterilized filter paper discs (6 mm diameter) were then impregnated with known amount of the test substance using micropipette and the residual solvents were completely evaporated. Discs containing the test materials (400 µg/disc) were placed onto nutrient agar medium uniformly seeded with the test microorganisms. Standard disc of Ciprofloxacin (30 µg/disc) and blank discs (impregnated with solvents followed by evaporation) were used as positive and negative control, respectively. These plates were then kept at low temperature (4 ^{o}C) for 24 hours to allow maximum diffusion of the test materials and Ciprofloxacin and then incubated at 37 ^{o}C for 24 hours to allow maximum growth of the organisms. The test material having antimicrobial activity inhibited the growth of the microorganisms and a clear, distinct zone of inhibition was visualized surrounding the discs. The antimicrobial activity of the test agents was determined by measuring the diameter of zone of inhibition expressed in mm (Bauer *et al.*, 1966).

Results and Discussion

The present study was designed to evaluate the membrane stabilizing, antioxidant activity, cytotoxicity and antimicrobial properties of different organic soluble materials of the methanol extract of *N. nouchali.* The extractives of *N. nouchali* at concentration 1.0 mg/mL significantly protected the lysis of mice erythrocyte membrane induced by hypotonic solution and heat, as compared to the standard acetyl salicylic acid (0.10 mg/mL) (Table 1). In hypotonic solution-induced haemolysis, the aqueous soluble fraction (AQSF) inhibited 66.55% haemolysis of RBC as compared to 71.9% produced by acetyl salicylic acid (Figure 1). The chloroform and petroleum ether soluble extractives also revealed significant inhibition of haemolysis of RBCs. On the other hand, in heat induced haemolysis, the aqueous soluble fraction (AQSF) inhibited 41.64% haemolysis of RBCs as compared to 42.1% demonstrated by acetyl salicylic acid (at 0.10 mg/ml).

Table 1. Effect of extractives of *N. nouchali* on hypotonic solution and heat-induced haemolysis of erythrocyte membrane.

Sample	Concentration (mg/mL)	Haemolysis inhibition (%)	
		Hypotonic solution induced	Heat induced
ME	1.0	49.48 ± 1.04	24.72 ± 0.38
PESF	1.0	40.61 ± 0.39	32.61 ± 0.41
CTCSF	1.0	55.46 ± 0.81	29.35 ± 0.97
CSF	1.0	52.56 ± 0.94	38.52 ± 0.82
AQSF	1.0	66.55 ± 0.81	41.64 ± 0.69
Acetyl salicylic acid	0.10	71.9 ± 0.99	42.1 ± 0.76

Here**,** ME = methanolic crude extract; PESF = petroleum ether soluble fraction; CTCSF = carbon tetrachloride soluble fraction; CSF= chloroform soluble fraction; AQSF = aqueous soluble fraction of the methanolic extract of *N. nouchali.*

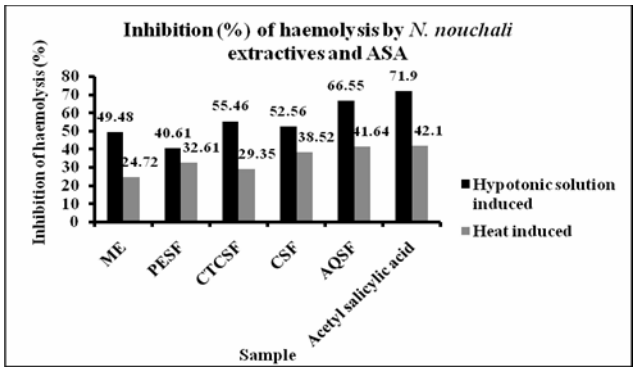

Figure 1. Comparison of inhibition (%) of haemolysis by heat and hypotonic solution-induced conditions.

Table 2. The total phenolic content and cytotoxic activities of different partitionates of *N. nouchali.*

Sample	Total Phenolic Content (mg of GAE/gm of dried extract)
ME	98.87 ± 0.28
PESF	81.08 ± 0.27
CTCSF	87.13 ± 0.22
CSF	96.88 ± 0.43
AQSF	104.04 ± 0.08

The total phenolic content varied for different partitionates of *N. nouchali* ranging from 81.08 mg to 104.04 mg of GAE/gm of dried extract (Table 2). The highest total phenolics were found in AQSF (104.04 mg of

GAE/gm of dried extract) and the lowest in PESF (81.08 mg of GAE/gm of dried extract).

In case of brine shrimp assay, the crude methanol extract (ME) and its all partitionates showed zero mortality as no nauplii died even after 24 hours of observation (result is not shown in table 2). So, no median lethal concentration (LC_{50}, the concentration at which 50% mortality of brine shrimp nauplii occurred) could be determined for test sample although the LC_{50} for vincristine sulphate was found to be 0.451 µg/ml.

The crude methanol extract, along with petroleum ether, carbon tetrachloride, chloroform and aqueous soluble partitionates of *N. nouchali* were screened against thirteen bacteria and three fungi and the results were compared with standard antibiotic, Ciprofloxacin. Both the aqueous soluble fraction (AQSF) and chloroform soluble fraction (CSF) exhibited the significant inhibition of microbial growth having zone of inhibition ranging from 8.3 mm to 19.5 mm and 8.0 mm to 17.2 mm, respectively (Table 3). The carbon tetrachloride soluble fraction (CTCSF) showed highest activity (zone of inhibition = 19.5 mm) against *Vibrio parahemolyticus*. The crude methanol extract (ME) exhibited mild antimicrobial activity against most of the test organisms while the petroleum ether soluble fraction (PESF) revealed moderate inhibition against *Shigella boydii*.

Table 3. Antimicrobial activity of *N. nouchali* extractives at 400 µg/disc.

Test microorganisms	Diameter of zone of inhibition (mm)					
	ME	PESF	CTCSF	CSF	AQSF	Ciprofloxacin
Gram positive bacteria						
Bacillus sereus	-	-	-	-	-	48.2±1.38
B. megaterium	12.0±0.82	8.2±0.82	17.1±0	11.1±1.12	12.3±2.25	47.9±1.79
B. subtilis	10.3±0.75	-	-	8.0±0.81	9.1±1.25	49.2±2.10
Staphylococcus aureus	-	-	8.0±0.89	-	11.6±1.63	48.0±2.32
Sarcina lutea	8.1±0.71	-	12.4±0.91	12.4±0.97	14.2±2.05	50.0±0
Gram negative bacteria						
Escherichia coli	-	-	8.0±1.41	9.7±0.28	12.0±2.49	49.0±2.81
Pseudomonas aeruginosa	-	-	-	-	8.6±1.04	47.4±1.78
Salmonella typhi	-	-	8.4±0.88	-	-	45.7±3.17
S. paratyphi	8.7±0.84	-	-	8.0±0.81	-	55.1±2.11
Shigella boydii	10.7±1.23	15.2±3.21	-	8.3±1.08	17.9±3.1	52.1±2.37
S. dysenteriae	11.0±0.92	8.6±0.89	12.0±1.64	12.8±1.16	13.7±2.21	47.8±0.91
Vibrio mimicus	8.3±0.79	-	-	-	8.3±0.88	48.5±2.28
V. parahemolyticus	8.9±0.91	-	19.5±1.32	17.2±0.98	9.0±0.69	48.0±1.09
Fungi						
Candida albicans	-	-	-	-	8.7±1.69	48.8±1.89
Aspergillus niger	-	-	-	-	-	41.4±2.41
Sacharomyces cerevisiae	11.6±1.45	-	8.1±0.85	8.0±1.02	18.3±2.48	46.7±1.98

Here**,** ME = methanolic crude extract; PESF = petroleum ether soluble fraction; CTCSF = carbon tetrachloride soluble fraction; CSF= chloroform soluble fraction; AQSF = aqueous soluble fraction of the methanolic extract of *N. nouchali.*

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