

**PHYTOCHEMICAL AND ANTIMICROBIAL INVESTIGATION OF *NYMPHAEA NAUCHALI* LEAF
NATIONAL FLOWER OF BANGLADESH**Rishikesh*¹, Drishti Rani Ghosh², Md. Sium-Ul Hossain Miah², Md. Anwarul Haque³, Anwarul Islam¹¹Department of Pharmacy, Bangladesh University, Dhaka-1207²Department of Pharmacy, University of Asia Pacific, Dhaka-1209³Department of Pharmacy, Rajshahi University, Rajshahi-6205**Received 15 July 2013; Revised 27 July 2013; Accepted 02 August 2013****ABSTRACT**

The methanolic extracts of *Nymphaea nauchali* was evaluated for antimicrobial activity against clinically significant bacteria *Staphylococcus aureus*, *Pseudomonas aureus*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Bacillus cereus*, *Escherichia coli*, *Xanthomonas campestris*, *Candida albicans*. The *in vitro* antimicrobial activity was performed by disc diffusion method. The methanolic leaf extract of *Nymphaea nauchali* was active against all the investigated bacterial strains. All the activities were compared by measuring the zone of inhibition with the standard antibiotic. The methanol extract of *Nymphaea nauchali* (Family: Nymphaeaceae) was studied for its phytochemical analysis. This study of the extracts indicates the presence of Protein, Sugar, Tannins, Alkaloids, Saponins, Flavonoids, Terpenoids, and Steroids.

Key words: *Nymphaea nauchali*, preliminary phytochemical, antimicrobial action.

INTRODUCTION:

Herbal medicine is the provenance of about 75-80% of the whole population, mainly in developing countries. It is reported to have minimal side effects^{1, 2}. There are more than 35,000 medicinal plant species have been used in various human populations around the world for medicinal purpose³. Ayurveda is ancient health care system and is practiced widely in India and other countries⁴. The importance of herbs in the management of human ailments cannot be over emphasized. Roughly 20% of the plants found in the world have been submitted to pharmacological biological test, and a substantial number of new antibiotics introduced on the market are obtained from natural or semi-synthetic resources⁵. The investigation of certain phytochemicals for their antimicrobial properties can be of great significance in therapeutic treatments.

Water Lily (common name for a family of aquatic plants Nymphaeaceae Salisb) is a aquatic herb with perennial rhizomes or rootstocks anchored with mud, floating or submerged leaves and solitary, showy flowers⁶. *Nymphaea* species with white flower is the **national flower of Bangladesh**, which is constituted by 70 species

of cosmopolitan distribution^{7, 8}.

In folk medicine, the *Nymphaea* is reported to be soothing with tranquilizing effects and is reputedly a detoxicant and aphrodisiac along with astringent, diuretic properties. It is used in Ayurvedic medicine for dyspepsia, enteritis, diarrhea, urinary problems, fevers and heart palpitations⁹. The leaves roots and flowers have a wide range of pharmacological activities and are used for diabetes, eruptive fevers and liver disorders. The leaves have been used in traditional Sudanese medicine as a remedy for dysentery, to treat tumours¹⁰. Hence, the aim of this study was to investigate the phytochemical constituents of this plant and to evaluate their antimicrobial activities. *Nymphaea nouchali* is considered a medicinal plant in Indian Ayurvedic medicine under the name *Ambal*; it was mainly used to treat indigestion¹¹. Recent experiments have confirmed that it has medicinal qualities as an antihepatotoxic and antidiabetic^{12, 13}. Like all waterlilies or lotuses, its tubers and rhizomes can be used as food items; they are eaten usually boiled or roasted. In the case of *N. nouchali*, its tender leaves and flower peduncles are also valued as food¹⁴.



Figure- 1: *Nymphaea nouchali*

MATERIAL AND METHODS:

Leaves of *Nymphaea nouchali* were collected from Munshiganja district, Dhaka wash twice with tap water and it was allowed to dry under shade. It was taxonomically identified with the help of National Herbarium of Bangladesh, Dhaka (Accession. No # 35250). The dried leaves were made into a fine powder.

Extraction of leaves:

About 250 gm of powdered leaves was taken in a clean flat-bottomed glass container and percolated with 3 liters of Methanol. The container with its content was sealed and kept for 7 days with occasional shaking and stirring .the mixture was the filtered successively through a piece of clean white cotton .The filtrate thus obtained are kept in a open air for the evaporation of the methanol. After 10 to 15 days all the methanol are evaporated and got the extract of methanol. Then it was allowed to do preliminary phytochemical analysis.

PRELIMINARY PHYTOCHEMICAL ANALYSIS:

The preliminary phytochemical assay was done for the *Nymphaea nouchali*-

Test for Tannins:

1. **Ferric chloride Test:** 0.5 ml of solution of extract was taken in a test tube. Then 0.1 ml of 5% Ferric chloride solutions was added. Greenish black precipitate was formed and indicated the presence of tannins.
2. **Potassium dichromate test:** 0.5 ml solution of extract was taken in a test tube .Then 0.1 ml of 10% Potassium

dichromate solution was added. A yellow precipitate was formed in the presence of tannins¹⁵.

Test for Flavonoids:

Added a few drops of concentrated hydrochloric acid to a small amount of an alcoholic extract of the plant material. Immediate development of a red colors indicates presence of Flavonoids.

Test for Saponnins:

1 ml solution of extract was diluted with distilled water to 20 ml and shaken in a graduated cylinder for 15 minutes. One – centimeter layer of foam indicates the presence of saponnins.

Test for Steroids:

1 ml of solution chloroform extract was taken and then added 0.2 ml Sulphuric acid . Red color indicates the presence of steroids.

Tests for Alkaloids:

1. **Mayer's Test:** 2 ml of solution of extract and 0.2 ml of dilute hydrochloric acid were taken in a test tube. Then 1 ml of Myer's reagent were added. Yellow color precipitate was formed and that was indicated as the presence of alkaloids.
2. **Dragendroff's test:** 2 ml of solution of extract and 0.2 ml of dilute hydrochloric acid were taken in a test tube. Then 1 ml of Dragendroff's reagent was added. Orange brown precipitate was formed and that was indicated as the presence of alkaloids. The alkaloidal fraction of the stem has also been reported to be active against all micro-organisms¹⁶.

Test for Terpenoids:

A few mg of the substance is treated with puncal reagent (ammonium heptamolybedate, ceric sulphate in concentrated Sulphuric acid) and heated at 150 °C blue colour shows the presence of terpenoids³.

Test for Sugar:

A few mg of the substance were added to the conc.Sulphuric acid in alcohol Heated gently if necessary, charring or black color shows the presence of sugar³.

Test for Protein:

A few mg of the substance were added to the Ninhydrin reagent, Heated gently if necessary, blue colour shows the presence of protein³.

ANTIMICROBIAL ACTION:

Principle of disc diffusion method:

In this classical method, antibiotics diffuse from a confined source through the nutrient agar gel and create a concentration gradient. Dried and sterilized filter paper discs (6 mm diameter) containing the test samples of

known amounts are placed on nutrient agar medium uniformly seeded with the test microorganisms. Standard antibiotic (Ciprofloxacin) discs and blank discs are used as positive and negative control. These plates are kept at low temperature (4°C) for 24 hours to allow maximum diffusion of the test materials to the surrounding media¹⁷. The plates are then inverted and incubated at 37°C for 24 hours for optimum growth of the organisms. The test materials having antimicrobial property inhibit microbial growth in the media surrounding the discs and thereby yield a clear, distinct area defined as zone of inhibition. The antimicrobial activity of the test agent is then determined by measuring the diameter of zone of inhibition expressed in millimeter^{17,18}.

Culture medium and their composition:

The following media is used normally to demonstrate the antimicrobial activity and to make subculture of the test organisms. Prepared Muller-Hunton medium was supplied from IPH (Institute of Public Health) its composition/100 ml is shown here; **Muller – Hunton medium:**

Ingredients	Amounts
Beef infusion	30 gm
Casamino acid	1.75 gm
Starch	0.15 gm
Bacto agar	1.70 gm
Distilled water q.s.	100 ml
pH	7.3 ±0.2 at 250 C

Nutrient agar medium (DIFCO) is the most frequently used and also used in the present study for testing the sensitivity of the organisms to the test materials and to prepare fresh cultures.

Preparation of medium:

To prepare required volume of this medium, calculated amount of Muller-Hinton agar was taken in a conical flask and distilled water was added to it to make the required volume. The contents were heated in a water bath to make a clear solution. The pH (at 25°C) was adjusted at 7.2-7.6 using NaOH or HCl. 10 ml and 5 ml of the medium. The conical flasks were then capped and sterilized by autoclaving at 15-lbs. pressure at 121°C for 20 minute. The slants were used for making fresh culture of bacteria and fungi that were in turn used for sensitivity study.

Sterilization procedures:

In order to avoid any type of contamination and cross contamination by the test organisms the antimicrobial screening was done in Laminar Hood and all types of

precautions were highly maintained. UV light was switched on one hour before working in the Laminar Hood. Petri dishes and other glassware were sterilized by autoclaving at a temperature of 121°C and a pressure of 15-lbs/sq. inch for 20 minutes. Micropipette tips, cotton, forceps, blank discs etc. were also sterilized.

Preparation of subculture:

In an aseptic condition under laminar air cabinet, the test organisms were transferred from the pure cultures to the agar slants with the help of a transfer loop to have fresh pure cultures. The inoculated strains were then incubated for 24 hours at 37°C for their optimum growth. These fresh cultures were used for the sensitivity test.

Preparation of the test plates:

The test organisms were transferred from the subculture to the test tubes containing about 10 ml of melted and sterilized agar medium with the help of a sterilized transfer loop in an aseptic area. The test tubes were shaken by rotation to get a uniform suspension of the

organisms. The bacterial suspension was immediately transferred to the sterilized Petri dishes. The Petri dishes were rotated several times clockwise and anticlockwise to assure homogenous distribution of the test organisms in the media^{19, 20}.

Preparation of discs:

Three types of discs were used for antimicrobial screening.

Standard discs:

These were used as positive control to ensure the activity of standard antibiotic against the test organisms as well as for comparison of the response produced by the known antimicrobial agent with that of the test sample. In this investigation, Ciprofloxacin (30µg/disc) standard disc was used as the reference

Blank discs:

These were used as negative controls which ensure that the residual solvents (left over the discs even after air-drying) and the filter paper were not active themselves.

Preparation of sample discs with test samples:

Measured amount of each test sample was dissolved in specific volume of solvent to obtain the desired concentrations in an aseptic condition. Sterilized metrical (BBL, Cocksville, USA) filter paper discs were taken in a blank Petri dish under the laminar hood. Then discs were soaked with solutions of test samples and dried.

Application of the test samples:

Standard Ciprofloxacin (30 µg/disc) discs were used as positive control to ensure the activity of standard antibiotic against the test organisms as well as for comparison of the response produced by the known antimicrobial agent with that of produced by the test sample. Blank discs were used as negative controls which ensure that the residual solvents (left over the discs even after air-drying) and the filter paper were not active themselves.

Diffusion and Incubation:

The sample discs, the standard antibiotic discs and the control discs were placed gently on the previously marked zones in the agar plates pre-inoculated with test bacteria and fungi. The plates were then kept in a refrigerator at 4⁰C for about 24 hours upside down to allow sufficient diffusion of the materials from the discs to the surrounding agar medium. The plates were then inverted and kept in an incubator at 37⁰C for 24 hours.

Determination of antimicrobial activity by measuring the zone of inhibition:

The antimicrobial potency of the test agents are measured by their activity to prevent the growth of the microorganisms surrounding the discs which gives clear zone of inhibition. After incubation, the Antimicrobial activities of the test materials were determined by measuring the diameter of the zones of inhibition in millimeter with a transparent scale.

RESULTS AND DISCUSSION:

Table 1: Phytochemical analysis of *Nymphaea Nauchali*

Name of Test	Extract of <i>Nymphaea Nauchali</i>
Test for Tannins	Presence
Test for Flavonoids	Presence
Test for Saponnins	Presence
Test for Steroids	Presence
Tests for Alkaloids	Presence
Test for Terpenoids	Presence
Test for Sugar	Presence
Test for Protein	Presence

The Methanolic extract exhibited antimicrobial activity against most of the test organisms. The crude Methanolic extract showed antimicrobial activity against the test organisms, average zone of inhibition (8.04mm) at a concentration of 200 µg/disc.

Table 2: Antimicrobial activity of extracts of *Nymphaea Nauchali*

Test microorganisms	Diameter of zone of inhibition (mm)	
	Methanolic Extract	Ciprofloxacin
<i>Bacillus sereus</i>	8.03	40
<i>Bacillus subtilis</i>	9.08	41
<i>Staphylococcus aureus</i>	7	40
<i>Escherichia coli</i>	7.05	42
<i>Pseudomonas aureus</i>	8	40
<i>Pseudomonas aeruginosa</i>	8	40
<i>Xanthomonas campestris</i>	10.08	40
<i>Candida albicans</i>	7.09	41

Plants are known to have beneficial the therapeutic effect documented in traditional system of medicine. Interest in a large number of traditional natural roducts has increased. The results obtained for the antimicrobial tests of *Nymphaea Nauchali* are presented in Table 2. Methanolic extract showed broader spectrum of activity, being active to both bacteria and fungi organisms. The methanol extract for example, 10.08 mm was maximum diameter zone recorded as for *Xanthomonas campestris* and 7 mm minimum diameter zone for *Staphylococcus aureus*.. All the eight microbes tested are susceptible to methanol extract with the inhibition zone range of 7 – 10.08 mm. The antifungal activity of plant extracts against *Candiada albicans* is common. In the present antimicrobial activity of plant extract towards drug resistant or clinically significant microbes are reported and it was observed that active constituent of plant material seep out in organic solvent to display biological activity. Further phytochemical studies for identification and elucidation of active constituent in plant material tested in expected to serve as lead in the development of novel bioactive antimicrobial compound. Many people in developed countries have begun to turn to alternative or complementary therapies including medicinal herbs ²¹.

CONCLUSION:

After measuring the diameter of the zones of inhibition in millimeter with a transparent scale, Methanolic extract exhibited high antimicrobial activity against gram positive and gram negative bacteria. We therefore, suggest further, the purification and characterization of the phytochemicals that would be obtained with a view to obtaining useful chemotherapeutic agent.

ACKNOWLEDGEMENTS:

The authors are thankful to Bangladesh University and University of Dhaka for their supports and cooperation.

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