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Qualitative and quantitative determination of secondary metabolites and antioxidant potential of *Nymphaea nouchali* flowers

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ABSTRACT

In the Indian ayurvedic system of medicine, *Nymphaea nouchali* is used for the treatment of diabetes, inflammation, liver disorders, cutaneous diseases, blenorrhagia, urinary disorders, menorrhagia, menstruation problem, as an aphrodisiac, bitter tonic, antimicrobial agent and anti antihepatotoxic effect. The aim of the present study is to examine *Nymphaea nouchali* flowers for phytochemical profile, *in vitro* antioxidant activities. Qualitative analysis of various phytochemical constituents and quantitative analysis of total phenolics and flavonoids were determined by the well-known test protocol available in the literature. Quantitative analysis of phenolic and flavonoids was carried out by Folins Ciocalteau reagent method and aluminium chloride method respectively. The *In vitro* antioxidant activity of ethanolic extract of the flowers was assessed against nitric oxide, hydrogen peroxide assay using standard protocols. Phytochemical analysis revealed the presence of phenols, flavonoids, tannins, saponins, alkaloids, fixed oil and fats. The total phenolics content of flowers ethanolic extract was (18.4 mg/100mg), followed by flavonoids (12.4mg/100mg). The activities of ethanolic flowers extract against nitric oxide and hydrogen peroxide were concentration dependent with IC_{50} values of 68.39 and 64.54 µg/ml respectively. The present study concluded that the crude extract of *Nymphaea nouchali* is a potential source of natural antioxidants and this justifies its use in folkloric medicine.

Keywords: *Nymphaea nouchali*, Phytochemical, Antioxidant, Nitric oxide, Hydrogen peroxide, Phenols, Flavonoids

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INTRODUCTION

Molecular oxygen is required to maintain life, but it can be toxic through the formation of reactive oxygen species (ROS). ROS includes superoxide radical, hydroxyl radical, singlet oxygen and H_2O_2 which have been found to play an important role in the initiation and progression of various diseases such as atherosclerosis, inflammatory injury, cancer and cardiovascular disease[1]. Oxidative stress, initiated by these free radicals, seek stability through electron pairing with biological macromolecules such as proteins, lipids and DNA in healthy human cells and cause protein and DNA damage along with lipid peroxidation. But organisms have multiple mechanisms to protect cellular molecules (DNA, RNA and proteins) against ROS induced damage. These include repair enzymes (DNA glycosylases, AP endonucleases etc), antioxidant enzymes (SOD, catalase, and glutathione peroxidase) and intra as well as extracellular antioxidants (glutathione, uric acid, ergothioneine, vitamin E, vitamin C and phenolic compounds[2]. However, this natural antioxidant mechanism can be inefficient for severe and/or continued oxidative stress. Based on this idea, there has been a strong demand of therapeutic and chemo preventive antioxidant agents with limited cytotoxicity to enhance the antioxidant capacity of the body and help attenuate the damage induced by ROS. Antioxidants are a loosely defined group of compounds characterised by their ability to be oxidised in place of other compounds present[3]. Antioxidants are molecules that inhibit the initiation of oxidation chain reactions thereby preventing damage to human body cells[4]. At present, synthetic antioxidants are available such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) but they were proven to be toxic for human beings[5]. Medicinal plants containing polyphenols have been reported for antioxidant and other pharmacological activities[6]. Plant based natural antioxidants are at most interest worldwide because of non toxic nature. *Nymphaea nouchali* (Water lily in English and Shapla in Bangla Fig.1) belongs to the family Nymphaeaceae. *N. nouchali* is commonly known as the red and blue water lily or by its synonym *Nymphaea stellata*. This plant is native from the Indian subcontinent to Australia. It is the national flower of Bangladesh and Srilanka. In its natural state, *N. nouchali* is found in static or slow-flowing aquatic habitats of little to moderate depth. *N. nouchali* is a day blooming plant with submerged

roots and stems. It is a large aquatic herb; leaves broad, petiole very long, flowers white, rose or red and fruit, a globose berry. Traditionally, whole plant is used for liver disorders and aphrodisiac property. Leaves, roots and flowers are used as cardio tonic, astringent, demulcent and as a remedy for kidney problems. Flowers were reported for antidiabetic, antihepatotoxicity and anti-inflammatory activities[7-12] and leaves were reported for antimicrobial activity[13]. Three steroids, namely 24-ethyl-5α-cholestan- 3-one, 5α-stigmast-22-en-3-one, stigmast-5,22-dien-3- one have been isolated from *N. nouchali* stem bark showing antimicrobial and cytotoxic activities[14]. The seeds, however, are said to be stomachic and restorative, and they are prescribed as a diet for diabetes mellitus in the Ayurvedic system of medicine[15]. Hence, the flowers of plant contain flavonoid, gallic acid, astragalin, quercetin and kaempferol[16]. And the seeds also contain protiens, pentason, mucilage etc[17]. But yet the flowers has not been subjected to systematic scientific investigation to assets its antioxidant activity. Therefore it was our intention to investigate antioxidant activity of this plant. For this purpose the factors responsible for the potent antioxidant ability of *N. nouchali* ethanolic flowers extract was evaluated by preliminary phytochemical assay, nitric oxide, hydrogen peroxide assay. The content of important phytoconstituents such as phenolics, flavanoids and tannins were also quantitatively determined.

Figure 1: Photograph of medicinal plant *Nymphaea nouchali*

MATERIALS AND METHODS

Plant materials

The flower of plant of *Nymphaea nouchali* was collected from rural area of Bhopal (M.P), India in the months of January 2017. The sample was identified by senior Botanist Dr. Pradeep Tiwari, Doctor Hari Singh Gour Vishwavidyalaya (M.P.) by comparing with the voucher specimen. Plant material (flower) selected for the study were washed thoroughly under running tap water and then were rinsed in distilled water; they were allowed to dry for some time at room temperature. Then the plant material was shade dried without any contamination for about 3 to 4 weeks. Dried plant material was grinded using electronic grinder. Powdered plant material was observed for their colour, odour, taste and texture. Dried plant material was packed in air tight container and stored for Phytochemical and biological studies.

Chemical reagents

All the chemicals used in this study were obtained from HiMedia Laboratories Pvt. Ltd. (Mumbai, India), Sigma-Aldrich Chemical Co. (Milwaukee, WI, USA), SD Fine-Chem Chem. Ltd. (Mumbai, India) and SRL Pvt. Ltd. (Mumbai, India).All the chemicals used in this study were of analytical grade.

Extraction Procedure

Defatting of plant material

Powdered plant material (flower) *Nymphaea nouchali* was shade dried at room temperature. The shade dried flower was coarsely powdered and subjected to extraction with petroleum ether (60-80°C) in a soxhlet apparatus. The extraction was continued till the defatting of the material had taken place.

Extraction

80 g. of *Nymphaea nouchali* dried flower were successive extracted with various solvent (chloroform, ethyl acetate, ethanol and aqueous) and using different drug: solvent ratios using hot continuous percolation for different time. The extracts were evaporated above their boiling points and stored in an air tight container free from any contamination until it was used. Finally the percentage yields were calculated of the dried extracts[18].

Qualitative phytochemical analysis of plant extract

The *N. nouchali* flowers extract obtained was subjected to the preliminary phytochemical analysis following standard methods by Khandelwal and Kokate [19, 20]. The extract was screened to identify the presence or absence of various active principles like phenolic compounds, carbohydrates, flavonoids, glycosides, saponins, alkaloids, fats or fixed oils, protein and amino acid and tannins.

Quantification of secondary metabolites

Quantitative analysis is an important tool for the determination of quantity of phytoconstituents present in plant extracts. For this TPC and TFC are determined. Extracts obtained from flower of *Nymphaea nouchali* plant material of subjected to estimate the presence of TPC and TFC by standard procedure.

Total Phenol Determination

The total phenolic content was determined using the method of Olufunmiso *et al*[21]. A volume of 1 ml of *N. Nouchali* flowers extracts or standard was mixed with 5 ml of Folin Ciocalteau reagent (previously diluted with distilled water 1:10 v/v) and 4 ml (75g/l) of sodium carbonate. The mixture was allowed to stand for 15 min under room temperature. The blue colour developed was read at 765 nm using UV/visible spectrophotometer. The total phenolic content was calculated from the standard graph of gallic acid and the results were expressed as gallic acid equivalent (mg/g).

Total Flavonoids Determination

The total flavonoid content was determined using the method of Olufunmiso *et al*[21]. 1 ml of 2% $AlCl_3$ methanolic solution was added to 1 ml of extract or standard and allowed to stand for 60 min at room temperature; the absorbance of the reaction mixture was measured at 420 nm using UV/visible spectrophotometer The content of flavonoids was calculated using standard graph of quercetin and the results were expressed as quercetin equivalent (mg/g).

In-vitro antioxidant assays

Nitric oxide scavenging activity

Nitric oxide was generated from sodium nitroprusside and was measured by the Griess reagent. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitric ions that can be estimated by using Griess reagent. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitric oxide[22]. Sodium nitroprusside (10 mmol/l) in phosphate buffer saline (PBS) was mixed with different concentrations of the extract and incubated at 25°C for 150min. The samples were added to Griess reagent (1% sulphanilamide, 2% $H_3PO_4$and 0.1% napthylethylenediamine dihydrochloride). The absorbance of the chromophore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with napthylethylenediamine was read at 546 nm and referred to the absorbance of standard solutions of ascorbic acid treated in the same way with Griess reagent as a positive control. All the tests were performed in triplicate and the graph was plotted with the mean values. The percentage of inhibition was measured by the following formula:

$$(\%)\ \text{Inhibition} = (A_{control}-A_{test})/A_{control}\times 100$$

Where $A_{control}$ is the absorbance of the control (without extract) and A_{test} is the absorbance in the presence of the extract/standard.

Free radical scavenging activity (frsa) using hydrogen peroxide

The hydrogen peroxide FRSA of the ethanolic extracts was done as suggested by Czochra and Widensk[23]. 2ml of hydrogen peroxide (43 m mol) and 1.0 ml of ethanolic sample [20-100 μl of ethanolic extract (4 mg/ml) of plant in ethanol] followed by 2.4 ml of 0.1 M phosphate buffer (pH 7.4) were added. The resulting solution was kept for 10 min and the absorbance was recorded at 230 nm. All readings were repeated three times. Blank was prepared without adding hydrogen peroxide and control was prepared without sample. Ascorbic acid was used as a standard compound. Free radical scavenging activity of hydrogen peroxide (%) was calculated.

Statistical Analysis

All the experiments were done in triplicates. The experimental results are expressed as mean±SEM of triplets. Statistical analysis was performed using Graph Pad Prism Software, Version 4.0.3 (Graph Pad Software, San Diego, CA, USA).

RESULTS

Extract yield

The yield of *N. Nouchali* ethanolic flowers extracts was 3.9 % w/w.

Qualitative phytochemical analysis

Preliminary phytochemical screening of *N. Nouchali* flower extracts revealed the presence of various components such as phenolic compounds, carbohydrates, flavanoids, glycosides, saponins, alkaloids, fats or fixed oils, protein and amino acid and tannins among which phenols and flavones were the most prominent ones and the results are summarized in Table 1.

Table 1: Phytochemical evaluation of *Nymphaea nouchali*

Chemical Tests	Chloroform	Ethyl acetate	Ethanolic	Aqueous
Alkaloids				
Hager's reagent	-	-	+	-
Wagner's reagent	-	-	-	-
Dragendorff's reagent	-	-	-	-
Glycosides (+Ve)				
Legal's test	-	-	-	-
Phenols/Tannins				
Ferric chloride	-	+	+	-
Flavonoids				
Lead acetate test	-	+	+	+
Alkaline reagent test	-	+	+	-
Saponins				
Foam test		+	-	-
Fixed oil/Fats				
Spot	+	-	-	-
Saponification	-	-	-	
Carbohydrates				
Molish test	-	-	-	-
Fehling's solution test	-	-	-	-
Amino acids				
Xantoprotein Test	-	-	-	-
Protein				
Biuret test	-	-	-	-

(+) Indicates 'Presence'; (-) Indicates 'Absence'

Quantitative phytochemical analysis

Among the secondary metabolites that were quantified, the total phenolic content was the highest with 18.4 mg/100mg of the ethanolic extract followed by the total flavonoids content with 12.4mg/100mg of the ethanolic extract The results are tabulated in Table 2.

Table 2: Total Phenolic and flavonoids content of *Nymphaea nouchali*

Estimation	*Nymphaea nouchali*		
	Ethyl acetate	Ethanolic	Aqueous
Total Phenol (mg/100mg)	14.4	18.4	---
Total Flavanoids (mg/100mg)	6.43	12.4	10.2

Nitric oxide radical scavenging activity

Fig. 2 shows the scavenging activity of *N. Nouchali* ethanolic flowers extract against nitric oxide radical released by sodium nitroprusside in a concentration dependent manner. A comparable scavenging activity was observed between the extract and the standard ascorbic acid. At 20 µg/ml, the percentage inhibitions of the *N. Nouchali* ethanolic flowers extracts and ascorbic acid were 36.11 % and 47.70 % respectively. The IC_{50} value of the standard was 24.63µg/ml while that of the ethanolic extract was 68.39µg/ml. The standard and the extract recorded a gradual dose-dependent inhibitory activity tested in an increasing order. And in the case of *N. Nouchali* ethanolic flowers extract, the maximum scavenging activity of 68.39% was observed at 100 µg/ml concentration.

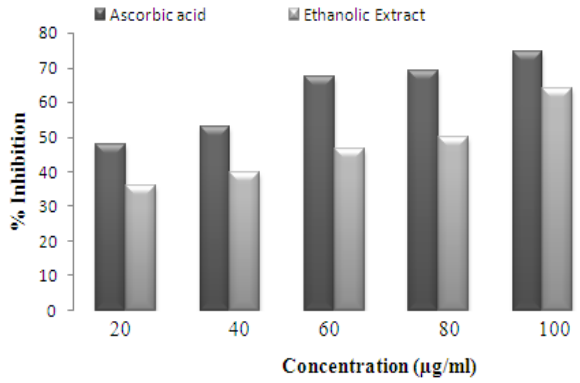

Figure 2: Nitric oxide scavenging activity of the ethanolic flower extract of *N. Nouchali* in comparison with ascorbic acid

Hydrogen peroxide free radical scavenging activity

The extract was capable of scavenging hydrogen peroxide in a concentration-dependent manner. The radical scavenging activity of *N. Nouchali* extract increased with increasing in concentrations (Fig.3).

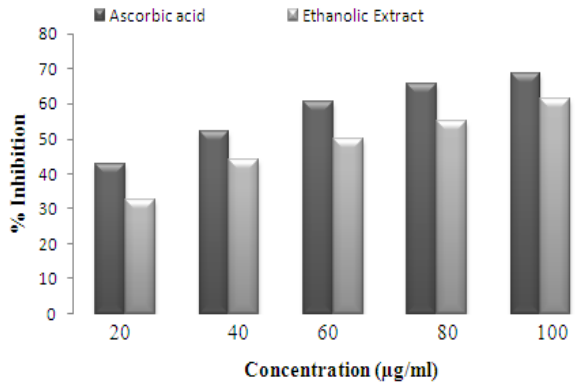

Figure 3: Hydrogen peroxide scavenging activity of the ethanolic flower extract of *N. Nouchali* in comparison with ascorbic acid

The IC_{50} value of the standard was 36.613µg/ml while that of the extract was 64.54µg/ml. The standard and the extract recorded a gradual dose-dependent inhibitory activity tested in an increasing order. And in the case of *N. Nouchali* ethanolic flowers extract, the maximum scavenging activity of 64.54% was observed at 100 µg/ml concentration.

DISCUSSION

Medicinal plants contain various phytochemical compounds that attribute to their medicinal properties. Polyphenols are the major phytochemical compounds which were reported for many pharmacological properties in previous studies that include antidiabetic, hepatoprotective, anticancer and antimicrobial activities[24]. The medicinal value of polyphenols in the plants is due to their higher antioxidant nature. The presence of phenolic compounds contributes to the antioxidative properties and thus the usefulness of these plants in herbal medicament. Flavonoids have also been shown to exhibit their actions through effects on membrane permeability and by inhibition of membrane-bound enzymes such as the ATPase and phospholipase A2[25] and this property may explain the mechanisms of antioxidative action of *N. Nouchali* ethanolic flowers extract. In the present study, phytochemical screening resulted in the presence of phenolic compounds, flavonoids, carbohydrates, alkaloids and tannins. Free radical scavenging activity of the plant extract contributes to the neutralization of free radicals thereby inhibiting chain reaction and stops cellular damage within body cells. Hence, Nitric oxide, hydrogen peroxide radical scavenging activity was performed that has given high radical scavenging activity of ethanolic extract fallowed by chloroform, ethyl acetate and aqueous extracts. All the above methods have proven the *N. Nouchali* ethanolic flowers extract to possess significant antioxidant activity which is due to the presence of various bioactive principles in it. Hence, it is evident that the polyphenols that has been detected in the present study are good antioxidants and their presence within the ethanolic extract of *N. nouchali* flowers has contributed to the antioxidant activities respectively.

CONCLUSION

The present study concluded that this medicinal plant viz. *N. nouchali* is a promising source of potential antioxidants and may be efficient as preventive agents in the pathogenesis of some diseases. However, the strength of the existing data is not enough to suggest a reasonable mode of action for antioxidant effects. Although antioxidant activities of the mentioned extracts were lower than standard reference compounds, this needs to be fully clarified by further assay methods and using additional concentrations of extracts. Further phytochemical studies are also required to isolate and characterize active ingredients that are responsible for its antioxidant activity and to explore the existence of synergism if any, among the compounds.

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