Innovare Academic Sciences

International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 6, Issue 6, 2014

Original Article

PHYTOCHEMICAL PROFILE, *IN VITRO* ANTIOXIDANT AND HEMOLYTIC ACTIVITIES OF VARIOUS LEAF EXTRACT OF *NYMPHAEA NOUCHALI* LINN: AN *IN VITRO* STUDY

LAKSHMI G, SMITHA N, AMMU S V, PRIYA C L, BHASKARA RAO K V *

Molecular and Microbiology Laboratory, Environmental Biotechnology Division, School of Bio Sciences and Technology, VIT University, Vellore, (T.N), India. Email: kokatibhaskar@gmail.com

Received: 15 May 2014 Revised and Accepted: 12 Jun 2014

ABSTRACT

Objective: The aim of the present study is to examine *Nymphaea nouchali* leaves for phytochemical profile, *in vitro* antioxidant and hemolytic activities.

Methods: The study was initiated by preliminary phytochemical screening to detect the presence of carbohydrates, flavonoids, alkaloids, saponins and tannins. Quantitative analysis of phenolic and flavonoids was carried out by Folins Ciocalteau reagent method. *In vitro* antioxidant activities were performed by DPPH free radical scavenging assay, Reducing power assay, Total antioxidant assay and Thiobarbituric acid assay. To check the toxicity of the extract on red blood cells, hemolytic activity was carried out by UV visible Spectrophotometric method. High Performance Liquid Chromatography (HPLC) analysis was performed to categorize type of phenolic compounds present in the bioactive extract.

Results: The bioactive extract exhibited the presence of carbohydrates, phenolic compounds, alkaloids and tannins. The extract showed potent reducing power and total antioxidant activities, while it has also shown 94% of DPPH radical scavenging activity and 88% of metal chelating activity with IC_{50} values of 42µg/mL and 28µg/mL respectively. The extract did not show harmful effect towards human erythrocytes through the test performed.

Conclusion: The present study concluded that the methanolic extract of *Nymphaea nouchali* leaves have shown higher antioxidant activity for the tests performed. The extract showed no hemolysis as well. HPLC analysis identified the presence of Epigallocatechin, Epicatechin gallate, Luteolin-7-o glucoside, Apigenin-6-C-glucoside and Myricetin-3-o rhanoside that could be contributing to the antioxidant nature of the extract. In future, bioactive compound should be isolated and purified to be used as a safe Phytomedicine.

Keywords: *Nymphaea nouchali,* DPPH radical scavenging activity, Hemolytic activity, HPLC analysis.

INTRODUCTION

Free radicals are highly reactive and unstable molecules which attacks healthy cell components to steal an electron to become stable. This electron scavenging creates a chain reaction of free radical formation which result in oxidative stress and cause damage to the cell membrane, proteins, blood vessel walls, fats or even to DNA [1-2]. Free radicals are of reactive oxygen species (ROS) and reactive nitrogen species (RNS) derivatives and some are non-radical reactive derivatives. Free radical formation occurs either by body's natural processes such as metabolic process in mitochondria and in cytochrome oxidase, lipid peroxidation, sometimes by microbial stimulation on neutrophils or environmental factors like exposure to radiations, metal catalyzed reactions, chemical fumes from automobiles, smoking of cigarettes also intitate the formation of free radicals [3]. Free radicals have been linked to ageing, tissue damage, arthritis, diabetes, cardiovascular disease, cataracts and some types of cancer.

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 belongs to the family Nymphaeaceae is an aquatic plant native of Indian Sub continent. 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 commonly known as water lily. Traditionally, whole plant is used for liver disorders. Leaves, roots and flowers are used as cardio tonic, astringent, demulcent and as a remedy for kidney problems. Flowers were reported for antioxidant, antidiabetic, antihepatotoxicity and anti-inflammatory activities [7-10] and leaves were reported for antimicrobial activity [11]. Leaves of *N. nouchali* has not been studied for its antioxidant potential as per existing scientific reports. Therefore present study is aimed to evaluate antioxidant and hemolytic activities of various leaf extracts of *N. nouchali* Linn.

Fig. 1: Photograph of medicinal plant *Nymphaea nouchali*

MATERIALS AND METHODS

Chemicals

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

Plant Collection and authentication

The leaves were collected from the pond of VIT University, Vellore district, Tamil nadu, India. The plant was authenticated by Dr. P. Jayaraman, Director, Institute of Herbal Botany Plant Anatomy Research Center, Chennai with Authentication no: PARC/2013/2143.

Plant Processing

The fresh leaves were shade dried, powered using blender and extracted by maceration technique using petroleum ether, chloroform, methanol, and distilled water. The filtrate was then kept on the rotary evaporator and the extract was stored in air tight container.

Phytochemical Screening

Phytochemical screening was carried out to to detect the presence of carbohydrates, flavonoids, alkaloids, saponins and tannins by using standard procedures [12].

Antioxidant activities

DPPH Radical Scavenging assay

The DPPH reagent which is 1, 1-diphenyl-2picryl hydrazyl radical was used for the determination of free radical scavenging activity of the extract. The stock solution (0.01g/mL) was prepared from which different concentration like 40, 60, 80 and 100 µg/µL was made using DMSO (Dimethyl Sulphoxide) and distilled water. The test was carried in triplicates. A volume of 2 mL of the above prepared concentrations was mixed with 1mL of DPPH which was incubated at 20ºC for 40 mins in dark. Absorbance was measured in 517nm using UV visible spectrophotometer with methanol as a blank and ascorbic acid as standard [13].

% DPPH radical was calculated using the given formula:

$$\%\ DPPH\ radical\ Scavenging\ activity = \frac{(Ac-At)}{(Ac)} \times 100$$

Ac- Absorbance of the control (DPPH)

At- Absorbance of test sample

Reducing Power assay

A volume of 2.5 mL of plant extract at different concentrations (40, 60, 80 and 100µg/µL) was mixed with equal volume of phosphate buffer and potassium ferricyanide and incubated at 50ºC for 20 mins. About 2.5 mL of trichloroacetic acid (TCA) was added to the above mixture. About 0.1ml of 1% ferric chloride solution was also added and incubated at 50ºC for 10 min. Absorbance was measured in700nm using UV visible spectrophotometer. Each experiment was carried out in triplicates [14].

Total antioxidant assay

Different concentrations of 40, 60, 80 and 100µg/µL of the extract were prepared. The reaction mixture was also prepared using 96.77 distilled water and 3.33 concentration hydrochloric acid and to this 0.35g of sodium phosphorus monobasic and 0.4g of ammonium molybdate was added and made the volume to 100 ml. About 1mL of extract with different concentrations was mixed with 3ml of reaction mixture and incubated at 95ºC for 1 hour. Absorbance was measured at 695nm using UV visible spectrophotometer with the blank containing 33mL of reaction mixture and 1ml distilled water [15].

Metal chelating assay

Metal chelating activity was performed as per the protocol of Dinis et al., 1994 [16]. To 1mL of different concentrations of the extract (40, 60, 80 and 100 µg/mL), 50µL of 2mM of $Fecl_2$ and 0.2mL of 5mm ferrozine solution was added. 0.1 ml ferricyanide was added and shaken for 10 min. Chelation of ferrous ions was measured at 562nm using UV visible spectrophotometer.

% of Chelating effect = (Ac- As/Ac) ×100

Ac=Absorbance reading of control; As= Absorbance reading of sample

Total phenolic content estimation

Different concentrations (40, 60, 80 and 100µg/µL) of the extract were prepared in triplicates. To the prepared concentrations of the extract, 2.5ml of Folin Ciocalteau reagent and 2.5 ml of 7.5% sodium carbonate was added. The mixture was incubated at 45°C for 15 min. Absorbance was measured using UV visible spectrophotometer at 765nm.The results was expressed gallic acid equivalence in µg [17].

Hemolytic activity

Preparation of Red blood cells suspension

About 5 mL of blood was collected from a healthy individual was collected in a tube containing heparin. The blood was centrifuged at 1500rpm for 3 mins. The supernatant was collected and plasma was discarded. The pellet was washed for 3 times using 0.75% Nacl and centrifuged at 1500rpm for 5mins. The cells were resuspended in normal saline to 0.5%.

To 0.5ml of cell suspension, 0.5 ml of different concentration of plant extract (40, 60, 80 and 100µg/mL) in phosphate buffer saline (PH 7.2) was added. The mixture was incubated at 37ºC for 30 mins and centrifuged at 1500 rpm for 10 mins. The free hemoglobin in the supernatant was measured using UV visible spectrophotometer at 540nm.The phosphate buffer saline and distilled water was used as minimal and maximal hemolytic control [18].

% hemolytic activity was calculated using the given formula:

$$\%\ \text{Hemolysis} = \frac{(At-An)}{(Ac-An)} \times 100$$

At- Absorbance of test sample

An- Absorbance of control (saline)

Ac-- Absorbance of control (water)

HPLC Analysis

High performance liquid chromatography was carried out for the detection of Phenolic compounds in the extract. The HPLC system was equipped with dual λ detector and a waters1525 binary pump and consists of a column (C18). The gradient elution was carried out at 35ºC with the help of solution A (50mM sodium phosphate at pH-3.3 and 10% methanol) and B (70% methanol) at a flow rate of 1mL/min. First 100% solution A,then for 15 min 70%A, again for 30 min 65%A, for next 20 min, 60%A, for another 5min, 50% and last 0% A for 25 min. The volume of the injection was 10µL of ethanolic extract dissolved in methanol. For analysis of phenolic compounds in the extract, the detected peaks at different wavelengths (250-510 nm) having different retention times were compared with respect to retention times of known standard compounds in the library [19].

RESULTS

Yield of the plant extract

Hundred grams of dried leaf powder of *Nymphaea nouchali* after extraction resulted in 12.8% yield. Yield of the extract was calculated in respect to the fresh mass to dry weight.

Phytochemical analysis

The extract showed the presence carbohydrates, phenolic compounds, alkaloids and tannins.

DPPH radical scavenging activity

DPPH is a free radical which is violet in color and is soluble only in methanol or ethanol. When DPPH reacts with an antioxidant, loses its color and becomes light purple to yellowish in color depending on the free radical scavenging ability of the antioxidant compound. Petroleum ether, chloroform, methanolic, and aqueous extracts of *N. nouchali* leaves were evaluated for DPPH radical scavenging activity. Among the four extracts tested, methanolic extract has shown 96% of radical scavenging with IC_{50} value of 42µg/ml (Figure 1).

Reducing Power assay

In the reducing power assay, ferric ions in the reaction mixture changes to ferrous ions. This reaction results in the change of the color of reaction mixture from yellow to bluish green. The intensity of bluish green color formation is directly proportional to the reducing power capacity of the extract. Methanolic extract has shown reducing power activity in a dose dependent manner (Figure 2).

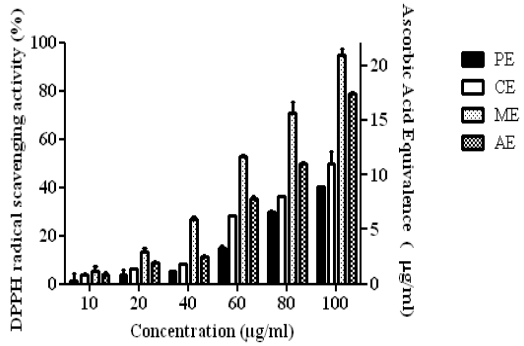

Fig. 1: DPPH radical scavenging activity; PE: Petroleum ether extract; CE: Chloroform extract; ME: Methanolic extract; AE: Aqueous extract; All the values are expressed in Mean ± SD (n=3).

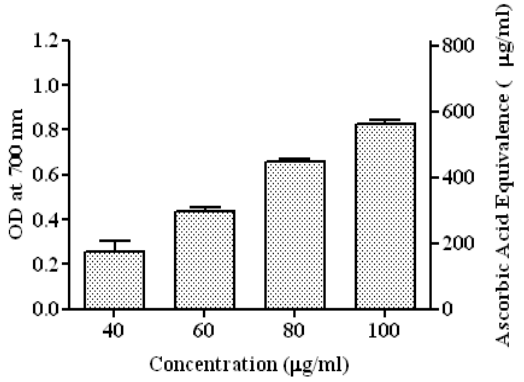

Fig. 2: Reducing power assay; The Data was represented as Mean±SD. Experiment was done in triplicates; n=3.

Total antioxidant assay

Total antioxidant activity of the extract was estimated by phosphomolybdate method. Phosphomolybdate MO (VI) gets reduced to MO (V) by the addition of the extract. Thus, resulting in green colored phosphomolybdenum complex formation. Intensity of color formation is directly proportional to the total antioxidant activity of the extract. Methanolic extract of *N. nouchali* leaves exhibited prominent total antioxidant activity in a dose dependent manner (Figure 3).

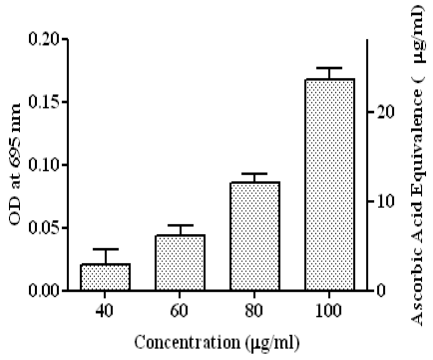

Fig. 3: Total antioxidant activity; The Data was represented as Mean±SD. Experiment was done in triplicates; n=3.

Metal chelating activity

Ferrozine forms complexes with Fe^{2+} that results in the formation of red color. While in the presence of chelating agents, Fe^{2+} complex gets captured by the chelating agents and hence complex formations gets disrupted. Intensity of red color reduction gives the chelating effect of tested plant extract. Methanolic extract of *N. nouchali* exhibited 88% of metal chelating activity as per increasing concentrations with an IC_{50} value of 28µg/mL (Figure 4).

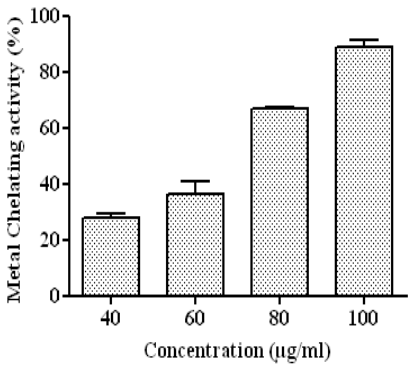

Fig. 4: Metal chelating activity; The Data was represented as Mean±SD. Experiment was done in triplicates; n=3.

Total phenolic content estimation

Methanolic extract of *N. nouchali* leaves has shown the presence of high amount of phenols 66.99 mg gallic acid equivalence per gram of the extract (Figure 5).

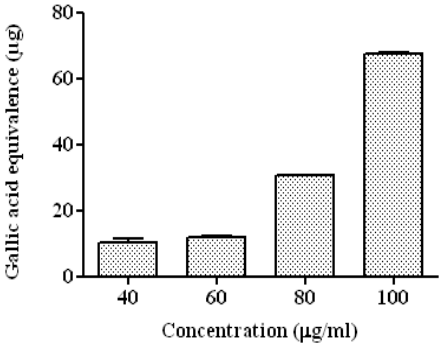

Fig. 5: Estimation of Total flavanoids content; The Data was represented as Mean±SD. Experiment was done in triplicates; n=3.

Hemolytic activity

The rupturing of healthy RBC cells is known as hemolysis. Hemolysis is the indicator of cytotoxicity towards RBC's cells. Phytochemical present in extract can show hemolytic activity. Mainly they can change the erythrocyte membrane structure. Through this *in vitro* method we can easily measure the amount of hemolysis [18]. *N. nouchali* leaves methanolic extract does not show any hemolytic activity against RBC cells.

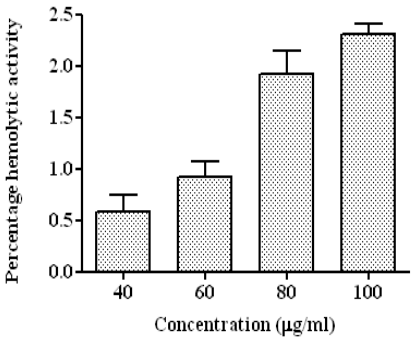

Fig. 5: Hemolytic activity; The Data was represented as Mean±SD. Experiment was done in triplicates; n=3

HPLC analysis

HPLC analysis was carried out with the methanolic extract of *N. nouchali* leaves to detect the polyphenolic compounds present in the

sample. Five poly phenols are detected by HPLC analysis which includes Epigallocatechin, Epicatechin gallate, Luteolin-7-o glucoside, Apigenin-6-C-glucoside and Myricetin-3-o rhanoside identified by comparing the sample retention times with reference retention times of the phenolic compounds library at different wavelengths ranging from 220-510 nm and were given in the table 1.

Table 1: HPLC analysis of polyphenols identification in the methanolic extract of *N. nouchali* leaves

S. No.	Compound name	Wave length (nm)	Sample Retention time	Reference Retention time
1.	Epigallocatechin	280	13.119	13.1
2.	Epicatechin gallate	280	21.105	22.9
3.	Luteolin-7-o glucoside	320	36.795	37.1
4.	Apigenin-6-C-glucoside	320	38.711	38.2
5.	Myricetin-3-o rhanoside	370	36.933	36.4

DISCUSSION

Medicinal plants conain various phytochemical compouds that attribute to their medicinal propertities. Polyphenols are the major phytochemical compounds which were reported for many pharmacological properties in previous studies that includes antidiabetic, hepatoprotective, anticancer and antimicrobial activities [20]. The medicinal value of polyphenols in the plants is due to their higher antioxidant nature. In the present study, phytochemical screening resulted in the presence of phenolic compounds, 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, DPPH radical scavenging activity was performed that has given high radical scavenging activity of methanolic extract fallowed by aqueous, chloroform and petroleum ether extracts. Therefore, methanolic extract was selected for further antioxidant studies. Transition metals especially iron is involved in the initiation of lipid peroxidation, hence metal chelating activity was performed which has shown promising amount of metal chelation of the methanolic extract of *N. nouchali* leaves. Extract also displayed good total antioxidant and reducing power capabilities. Further, HPLC analysis was carried out to know the polyphenols present within the extract which is responsible for the antioxidant activities. HPLC analysis identified the presence of Epigallocatechin, Epicatechin gallate, Luteolin-7-o glucoside, Apigenin-6-C-glucoside and Myricetin-3-o rhanoside. Epigallocatechin gallate is reported previously for antioxidant activity [21]. Luteolin-7-o glucoside from *Helichrysum compactum* was reported for antioxidant and antibacterial activities [22]. Apigenin-6-C-glucoside from *Croton zambesicus* was reported for antioxidant activity [23]. Hence, it is evident that the polyphenols that has been detected in the present study are good antioxidants and their presence within the methanolic extract of *N. nouchali* leaves has contributed to the antioxidant activities respectively.

CONCLUSION

The present study concluded that the methanolic extract of *Nymphaea nouchali* leaves have shown higher antioxidant activities for the tests performed. The extract showed no hemolysis as well. HPLC analysis identified the presence of Epigallocatechin, Epicatechin gallate, Luteolin-7-o glucoside, Apigenin-6-C-glucoside and Myricetin-3-o rhanoside that could be contributing to the antioxidant nature of the extract. In future, bioactive compound should be isolated and purified to be used as a safe Phytomedicine.

ACKNOWLEDGEMENT

We are grateful to VIT University, Vellore, Tamil Nadu, India and School of Biosciences (SBST) for their support throughout the research work.

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