TOTAL PHENOLICS, FLAVONOIDS AND IN VITRO ANTIOXIDANT ACTIVITY OF NYMPHAEA PUBESCENS WILD RHIZOME

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ABSTRACT
Antioxidant activity of rhizome of Nymphaea pubescens was studied for its free radical scavenging property on different in vitro models viz, DPPH, hydroxyl, superoxide, ABTS and reducing power by using methanol extract. Total phenolic and flavonoid contents were estimated. In vitro antioxidant activity revealed that the methanol extract showed more potent activity. In vitro antioxidant activity was compared with standard ascorbic acid/ trolox.

Keywords: Nymphaea pubescens, reducing power, DPPH, ABTS, flavonoid, in vitro.

INTRODUCTION
Oxidative stress results from an imbalance between the generation of reactive oxygen species and endogenous antioxidant systems. ROS are major sources of primary catalysts that initiate oxidation in vivo and in vitro.1-2 These ROS creates oxidative stress which results in numerous diseases and disorders such as cancer, cardiovascular disease, neural disorders, Alzheimer’s disease, mild cognitive impairment, Parkinson’s disease, alcohol induced liver disease, ulcerative colitis, ageing, atherosclerosis.3-6 The compounds especially from natural sources are providing protection against free radicals. This has attracted a great deal of research interest in natural antioxidants. It is necessary to screen out the medicinal plants for their antioxidant potential.7-9
Nymphaea pubescens Wild (Nymphaeaceae) is a perennial aquatic rhizomatous stoloniferous herb. It is commonly known as water lily, which includes about fifty species and widely distributed in tropical and temperate regions, inhabiting stagnant fresh water, ponds, lakes and swamps.\textsuperscript{[10]} The flowers of Nymphaea pubescens was used as blood purifier and in the treatment of jaundice. Fruits are given with salt for snake bite poisoning followed by blood in urine. Root shock given for cystitis, nephritis, fever, insomnia, jaundice and haemorrhoids.\textsuperscript{[11]} In siddha system of medicine the whole plant was used in the treatment of eyedisorders, diabetes and dyspepsia.\textsuperscript{[12-14]} Nymphaea species was traditionally used for treating cancer. Antiproliferative activity of ethanolic flower extract from Nymphaea pubescens against human cervical and breast carcinoma \textit{in vivo} were studied.\textsuperscript{[10]} Antidiabetic, hypolipidaemic and antioxidant effects of ethanol extract of Nymphaea pubescens rhizomew were investigated by Shajeela et al.\textsuperscript{[15]} Taking into consideration of the medicinal importance of Nymphaea pubescens rhizome, the methanol extract of rhizome of Nymphaea pubescens was analysed for the \textit{in vitro} antioxidant activity using various models viz, DPPH, hydroxyl, superoxide and ABTS radical cation scavenging activities.

MATERIALS AND METHODS

The rhizome of Nymphaea pubescens Wild. were freshly collected from Injikuzhi, Agasthiarmalai Biosphere Reserve, Western Ghats, Tamil Nadu. The plant specimen was identified and authenticated in Botanical Survey of India, Southern Circle, Coimbatore, Tamil Nadu, India. A voucher specimen was deposited in Ethnopharmacology unit, Research department of Botany, V.O.Chidambaram College, Tuticorin, Tamil Nadu.

Preparation of Plant extract

Rhizomes of the plant were dried in shade for one week, powdered and extracted with methanol using cold extraction in shaker for 48h at room temperature. The methanol extracts were concentrated in a rotary evaporator to obtain concentrated methanol extract which was then used for the estimation of total phenolic, flavonoid and the assessment of antioxidant activity.

Estimation of Total Phenolics

Total phenolic contents were determined according to Lachman \textit{et al.}\textsuperscript{[16]} 1mL of sample extract was transferred into a 50mL volumetric flask and diluted approximately with 5mL distilled water. Then, 2.5mL Folin-Ciocalteau reagent and 7.5mL of 20\% (w/w) Na\textsubscript{2}CO\textsubscript{3} were added and made up to 50mL with distilled water. It was agitated and left to stand for 2hrs.
Absorbance of the sample was measured on the spectrophotometry at 765nm against a blank prepared with distilled water. Gallic acid was used for calibration. The results were expressed as Gallic acid equivalent (GAE) in g/100g.

**Estimation of Flavonoids**

The total flavonoid content was determined according to Eom et al.\[17\] An aliquot of 0.5mL of sample (1mg/mL) was mixed with 0.1mL of 10% aluminium chloride and 0.1mL of potassium acetate (1M). In this mixture, 4.3mL of 80% methanol was added to make 5mL volume. The mixture was vortexed and the absorbance was measured spectrophotometrically at 415nm. The value of optical density was used to calculate the total flavonoid content present in the sample.

**DPPH radical scavenging activity**

The DPPH is a stable free radical and is widely used to assess the radical scavenging activity of antioxidant component. This method is based on the reduction of DPPH in methanol solution in the presence of a hydrogen donating antioxidant due to the formation of the non radical form DPPH-H. \[18\]

The free radical scavenging activity of all the extracts was evaluated by 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) according to the previously reported method.\[18\] Briefly, an 0.1mm solution of DPPH in methanol was prepared, and 1mL of this solution was added to 3mL of the solution of all extracts in methanol at different concentration(125,250,500 &1000µg/mL).The mixtures were shaken vigorously and allowed to stand at room temperature for 30 minutes. Then the absorbances were measured at 517 nm using a UV-VIS spectrophotometer (Genesys 10UV: Thermo electron corporation).Ascorbic acid was used as the reference. Lower absorbance values of reaction mixture indicate higher free radical scavenging activity. The capability to scavenging the DPPH radical was calculated by using the following formula.

\[
\text{DPPH scavenging effect (\% inhibition)} = \{(A_0 - A_1)/A_0\} \times 100
\]

Where, \(A_0\) is the absorbance of the control reaction, and \(A_1\) is the absorbance in presence of all of the extract samples and reference. All the tests were performed in triplicates and the results were averaged.
Hydroxyl radical scavenging activity

The scavenging capacity for hydroxyl radical was measured according to the modified method of Halliwell et al.\textsuperscript{19} Stock solutions of EDTA (1mM), FeCl\textsubscript{3} (10mM), Ascorbic Acid (1mM), H\textsubscript{2}O\textsubscript{2} (10mM) and Deoxyribose (10 mM), were prepared in distilled deionized water.

The assay was performed by adding 0.1mL EDTA, 0.01mL of FeCl\textsubscript{3}, 0.1mL H\textsubscript{2}O\textsubscript{2}, 0.36mL of deoxyribose, 1.0mL of the extract of different concentration (125, 250, 500 & 1000 µg/mL) dissolved in distilled water, 0.33mL of phosphate buffer (50mM, pH 7.9), 0.1mL of ascorbic acid in sequence. The mixture was then incubated at 37\textdegree C for 1 hour. 1.0mL portion of the incubated mixture was mixed with 1.0mL of 10\% TCA and 1.0mL of 0.5\% TBA (in 0.025M NaOH containing 0.025\% BHA) to develop the pink chromogen measured at 532nm. The hydroxyl radical scavenging activity of the extract is reported as % inhibition of deoxyribose degradation is calculated by using the following equation

\[
\text{Hydroxyl radical scavenging activity} = \left( \frac{A_0 - A_1}{A_0} \right) \times 100
\]

Where, \(A_0\) is the absorbance of the control reaction, and \(A_1\) is the absorbance in presence of all of the extract samples and reference. All the tests were performed in triplicates and the results were averaged.

Superoxide radical scavenging activity

The superoxide anion scavenging activity was measured as described by Robak and Gryglewski.\textsuperscript{20} The superoxide anion radicals were generated in 3.0 mL of Tris–HCl buffer (16 mM, PH 8.0), containing 0.5 mL of NBT (0.3mM), 0.5 mL NADH (0.936mM) solution, 1.0mL extract of different concentration (125, 250, 500 & 1000 µg/mL), and 0.5mL Tris–HCl buffer (16mM, PH 8.0). The reaction was started by adding 0.5 mL PMS solution (0.12mM) to the mixture, incubated at 25\textdegree C for 5 min and the absorbance was measured at 560 nm against a blank sample, ascorbic acid. The percentage inhibition was calculated by using the following equation

\[
\text{Superoxide radical scavenging activity} = \left( \frac{A_0 - A_1}{A_0} \right) \times 100
\]

Where, \(A_0\) is the absorbance of the control reaction, and \(A_1\) is the absorbance in presence of all of the extract samples and reference. All the test were performed in triplicates and the results were averaged.
Antioxidant Activity by Radical Cation (ABTS. +)
ABTS assay was based on the slightly modified method of Re et al.[21] ABTS radical cation (ABTS+) was produced by reacting 7mM ABTS solution with 2.45mM potassium persulphate and allowing the mixture to stand in the dark at room temperature for 12-16 h before use. The ABTS+ Solution was diluted with ethanol to an absorbance of 0.70+0.02 at 734nm. After addition of 100µL of sample or trolox standard to 3.9 mL of diluted ABTS+ solution, absorbance was measured at 734 nm by Genesis 10s UV-VIS (Thermo scientific) exactly after 6 minutes. Results were expressed as trolox equivalent antioxidant capacity (TEAC).

\[
\text{ABTS radical cation activity} = \frac{(A_0 – A_1)/A_0) \times 100}\]

Where, \(A_0\) is the absorbance of the control reaction, and \(A_1\) is the absorbance in presence of all of the extract samples and reference. All the tests were performed in triplicates and the results were averaged.

Reducing Power
The reducing power of the extract was determined by the method of Singh et al. [22] with minor modification to Oyaizu.[23] 1.0mL of solution containing 125, 250, 500 & 1000 µg /mL of extract was mixed with sodium phosphate buffer (5.0mL, 0.2 M, pH6.6) and potassium ferricyanide (5.0mL, 1.0%): The mixture was incubated at 50°C for 20 minutes. Then 5mL of 10% trichloroacetic acid was added and centrifuged at 980g (10 minutes at 5°C) in a refrigerator centrifuge. The upper layer of the solution (5.0mL) was diluted with 5.0mL of distilled water and ferric chloride and absorbance read at 700nm. The experiment was performed thrice and results were averaged.

RESULTS
Total phenolic content and flavonoid content
Total phenolic content and flavonoid content of the extract were found to be 0.48 g 100g\(^{-1}\) and 0.98 g 100g\(^{-1}\) respectively.

DPPH radical scavenging activity
DPPH radical scavenging activity of methanol extract of Nymphaea pubescens rhizome was shown in figure 1. The extract exhibited potent DPPH radical scavenging activity in concentration dependent manner.
Hydroxyl radical scavenging activity
Hydroxyl radical scavenging activity was determined (Figure 2) to be increased with the increase in the concentration of rhizome extract from 125 to 1000 µg/mL. The percentage of inhibition of the hydroxyl radical was varying from 16.16% in 125µg/mL of extract to 61.33% in 1000 µg/mL of rhizome extract.

Superoxide radical scavenging activity
The *Nymphaea pubescens* rhizome extract was subjected to the superoxide scavenging assay and the results was shown in figure 3. It indicates that *Nymphaea pubescens* (1000µg/mL) exhibited the maximum superoxide radical scavenging activity of 59.26% which is lower than the standard ascorbic acid whose scavenging effect is 95.34%.

ABTS radical scavenging activity
The effect of *Nymphaea pubescens* rhizome extract and standard (trolox) on ABTS radical cation was compared and shown in figure 4. The scavenging effect increases with the concentration of standard and samples. At 1000µg/mL concentration of *Nymphaea pubescens* possessed 79.66% scavenging activity on ABTS. All the concentration of *Nymphaea pubescens* showed higher activity than the standard trolox.

Reducing power
The reducing power of *Nymphaea pubescens* was compared with the standard ascorbic acid. The reducing power increases with the increasing concentration. The reducing power of the samples was shown in figure 5.

![Figure- 1: DPPH radical scavenging activity of methanol extract of *Nymphaea pubescens*](image-url)
Figure- 2: Hydroxyl radical scavenging activity of methanol extract of *Nymphaea pubescens*

Figure-3: Superoxide radical scavenging activity of methanol extract of *Nymphaea pubescens*

Figure-4: ABTS radical cation scavenging activity of methanol extract of *Nymphaea pubescens*
**DISCUSSION**

The antioxidant activity of several neutrally occurring compounds has been known for decades. Recently, many types of plants have been considered as source of reactive oxygen species inhibitors. They can be used as food additives and can also provide protection against tissue oxidation.\(^{[24]}\)

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**Figure- 5: Reducing power ability of methanol extract of *Nymphaea pubescens***

**Figure- 6: IC\textsubscript{50} values of methanol extract of *Nymphaea pubescens***

**IC\textsubscript{50} value**

IC\textsubscript{50} values of *Nymphaea pubescens* extract and standard ascorbic acid for DPPH, hydroxyl, superoxide radical scavenging and standard trolox for ABTS radical cation scavenging activity was found to be 21.47 µg/mL and 18.26 µg/mL; 26.33 µg/mL and 18.46 µg/mL; 31.59 µg/mL and 72.08 µg/mL; 26.33 µg/mL and 20.67 µg/mL respectively (figure 6).
Antioxidant compounds scavenge free radicals such as peroxide, hydro peroxide or lipid peroxyl and thus reduce the level of oxidative stress and slow or prevent the development of complications associated with oxidative stress related diseases.[25] Many synthetic antioxidants have shown toxic and mutagenic effects, which have shifted attention towards naturally occurring antioxidants.

Radical scavenging activities are very important due to the deleterious role of the free radicals in biological systems. Many secondary metabolites which include flavonoids, phenolic compounds etc serve as source of antioxidants and do scavenging activities.[26-27] In the present study, it is evident that the extract of the studied species, *Nymphaea pubescens* possess effective antioxidant activity. This nature perhaps due to the presence of respective phytochemicals like flavonoids, phenolics etc in this species.[28]

*In vitro* antioxidant activity of the methanol extract of *Nymphaea pubescens* was investigated in the present study by DPPH, hydroxyl, superoxide ABTS radical cation scavenging activity and reducing power DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. Antioxidant on interaction with DPPH, transfer electron or hydrogen atom to DPPH thus neutralizing its free radical character and convert it to 1-1 diphenyl-2-picryl hydrazine and the degree of discoloration indicates the scavenging activity of the drug.[29] The decrease in absorbance of DPPH radical caused by antioxidant is due to the reaction between antioxidant molecule and radical progress which results in the scavenging of the radical by hydrogen donation. It is visually noticeable as a change in colour from purple to yellow. Hence, DPPH is usually used as a substance to evaluate the antioxidant activity.[30-31] In the present study, the DPPH scavenging effect was increasing with the increase in the concentration of the extracts from 125- 1000µg/mL.

The cell damaging action of hydroxyl radical is well known as it is the strongest among free radicals and it has short half life.[32] *Nymphaea pubescens* exhibited the greatest scavenging effect of –OH but less than the standard ascorbic acid. –OH is known to be capable of abstracting hydrogen peroxide from membranes and they bring about lipid peroxidation. It is thus anticipated that *Nymphaea pubescens* would show antioxidant effects against lipid peroxidation on biomembranes and would scavenge –OH radicals at the stage of initiation and termination.
Superoxide dismutase catalyses the dismutation of the highly reactive superoxide anion to oxygen and hydrogen peroxide. Superoxide anion is the first reduction product of oxygen\[^{[33]}\] which is measured in terms of inhibition of generation of O\(_2\). In the present study the results clearly indicates that the plant extract has a noticeable effect as scavenging superoxide radical.

The ABTS assay is based on the inhibition of the absorbance of the radical cation ABTS, which has a characteristic long wavelength absorption spectrum. The ABTS chemistry involves direct generation of ABTS radical mono cation with no involvement of any intermediary radical. It is decolorization assay, thus the radical cation is performed prior to addition of antioxidant test system, rather than the generation of the radical taking place continually in the presence of antioxidant. In the present study, the *Nymphaea pubescens* extract showed potent antioxidant activity in ABTS method which is comparable to the standard used, trolox.

The reducing capacity of a compound may serve as a significant indicator of it’s potential antioxidant activity. Increase in absorbance of the reaction mixture indicates the reductive capabilities of *Nymphaea pubescens* extract in concentration dependent manner when compared to the standard ascorbic acid.

Polyphenolic compounds could be taken into account for the strong free radical scavenging activity, which is in agreement with previous reports. Gulcin *et al* \[^{[34]}\] reported excellent correlation for medicinal plants when antioxidant activity was compared with total polyphenolic content. Hazara *et al* \[^{[35]}\] and Rajan *et al* \[^{[36]}\] also suggested that polyphenolic content could be related to the antioxidant activities.

In conclusion, the high antioxidant activity exhibited by *Nymphaea pubescens* rhizome extract provided justification for the therapeutic use of this plant due to the phytochemical constituents. The present study suggests that this extract could be a potential source of natural antioxidant that could be of great importance for the treatment of radical related diseases and age associated diseases.
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REFERENCES


