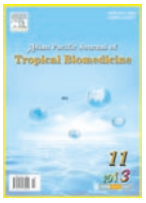
Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Biomedicine

journal homepage: www.elsevier.com/locate/apjtb



Document heading doi:10.1016/S2221-1691(13)60174-4

Phytochemical analysis and *in vitro* antioxidant acitivity of hydroalcoholic seed extract of *Nymphaea nouchali* Burm. f.

Mabel Parimala[1], Francis Gricilda Shoba[1*]

[1]*P.G. & Research Department of Zoology, Voorhees College, Vellore, Tamilnadu, India*

PEER REVIEW

Peer reviewer

Dr. A. Hannah Rachel Vasanthi, Associate Professor, Dept. of Biotechnology, School of Life Sciences, Pondicherry University, Pudhucherry-605014, India.
Tel: 0413-2654745, 09443135842
E-mail: hrvasanthi@gmail.com

Comments

The present work on the phytoconstituents of *N. nouchali* and its antioxidant activity is a good attempt to identify the potential role on mitigating oxidative stress. The results are interesting and is worth for further studies on *in-vivo* assay models.
Details on Page 894

ABSTRACT

Objective: To evaluate the phytochemical constituents and the antioxidant activity of hydroalcoholic extract of *Nymphaea nouchali* seed locally prescribed as a diet for diabetes mellitus.
Methods: The antioxidant and free radical scavenging activity of hydroalcoholic extract of the plant was assessed against 1,1 diphenyl-2-picryl hydrazyl (DPPH), nitric oxide and lipid peroxidation using standard protocols. Total phenolics, flavonoids and tannins were also determined.
Results: Phytochemical analysis revealed the presence of phenols, flavones, tannins, protein, reducing sugars, glycosides, saponins, alkaloids and steroids. The activities of plant extract against DPPH, nitric oxide and lipid peroxidation was concentration dependent with IC_{50} value of 42.82, 23.58 and 54.65 μg/mL respectively. The total antioxidant capacity was high with 577.73 mg vitamin E/g of the extract and showed a moderately high vitamin C content of 197.22 mg/g. The total tannin content of hydroalcoholic seed extract was high (195.84 GE/g), followed by phenolics (179.56 GE/g) and flavonoids (23.55 QE/g).
Conclusion: Our findings provide evidence that the crude extract of *Nymphaea nouchali* is a potential source of natural antioxidants and this justifies its use in folkloric medicine.

KEYWORDS

Nymphaea stellata, Phytochemical, Antioxidant, Scavenging, Free radicals, DPPH, Nitric oxide, Lipid peroxidation, Phenols, Flavonoids

1. Introduction

Molecular oxygen is required to sustain 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/or 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

*Corresponding author: Dr. F. Gricilda Shoba, Associate Professor, P.G. & Research Department of Zoology, Voorhees College, Vellore-632 001. Tamilnadu, India.
Tel: 91-9486423253
E-mail: gricildashoba@gmail.com
Fundation Project: Supported by the University Grants Commission, Maulana Azad National Fellowship Scheme 2012-2013, Government of India. Grant No. MANF-2012-13-CHR-TAM-15932.

Article history:
Received 24 Aug 2013
Received in revised form 29 Aug, 2nd revised form 5 Sep, 3rd revised form 13 Sep 2013
Accepted 15 Oct 2013
Available online 28 Nov 2013

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]. There are mainly two strategies proposed for this, one would be to use the antioxidants with direct radical scavenging activity, and the other approach is to identify antioxidants that would increase the expression of antioxidant enzymes[4]. There are some synthetic antioxidants like butylated hydroxyl toulene, butylated hydroxyl anisole and tetra butyl hydroquinone that have been commonly used. However, it has been suggested that these compounds are carcinogens[5]. This led to an increased interest in natural antioxidants from plant sources.

Plant based antioxidants are preferred to the synthetic ones because of their multiple mechanisms of actions and non-toxic nature. These facts have inspired widespread screening of plants for possible medicinal and antioxidant properties; the isolation and characterization of diverse phytochemicals and the utilization of antioxidants of natural origin to prevent diseases[6].

Nymphaea nouchali Burm. f. (*N. nouchali*, syn. *Nymphaea stellata* Willdenow) belongs to the family Nymphaeaceae. *N. nouchali* is commonly known as the red and blue water lily or by its synonym *Nymphaea stellata*. It is called as Nilotpalam, Allithamarai or Vellambal in Tamil. This plant is native from the Indian subcontinent to Australia. It is the national flower of Bangladesh and Srilanka. It is a large aquatic herb; leaves broad, petiole very long, flowers white, rose or red and fruit, a globose berry. Experimental studies have proven the leaves to possess aphrodisiac property[7]. Flowers of this plant has been extensively investigated and found to possess antioxidant[8], antidiabetic[9], anti-inflammatory[10] and antihepatotoxic activities[11]. 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. stellata* stem bark showing antimicrobial and cytotoxic activities[12]. 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[13]. The seeds are known to protect liver from the hepatotoxic effects of carbon tetrachloride[14]. Protein, pentosan, mucilage and tannins are reported in the seeds[15]. But no extensive chemical investigation has been done so far in the seeds except for a new steroid which was isolated from the methanolic fraction recently and was designated as nymphasterol[16].

Hence, this is the first attempt ever made to investigate on the antioxidant study of the seeds of *N. nouchali* Burm. f. The aim of this study was to assess the *in vitro* antioxidant activity of hydroalcoholic extract of the seeds of *N. nouchali*. For this purpose the factors responsible for the potent antioxidant ability of *N. nouchali* hydroethanolic seed extract was evaluated by preliminary phytochemical assay, 1,1-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity, nitric oxide scavenging activity and lipid peroxidation inhibitory activity. The content of important phytoconstituents such as phenolics, flavanoids and tannins were also quantitatively determined.

2. Materials and methods

2.1. Plant materials

The seeds of *N. nouchali* were collected from a pond called "Paatukulam" in the Leepuram village of Kanyakumari District located at the southernmost tip of the peninsular India. The seeds were collected, washed thoroughly with fresh running water, dried under shade with room temperature (25±1) °C for few weeks and coarsely powdered in a blender. The plant was identified and authenticated by Prof. Jayaraman, Plant Anatomy Research Centre, Chennai by comparing with the voucher specimen.

2.2. Chemical reagents

DPPH, sodium nitroprusside, ascorbic acid, α-tocopherol, gallic acid and quercetin were purchased from HiMedia Laboratories, Mumbai. Other chemicals such as ferric chloride, sodium carbonate, aluminium chloride, potassium acetate, phosphate buffer, trichloroacetic acid and 2-thiobarbituric acid were purchased from Sisco Research Laboratories, Mumbai. All the chemicals used in this study were of analytical grade.

2.3. Extraction

Extraction was performed by hot percolation method using soxhlet apparatus. About 500 g of the coarsely powdered raw materials of *N. nouchali* seeds was extracted in 70% hydroethanol by continuous hot extraction method at 50 °C. The extract with 70% hydroethanol was decanted from the soxhlet apparatus and the filtrate was evaporated for the total elimination of alcohol using a rotaflash vacuum evaporator. The concentrated liquid extract obtained was then transferred to a china dish and kept in a water bath at 50 °C to concentrate to dryness. The residual extract, *N. nouchali* hydroethanolic seed extract was transferred and stored in an air tight container free from any contamination until it was used. The % yield was calculated.

2.4. Ash content

Total ash and acid insoluble ash have been analyzed as per the protocols given in Ayurvedic pharmacopoeia[17].

2.4.1. Total ash

A little amount of *N. nouchali* seed was taken in a silica crucible. It was incinerated by gradually increasing the heat not exceeding dull red heat (450 °C) until free from carbon, cooled and weighed. The percentage of ash was calculated with

reference to air-dried drug. The procedure was repeated to get the constant weight.

2.4.2. Acid insoluble ash

The total ash obtained was boiled for 5 min with 25 mL of (10% w/v) dilute hydrochloric acid and filtered through ashless filter paper (Whatmann 4.1). The filter paper was ignited in the silica crucible, cooled and insoluble ash was weighed.

2.5. Preliminary phytochemical analysis

The *N. nouchali* hydroethanolic seed extract obtained was subjected to the preliminary phytochemical analysis following standard methods by Harbone[18]. The extract was screened to identify the presence or absence of various active principles like phenolic compounds, reducing sugars, flavones, glycosides, saponins, alkaloids, anthraquinones, quinones, proteins and tannins.

2.6. Quantification of primary metabolites

2.6.1. Total protein content

The total protein content was estimated according to Lowry *et al*[19]. To 1 mL of the plant seed extract or standard, 5 mL of alkaline copper sulphate reagent was added, mixed well and allowed to stand for 10 min and then 0.5 mL of Folins-Ciocalteau's reagent was added and mixed well. The mixture was allowed to stand under dark for 30 min. The blue colour developed was read at 660 nm using UV/visible spectrophotometer (Perkin Elmer, Lambda 25, USA). The protein content of the extract was calculated from the standard graph of Bovine Serum Albumin and the results were expressed as % w/w.

2.6.2. Total carbohydrate content

The total carbohydrate content was evaluated by following the method of Hedge and Hofreiter[20]. To 0.5 mL of *N. nouchali* hydroethanolic seed extract or standard, 0.5 mL water was added to make the volume to 1mL. A volume of 4 mL of anthrone reagent was added. The mixture was heated for 8 min in boiling water bath and cooled. The green colour developed was read at 630 nm using UV/visible spectrophotometer (Perkin Elmer, Lambda 25, USA). The carbohydrate content of the extract was calculated from the standard graph of glucose and the results were expressed as % w/w.

2.6.3. Total lipid content

The total lipid content was estimated by the method of Zlatkis *et al*. with minor modifications[21]. About 0.1 mL of the *N. nouchali* hydroethanolic seed extract supernatant or standard was made up to 5 mL with working ferric chloride acetic acid reagent and the tubes were kept at room temperature for 10 min. Three millilitres of 85% concentrated sulphuric acid was added. The mixture was kept at ice cold condition for 20 min. The pink colour formed was read at 540 nm using UV/visible spectrophotometer (Perkin Elmer, Lambda 25, USA). The lipid content of the extract was calculated from the standard graph of cholesterol and the results were expressed as % w/w.

2.7. Quantification of secondary metabolites

2.7.1. Total phenol determination

The total phenolic content was determined using the method of McDonald *et al*[22]. A volume of 1 mL of *N. nouchali* hydroethanolic seed extract or standard was mixed with 5 mL of Folin Ciocalteau reagent and 4 mL 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 (Perkin Elmer, Lambda 25, USA). The total phenolic content was calculated from the standard graph of gallic acid and the results were expressed as gallic acid equivalent (mg/g).

2.7.2. Total flavonoid determination

The total flavonoid content was determined using the method of Chang *et al*[23]. A volume of 0.5 mL of *N. nouchali* hydroethanolic seed extract or standard was separately mixed with 4.5 mL of methanol. To the mixture, 0.1 mL of 10% aluminium chloride and 0.1 mL of 1 mol/L sodium acetate was added. Then allowed the mixture at room temperature for 30 min; the absorbance of the reaction mixture was measured at 415 nm using UV/visible spectrophotometer (Perkin Elmer, Lambda 25, USA). The content of flavonoids was calculated using standard graph of quercetin and the results were expressed as quercetin equivalent (mg/g).

2.7.3. Total tannin determination

The total tannin content was determined using the method of Schanderl[24]. One millilitre of the *N. nouchali* hydroethanolic seed extract or standard was taken and the volume was made up to 1 mL with distilled water. To the mixture, 0.5 mL Folin's phenol reagent followed by 5 mL of 35% sodium carbonate was added and kept at room temperature for 5 min. The blue colour formed was read at 640 nm using UV/visible spectrophotometer (Perkin Elmer, Lambda 25, USA). The content of tannins was calculated using standard graph of gallic acid and the results were expressed as gallic acid equivalent (mg/g).

2.8. In vitro antioxidant assays

2.8.1. DPPH radical scavenging activity

DPPH radical scavenging assay was performed by the method of Koleva *et al*[25]. About 10 μL of each concentration (1.95-1000.00 μg/10 μL) of *N. nouchali* hydroethanolic seed extract or standard was added to 190 μL DPPH solution. After vortexing, the mixture was incubated for 20 min at 37 °C. The decrease in absorbance of test mixture due to quenching of DPPH free radical was measured at 517 nm. The IC_{50} value was determined as the concentration of the test mixture that gave 50% reduction in the absorbance from control blank. Vitamin E

was used as a reference standard. The percentage inhibition was calculated as following:

(%) Inhibition=[(control-test)/control]×100

2.8.2. Nitric oxide radical scavenging activity

Nitric oxide radical scavenging assay was performed by the method of Green *et al*[26]. Sodium nitroprusside (5 mmol/L) in phosphate buffered saline was mixed with 3 mL of different concentrations (1.95-1000.00 μg/mL) of the *N. nouchali* hydroethanolic seed extract dissolved in water and incubated at 25 °C for 150 min. The samples from the above were allowed to react with Griess reagent. The absorbance of the chromophore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with naphthylethylenediamine was read at 546 nm using UV/visible spectrophotometer (Perkin Elmer, Lambda 25, USA). Quercetin was used as a reference standard. The percentage of nitric oxide radical scavenging activity was calculated by the formula below and the results were computed. The IC_{50} value was determined.

(%) Inhibition=[(control-test)/control]×100

2.8.3. Lipid peroxidation inhibition assay

Lipid peroxidation inhibition assay was performed by the method of Okhawa *et al*[27]. The test system contained 1 mL of tissue homogenate with *N. nouchali* hydroethanolic seed extract (1.95-1000.00 mg/mL). To 1 mL of tissue homogenate, lipid peroxidation was initiated by the addition of 0.1 mL of $FeSO_4$ (25 mmol/L), 0.1mL of ascorbate (100 mmol/L) and 0.1 mL of KH_2PO_4 (10mmol/L) and the volume was made up to 3 mL with distilled water and incubated at 37 °C for 1 h. Then, 1 mL of 5% trichloroacetic acid and 1 mL of 2-thiobarbituric acid was added to this reaction mixture and the tubes were boiled for 30 min in a boiling water bath. This was centrifuged at 3 500 r/min for 10 min. The extent of inhibition of lipid peroxidation was evaluated by the estimation of thiobarbituric acid reactive substances (TBARS) level by measuring the absorbance at 532 nm using UV/visible spectrophotometer (Perkin Elmer, Lambda 25, USA). Quercetin was used as a reference standard. The IC_{50} value was determined. The percentage inhibition of lipid peroxidation was calculated by the following formula:

(%) Inhibition=[(control-test)/control]×100

2.8.4. Total antioxidant capacity

The total antioxidant capacity was determined as prescribed by Prieto *et al*.[28] with minor modifications. An aliquot of *N. nouchali* hydroethanolic seed extract or standard was combined with α-tocopherol reagent solution. The tubes were capped and incubated in a boiling water bath at 95 °C for 60-90 min. Samples were cooled to room temperature, the absorbance of each was measured at 695 nm using UV/visible spectrophotometer (Perkin Elmer, Lambda 25, USA). The content of total antioxidant capacity was calculated using a standard graph of α-tocopherol and the results were expressed as vitamin E equivalent (mg/g).

2.8.5. Determination of vitamin c content

The vitamin C content was determined as prescribed by Omaye *et al*.[29] with slight modifications. To 1 mL of *N. nouchali* hydroethanolic seed extract or standard, 0.1 mL of DTC reagent was added and incubated at 37 °C for 3 h. After incubation, 1.25 mL of 85% H_2SO_4 was added under ice-cold condition. The mixture was kept at room temperature for 30 min. The absorbance was measured at 540 nm using UV/visible spectrophotometer (Perkin Elmer, Lambda 25, USA). The content of vitamin C was calculated using a standard graph of ascorbic acid.

2.9. 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).

3. Results

3.1. Extract yield

The yield of *N. nouchali* hydroethanolic seed extract was 6.2% w/w.

3.2. Ash content

The total ash content of *N. nouchali* seed was found to be 1.6% w/w. The acid-insoluble ash content of the seeds was estimated to be 0.9% w/w.

3.3. Qualitative phytochemical analysis

Preliminary phytochemical screening of *N. nouchali* hydroethanolic seed extract revealed the presence of various components such as proteins, carbohydrates, reducing sugars, glycosides, phenols, tannins, flavones, saponins and alkaloids among which phenols, tannins and flavones were the most prominent ones and the results are summarized in Table 1.

Table 1

Phytoconstituents in *N. nouchali* hydroethanolic seed extract.

Phytoconstituents	Presence/absence
Protein	+++
Carbohydrate	++
Reducing sugar	+++
Glycosides	+++
Phenol	+++
Tannin	+++
Flavones	+++
Saponin	++
Steroid	+
Alkaloid	++
Anthraquinone	-
Quinone	-

+: Presence (+ mild, ++ moderate, +++ high), -: Absence.

3.4. Quantitative phytochemical analysis

The percentage composition of the primary metabolites of the *N. nouchali* hydroethanolic seed extract showed total protein, total carbohydrate and total lipid content to be (17.47±1.32)% w/w, (13.92±1.19)% w/w and (0.10±0.01)% w/w respectively. Among the secondary metabolites that were quantified, the total tannin content was the highest with (195.84±0.80) GE/g of the extract followed by the total phenolic content with (179.56±1.59) GE/g of the extract and the total flavonoid content with (23.55±1.46) QE/g of the extract. The results are tabulated in Table 2.

Table 2

Quantified phytochemical compounds.

Compounds	Amount	Equivalents
Protein	17.47%	ND
Carbohydrate	13.92%	ND
Lipid	0.10%	ND
Total phenolics	179.56±1.59 GE/g*	Gallic acid
Total flavonoids	23.55±1.46 QE/g*	Quercetin
Total tannins	195.84±0.8 GE/g*	Gallic acid

*:Values are expressed as mean±SEM (n=3).

ND: Not detected; GE: Gallic acid equivalent; QE: Quercetin equivalent.

3.5 DPPH scavenging activity

The results of DPPH radical scavenging activity of the *N. nouchali* hydroethanolic seed extract and the standard vitamin E are presented in Figure 1. The percentage inhibitory activity of free radicals by 50% has been used widely as a parameter to measure antioxidant activity. In this study, both the plant extract and the standard vitamin E significantly scavenged the DPPH radical with increasing concentrations. The percentage inhibition of the DPPH radical by the *N. nouchali* hydroethanolic seed extract and vitamin E at 1.95 μg/mL was 8.56% and 16.82% while the IC_{50} values were 42.82 μg/mL and 27.82 μg/mL, respectively. The scavenging activity of the *N. nouchali* hydroethanolic seed extract was found to be lower than that of vitamin E standard. The percentage of inhibition reached maximum of 89.47% at 1000 μg/mL of *N. nouchali* hydroethanolic seed extract. However with the addition of a larger amount of *N. nouchali* hydroethanolic seed extract to the DPPH assay mixture, the degree of inhibition decreased, indicating a pro-oxidant effect.

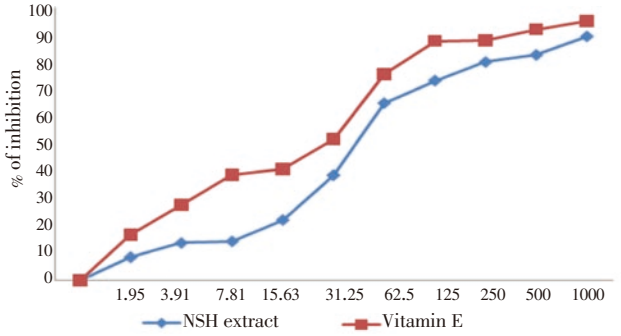


Figure 1. DPPH radical scavenging activity of the hydroalcoholic seed extract of *N. nouchali* in comparison with vitamin E.

NSH: *N. nouchali* hydroethanolic seed extract.

3.6. Nitric oxide radical scavenging activity

Figure 2 shows the scavenging activity of *N. nouchali* hydroethanolic seed 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 quercetin. At 1.95 μg/mL, the percentage inhibitions of the *N. nouchali* hydroethanolic seed extract and quercetin were 21.87% and 30.20% respectively. The IC_{50} value of the standard was 13.54 μg/mL while that of the extract was 23.58 μg/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* hydroethanolic seed extract, the maximum scavenging activity of 91.54% was observed at 1000 μg/mL concentration.

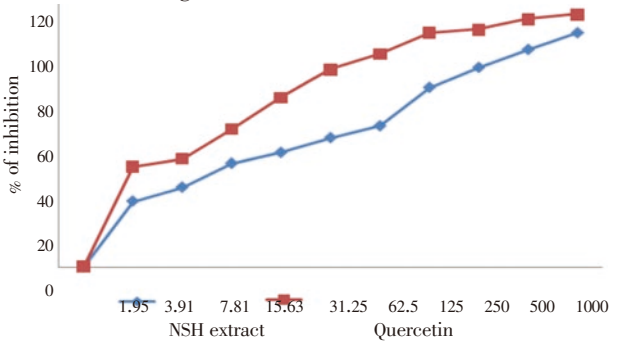


Figure 2. Nitric oxide scavenging activity of the hydroalcoholic seed extract of *N. nouchali* in comparison with quercetin.

NSH: *N. nouchali* hydroethanolic seed extract.

3.7. Lipid peroxidation inhibition assay

The lipid peroxide inhibitory activity of the extract against TBARS initiated by ferric chloride was investigated and the result is shown in Figure 3. The percentage inhibitory activity of the *N. nouchali* hydroethanolic seed extract was compared with the standard quercetin. The extract showed the prevention of formation of TBARS in a dose dependent manner. At 1.95 μg/mL concentration of the extract and quercetin, the percentage of inhibition was 24.69% and 37.50% respectively. The IC_{50} value of the extract was 54.65 μg/mL while that of the standard was lesser at 21.34 μg/mL. The extract showed maximum of 87.90% inhibition at 1000 μg/mL concentration. The *N. nouchali* hydroethanolic seed extract was found to be only a mild inhibitor of lipid peroxidation *in vitro* in contrast to the standard quercetin which showed a maximum of 94.87% inhibition at 1000 μg/mL concentration.

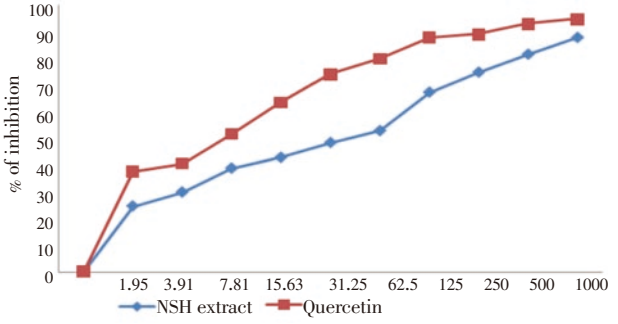


Figure 3. Lipid peroxidation inhibition activity of the hydroalcoholic seed extract of *N. nouchali* in comparison with quercetin.

NSH: *N. nouchali* hydroethanolic seed extract.

3.8. Total antioxidant capacity

The total antioxidant capacity of *N. nouchali* hydroethanolic seed extract was found to be (577.73±1.79) mg vitamin E/g of the extract.

3.9. Determination of vitamin C content

The *N. nouchali* hydroethanolic seed extract showed a high vitamin C content of (197.22±0.90) mg/g of the extract.

4. Discussion

Ash content of the plant material is the residue remaining after the moisture has been removed and the organic matter has been burnt away at 550 ℃. Total ash is determined by weighing the dry mineral residue of the plant material heated at elevated temperatures. Acid insoluble ash is an index of mineral matter especially dirt or sand (silica) and determined after digesting of total ash in 10% HCl. Both total ash and acid insoluble ash contents are useful parameters to illustrate the quality as well as the purity of herbal medicine[30]. The values of *N. nouchali* seeds were all well below the European Pharmacopoeia maximum acceptable limit of 14% w/w[31].

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[32], and this property may explain the mechanisms of antioxidative action of *N. nouchali* hydroethanolic seed extract. Flavonoids serve as health promoting compound as a results of its anion radicals[33]. Tannins are known to be useful in the treatment of inflamed or ulcerated tissues and they have remarkable activity in cancer prevention and as anticancer agents[34]. Thus, *N. nouchali* hydroethanolic seed extract containing this compound may also serve as a potential source of bioactive compounds in the treatment of cancer. Moreover, the plant extract has revealed saponins, proven to possess antioxidant and antiperoxidative properties[35]. *N. nouchali* hydroethanolic seed extract did not show the presence of anthraquinones whose derivatives tend to have laxative effects and its prolonged use and abuse leads to melanosis coli[36]. The absence of quinones in the extract is in a way beneficial, since these are oxidants and electrophiles which interact with biological systems to initiate and propagate a toxic response[37].

An antioxidant, in general, is a substance capable of preventing or slowing the oxidation of other molecules. Antioxidants interfere with the oxidative processes by scavenging free radicals, chelating free catalytic metals, acting as electron donors, preventing the formation of peroxides, breaking the autoxidative chain reaction and/or reducing localized O_2 concentrations[38]. Antioxidant activities of the plant extracts are generally studied and evaluated by using *in vitro* systems wherein ROS are generated by certain chemical reactions which liberate free radicals. Therefore, the quenching or scavenging ability or the inhibition of the generation of radicals is being taken as the antioxidant capacity of the plant extract.

The DPPH radical is widely used as the model system to investigate the scavenging activities of several natural compounds such as phenolic or crude extracts of plants. DPPH is a relatively stable free-radical at room temperature and accepts an electron or hydrogen radical to become stable diamagnetic molecule[39]. DPPH radical is scavenged by antioxidants present in the *N. nouchali* hydroethanolic seed extract through the donation of proton forming the reduced DPPH. Radical scavenging activity of *N. nouchali* hydroethanolic seed extract increased with increasing percentage of the free radical inhibition. The maximum inhibition which occurred at 1 000 μg/mL indicates the maximum free radical scavenging potential of *N. nouchali* hydroethanolic seed extract by their hydrogen donating ability.

Nitric oxide radical scavenging capacity of *N. nouchali* hydroethanolic seed extract was detected by sodium nitroprusside in aqueous solution which at physiological pH spontaneously generated nitric oxide. Scavengers of nitric oxide from the *N. nouchali* hydroethanolic seed extract competed with oxygen, leading to reduced production of nitrite ions, but showed only a mild inhibitory activity. This is similar to a previous study with aqueous and alcoholic extract of *N. alba* flowers where the maximum inhibition was 15.72% and 47.25% only at 125 μg/mL concentration[40].

Assay of TBARS measures malondialdehyde present in the sample, as well as malondialdehyde generated from lipid hydroperoxides by the hydrolytic conditions of the reaction[41]. *N. nouchali* hydroethanolic seed extract was found to inhibit lipid peroxidation to a certain extent. Though this method is slower than DPPH, the test environment is more similar to the *in vivo* conditions[42].

The total antioxidant capacity of the *N. nouchali* hydroethanolic seed extract is identified by an assay which is based on the reduction of Mo(VI)-Mo(V) by the extract and subsequent formation of a green phosphate/Mo(V) complex at acidic pH. This assay has successfully helped in the quantification of vitamin E content[28].

All the above methods have proven the *N. nouchali* hydroethanolic seed extract to possess significant antioxidant activity which is due to the presence of various bioactive principles in it. The milder effect is because of its crude nature when compared to a pure compound. Plant compounds including polyphenols, carotenoids, vitamin E and vitamin C are exogenous antioxidants which satisfy the basic two conditions of antioxidants by either delaying or preventing free

radical-mediated oxidation (or autooxidation) of an oxidizable compound such as DNA, proteins, lipids or carbohydrates), at low concentration compared to the substrate, generating a more stable radical[43]. An extensive study has been made previously in *N. nouchali* flowers for phytoconstituent quanitification and its relation to antioxidant capacity. The methanolic extracts of dry flowers have been reported to exhibit high antioxidant activities with high total phenolic content and due to the presence of phenolic compounds such as flavonoids, phenolic acids and tannins[8]. The hydroalcoholic extract of dried flowers have also found to possess antioxidant activity which is due to compounds such as gallic acid, astragalin and kaempferol which contains phenolic moieties in them[44].

Polyphenols in the *N. nouchali* hydroethanolic seed extract have scavenged the free radicals (R.) possessing an unpaired electron either by donation of hydrogens or electrons, resulting in comparatively stable phenoxyl (PhO.) radicals (neutral (PhO.) or cationic (PhO^+.) molecules, which are stabilized by delocalization of unpaired electrons around the aromatic ring. But the potential scavenging abilities of phenolics mainly depend on the number and the position of hydrogen donating hydroxyl groups on the aromatic cycles of the phenolic molecules. In addition they regenerate vitamin E which in turn, scavenges lipid peroxyl radicals[45]. Polyphenols thereby, could exert their antioxidant activity by inhibiting the catalytic activity of many enzymes eliciting ROS formation, including xanthine oxidase, lipoxygenase, cyclooxygenase and nicotinamide adenine dinucleotide phosphate-oxidase[46].

Flavanoids possess two aromatic rings and are better scavengers. An important effect of flavonoid is the scavenging of oxygen-derived free radicals. Flavonoids such as quercetin diminish oxidative damage by enhancing the concentration of glutathione. In addition, quercetin blocks Ca^{2+} influx, thus blocking Ca^{2+}-channels responsible for cell death. Multiple mechanisms of action have been also recently proposed in mediating cardiovascular effects of flavonoids. These include metal complexation, inhibition of xantine oxidase activity (which generates superoxide anions) as well as chemical quenching of ROS[47]. Selected flavonoids scavenge the highly reactive oxygen derived radicals called peroxynitrite. The *N. nouchali* hydroethanolic seed extract which has recorded the presence of flavonoids may also have a similar effect.

The tannin content of *N. nouchali* hydroethanolic seed extract also has a correlation to its antioxidant activity. Tannins do not act as pro-oxidants and in fact react very rapidly to quench the hydroxyl radical. The tannins in *N. nouchali* hydroethanolic seed extract may contain both hydrolysable and condensed tannins, since both have got a wide array of antioxidant mechanism like free radical scavenging activity, chelation of transition metals, inhibition of pro-oxidative enzymes and lipid peroxidation[48]. Hence the high content of tannin in *N. nouchali* hydroethanolic seed extract explains very well its antioxidant action. *In vivo* studies have shown that tannin-protein complexes in the gastrointestinal tract provide persistent antioxidant activity[49].

The total antioxidant activity was found to be very high in *N. nouchali* hydroethanolic seed extract and is proportionate to the amount of vitamin E content in the extract. α-Tocopherol (vitamin E) deactivates photosynthesis-derived reactive oxygen species and prevents the propagation of lipid peroxidation by scavenging lipid peroxyl radicals. α-Tocopherol can also inhibit oxidation of protein[50]. The vitamin C content also contributes to the antioxidant activity on *N. nouchali* hydroethanolic seed extract, since vitamin C is a very important, and powerful antioxidant that works in aqueous environments of the body. Ascorbic acid, behaves as a vinylogous carboxylic acid, wherein the double bond transmits electron pairs between the hydroxyl and the carbonyl. Ascorbate acts as an antioxidant by being available for energetically favourable oxidation. ROS oxidize ascorbate first to monodehydroascorbate and then dehydroascorbate. The ROS are reduced to water, while the oxidized forms of ascorbate are relatively stable and unreactive, and do not cause cellular damage[51]. This encourages the use of *N. nouchali* hydroethanolic seed extract *in vivo* without being toxic.

Thus, the *N. nouchali* hydroethanolic seed extract rich in antioxidants can be employed as a strategy in the prevention of several chronic human diseases since the antioxidants in their natural matrices are generally assumed to be safe, and their concentration is physiologic. The antioxidant potential of the *N. nouchali* hydroethanolic seed extract can therefore be mainly attributed and correlated to its high tannin content, high phenolic content and vitamin E content acting in an additive and synergistic manner. Hence several antioxidative mechanisms are proposed as being responsible for maintaining optimal health and also preventing diseases. The protection against free radical-mediated lipid peroxidation, DNA and protein oxidation, and oxidative stress-related mitochondrial dysfunction may constitute the principal way of antioxidants in the *N. nouchali* hydroethanolic seed extract to prevent the initiation and progression of several diseases including diabetes, cancer, cardiovascular complications, neurodegenerative diseases and the side effects of aging[52].

To conclude, the findings of the present study support 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.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

The first author thanks the Maulana Azad National Fellowship of University Grants Commission, Government of India, for the financial support.

Comments

Background

Etiopathogenesis of chronic disorders like diabetes, cancer arthritis *etc*. are due to oxidative stress which can be mitigated by natural antioxidants. The present study on the phytoconstituents of *N. nouchali* and its antioxidant activity is a good attempt to identify the potential role on mitigating oxidative stress.

Research frontiers

The present paper on the *in-vitro* antioxidant activity of the extract of *N. nouchali* is interesting to corroborate its traditional medicinal use. Moreover, the quantification of the phytoconstituents which may be responsible for the bioactivity is worth studying.

Related reports

The work has been designed following appropriate research protocols. The results are interesting to be studied further.

Innovations and breakthroughs

Although there exists traditional use and scientific claims on the bioactivity of *N. nouchali*, the present study adds value to the use of the plant.

Applications

Further studies on *in-vivo* assay models and the isolation and charcterisation of the bioactive molecules in the plant would help in developing novel drugs for chronic disorders.

Peer review

The present work on the phytoconstituents of *N. nouchali* and its antioxidant activity is a good attempt to identify the potential role on mitigating oxidative stress. The results are interesting and is worth for further studies on *in-vivo* assay models.

References

[1] Sreeramulu D, Reddy CVK, Chauhan A, Balakrishna N, Raghunath M. Natural antioxidant activity of commonly consumed plant foods in India: Effect of domestic processing. *Oxid Med Cell Longev* 2013; doi: 10.1155/2013/369479.

[2] Hamid AA, Aiyelaagbe OO, Usman LA, Ameen OM, Lawal A. Antioxidants: Its medicinal and pharmacological applications. *Afr J Pure Appl Chem* 2010; **4**(8): 142-151.

[3] Matough FA, Budin SB, Hamid ZA, Alwahaibi N, Mohamed J. The role of oxidative stress and antioxidants in diabetic complications. *Sultan Qaboos Univ Med J* 2012; **12**(1): 5-18.

[4] Talalay P. Chemoprotection against cancer by induction of phase 2 enzymes. *Biofactors* 2000; **12**(1-4): 5-11.

[5] Branen AL. Toxicology and biochemistry of butylated hydroxyanisole and butylated hydroxytoluene. *J Am Oil Chem Soc* 1975; **52**(2): 59-63.

[6] Mahantesh SP, Gangawane AK, Patil CS. Free radicals, antioxidants, diseases and phytomedicines in human health: Future perspects. *World Res J Med Arom Plants* 2012; **1**(1): 6-10.

[7] Raja MKMM, Agilandeswari D, Madhu BH, Mallikarjuna M, Sowjanya PJS. Aphrodisiac activity of ethanolic extract of *Nymphaea stellata* leaves in male rats. *Contemp Invest Observ Pharm* 2012; **1**(1): 24-30.

[8] Nagavani V, Rao TR. Evaluation of antioxidant potential and qualitative analysis of major polyphenols by RP-HPLC in *Nymphaea nouchali* Burm flowers. *Int J Pharm Pharm Sci* 2010; **2**(4): 98-104.

[9] Rajagopal K, Sasikala K. Antihyperglycaemic and antihyperlipidaemic effects of *Nymphaea stellata* in alloxan-induced diabetic rats. *Singapore Med J* 2008; **49**(2): 137-141.

[10] Jahan I, Mamun MAA, Hossen MA, Sakir JAMS, Shamimuzzaman M, Uddin MJ, *et al*. Antioxidant, analgesic and anti-inflammatory activities of *Nymphaea nouchali* flowers. *Res J Pharmacol* 2012; **6**(5-6): 62-70.

[11] Adewusi EA, Afolayan AJ. A review of natural products with hepatoprotective activity. *J Med Plants Res* 2010; **4**(13): 1318-1334.

[12] Chowdhury BN, Haque MM, Sohrab MH, Afroz F, Al-mansur MA, Sultana T, et al. Steroids from the stem of *Nymphaea stellata*. *J Bangladesh Acad Sci* 2013, **37**(1): 109-113.

[13] Raja MKMM, Sethiya NK, Mishra SH. A comprehensive reiview on *Nymphaea stellata*: A traditionally used bitter. *J Adv Pharm Tech Res* 2010; **1**(3): 311-319.

[14] Verma A, Ahmed B. Anti-hepatotoxic activity of *Nymphaea stellata* seeds in carbon tetrachloride induced toxicity. *Indian J Nat Prod* 2009; **5**(4): 1-4.

[15] Rani DD, Kumar SA, Shuaib M, Sudhir SG. *Nymphaea stellata*: A potential herb and its medicinal importance. *J Drug Deliv Ther* 2012; **2**(3): 41-44.

[16] Verma A, Ahmed B, Upadhyay R, Soni N. Nymphasterol, a new steroid from *Nymphaea stellata*. *Med Chem Res* 2012; **21**(6): 783-787.

[17] Ministry of Health and Family Welfare. *The Ayurvedic pharmacopoeia of India, 1999*. India: Government of India, Ministry of Health and Family Welfare, Department of Indian System of Medicine and Homeopathy; 1999.

[18] Harbone JB. *Phytochemical methods*. London: Chapman and Hall; 1973.

[19] Lowry OH, Rosenberg NJ, Farr AL, Randal RJ. Protein measurement with the Folin-Phenol reagent. *J Biol Chem* 1951; **193**(1): 265-275.

[20] Hedge JE, Hofreiter BT. *Carbohydrate chemistry*. 17 ed. New York: Academic Press; 1962, p. 17.

[21] Zlatkis A, Zak B, Boyle GJ. A new method for the direct determination of serum cholesterol. *J Lab Clin Med* 1953; **41**(3): 486-492.

[22] McDonald S, Prenzler PD, Autolovich M, Robards K. Phenolic content and antioxidant activity of olive oil extracts. *Food Chem* 2001; **73**(1): 73-84.

[23] Chang C, Yang M, Wen H, Chern J. Estimation of total flavonoid content in propolis by two complementary colorimetric methods. *J Food Drug Anal* 2002; **10**(3): 178-182.

[24] Schanderl SH. Methods in food analysis. New York: Academic Press; 1970, p. 709.

[25] Koleva II, van Beek TA, Linssen JP, de Groot A, Evstatieva LN. Screening of plant extracts for antioxidant activity: a comparative study on three testing methods. *Phytochem Anal* 2002; **13**(1): 8-17.

[26] Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite and [15N] nitrate in biological fluids. *Anal Biochem* 1982; **126**(1): 131-138.

[27] Okhawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem* 1979; **95**(2): 351-358.

[28] Prieto P, Pineda M, Agulilar M. Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E. *Anal Biochem* 1999; **269**(2): 337-341.

[29] Omaye ST, Turnbull JD, Sauberlich HE. Selected methods for the determination of ascorbic acid in animal cells, tissues and fluids. *Methods Enzymol* 1979; **62**: 3-11.

[30] Rao Y, Xiang B. Determination of total ash and acid-insoluble ash of Chinese herbal medicine *Prunellae spica* by near infrared spectroscopy. *Yakugaku Zasshi* 2009; **129**(7): 881-886.

[31] Muhammad S, Naveed M, Haroon K, Saeed AK. Analysis of toxic heavy metals in branded Pakistani herbal products. *J Chem Soc Pakistan* 2010; **32**(4): 471-475.

[32] Li H, Wang Z, Liu Y. [Review in the studies on tannins activity of cancer prevention and anticancer]. *Zhong-Yao-Cai* 2003; **26**(6): 444-448. Chinese.

[33] Hausteen B. Flavonoids, a class of natural products of high pharmacological potency. *Biochem Pharm* 1983; **32**: 1141-1148.

[34] Sun Y, Zhang T, Wang B, Li H, Li P. Tannic acid, an inhibitor of poly (ADP-ribose) glycohydrolase, sensitizes ovarian carcinoma cells to cisplatin. *Anticancer Drugs* 2012; **23**(9): 979-990.

[35] Elekofehinti OO, Adanlawo IG, Komolafe K, Ejelonu OC. Saponins from *Solanum anguivi* fruits exhibit antioxidant potential in Wistar rats. *Ann Biol Res* 2012; **3**(7): 3212-3217.

[36] Müller-Lissner SA. Adverse effects of laxatives: Fact and fiction. *Pharmacol* 1993; **47**(1): 138-145.

[37] Monks TJ, Jones DC. The metabolism and toxicity of quinones, quinonimines, quinone methides, and quinone-thioethers. *Curr Drug Metab* 2002; **3**(4): 425-438.

[38] Brewer MS. Natural antioxidants: Sources, compounds, mechanisms of action, and potential applications. *Compr Rev Food Sci Food Saf* 2011; **10**: 221-247.

[39] Halliwell B, Gutteridge JMC. *Free radicals in biology and medicine*. UK: Oxford University Press; 2007.

[40] Madhusudhanan N, Lakshmi T, Kumar SG, Ramakrishnan, Konda VGR, Roy A, et al. *In vitro* antioxidant and free radical scavenging activity of aqueous and ethanolic flower extract of *Nymphaea alba*. *Int J Drug Dev Res* 2011; **3**(3): 252-258.

[41] Trevisan M, Browne R, Ram M, Muti P, Freudenheim J, Carosella AM, et al. Correlates of markers of oxidative status in the general population. *Am J Epidemiol* 2001; **154**(4): 348-356.

[42] Kulisic T, Radonic A, Katalinic V, Milosa M. Use of different methods for testing antioxidative activity of oregano essential oil. *Food Chem* 2004; **85**: 633-640.

[43] Halliwell B. How to characterise a biological antioxidant. *Free Radic Res Commun* 1990; **9**: 1-32.

[44] Rakesh SU, Patil PR, Salunkee VR. Free radical scavenging activity of hydroalcoholic extracts of dried flowers of *Nymphaea stellata* Willd. *Int J Pharm Bio Sci* 2010; **1**: 1-9.

[45] Rice-Evans CA, Miller NJ, Paganga G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radic Biol Med* 1996; **20**: 933-956.

[46] Atmani D, Chaher N, Berboucha M, Debbache N, Boudaoud H. Flavonoids in human health: From structure to biological activity. *Curr Nutr Food Sci* 2009; **5**: 225-237.

[47] Brunetti C, Di Ferdinando M, Fini A, Pollastri S, Tattini M. Flavonoids as antioxidants and developmental regulators: Relative significance in plants and humans. *Int J Mol Sci* 2013; **14**(2): 3540-3555.

[48] Koleckar V, Kubikova K, Rehakova Z, Kuca K, Jun D, Jahodar L, et al. Condensed and hydrolysable tannins as antioxidants influencing the health. *Mini Rev Med Chem* 2008; **8**(5): 436-447.

[49] Riedl KM, Hagerman AE. Tannin protein complexes as radical scavengers and radical sinks. *J Agric Food Chem* 2001; **49**: 4917-4923.

[50] Estévez M, Heinonen M. Effect of phenolic compounds on the formation of alpha-aminoadipic and gamma-glutamic semialdehydes from myofibrillar proteins oxidized by copper, iron, and myoglobin. *J Agric Food Chem* 2010; **58**(7): 4448-4455.

[51] Flora SJS. Structural, chemical and biological aspects of antioxidants for strategies against metal and metalloid exposure. *Oxid Med Cell Longev* 2009; **2**: 63-67.

[52] Bouayed J, Bohn T. Dietary derived antioxidants: Implications on health. In: *Nutrition, well-being and health*. USA: InTech; 2012.