ISSN: 2520-7695 (Print) and XXXX-XXXX (Online)
Medical and Health Sciences Research Journal
Med. Health Sci. Res. J., Jan-Apr 2017, 1(1): 70-75
Journal Homepage: http://www.mhsrjournal.com/
http:/www.wollegajournals.com/

Original Research

In vitro Antioxidant Activity of Methanolic Extract of *Nelumbium speciosum*

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Abstract

The present study was conducted to evaluate the antioxidant activity of medicinal plant *Nelumbium speciosum.* The objectives of this study are to find the presence of secondary metabolites by preliminary phytochemical investigation and FTIR analysis in the *N. speciosum* methanolic leaf extract. This antioxidant activity study was tested for their free radical scavenging properties using ascorbic acid as standard antioxidant. Antioxidant activity of methanolic leaf extract of *N. speciosum* was determined by using different methods namely DPPH radical scavenging assay, ferric reducing assay, nitric oxide radical scavenging assay, superoxide radical scavenging assay and ABTS radical scavenging assay, and its IC_{50} values were found to be 33.80, 662.20, 265.55, 219.5 and 20.34µg/ml. The extracts exhibited marked dose dependent *in vitro* antioxidant activity.

Article Information

Article History:
Received : 02-02-2017
Revised : 03-04-2017
Accepted : 20-04-2017

Keywords:
Nelumbium speciosum
Antioxidant activity
Free radicals

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INTRODUCTION

Antioxidant, a molecule which neutralizes harmful free radical compounds that damage living cells. Antioxidants can act as enzymes in the body tissue, and vitamin supplements. They are added to metals, foodstuffs, and oils routinely to prevent the damage caused by free radical. Oxidation is one of the destructive processes, wherein it breaks down the molecules and causes various diseases. During transformation oxygen produces reactive oxygen species (ROS) such as hydroxyl radicals, superoxide, and hydrogen peroxide. Molecular oxygen is essential for all living organisms, but aerobic species are suffered by injury if they exposed to concentration more than 21%. Free radicals induce oxidative damage to the bio-molecules such as protein, lipid, lipoproteins and DNA. Antioxidants are act as inhibitors of the oxidation process and are found to inhibit the oxidant chain reactions at a very small concentration and it eliminates the pathological processes. Antioxidants are considered as a possible protection agent for reducing oxidative of human body from the ROS and retard the progress of many chronic diseases as well as lipid peroxidation (Peryor, 1991; Kinsella *et al*., 1993; Lai *et al*., 2001).

About 90 percent of medicinal plants are found growing wild in different climatic regions of the country. Traditional medical knowledge of medicinal plants and their use by indigenous culture are not only useful for conservation of cultural traditions and biodiversity but also for community health care and drug development in the present and future (Idu, *et al*., 2009). Medicinal plants are a rich source of antioxidants. Antioxidants are important for human health and nutrition. They play a major role in the genesis of various diseases such as cancer, ageing, rheumatoid arthritis, atherosclerosis and inflammation. These medicinal plants provide rich antioxidants include vitamin C, carotenoids and Phenolic compounds. Some synthetic antioxidants also available but these synthetic antioxidants are suspected to cause a liver damage. Therefore, nowadays medicinal plants are used as an antioxidant and it is evaluated by various screening methods (Kokate, 1999; Nikhat *et al*., 2009; Kekuda *et al*., 2013).

The *N. speciosum* has been used in the Orient as a medicinal herb for well over 1,500 years. In Asian culture predominantly in China, all plant parts of *N. speciosum* are consumed as food or used for medicinal purposes, including rhizomes, nodes, roots, seeds, young shoots, leaf, stalks, petals, stamens, and pericarps (or fruit receptacles, seedpods) (Wang and Zhang, 2004). All parts of the plant are used, they are astringent, cardiotonic, febrifuge, hypotensive, resolvent, stomachic, styptic, tonic and vasodilator. The leaf juice is used for the treatment of diarrhoea, and Glycyrrhiza spp. leaf decoction is used for the treatment of sunstroke. The dried leaf is used in summer heat, to invigorate the function of the spleen and to arrest bleeding by reducing heat in the blood. The leaf extract has diuretic and astringent properties, and is used to treat fever, sweating and as a styptic (Anon, 1977). The leaves are used in the treatment of haematemesis, epistaxis, haemoptysis, haematuria, metrorrhagia, hyperlipidaemia and obesity. Primarily they are used for clearing heat, removing heatstroke, cooling the blood and to stop bleeding (Luo, 2005). The stem is used in Ayurvedic medicine as a diuretic, anthelmintic and to treat strangury, vomiting, leprosy, skin diseases, nervous exhaustion and diarrhoea (Sridhar, 2007). The plant has a folk history in the

treatment of cancer, modern research has isolated certain compounds from the plant that show anticancer activity. In the present study, methanol extract of *Nelumbium speciosum* leaf was subjected for the antioxidant screening using different *invitro* methods.

MATERIALS AND METHODS

Plant Collection and Extraction

The leaves of *N. speciosum* were collected from ponds in Trichirappalli. Collected plant materials were shade dried, powdered and used for extraction. The dried powder from leaves (30 g) were extracted thrice with 2 L of 80% methanol at 80°C for 3 h. The extracts were combined and concentrated to dryness under reduced pressure. The dried *N. Speciosum* was kept in airtight bottles at −20°C until use.

Phytochemical Analysis

Phytochemical analysis for major phytoconstituents of the plant extract was undertaken using standard methods. The plant extracts were screened for the presence of biologically active compounds like Alkaloids, phenols, terpenoids etc.

Qualitative Phytochemical Analysis

Test for Alkaloids

Dragendoff's Test: In a test tube containing 1 ml of extract, a few drops of dragendoff's reagent was added and the colour developed was noticed. Appearance of orange colour indicates the presence of Alkaloids.

Test for terpenoids: In a test tube containing 1 ml of extract, a few drops of thionyl chloride were added. Appearance of pink colour indicates the presence of terpenoids.

Test for steroids

Leibermann-barchard Test

The powder was dissolved in two ml of chloroform in a dry test tube. 10 droops of acetic anhydride and two drops of concentrated sulphuric acid were added. The solution becomes red then blue finely bluish indicates the presence of steroids.

Test for Coumarins: 1 ml of extract, 1 ml of 10% sodium hydroxide was added. The presence of coumarins is indicated by the formation of Yellow colour.

Test for Tannins: To few mg of powder, 10% alcoholic Ferric chloride was added, formation of dark blue or greenish black colour shows the presence of Tannins.

Test for Flavonoids: To few mg of powder, Magnesium and 1-2 drops of concentrated hydrochloric acid were added. Formation of the red or pink colour shows the presence of flavonoids.

Test for Phenols: To 1 ml of extract add 2 ml of distilled water and few drops of 10% ferric chloride. Appearances of blue or green colour indicate the presence of phenols.

Test for Quinones: To 1 ml of Methanolic extract add 2 drops of concentrated hydrochloric acid formation of red colour indicates presence of quinines.

Test for Sugar: 1 ml of extract with Fehling's solution was added Appearance of red colour indicates the presence of sugar.

Fourier Transform Infrared Spectrophotometer (FTIR)

Fourier Transform Infrared Spectrophotometer (FTIR) is perhaps the most powerful tool for identifying the types of chemical bonds (functional groups) present in compounds. The wavelength of light absorbed is characteristic of the chemical bond as can be seen in the annotated spectrum. By interpreting the infrared absorption spectrum, the chemical bonds in a molecule can be determined. The powdered sample of plant specimen (*N. speciosum*) was loaded in FTIR spectroscope (Shimadzu, IR Affinity 1, Japan), with a Scan range from 400 to 4000 cm $^{-1}$ with a resolution of 4 cm^{-1}.

DPPH Radical Scavenging Activity

The free radical scavenging capacity of *N. speciosum* methanolic extract was determined using DPPH. DPPH solution (0.004% w/v) was prepared in 95% methanol. Extracts of *N. speciosum* was mixed with 95% methanol to prepare the stock solution (10 mg/100ml). The concentration of extract solution was 10 mg /100 ml or 100µg/ml. From stock solution 2ml, 4ml, 6ml, 8ml and 10ml of this solution were taken in five test tubes and by serial dilution with same solvent were made the final volume of each test tube up to 10 ml whose concentration was then 20µg- 100µg/ml. Freshly prepared DPPH solution (0.004% w/v) was added in each of these test tubes containing extracts (20-100µg/ml) and after 10 min, the absorbance was taken at 517 nm using a spectrophotometer. Ascorbic acid was used as a reference standard and dissolved in distilled water to make the stock solution with the same concentration (10 mg/100ml or 100 µg/ml) of extracts. Control sample was prepared containing the same volume without any extract and reference ascorbic acid. 95% methanol was used as blank. % scavenging of the DPPH free radical was measured using the following equation (Blois, 1958).

% DPPH scavenging activity = control- test/ control X 100

The antioxidant activity of the extract was expressed as IC_{50} and compared with standard. The IC_{50} value is defined as the concentration (in µg/ml) of extracts that scavenge 50% of DPPH radicals.

Reducing Power Assay

Reducing power assay (Nikhat *et al*., 2009) works under the following principle. Substances, which have reduction potential, react with potassium ferricyanide (Fe^{3+}) to form Potassium ferrocyanide (Fe^{2+}), which then reacts with ferric chloride to form ferric ferrous complex that has an absorption maximum at 700 nm. This experiment was carried out as described previously (Yildrim *et al*., 2001). Briefly, different concentrations of extracts (20-100 µg/ml) was mixed with 2.5 ml phosphate buffer (0.2M, pH 6.6) and 2.5 ml potassium ferricyanide [K_3Fe (CN_6)] (10g/l), then mixture was incubated at 50-degree C for 20 min. Two and one-half, 2.5 ml of trichloroacetic acid (100g/l) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. Finally, 2.5 ml of the supernatant solution was mixed with 2.5 ml of distilled water and 0.5 ml Fecl3 (1g/l) and absorbance measured at 700nm in UV-Visible Spectrophotometer (Systronics UV-Visible Spectrophotometer 119, India). Ascorbic acid was used as standard and phosphate buffer used as blank solution. The absorbance of the final reaction mixture of two parallel experiments was expressed as mean ± standard

deviation. Increased absorbance of the reaction mixture indicates stronger reducing power.

% increase in Reducing Power = A test/ A blank- 1 X 100

Nitric Oxide Scavenging Activity

The Nitric oxide scavenging activity of extract of *N. speciosum* were estimated by Griess reagent method employed by Marcocci *et al*. (1994). Nitric oxide (NO) was generated from sodium nitroprusside (SNP) and was measured by the SNP in aqueous solution at physiological pH spontaneously generates NO, which interacts with oxygen to produce nitrite ions that can be estimated by the use of Griess reagent. Scavengers of NO compete with oxygen leading to reduced production of NO. Nitric oxide scavenging activity was measured spectrophotometrically. SNP (5 mmol L–1) in phosphate buffered saline pH 7.4 was mixed with different concentrations of the extract (20–100 µg ml –1) prepared in methanol and incubated at 25 °C for 30 min. A control without the test compound, but with an equivalent amount of methanol, was taken. After 30 min, 1.5 ml of the incubated solution was removed and diluted with 1.5 ml of Griess reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% N-1-naphthylethylenediamine dihydrochloride). Absorbance of the chromophore formed during diazotization of the nitrite with sulphanilamide and subsequent coupling with N-1-naphthylethylene diamine dihydrochloride was measured at 546 nm and the percentage scavenging activity was measured regarding the standard.

% Nitric oxide scavenging activity
=Control- test/ control X 100

Superoxide Radical Scavenging Activity

The Superoxide Radical Scavenging Activity was measured by Nishimiki *et al*. (1972) method. Superoxide anions were generated using PMS / NADH system. The superoxide anions are subsequently made to reduce nitro blue tetrazolium (NBT) which yields a chromogenic product, which is measured at 560 nm. Test solution (20-100 µg/ml) in 0.1M phosphate buffer pH 7.4, 625 µl of 468 µM NADH solution, 625 µl of 150 µM NBT solution and 625µl of 60 µM PMS solution were added to a test tube and incubated at room temperature for 5 min. The absorbance was read at 560 nm. Linear graph of concentration Vs percentage inhibition was prepared and IC_{50} values were calculated.

% Superoxide radical scavenging activity
= control- test/ control X 100

ABTS Radical Scavenging Assay

ABTS radical cations were produced by reacting ABTS and APS. The ABTS scavenging capacity of the extract was compared with that of BHT and ascorbic acid and percentage inhibition calculated as For ABTS assay, the procedure followed with some modifications. The stock solutions included 7 mM ABTS solution and 2.4 mM potassium persulfate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 14 h at room temperature in the dark. The solution was then diluted by mixing 1 ml ABTS solution with 60 ml methanol to obtain an absorbance of 0.706 ± 0.01 units at 734 nm using a spectrophotometer. Fresh ABTS solution was prepared for each assay. Plant extracts (20-100 µg/ml) were allowed to react with 1 ml of the ABTS solution and the absorbance was taken at 734 nm after 7 min using a spectrophotometer.

% ABTS radical scavenging activity
= control- test/ control X 100

Estimation of Total Phenolic Content

Assay used for the determination of total phenolic content employs Folin and Ciocalteu's phenol reagent which response depending on the chemical structure of phenolics (i.e. the higher the number of functional –OH group). Total soluble phenolic compounds in the methanolic extracts were measured according to the method of Singleton and Rossi (1965) and expressed as gallic acid equivalents. A sample of the methanolic extract was added to distilled water for a final volume of 2 ml. After, it was mixed with 0.3 ml of a saturated sodium carbonate (Na_2CO_3) solution and 0.1ml of 1N Folin–Ciocalteu's phenol reagent. The mixture was placed for 1 h at room temperature in the dark. The absorbance was measured at 725 nm against the blank. The total phenolic content was expressed as mg of gallic acid equivalents.

Estimation of Total Flavonoid Content

The quantity of flavonoids present in the extracts of *N. speciosum* was estimated by Aluminium chloride colorimetric method (Zhishen *et al*., 1999). A dilute concentration of extract (0.5ml) was mixed with 0.5ml of methanol, 4ml of water, 0.3ml of $NaNO_2$ (5%) and incubated for 5 min at room temperature. After incubation, 0.3ml of $AlCl_3$ (10%) was added and again incubated at room temperature for 6 min. Later, 2ml of 1M NaOH and 2.4ml of distilled water were added and the absorbance was measured at 510nm using UV-Visible spectrophotometer. A calibration curve was constructed using different concentrations of Catechin (0-120µg/ml) and the flavonoid content was expressed as µg Catechin equivalents (CE) from the graph. Statistical Analysis All data were expressed as Mean ± Standard Deviation (SD) of the number of experiments (n =3). The IC_{50} value was calculated using linear regression analysis of the percent inhibition obtained using different concentrations. The regression equation was obtained and the concentration required to produce 50% effect (IC_{50}) was calculated.

Statistical Analysis

All data were expressed as Mean ± Standard Deviation (SD) of the number of experiments (n =3). The IC_{50} value was calculated using linear regression analysis of the percent inhibition obtained using different concentrations. The regression equation was obtained and the concentration required to produce 50% effect (IC_{50}) was calculated.

RESULT AND DISCUSSION

Phytochemical Analysis of the Leaf Sample of *N. speciosum*

The phytochemical substances that were present in the shade dried leaf of *N. speciosum* sample are presented in Table 1. The results of phytochemical analysis showed that the leaf of *N. speciosum* have different classes of bioactive constituents. Steroids, coumarin, tannins, flavonoids, anthraquinones, and total phenol were found to be present in methanolic extractant.

In the present investigation, the phytochemical analysis of the powdered leaves of *N. speciosum* methanolic extract showed the presence of a lot of

secondary plant metabolites which are responsible for its numerous medicinal effects. Plant tissues synthesize a wide variety of antioxidants which include ascorbic acids, proline, flavonoids, flavones, anthocyanin, catechin, etc. These compounds exhibit free radical scavenging activity and have received increasing attention for their potential role in the prevention of human disease as well as in food quality improvement (Shahidi and Wanasundara,1992; Vinson *et al.,* 2001).

Table 1: Preliminary phytochemical investigation in the *Nelumbium speciosum*

No	Phytochemical compound	Methanol
1	Anthroquinone	+++
2	Terpenoids	+
3	Alkaolids	+++
4	Phenolic Compounds	+++
5	Tannins	+
6	Flavonoids	++
7	Quinones	-
8	Steroids	+
9	Coumarins	+

Keys: + = Present in small concentrations
++ = Present in moderately high concentrations
+ + + = Present in high concentrations

Methanolic extract of *N. speciosum* leaves was analysed by FTIR spectroscopy to identify the functional groups present in the extract. The IR spectrum showed a peak at 3371cm-1 corresponding to the phenolic group (O-H stretch) and a stretch at 3985cm-1to alkanes. A peak at 1581 cm-1 suggested aromatic ring and a strong bend at 1411 indicated C-OH bending and peaks at 1257cm-1, 1045cm-1, 875cm-1 pointed out C-N bonding, C-OH and aromatic CH respectively (Figure 1).

Phenolic compounds are the hydroxylated derivatives of benzoic and cinnamic acids and contribute to overall antioxidant activities in the plants (Vinson *et al.,* 2001). Role of phenolic compounds as antioxidants have been well documented. Structural chemistry of polyphenols suggests their free radical scavenging properties (Rice-Evans *et al.,* 1997). The activity of antioxidants is determined by its reduction potential, ability to stabilize and delocalize the unpaired electrons, reactivity with other antioxidants, and transition metal chelating potential. Polyphenols have all the above characters and thus play a significant role in antioxidative defense systems in plants. Many phenylpropanoid compounds such as flavonoids, isoflavonoids, anthocyanins and polyphenols are induced in response to wounding, nutritional and cold stresses, and high visible light.

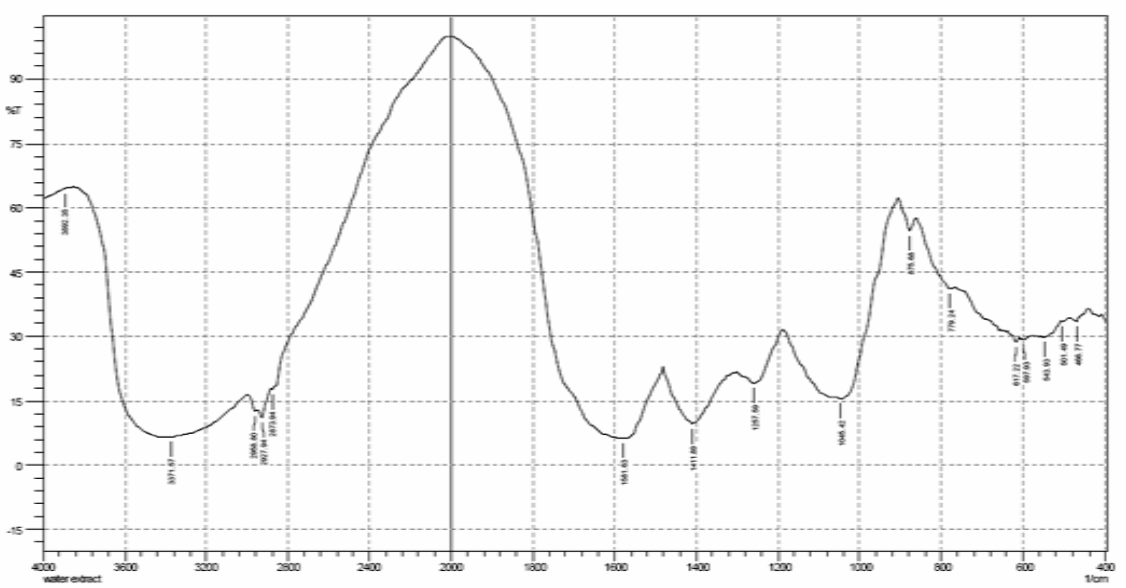

Figure 1: Fourier transform infrared spectrum of *Nelumbium speciosum*

N. speciosum was ground with KBr powder and then pressed into a polymer film for FTIR measurement in the frequency range of 400–4000cm−1 at a resolution of 4 cm−1 and spectrum results showed polyphenols with aromatic ring and C-OH bending.

Kareru *et al.* (2008), carried the spectral analysis for saponins in the crude dry powder of 11 plants and detected that *Albizia anthelmintica, Senna singueana, Maytenus senegalensis, Senna didymomotrya, Terminalia brownii*, and *Prunus africana* were likely to be bidesmosidic, oleanane-type triterpenoids, while those detected in Entada leptostachya and Rapanea rhododendroides might be monodesmosidic saponins.

Muruganantham *et al.* (2009) carried out the FTIR and EDS spectral analysis of plant parts like leaf, stem, and root of the medicinal plants, *Eclipta alba* and *Eclipta prostrate* and reported the presence of characteristic functional groups of carboxylic acids, amines, amides, sulphur derivatives, polysaccharides, nitrates, chlorates, and carbohydrate that are responsible for various medicinal properties of both herbal plants.

Five assays including DPPH, $ABTS^{+}$ radical, Nitric oxide, super oxide radical scavenging, and reducing power were employed to evaluate the anti-oxidant effects of methanolic leaf extract of *N. speciosum*. DPPH radical-scavenging assay results are shown in Table 2.

Table 2: Antioxidant activity of methanolic leaf extract of *N. speciosum*

No	Concentration (µg/ml)	DPPH radical scavenging activity	Reducing power assay	Nitric oxide scavenging activity	Superoxide radical scavenging activity	ABTS radical scavenging activity
1	20	40.02± 1.14	1.05±3.21	3.25±0.25	30.45±2.01	47.12±2.03
2	40	54.32±2.36	2.24±4.02	7.25±2.02	65.21±3.25	62.36±3.25
3	60	68.85±0.01	3.50±2.50	10.56±3.21	98.24±5.01	78.54±4.65
4	80	83.04±4.01	5.22±2.54	14.58±2.01	152.01±2.01	85.36±2.64
5	100	96.25±3.21	7.01±1.05	18.25±1.04	175.25±3.24	95.67±1.02

The DPPH assay is widely used to evaluate antioxidant activity in food components. The reduction of the stable purple free radical DPPH to the yellow hydrazine is achieved by trapping the unpaired electrons, and the degree of discoloration indicates the scavenging activity of samples. The antioxidant activity of phenolic compounds is due to their redox properties, which play an important role in absorption and neutralization of free radicals (Pietta *et al*., 1998; Shahidi and Wanasundara, 1992; Kekuda *et al*., 2013). The DPPH assay has been largely used as a quick, reliable and reproducible parameter to search the *in vitro* general antioxidant acitivity of pure compounds as well as plant extracts (Koleva *et al.,* 2002). DPPH radical is scavenged by antioxidants through the donation of proton forming the reduced DPPH. DPPH is one of the compounds that possess a proton free radical and showed a maximum absorption at 517 nm. When DPPH encounter proton radical scavengers, its purple colour fades rapidly (Khushad *et al*., 2003; Buhler and Miranda, 2000; Gooijer *et al*., 1997). The calculated IC_{50} value of *N. speciosum* was determined to be 33.80 µg/mL and the IC_{50} value of ascorbic acid as a positive control was 3.25 µg/ml.

Reducing capacity of extracts was measuring by ferrous ion (Fe^{3+}) to Ferric ion (Fe^{2+}) conversion. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity (Yen and Chen, 1995). Methanolic extract (7.01%) produced lower ferric reducing power when compared to standard which produced 85.05% inhibition at 100µg/ml concentration. The IC_{50} value of methanolic extract of *N. speciosum* is 662.20µg/ml.

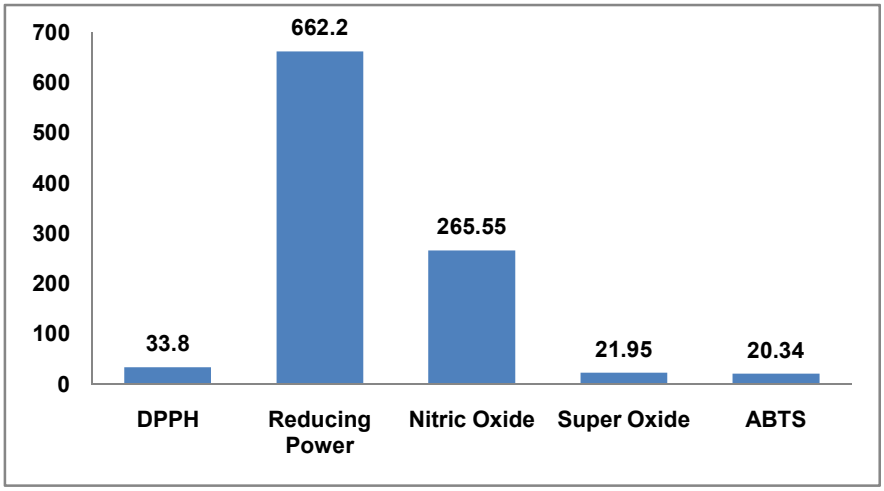

Figure 2: IC_{50} values of Antioxidant activity of methanolic leaf extract of *N. speciosum*

Scavengers of nitric oxide compete with oxygen and lead to reduced production of nitric oxide. In general, physiological pH sodium nitroprusside spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions. These nitrite ions are estimated by making use of Griess reagent. Table 2 illustrates the percentage of inhibition of nitric oxide generation by the extracts of *N. speciosum.* The IC_{50} value of methanolic extract of *N. speciosum* is 265.55µg/ml.

In Superoxide radical scavenging assay, superoxide derived from dissolved oxygen by PMS/NADH coupling reaction and it reduces NBT. Color reduction indicated the consumption of superoxide anion. Extracts of *N. speciosum* have been showed significant inhibition of superoxide anion generation. Extracts of *N. speciosum* showed best superoxide radical scavenging power. The IC_{50} value of methanolic extract of *N. speciosum* is 21.95 µg/ml.

ABTS (2, 2'-azinobis-3-ethylbenzothiozoline- 6-sulphonic acid) assay is based on the scavenging of light by ABTS radicals (Arnao *et al.,* 2001). An antioxidant with an ability to donate a hydrogen atom will quench the stable free radical, a process which is associated with a change in absorption which can be followed spectrophotometrically. The relatively stable ABTS radical has a green color and is quantified spectrophotometrically at 734nm. Table 2 illustrate the effect of plant extracts and ascorbic acid on converting ABTS+ to ABTS, which was measured at 734 nm. The IC_{50} value of methanolic extract of *N. speciosum* is 20.34µg/ml.

Total phenolic compound of the *N. speciosum* methanolic extract was 165mg/g which is equivalent to gallic acid. This is one of the compounds responsible for antioxidant activity. The flavonoid content was estimated by aluminium chloride colorimetric estimation method and the content of total flavonoids was estimated in terms of µg CE/mg of dry extract. *N. speciosum* exhibited 415µg CE/mg and this indicated that high flavonoid levels in *N. speciosum* may play an important antioxidant role.

CONCLUSIONS

This study demonstrated the antioxidant effects of methanolic extract from *N. speciosum* leaves in *in vitro* levels. *N. speciosum* exhibit marked *in vitro* antioxidant activity like ascorbic acid, and it is obvious that the constituents like phenolic compound, flavonoids, and alkaloids present in the extract may be responsible for such activity. Therefore, *N. speciosum* leaves extract could be used in food systems and/or as an antioxidant ingredient for functional food applications. Further studies are warranted for the isolation and characterization of antioxidant compounds, and *in vivo* studies are needed for understanding their mechanism of action as antioxidants.

Conflict of Interest

None declared.

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