

Comparative assessment of the antioxidant activity and free radical scavenging potential of different parts of *Nerium indicum*

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Abstract

Reactive oxygen species (ROS) cause damage to cellular components. Antioxidant compounds scavenge or neutralize the ROS and thus have significant role in human health. The present study 70% methanol extracts of *Nerium indicum* leaf, stem and root were evaluated for in vitro total antioxidant, radical scavenging activity along with phenolic and flavonoid contents. The extracts were examined for the scavenging activity of hydroxyl radical, nitric oxide, singlet oxygen, hypochlorous acid, superoxide, peroxyxynitrite, hydrogen peroxide. The extracts were also tested for their potential as an iron chelating agent, inhibition of lipid peroxidation and total reducing potential. The present study indicates that the total antioxidant, DPPH (2,2-diphenyl-1-picrylhydrazyl) radical and singlet oxygen scavenging potential is in the order of stem>root>leaf. The hydroxyl radical scavenging, hydrogen peroxide scavenging and hypochlorous acid scavenging activity is in the order leaf>stem>root, whereas superoxide scavenging and lipid peroxidation inhibition assay is root>leaf>stem. Miscellaneous results were obtained in the scavenging of other radicals by the extracts, viz., leaf>root>stem for peroxyxynitrite and iron chelation activity, root>stem>leaf for reducing power and stem>leaf>root for nitric oxide inhibition. The phenolic and flavonoid content is in the following order root>stem>leaf and leaf>stem>root respectively. The present study revealed that the leaf, stem and root extracts of *N. indicum* are effective free radical scavenger and might be used as a natural source of potent antioxidant.

Key-words: *Nerium indicum*, antioxidant, flavonoids, phenolics, free radicals.

Introduction

It is an enigma in metabolism that oxygen, which is required by the majority of complex life-forms for their endurance, is highly reactive and damages biological system by generating reactive oxygen species (ROS) [1]. Oxidative stress, which is an imbalance between production of oxidant and antioxidant defences in favour of the former, has been implicated in the etiology of several major human ailments including neural disorders, cardiovascular diseases, cancer, diabetes and arthritis [2]. In addition to diets rich in saturated fatty acids and carbohydrates, the adverse environmental conditions such as smog and UV radiation, increase oxidative damage in the body. Natural antioxidants are phyto-micronutrients that have gained importance in recent years due to their ability to delay the oxidation process, inhibiting the polymerization chain initiated by free radicals and other subsequent oxidizing reactions [3]. In recent years, research on medicinal plants has drawn enormous global attention. The natural antioxidants contained in foods, fruits, beverages, spices and supplements have received much attention from nutraceutical and cosmetic interest.

Nerium indicum Mill (syn. *N. oleander* L. and *N. odorum* Aiton) belongs to Apocynaceae family which is currently the only species classified under the genus *Nerium*. Locally known as "Sheth Karabi" (Bengali) and "Kaner" (Hindi), *N. indicum* is an erect, smooth, evergreen shrub used as traditional medicine in different parts of the world especially in India [4]. Polysaccharides from *N. indicum* have shown anti-tumor, immune-stimulating, and neuroprotective effects [5]. The flowers and leaves of *N. indicum* were used as traditional Chinese medicine to relieve pain and as cardiac muscle stimulant [6]. Antimicrobial activities of leaves and roots [7] and anticancer properties of the whole plant have been reported previously [8]. However, the complete antioxidant and free radical scavenging properties of *N. indicum* has not been reported yet.

The objective of the present study was to evaluate the antioxidant potential and free radical scavenging activity of 70% methanol extracts of *N. indicum* leaf, stem and root. The extracts were examined for different ROS scavenging activities including hydroxyl, superoxide, nitric oxide, hydrogen peroxide, peroxyxynitrite, singlet oxygen and hypochlorous acid, and for phenol and flavonoid contents, lipid peroxidation inhibition, iron chelating capacity, DPPH and total antioxidant activity.



Materials and Methods

Chemicals

2,2-azobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was obtained from Roche Diagnostics, Mannheim, Germany. 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was obtained from Fluka, Buchs, Switzerland. Potassium persulfate ($K_2S_2O_8$), ethylenediamine tetraacetic acid (EDTA), ascorbic acid, 2-deoxy-2-ribose, trichloroacetic acid (TCA), mannitol, nitro blue tetrazolium (NBT), reduced nicotinamide adenine dinucleotide (NADH), phenazine methosulfate (PMS), sodium nitroprusside (SNP), sulfanilamide, naphthylethylenediamine dihydrochloride (NED), L-histidine, lipoic acid, sodium pyruvate, quercetin and ferrozine were obtained from Sisco Research Laboratories Pvt. Ltd., Mumbai, India. Hydrogen peroxide, potassium hexacyanoferrate, Folin-Ciocalteu (FC) reagent, sodium carbonate (Na_2CO_3), butylated hydroxytoluene (BHT), sodium hypochloride (NaOCl), aluminium chloride ($AlCl_3$), ammonium iron (II) sulfate hexahydrate ($(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$), potassium nitrite (KNO_2), N,N-dimethyl-4-nitrosoaniline and xylenol orange were obtained from Merck, Mumbai, India. 1,1-Diphenyl-2-Picrylhydrazyl (DPPH), Gallic acid and curcumin were obtained MP Biomedicals, France. Ferrous sulfate and catalase were obtained from HiMedia Laboratories Pvt. Ltd., Mumbai, India. Evans Blue was purchased from BDH, England. Manganese dioxide was obtained from SD Fine Chemicals, Mumbai, India. Diethylene-triamine-pentaacetic acid (DTPA) was obtained from Spectrochem Pvt. Ltd., Mumbai, India. Thiobarbituric acid (TBA) was obtained from Loba Chemie, Mumbai, India. Sodium nitrite was obtained from Qualigens Fine Chemicals, Mumbai, India.

Plant material

White flowered variety of *N. indicum* was collected from the campus area of University of North Bengal, India, during the month of August. The plant was identified by taxonomist Prof. A. P. Das of Department of Botany, University of North Bengal and the herbarium sheet of the sample plant was stored at the Botany Department Herbarium, University of North Bengal with accession number of 9618.

Sample preparation

The whole plant was separated into three major parts: leaf, stem and root. The parts were washed properly with double distilled water. The parts were then shade dried at room temperature for 2 weeks and grinded to powder. The powder (100 g) was mixed with 70% methanol (1000 ml) and kept in a shaking incubator overnight (12h, 37°C, 160 rpm). Then the mixture was centrifuged at 5000 rpm for 15 minutes. The pellet was mixed with 70% Methanol (1000 ml) and kept overnight at the shaking incubator and centrifuged. The supernatant liquid was collected from both the phases and filtered. The resultant filtrate was concentrated in a rotary

evaporator under reduced pressure. The concentrated extract was lyophilized and stored at -20°C until further use.

Total antioxidant activity assay

Antioxidant activity of the extract was assayed depending on the ability of the sample to scavenge $ABTS^{+}$ radical cation compared to trolox standard [9]. The $ABTS^{+}$ radical cation was pregenerated by mixing 7 mM ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and incubated for 12–16 h in dark at room temperature until the reaction was completed and absorbance was stable. $ABTS^{+}$ was diluted with water at room temperature to equilibrated its absorbance to 0.70 (± 0.02). 10 μ l (0.05–10mg/ml) sample solution was mixed with 1 ml $ABTS^{+}$ solution and the absorbance was measured at 734 nm after 6 min. All experiments were repeated six times. The percentage inhibition of absorbance was calculated and plotted as a function of the concentration of standard and sample to determine the TEAC. TEAC was calculated by dividing the gradient of the plot for the sample by the gradient of the plot for trolox.

DPPH radical scavenging assay

The complementary study for the antioxidant capacity of the plant extracts were confirmed by the DPPH scavenging assay according to a standard method [10] with slight modification. Different concentrations (0-100 μ g/ml) of the extracts and the standard ascorbic acid were mixed with equal volume of ethanol. Then 50 μ l of DPPH solution (1mM) was pipetted into the previous mixture and stirred thoroughly. The resulting solution was kept standing for 2 minutes before the optical density (OD) was measured at = 517 nm. The measurement was repeated with six sets. The percentage radical scavenging activity was calculated from the following formula:

$$\% \text{ of scavenging [DPPH]} = [(A_0 - A_1) / A_0] * 100$$

Where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of the samples and standard.

Hydroxyl Radical Scavenging Assay

The assay was performed according to a standard method [11]. Quantification of the degradation product of 2-deoxyribose by condensation with TBA is the basic principle behind the assay. Hydroxyl radical was generated by the Fe^{3+} -ascorbate-EDTA- H_2O_2 system which is known as the Fenton reaction. The final volume of the reaction mixture contained 1 ml, 2-deoxy-2-ribose (2.8 mM); KH_2PO_4 -KOH buffer (20 mM, pH 7.4); $FeCl_3$ (100 μ M); EDTA (100 μ M); H_2O_2 (1.0 mM); ascorbic acid (100 μ M) and various concentrations (0–200 μ g/ml) of the test sample i.e. the plant extract. The reaction mixture was kept in incubation for 1 h at 37°C and after incubation 0.5 ml of the reaction mixture was mixed with 1 ml 2.8% TCA and 1 ml 1% aqueous TBA was added to it. The solution was incubated at 90°C for 15 min to develop the colour. After incubation, the solution was cooled down to room temperature



and the absorbance was measured at 532 nm against an appropriate blank solution. All tests were performed six times. Mannitol which is a classical OH scavenger was used as a positive control. Percentage of inhibition was evaluated by the following equation:

$$\% \text{ of inhibition} = [(A_0 - A_1) / A_0] * 100$$

Where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of the samples and standard.

Superoxide radical scavenging assay

This experiment was performed based on the reduction of NBT according to a previously reported method [11]. Superoxide radicals are generated by the non-enzymatic PMS/NADH system. The superoxide radicals then reduce NBT into a purple-coloured formazan. The 1 ml reaction mixture contained phosphate buffer (20 mM, pH 7.4), NADH (73 μ M), NBT (50 μ M), PMS (15 μ M) and various concentrations (0–50 μ g/ml) of sample solution. The reaction mixture was incubated at room temperature for 5 min. After incubation, the absorbance was taken at 562 nm against an appropriate blank solution. All tests were performed six times. Quercetin was used as positive control. The percentage inhibition of superoxide anion generation was calculated using the following formula:

$$\% \text{ of inhibition} = [(A_0 - A_1) / A_0] * 100$$

Where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of the samples and standard.

Nitric oxide radical scavenging assay

At physiological pH, nitric oxide generated from aqueous SNP solution interacts with oxygen to produce nitrite ions, which may be quantified according to the Griess Illosvoy reaction [12]. The reaction mixture contained 10 mM SNP, phosphate buffered saline (pH 7.4) and various doses (0–70 μ g/ml) of the test solution in a final volume of 3 ml. After incubation for 150 min at 25°C, 1 ml sulfanilamide (0.33% in 20% glacial acetic acid) was added to 0.5 ml of the incubated solution and allowed to stand for 5 min. Then 1 ml of NED (0.1% w/v) was added and the mixture was incubated for 30 min at 25°C. The pink chromophore generated during diazotization of nitrite ions with sulphanilamide and subsequent coupling with NED was measured spectrophotometrically at 540 nm against a blank sample. Curcumin was used as a standard. All tests were performed six times. The percentage inhibition of nitric oxide radical generation was calculated using the following formula:

$$\% \text{ of inhibition} = [(A_0 - A_1) / A_0] * 100$$

Where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of the samples and standard.

Hydrogen peroxide scavenging assay

The assay was performed according to a previously described standard method [13] with slight modifications. An aliquot of 50 mM H_2O_2 and various concentrations (0–2.0 mg/ml) of samples were mixed (1: 1 v/v) and incubated for 30 min at room temperature. After incubation, 90 μ l of the H_2O_2 -sample solution was mixed with 10 μ l HPLC-grade methanol and 0.9-ml FOX reagent was added (previously prepared by mixing 9 volumes of 4.4-mM BHT in HPLC-grade methanol with 1 volume of 1-mM xylenol orange and 2.56-mM ammonium ferrous sulfate in 0.25 M H_2SO_4). The reaction mixture was then vortexed and incubated at room temperature for 30 min. The absorbance of ferric-xylenol orange complex was measured at 560 nm. All tests were carried out six times and sodium pyruvate was used as the reference compound. The percentage of scavenging of hydrogen peroxide of fruit extracts and standard compound:

$$\% \text{ of scavenged } H_2O_2 = [(A_0 - A_1) / A_0] * 100$$

Where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of the samples and standard.

Peroxynitrite scavenging activity

A previously described standard method [14] was followed to synthesize Peroxynitrite ($ONOO^-$). 5 ml 0.6 M KNO_2 was mixed with an acidic solution (0.6 M HCl) of 5 ml H_2O_2 (0.7 M) on ice bath for 1 min and 5 ml of ice-cold 1.2 M NaOH was added to the solution. The solution was subjected to treatment with granular MnO_2 prewashed with 1.2 M NaOH to remove the excess H_2O_2 . The reaction mixture was left overnight at -20°C. Peroxynitrite solution was collected from the top of the frozen mixture and the concentration was measured spectrophotometrically at 302 nm ($= 1670 M^{-1} cm^{-1}$).

To measure peroxynitrite scavenging activity an Evans Blue bleaching assay was used. The assay was performed by a standard method with a slight modification [15]. The reaction mixture contained 50 mM phosphate buffer (pH 7.4), 0.1 mM DTPA, 90 mM NaCl, 5 mM KCl, 12.5 μ M Evans Blue, various doses of plant extract (0–200 μ g/ml) and 1 mM peroxynitrite in a final volume of 1 ml. The absorbance was measured at 611 nm after incubation at 25°C for 30 min. The percentage of scavenging of $ONOO^-$ was calculated by comparing the results of the test and blank samples. All tests were performed six times. Gallic acid was used as the reference compound. The percentage of scavenging of peroxynitrite anion was calculated using the following equation:

$$\% \text{ of scavenged peroxynitrite} = [(A_0 - A_1) / A_0] * 100$$

Where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of the samples and standard.

Singlet oxygen scavenging assay



The assay was performed according to previously reported spectrophotometric method with minor modifications [16]. The production of singlet oxygen (1O_2) was determined by monitoring the bleaching of N, N-dimethyl-4-nitrosoaniline (RNO). Singlet oxygen was generated by a reaction between NaOCl and H_2O_2 and the bleaching of RNO was read at 440 nm. The reaction mixture contained 45 mM phosphate buffer (pH 7.1), 50 mM NaOCl, 50 mM H_2O_2 , 50 mM L-histidine, 10 μ M RNO and various concentrations (0–100 μ g/mL) of sample in a final volume 2 ml. The solution mixture was incubated at 30°C for 40 min and decrease in the absorbance of RNO was measured at 440 nm. The scavenging activity of sample was compared with that of lipoic acid, used as a reference compound. All tests were performed six times. Singlet oxygen scavenging activity was calculated by the following formula:

$$\% \text{ of scavenged singlet oxygen} = [(A_0 - A_1) / A_0] * 100$$

Where A_0 was the absorbance of the control, and A_1 was the absorbance in the presence of the sample of fruit extracts and standard.

Hypochlorous acid scavenging assay

Hypochlorous acid (HOCl) was freshly prepared just before the experiment, by adjusting the pH of a 10% (v/v) solution of NaOCl to 6.2 with 0.6 M H_2SO_4 , and the concentration of HOCl was determined by measuring the absorbance at 235 nm using the molar extinction coefficient of 100 $M^{-1} cm^{-1}$. The assay was carried out according to a previously described standard method [11]. The scavenging activity was evaluated by measuring the decrease in absorbance of catalase at 404 nm. The reaction mixture contained, in a final volume of 1 ml, 50 mM phosphate buffer (pH 6.8), catalase (7.2 μ M), HOCl (8.4 mM) and increasing concentrations (0–200 μ g/ml) of plant extract. The assay mixture was incubated at 25°C for 20 min and the absorbance was measured against an appropriate blank. All tests were performed six times. Ascorbic acid was used as the reference compound. The percentage of scavenging of HOCl was calculated using the following equation:

$$\% \text{ of scavenged HOCl} = [(A_0 - A_1) / A_0] * 100$$

Where A_0 was the absorbance of the control, and A_1 was the absorbance in the presence of plant extracts and standard.

Fe²⁺ chelation

The ferrous ion chelating activity was evaluated by a standard spectrophotometric method [17] with minor changes. The reaction was carried out in HEPES buffer (20 mM, pH 7.2). Various concentrations of plant extracts (0–300 μ g/ml) were mixed with 12.5 μ M ferrous sulphate solution. The reaction was initiated by the addition of ferrozine (75 μ M). The mixture was shaken vigorously and incubated for 20 min at room temperature, and the absorbance was measured at 562 nm. All tests were performed for six times. EDTA was used as a positive control.

Measurement of Reducing Power

The method described by Oyaizu was followed with slight modification to determine the Fe^{3+} reducing power of the plant extract [18]. Different concentrations (0–1.0 mg/ml) of extract (0.5 ml) were mixed with 0.5 ml phosphate buffer (pH 6.6) and 0.5 ml 0.1% potassium hexacyanoferrate. The solution was incubated at 50°C in a water bath for 20 min. 0.5 ml of TCA (10%) was added after incubation to terminate the reaction. The upper portion of the solution (1 ml) was mixed with 1 ml distilled water, and 0.1 ml $FeCl_3$ solution (0.01%) was added. The reaction mixture was left for 10 min at room temperature and the absorbance was measured at 700 nm against an appropriate blank solution. All tests were performed six times. A higher absorbance of the reaction mixture indicated greater reducing power. BHT was used as a positive control.

Lipid Peroxidation Inhibition Assay

This assay was carried out according to a previously described method [19], with slight modification. Brain homogenate was prepared by centrifuging Swiss albino mice brain (20 ± 2 g) with 50 mM phosphate buffer (pH 7.4) and 120 mM KCl, at 3000 rpm for 10 min. A 100 μ l aliquot of the supernatant homogenate was mixed with the plant extract of various concentrations (0–25 μ g/ml), followed by addition of 0.1 mM $FeSO_4$ and 0.1 mM ascorbic acid, and incubated for 1 h at 37°C. 500 μ l 28% TCA was used to stop the reaction and then 380 μ l 2% TBA was added followed by heating at 95°C for 30 min, to generate the colour. Then the samples were cooled on ice, centrifuged at 8000 rpm for 2 min and the absorbance of the supernatant was taken at 532 nm. All tests were performed six times. Trolox was used as the standard.

Quantification of total phenolic content

Slightly modified method of Singleton and Rossi [20] was followed to determine the total phenolic content using FC reagent. Briefly, 0.1ml of extract was mixed with 0.75 ml of FC reagent (previously diluted 1000-fold with distilled water). The reaction mixture was incubated for 5 min at 22°C; then 0.06% Na_2CO_3 solution was added to the mixture. After incubation at 22°C for 90 min, the absorbance was measured at 725 nm. The phenolic content was evaluated from a gallic acid standard curve.

Quantification of total flavonoid content

Total flavonoid content was quantified according to a standard method using quercetin as a standard [21]. The plant extract of 0.1 ml was added to 0.3 ml distilled water followed by 0.03 ml 5% $NaNO_2$. After 5 min at 25°C, 0.03 ml 10% $AlCl_3$ was added. After another 5 min, the reaction mixture was treated with 0.2 ml 1 mM NaOH. Finally the reaction mixture was diluted to volume (1 ml) with water. Then the absorbance was measured at 510 nm. The flavonoid content was calculated from a quercetin standard curve.

Statistical analysis



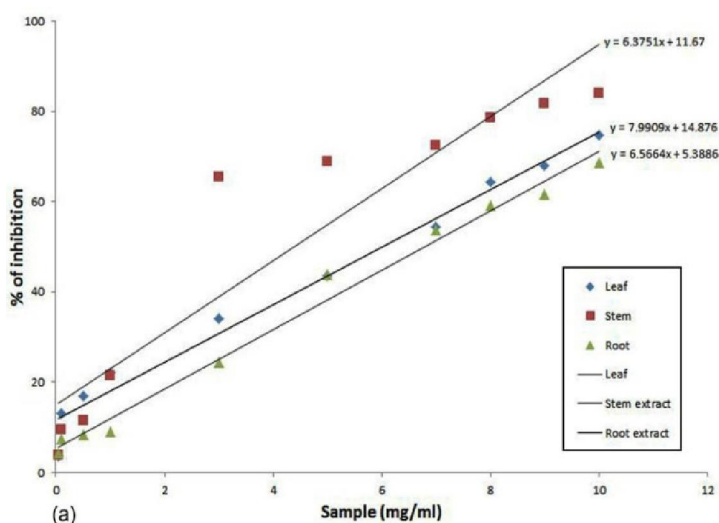
All data are reported as the mean \pm SD of six measurements. Statistical analysis was performed using KyPlot version 2.0 beta 15 (32 bit). The IC_{50} values were calculated by the formula

$$Y = 100 \cdot A1 / (X + A1),$$

where $A1 = IC_{50}$, $Y =$ response ($Y = 100\%$ when $X = 0$), $X =$ inhibitory concentration. The IC_{50} values were compared by paired t tests. $p < 0.05$ was considered significant.

Results

Total antioxidant activity



The overall antioxidant activity of *N. indicum* was measured by using TEAC assay which is based on interaction between antioxidant and $ABTS^{\cdot+}$ radical cation which has a characteristic color showing maxima at 734 nm. Interaction with the extract or standard trolox suppressed the absorbance of the $ABTS^{\cdot+}$ radical cation and the results, expressed as percentage inhibition of absorbance, are shown in figure 1(a) and figure 1(b), respectively. The TEAC value of the *N. indicum* leaf, stem and root extract were 0.316 ± 0.002 , $0.396 \pm .001$ and 0.325 ± 0.003 respectively.

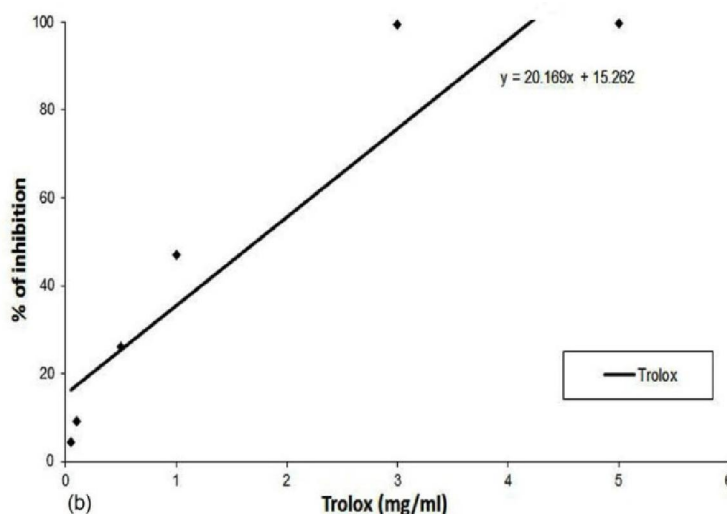


Figure 1 - Total antioxidant activity

Total antioxidant activity of (a) *N. indicum* leaf, stem and root extracts and (b) standard trolox on decolourization of ABTS radical cation. All data are expressed as mean \pm S.D. ($n = 6$).

DPPH

The leaf, stem and root extracts of *N. indicum* showed excellent dose-dependent scavenging activity of DPPH radical. The IC_{50} values (Table 1) of the leaf, stem and root extracts and standard

ascorbic acid were $217.15 \pm 18.39 \mu\text{g/ml}$, $63.56 \pm 1.63 \mu\text{g/ml}$, $166.18 \pm 6.84 \mu\text{g/ml}$ and $5.29 \pm 0.28 \mu\text{g/ml}$ respectively. At $100 \mu\text{g/ml}$, the percentage of inhibition of the leaf, stem and root extracts were 33.14%, 64.16% and 38.03% whereas at $45 \mu\text{g/ml}$ the standard ascorbic acid shows 27.93% inhibition (Figure 2).

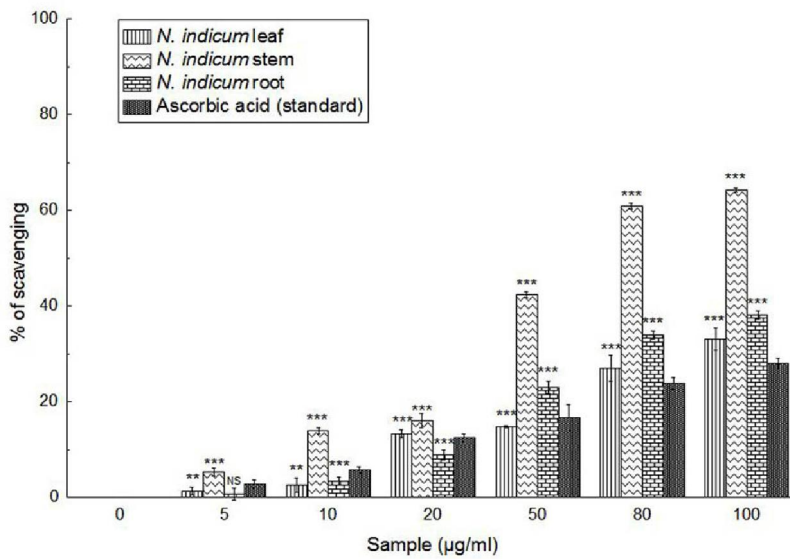


Figure 2 - DPPH radical scavenging activity
 DPPH radical scavenging activity of leaf, stem and root of *N. indicum* and standard ascorbic acid. The results are mean ± S. D. (n=6). NS = non-significant, **p < 0.01 and ***p < 0.001 vs 0 µg/ml.

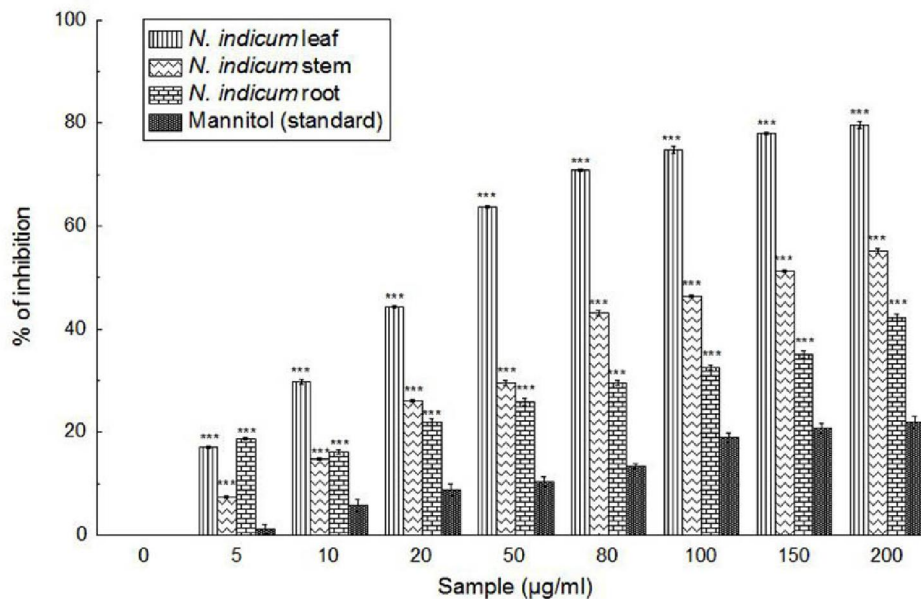


Figure 3 - Hydroxyl radical scavenging assay
 Hydroxyl radical scavenging activities of the *N. indicum* leaf, stem and root extract and the reference compound mannitol. The results are mean ± S. D. of six parallel measurements. ***p < 0.001 vs 0 µg/ml.



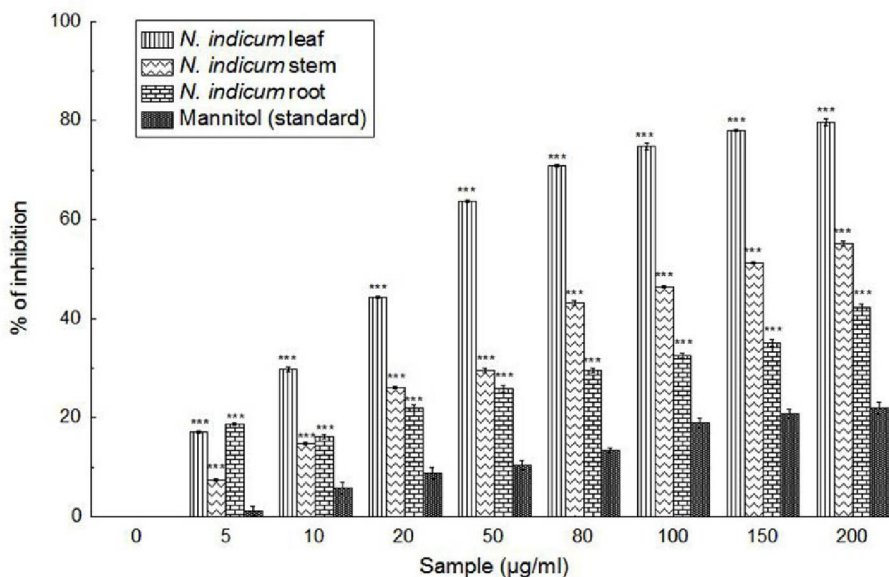


Figure 3 - Hydroxyl radical scavenging assay
Hydroxyl radical scavenging activities of the *N. indicum* leaf, stem and root extract and the reference compound mannitol. The results are mean \pm S. D. of six parallel measurements. *** $p < 0.001$ vs 0 $\mu\text{g/ml}$.

Hydroxyl radical scavenging assay

The abilities of the three extracts and standard mannitol to inhibit hydroxyl radical-mediated deoxyribose degradation in an Fe^{3+} -EDTA-ascorbic acid and H_2O_2 reaction mixture was exhibited by this assay. The results are displayed in figure 3. The IC_{50} values (Table 1) of the *N. indicum* leaf, stem and root extracts and standard in this assay were $29.65 \pm 0.21 \mu\text{g/ml}$, $118.68 \pm 1.11 \mu\text{g/ml}$, $208.16 \pm 2.70 \mu\text{g/ml}$ and $571.45 \pm 20.12 \mu\text{g/ml}$ respectively. The IC_{50} value of the extract was less than that of the standard. At 200 $\mu\text{g/ml}$, the percentage inhibition values for leaf stem and root extracts were 79.53%, 55.26% and 42.31% respectively.

Superoxide radical scavenging

Superoxide radicals, generated from the PMS-NADH coupling, can be measured by their ability to reduce NBT. The decrease in absorbance at 560 nm with the plant extract and the reference compound quercetin indicate their abilities to quench superoxide radicals in the reaction mixture. As shown in Figure 4, the IC_{50} values (Table 1) of the leaf, stem and root extracts and quercetin on superoxide scavenging activity were $224.35 \pm 3.45 \mu\text{g/ml}$, $268.33 \pm 6.04 \mu\text{g/ml}$, $170.69 \pm 2.41 \mu\text{g/ml}$ and $42.06 \pm 1.35 \mu\text{g/ml}$ respectively. The IC_{50} value of all the extract was less than that of the standard. At 120 $\mu\text{g/ml}$, the percentage inhibition of *N. indicum* leaf, stem, root extract and standard quercetin was plant extract was 31.55%, 30.20%, 33.86% and 50.66% respectively.

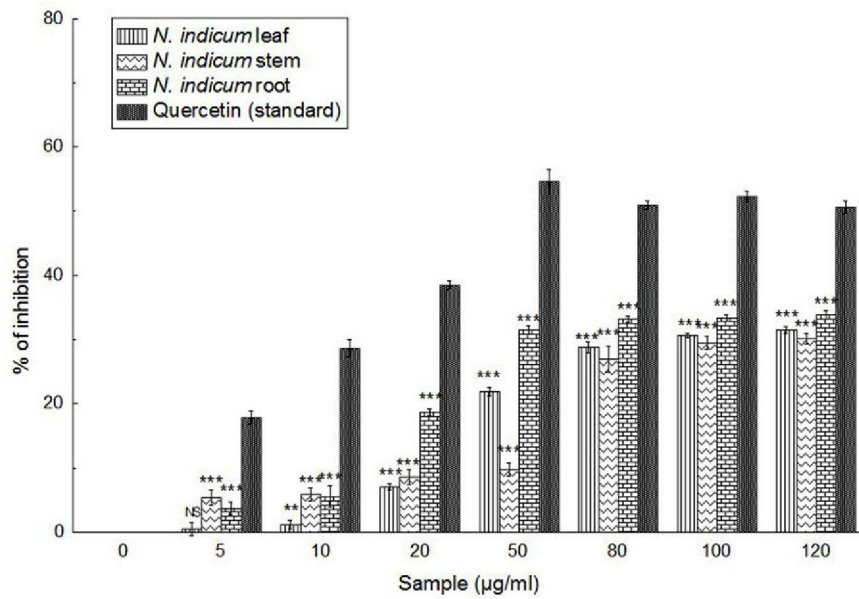


Figure 4 - Superoxide radical scavenging assay. Scavenging effect of *N. indicum* leaf, stem and root extracts and the standard quercetin on superoxide radical. All data are expressed as mean ± S.D. (n = 6). NS = non-significant, **p < 0.01 and ***p < 0.001 vs 0 µg/ml.

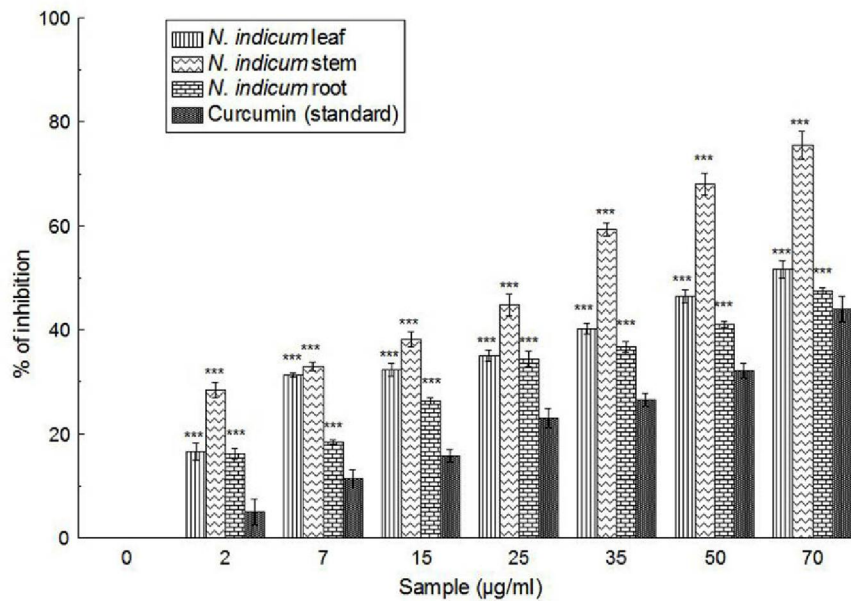


Figure 5 - The nitric oxide radical scavenging activity of *N. indicum* leaf, stem and root extracts and the standard curcumin. The data represent the percentage nitric oxide inhibition. Each value represents mean ± S.D. (n = 6). ***p < 0.001 vs 0 µg/ml.



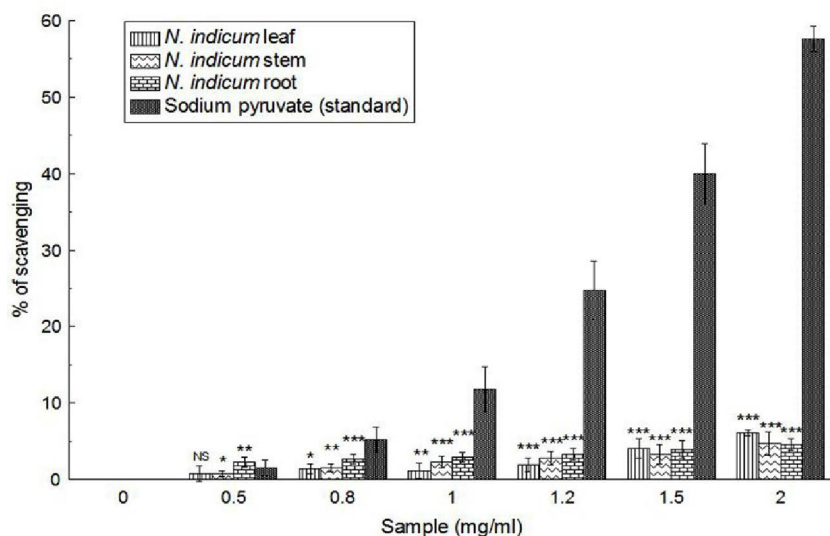


Figure 6 - H₂O₂ scavenging assay

H₂O₂ scavenging activity of *N. indicum* leaf, stem and root extracts and sodium pyruvate. All data are expressed as mean \pm S.D. (n = 6). NS= non-significant, *p < 0.05, **p < 0.01 and ***p < 0.001 vs 0 mg/ml.

Hydrogen peroxide scavenging

FOX reagent method was followed to assay the hydrogen peroxide scavenging activity of the plant extracts. Figure 6 shows that the plant extract is a very poor scavenger of H₂O₂ compared to standard sodium pyruvate. The IC₅₀ values (Table 1) of the leaf, stem and root extracts and the standard were 40.42 \pm 4.40 mg/ml, 39.87 \pm 5.67 mg/ml, 37.05 \pm 2.99 mg/ml and 3.24 \pm 0.30 mg/ml respectively (Table 1). At a concentration of 2 mg/ml, the scavenging percentages of *N. indicum* leaf, stem, root extracts and standard were 6.027%, 5.08%, 4.51% and 57.7% respectively.

Peroxynitrite scavenging

Figure 7 shows the peroxynitrite scavenging activity of all the extracts in a concentration dependent manner. The calculated IC₅₀ values of *N. indicum* leaf, stem and root extracts were 1672.80 \pm 56.68 μ g/ml, 2172.26 \pm 133.97 μ g/ml and 1869.97 \pm 122.30 μ g/ml respectively which was higher than that of the reference compound gallic acid (IC₅₀ = 876.24 \pm 56.96 μ g/ml) (Table 1). At 200 μ g/ml, the scavenging percentages of the *N. indicum* leaf, stem and root were 10.26%, 8.61% and 8.88% whereas for the standard gallic acid the scavenging percentage is 15.44%.

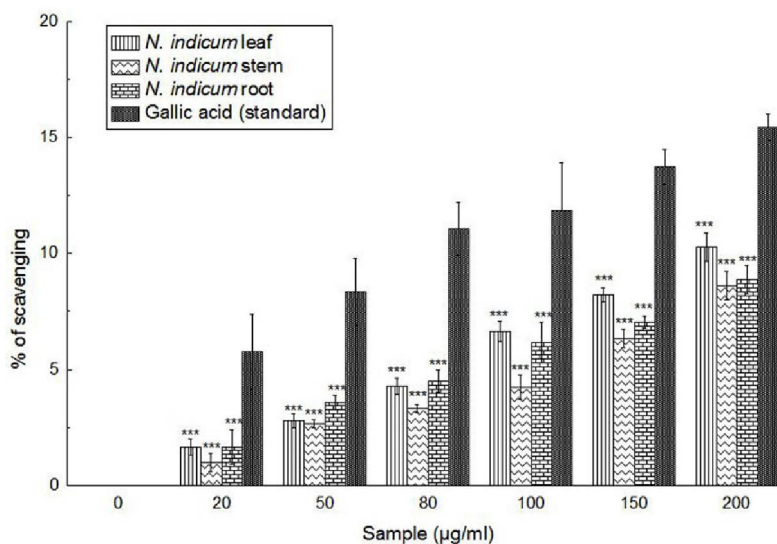


Figure 7 - Peroxynitrite anion scavenging assay

The peroxynitrite anion scavenging activity of *N. indicum* leaf, stem and root extracts and the standard gallic acid. Each value represents mean \pm S.D. (n = 6). ***p < 0.001 vs 0 μ g/ml.

Singlet oxygen scavenging

Leaf, stem and root extracts of *N. indicum* showed a moderate dose-dependent scavenging effect of singlet oxygen species with IC₅₀ values (Table 1) of

365.76 ± 5.52 µg/ml, 391.55 ± 7.53 µg/ml and 275.08 ± 7.5 µg/ml respectively (Figure 8). Lipoic acid was used as a reference compound and 46.15 ± 1.16 µg/ml lipoic acid was needed for 50% inhibition. At 200 µg/ml, the percentage scavenging of the leaf, stem and root extracts were 37.74%, 38.81% and 38.22% whereas that of lipoic acid was 75.38%.

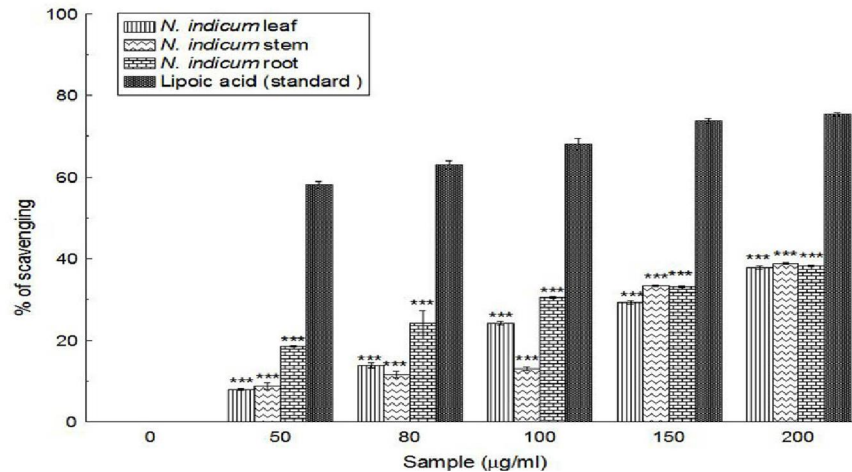


Figure 8 - Singlet oxygen scavenging assay.

Effects of *N. indicum* leaf, stem and root extracts and the standard lipoic acid on the scavenging of singlet oxygen. The results are mean ± S.D. (n=6). ***p < 0.001 vs µg/ml.

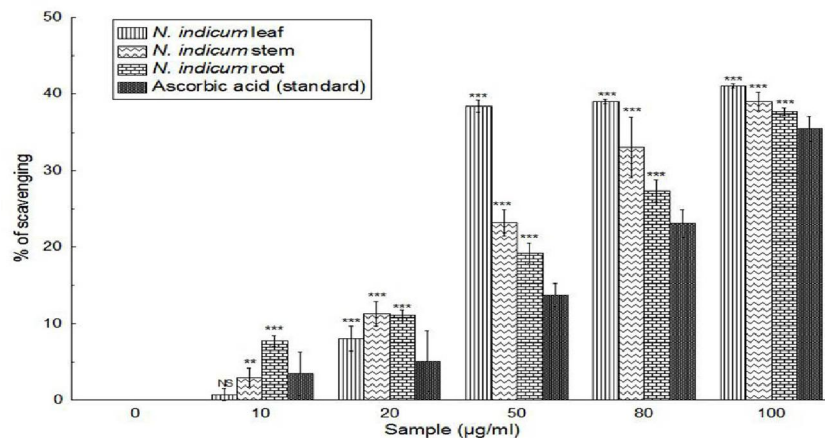


Figure 9 - Hypochlorous acid scavenging activities of leaf, stem and root extracts of *N. indicum* and the standard ascorbic acid.

All data are expressed as mean ± S.D. (n = 6). NS= non-significant, **p < 0.01 and ***p < 0.001 vs 0 µg/ml.

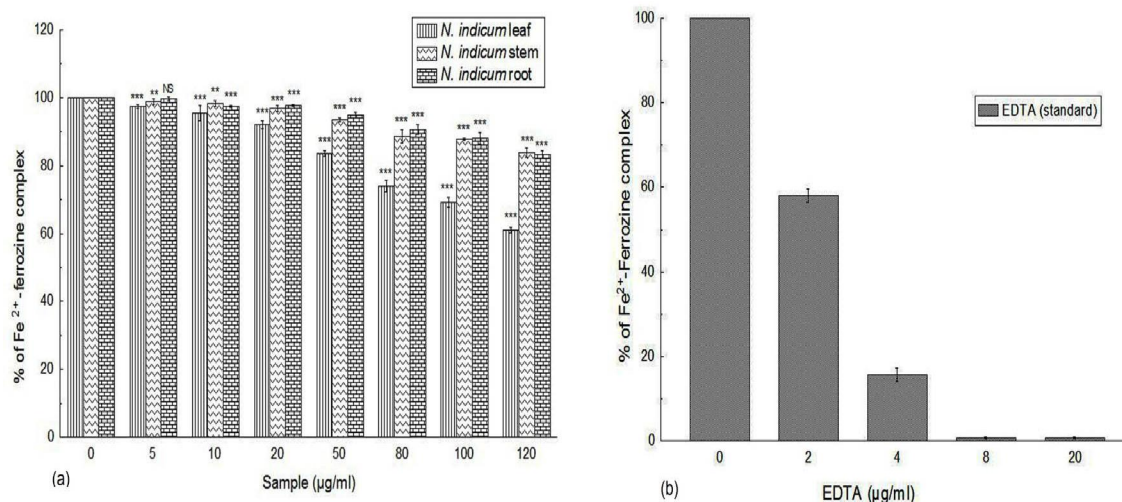


Figure 10 - Fe²⁺ chelation assay
Effects of (a) *N. indicum* leaf, stem and root extracts and (b) standard EDTA on Fe²⁺-ferrozine complex formation. The results are mean \pm S.D. (n=6). NS= non-significant, **p < 0.01 and ***p < 0.001 vs 0 µg/ml.

Hypochlorous acid scavenging

Figure 9 shows how effectively the leaf, stem and root extracts of *N. indicum* dose-dependently scavenge hypochlorous acid compared to that of ascorbic acid. The results indicate that the leaf (IC₅₀ = 124.74 \pm 1.91 µg/ml), stem (IC₅₀ = 162.25 \pm 10.31 µg/ml) and root (IC₅₀ = 267.63 \pm 3.60 µg/ml) extracts scavenged hypochlorous acid more efficiently than ascorbic acid (IC₅₀ = 235.95 \pm 5.75 µg/ml) (Table 1). At 100 µg/ml, the percentage scavenging of leaf, stem and root extracts were 41.10%, 39.03% and 37.67% whereas that of standard (ascorbic acid) was 34.6%.

Iron chelation

Ferrozine together with Fe²⁺ ion generates a violet colored complex. In the presence of a chelating agent, the ferrozine formation is interrupted and the intensity of the violet colour decrease with increase in the concentration of the chelating agent. The result (Figures 10(a) and 10(b)) demonstrated that the formation of ferrozine-Fe²⁺ complex is inhibited in the presence of the plant

extracts and reference compound. The IC₅₀ values (Table 1) of the leaf, stem and root extracts and EDTA were 216.70 \pm 9.82 µg/ml, 659.95 \pm 48.64 µg/ml, 698.38 \pm 39.00 µg/ml and 1.27 \pm 0.05 µg/ml respectively. At 120 µg/ml, the percentage of inhibition of the leaf, stem and root extracts were 38.91%, 16.06% and 16.78% whereas at 45 µg/ml the standard EDTA shows 99.5% inhibition.

Reducing power

As illustrated in Figure 11, reductive capability was measure by Fe³⁺ to Fe²⁺ transformation method in the presence of the leaf, stem and root extracts of *N. indicum* and reference compound ascorbic acid. The root extract displayed the greatest reducing capacity followed by the stem and leaf extracts. At 0.1mg/ml, the absorbance of *N. indicum* leaf, stem, root extracts and BHT were 0.45, 0.52, 0.85 and 0.46 respectively.

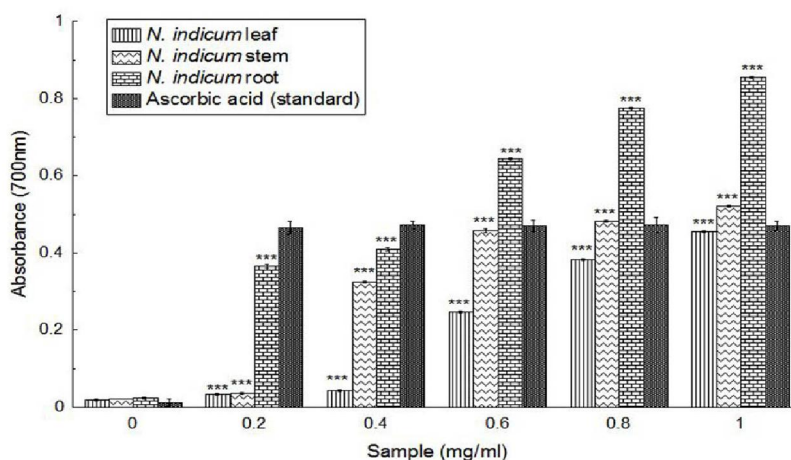


Figure 11 - Reducing Power Assay.

The reductive abilities of *N. indicum* leaf, stem, and root extracts and the standard ascorbic acid. Each value represents mean \pm S.D. (n = 6). ***p < 0.001 vs 0 mg/ml.

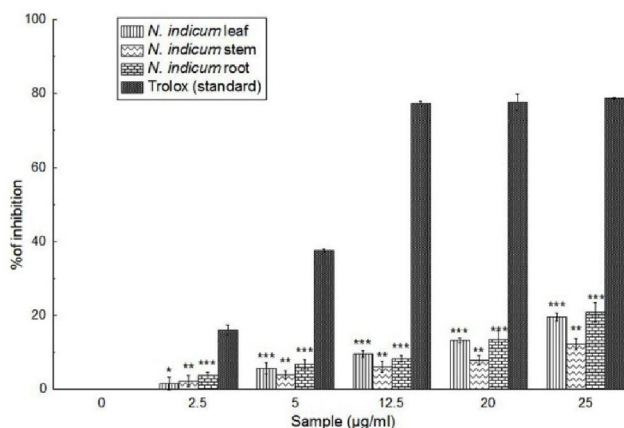


Figure 12 - Inhibition of Lipid peroxidation by *N. indicum* leaf, stem and root extracts and the standard trolox.

The data is expressed as the percentage of lipid peroxidation inhibition of brain homogenate, induced by Fe²⁺ ascorbic acid. Each value represents mean \pm S.D. (n=6). *p < 0.05, **p < 0.01 and ***p < 0.001 vs 0 µg/ml.

Lipid peroxidation

The IC₅₀ values (Table 1) of the *N. indicum* leaf, stem and root were 113.77 \pm 8.89 µg/ml, 199.17 \pm 33.51 µg/ml and 110.03 \pm 12.75 µg/ml respectively. Trolox was used as a standard which showed an IC₅₀ value of 6.76 \pm 0.17 µg/ml showing that the inhibitory efficiency

of the plant extract is poor compared to standard trolox. At 25 µg/ml concentration (Figure 12) the potential of the *N. indicum* leaf, stem and root to inhibit lipid peroxidation were 19.49%, 12.07% and 20.81% which is lower compared to trolox (78.87%).

Determination of total phenolic content

Phenolic compounds may contribute directly to antioxidative action. The total phenolic content of the *N. indicum* leaf, stem and root were 72.62 ± .08 mg/ml, 81.54 ± 0.05 mg/ml and 87.38 ± 0.16 mg/ml gallic acid equivalent per 100 mg plant extract respectively (Table 1).

Determination of total flavonoid content

The total flavonoid content of the 70% methanolic extract of *N. indicum* leaf, stem and root were 93.06 ± 0.03 mg/ml, 67.4 ± .06 mg/ml and 64.08 ± .002 mg/ml quercetin equivalent per 100 mg plant extract respectively (Table 1).

Table 1: Comparison of the antioxidant and free radical scavenging capacities of 70% methanolic crudes of *Nerium indicum* leaf, stem and root

Name of Assay	70% methanolic extract			Standard	Values of Standard compounds
	<i>N. indicum</i> leaf	<i>N. indicum</i> stem	<i>N. indicum</i> root		
TEAC Values	0.31 ± 0.002	0.325 ± 0.003	0.396 ± .001	-	-
† Phenolic content	72.62 ± .08	81.54 ± 0.05	87.38 ± 0.16	-	-
‡ Flavonoid content	93.06 ± 0.03	67.4 ± .06	64.08 ± .002	-	-
Ø IC ₅₀ values of the extracts for free radical scavenging capacity for:					
DPPH	217.15 ± 18.39 ^{NS}	63.56 ± 1.63 ^{***}	166.18 ± 6.84 ^{***}	Ascorbic acid	5.29 ± 0.28
Hydroxyl radical (OH [•]) scavenging	29.65 ± 0.21 ^{***}	118.68 ± 1.11 ^{***}	208.16 ± 2.70 ^{***}	Mannitol	571.45 ± 20.12
Superoxide anion (O ₂ ^{•-}) scavenging	224.35 ± 3.45 ^{***}	268.33 ± 6.04 ^{***}	170.69 ± 2.41 ^{***}	Quercetin	42.06 ± 1.35
Nitric oxide radical (NO) scavenging	46.56 ± 3.42 ^{***}	23.56 ± 1.16 ^{***}	62.43 ± 4.55 ^{***}	Curcumin	90.82 ± 4.75
Hydrogen peroxide (H ₂ O ₂) scavenging	40.42 ± 4.40 ^{***}	39.87 ± 5.67 ^{***}	37.05 ± 2.99 ^{***}	Sodium pyruvate	3.24±0.30
Peroxynitrite (ONOO ⁻) scavenging	1672.80 ± 56.68 ^{***}	2172.26±133.97 ^{***}	1869.97 ± 122.30 ^{***}	Gallic acid	876.24 ± 56.96
Singlet oxygen (¹ O ₂) scavenging	365.76 ± 5.52 ^{***}	391.55 ± 7.53 ^{***}	275.08 ± 7.5 ^{***}	Lipoic acid	46.15 ± 1.16
Hypochlorous acid (HOCl) scavenging	124.74 ± 1.91 ^{***}	162.25 ± 10.31 ^{***}	267.63 ± 3.60 ^{***}	Ascorbic acid	235.96 ± 5.75
Iron chelating activity	216.70 ± 9.82 ^{***}	659.95 ± 48.64 [†]	698.38 ± 39.00 [†]	EDTA	1.27 ± 0.05
Lipid peroxidation	113.77 ± 8.89 ^{**}	199.17 ± 33.51 [†]	110.03 ± 12.75 ^{**}	Trolox	6.76 ± 0.17

† Phenolic content (mg/ml Gallic acid equivalent per 100 mg plant extract)

‡ Flavonoid content (mg/ml Quercetin equivalent per 100 mg plant extract)

Ø Units of IC₅₀ for all activities are µg/ml, except H₂O₂ scavenging, peroxynitrite scavenging and iron chelating where the units are mg/ml. Data are expressed as mean ± S.D(n=6).

NS = non-significant; * p < 0.05; ** p < 0.01; *** p < 0.001

Discussion

Though complementary and alternative medicine (CAM) has recently come to the lime light, but for time immemorial, people have cured themselves by using local plants. Ayurveda which is an integral part of Indian culture dates back to 1500-800 BC, describes usage of thousands of species of plants to cure numerous ailments. Adverse side effects of modern synthetic drugs forced the scientists to search for the plant derived bioactive chemicals, a source of CAM to balance with the constant need for new and effective therapeutic agents. In search of multifunctional antioxidant compound thousands of plants have been screened which either would prevent the reactive species from being formed or remove them before they can degrade vital components of the cell. In addition, the publications on antioxidants and oxidative stress has nearly quadrupled in the past decade [22] aiming towards their importance in medicine.

The reaction between ABTS and potassium persulfate results in the production of a blue colored chromophore, ABTS^{•+}. After addition of the plant extract this preformed radical cation was converted to ABTS in a dose dependant manner. The results were compared with those obtained using trolox and the TEAC value demonstrates that the extracts possess convincing anti-oxidant property. The effect of the extracts in the scavenging assay of DPPH radical furthermore assured the fact that the extracts smoothly act as antioxidants, since the study on TEAC and DPPH scavenging can be observed as complementary to each other [23].

Hydroxyl radical is most potent among the ROS to damage the cellular components, causing lipid peroxidation, DNA damage and evoking carcinogenesis, mutagenesis and cytotoxicity [24]. At pH 7.4 ascorbic acid and H₂O₂ was mixed and incubated with Fe³⁺-EDTA premixture which generated hydroxyl radicals. This cause 2-deoxy-2-

ribose damage and generate malondialdehyde (MDA) like product. By heating MDA with TBA at low pH gives rise a pink chromogen. The hydroxyl radicals are removed from the sugar and prevented the reaction forming the pink chromophore with increasing concentrations of the plant extract. The results, as can be found from Figure 3 and Table 1, indicate that *N. indicum* extract is better hydroxyl radical scavenger than standard mannitol, with the leaf extract being the best in comparison to stem extract followed by the root extract.

Superoxide anion is formed as by product of mitochondrial respiration and several other enzymes. It initiates lipid oxidation by generating singlet oxygen and is detrimental for different biomolecules [25]. Most organisms living in oxygenated environment possess isoforms of the superoxide scavenging enzyme superoxide dismutase (SOD). It has been found that absence of cytosolic SOD (CuZnSOD) results in liver cancer, muscle atrophy, haemolytic anemia, cataracts and thymic involution [25]. *N. indicum* extracts has shown to have moderately superoxide scavenging activity in respect to the standard quercetin.

The binary molecule nitric oxide has been found to be associated with inflammatory conditions and different carcinomas. Direct tissue toxicity and septic shock associated vascular collapse are related to constant production of nitric oxide radical. Conditions such as multiple sclerosis, arthritis, juvenile diabetes and ulcerative colitis shows involvement of its chronic level expression [26]. Nitric oxide is generated from sodium nitroprusside reacting with oxygen to form nitrite. The extract inhibits the nitrite formation by directly competing with oxygen in the reaction with nitric oxide. The nitric oxide scavenging assay of *N. indicum* and standard curcumin proved that overall all three parts of the plant is more potent in scavenging nitric oxide than that of the standard. Our test showed that the stem of *N. indicum* has more nitric oxide scavenging activity than the leaf and root of the same plant.

Hydrogen peroxide which is the simplest peroxide is a strong oxidizing agent which can inactivate enzymes directly by oxidation of essential thiol (-SH) groups. It can cross cell membranes rapidly; once inside the cell, it can probably react with Fe^{2+} and possibly Cu^{2+} ions to form hydroxyl radicals and this may be the origin of many of its toxic effects [26]. All obligate and facultative aerobes contains catalyse peroxidases enzyme which decompose hydrogen peroxide to water and oxygen. From our experiment it is evident that the hydrogen peroxide scavenging activity of all three parts of *N. indicum* is negligible compared to that of the standard sodium pyruvate.

Free radical superoxide and nitric oxide react to form peroxynitrite anion which due to its oxidizing properties can harm cellular components [27]. Peroxynitrite bleaches Evans Blue by oxidizing it. The present study indicates that all the extracts inhibit evans blue bleaching by scavenging peroxynitrite and its activity is slightly less than that of the standard gallic acid.

Singlet oxygen is generally formed in the skin upon UV-radiation by photosensitizer pigments induces hyper-oxidation, oxygen cytotoxicity and decreases the antioxidant activity [28]. Singlet oxygen oxidizes LDL cholesterol and results in various cardiovascular disorders. In our current experiment we found *N. indicum* extracts to scavenge singlet oxygen moderately but less than the standard lipoic acid.

Hypochlorous acid is generated by myeloperoxidase-mediated peroxidation of chloride ions in neutrophils at the sites of inflammation [29]. Hypochlorous acid reacts with various types of biomolecules including nucleic acid [30], lipid and protein [31]. *N. indicum* hydromethanolic extracts showed greater hypochlorous acid scavenging activity than that of the standard ascorbic acid. The leaves possess more activity than the stem followed by the root.

The dual oxidation state property of iron (Fe^{2+} or Fe^{3+}) enables iron to accept or donate electron through redox reactions which is crucial for various biological processes but may prove harmful to cells in certain respect. In excess, iron can react with superoxide anion (O_2^-) and hydrogen peroxide and convert them into hydroxyl radical ($OH\cdot$) through Haber-Weiss reaction which cause severe injury to membranes, proteins and nucleic acid [32]. As a result lipid hydroperoxides are decomposed into peroxy and alkoxy radicals responsible for the chain reaction of lipid peroxidation [33]. The results from Figure 10 and Table 1 suggest that the decrease in the concentration dependent colour formation with ferrozine in presence of extract indicating its iron chelating property where the leaf extract has the greatest iron chelating capacity compared to the stem and root extracts. But still, compared to the standard EDTA the leaf extract possess least activity.

Generation of ferry-perferryl complex or hydroxyl radicals catalyzed by iron accelerates the process of peroxidation by decomposing lipid hydroperoxides into peroxy and alkoxy radicals. Hydroxyl radical is extremely reactive. It reacts with polyunsaturated fatty acid moieties of cell membrane and yield carbonyl products like malondialdehyde (MDA), which generate a pink chromogen with TBA. On addition of the plant extracts or the standard trolox, production of MDA is inhibited and, hence indicating the ability of the sample, although less than the standard, to inhibit lipid peroxidation. The result from their reducing abilities also correlates with the interpretation.

Though the activities of a potent antioxidants comprises various specialized features such as scavenging ROS and inhibiting lipid peroxidation but the overall antioxidant capacity of a compound may be attributed to the fact of possessing more antioxidant property with increasing reducing potential. The stem and root extracts have shown more antioxidant property than the standard ascorbic acid where the root showed far more total reducing capacity than that of the standard.

All the extracts have been found to contain significant amount of total flavonoid and phenolic contents. Both of these compounds have good antioxidant potential and their effects on health and disease prevention are considerable. Flavonoids are polyphenolic plant secondary metabolite characterized by a common benzopyrone ring which functions primarily as antioxidants and also have cardio protective role [34]. The mechanism of action of flavonoids is through scavenging or chelation of free radicals [35]. Natural phenolic content in plants are also very important which attribute hugely to their pharmacological values. They are potent vasodilators and are active antioxidants as their hydroxyl groups confer scavenging ability [36].



Conclusions

The present study reveals that the 70% methanolic extract of *N. indicum* leaf, stem and root possess excellent antioxidant and free radical scavenging capability along with high total phenolic and flavonoid content. It also possesses good reducing power, iron chelating capacity and lipid peroxidation inhibition ability which are necessary for healthy condition of the body. These in vitro studies indicate that *N. indicum* can act as a natural antioxidant. However, the active components responsible for the antioxidative activity of the plant are currently unrevealed. Therefore, these bioactive compounds need to be isolated, identified, characterized and tested for its antioxidant properties both in the in vivo and in vitro models.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

PD and DC performed the study and completed the analytic works. Acquisition of data and drafting of the manuscript was done by PD. TKC and NM revising the manuscript and finally approved the manuscript for submission. NM Supervised the study design.

Acknowledgements

The authors would like to thank Mr. Avinash Chaudhuri for his contribution towards collection of plant material and Mr. Ranjit Das for technical assistance. The authors are also grateful to Mr. Rhitajit Sarkar and Mr. Bibhabasu Hazra for providing their expert views and guidance throughout the experiments.

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