

**HEMIDESMUS INDICUS, AN AGE-OLD PLANT: STUDY OF ITS *IN VITRO* ANTIOXIDANT AND FREE RADICAL SCAVENGING POTENTIALS**

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**Summary**

In recent times, there is a lot of interest in ‘phytonutrients’ from plants, especially those used in traditional medicinal systems. The aim of the present study was to evaluate the *in vitro* antioxidant activities of *Hemidesmus indicus* root extract. A 70% methanolic extract of *Hemidesmus indicus* root was used in the *in vitro* study of total antioxidant activity and scavenging of radicals like hydroxyl, superoxide, nitric oxide, peroxy nitrite, hydrogen peroxide, singlet oxygen & hypochlorous acid, and for iron chelating capacity, reducing power, lipid peroxidation inhibition and phenolic & flavonoid contents. Total antioxidant activity in terms of trolox equivalent antioxidant capacity (TEAC) was  $0.67 \pm 0.01$ . The IC<sub>50</sub> values for scavenging of free radicals were  $257.78 \pm 7.22$  µg/ml,  $58.4 \pm 3.93$  µg/ml and  $90.82 \pm 4.75$  µg/ml for hydroxyl, superoxide and nitric oxide, respectively, and those for the singlet oxygen, hypochlorous acid scavenging and lipid peroxidation inhibition activities were  $421.47 \pm 54.81$  µg/ml,  $276.45 \pm 6.49$  µg/ml and  $25.93 \pm 1.27$  µg/ml, respectively. IC<sub>50</sub> value for the iron chelating property was found to be  $655.48 \pm 10.9$  µg/ml. The reducing power increased with increasing amounts of extract. The plant extract (100 mg) yielded  $55.7 \pm 0.001$  mg/ml gallic acid-equivalent phenolic content and  $174.6 \pm 0.007$  mg/ml quercetin-equivalent flavonoid content. The present study provides evidence that a 70% methanol extract of *Hemidesmus indicus* root is a potential source of natural antioxidants.

**Key Words:** *Hemidesmus indicus*, free radical, phenolic content, lipid peroxidation

## Introduction

According to the World Health Organization (WHO), most people of the developing countries rely on traditional medicines, as hundreds of species are recognized as having medicinal values and the 'phytomedicines' are beginning to link traditional and modern medicines. For past few decades, a large number of studies have shown that hypercholesterolemia, diabetes, hypertension, smoking, ageing and nitrate intolerance give rise to the generation of free radicals from endothelial, adventitial and also vascular smooth muscle cells. This gives rise to "oxidative stress" that is initiated by stabilization of free radicals through electron pairing with biological macromolecules such as proteins, lipids and DNA and cause protein and DNA damage along with lipid peroxidation. These changes contribute to cancer, atherosclerosis, cardiovascular diseases and inflammatory diseases (1). Protective activity of antioxidants, mostly through scavenging of free radicals and binding of iron that initializes free radical generation has been found to be beneficial against these diseases (2). Synthetic antioxidants such as butylated hydroxyl anisole (BHA) and butylated hydroxyl toluene (BHT) have been restricted for use due to their possible carcinogenic properties (3) and as a result, natural antioxidants have come into the spotlight. Already, the presence of natural antioxidants from different kind of plant materials and the importance of those have been found to be of great interest (4, 5). The traditional Indian medicinal system, *Ayurveda* has used various indigenous plants in several therapeutic purposes like cardioprotective, chemopreventive and rejuvenative effects.

*Hemidesmus indicus* [syn. *Periploca indica* (Family – Asclepiadaceae)] is a species of plant that is found in India, Sri Lanka and South-East Asian countries. It is a slender, laticiferous, twining, sometimes prostrate or semi-erect shrub. Roots are woody and aromatic. The root decoction cooling, antipyretic, alexiteric, antidiarrhoeal, and used in treatment of fevers, asthma, bronchitis, blood disorders, leucorrhoea, eye troubles, epileptic fits, poisoning, dysentery, diarrhea etc. The chemical constituents of the root are found to be hemidesmin 1, hemidesmin 2, a-amyrin, b-amyrin, lupeol, 2-hydroxy-4methoxy-benzoic acid and some triterpenes (6, 7, 8, 9). Root has anti-microbial (6), anti-snake venom (8), anti-bacterial (10), antinociceptive (11), anti-hyperglycemic properties (12) and potent anti-inflammatory activity (13). Ethanolic extract of root was found to be antihepatotoxic (14). Several pregnane and steroidal glycosides have also been isolated from the plant (15, 16). The antioxidant properties of methanolic extract of the root bark have also been evaluated (17).

The present study was performed to evaluate the antioxidant potential and free radical scavenging activities of a 70% methanolic extract of *H. indicus* root. The extract was examined for different reactive oxygen species (ROS) scavenging activities including hydroxyl, superoxide, nitric oxide, peroxy nitrite, hydrogen peroxide, singlet oxygen and hypochlorous acid, and for phenolic and flavonoid contents, iron chelating capacity and total antioxidant activity.

## Methods

**Chemicals:** 2,2'-azinobis-(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 ( $K_2S_2O_8$ ), 2-deoxy-2-ribose, mannitol, sodium nitroprusside (SNP), lipoic acid, quercetin and ferrozine were obtained from Sisco Research Laboratories Pvt. Ltd, Mumbai, India. Folin-ciocalteu reagent, xylenol orange and *N,N*-dimethyl-4-nitrosoaniline were obtained from Merck, Mumbai, India. Gallic acid and curcumin was obtained from MP Biomedicals, France. Catalase was obtained from HiMedia Laboratories Pvt. Ltd, Mumbai, India. Evans blue was purchased from BDH, England.

Diethylene-triamine-pentaacetic acid (DTPA) was obtained from Spectrochem Pvt. Ltd, Mumbai, India. Thiobarbituric acid (TBA) was obtained from Loba Chemie, Mumbai, India.

**Plant material:** The root of the *H. indicus* plant was collected from the Bankura district of West Bengal, India and authenticated through the Central Research Institute of Ayurveda, Kolkata, India, where a specimen (CRHS 116/08) was submitted.

**Extraction:** The powder (100 g) of the dried root of *H. indicus* was stirred using a magnetic stirrer with a 7:3 mixture of methanol:water (500 ml) for 15 hours; the mixture was then centrifuged at 2850 x g and the supernatant decanted. The process was repeated again by adding the solvent with the precipitated pellet. The supernatants were collected, concentrated in a rotary evaporator and lyophilized. The dried extract was stored at -20°C until use.

**Animals:** The Animal Ethical Clearance Committee of the Institute (Registration number: 95/1999/ CPCSEA) approved the use of adult male Swiss Albino mice, weighing 20-25 gm for experimentation. Each polypropylene cage contained 4 mice at a time; supplied with *ad libitum* laboratory diet and water. The mice were kept at 25 ± 2°C and 60 ± 5% humidity and normal photo cycle (12 h dark/12 h light).

**Total antioxidant activity:** Antioxidant capacity was done based on the scavenging of ABTS<sup>•+</sup> radical cation by the sample in comparison to trolox standard (18). ABTS solution was mixed with potassium persulfate to generate ABTS<sup>•+</sup> radical cation. Then 10 µl sample solution was mixed with 1 ml ABTS<sup>•+</sup> solution and the absorbance was measured at 734 nm. All experiments were repeated six times. The percentage inhibition of absorbance was calculated and plotted as a function of concentration of standard and sample to determine the trolox equivalent antioxidant concentration (TEAC).

**Hydroxyl radical scavenging:** The scavenging assay for hydroxyl radical was performed by a standard method (18). Hydroxyl radical was generated by the Fenton reaction using a Fe<sup>3+</sup>-ascorbate-EDTA-H<sub>2</sub>O<sub>2</sub> system. The assay quantifies the 2-deoxyribose degradation product, by its condensation with TBA. All tests were carried out six times. Mannitol, a classical ·OH scavenger, was used as a positive control. Percent inhibition was evaluated by comparing the results of the test and blank solution.

**Superoxide radical scavenging:** Measurements of superoxide anion scavenging activities of the sample and standard quercetin were done based on the reduction of NBT according to a previously described method (18). Superoxide radical is generated by a non-enzymatic system of phenazine methosulfate-nicotinamide adenine dinucleotide (PMS/NADH). These radicals reduce nitro blue tetrazolium (NBT) into a purple colored formazan which was measured spectrophotometrically at 562 nm. All tests were performed six times.

**Nitric oxide radical scavenging:** Sodium nitroprusside (SNP) gives rise to nitric oxide that under interaction with oxygen produce nitrite ions measured by Griess Illosvoy reaction (18). The chromophore generated was spectrophotometrically measured at 540 nm against blank sample. All tests were performed six times. Curcumin was used as a standard.

**Peroxynitrite anion scavenging:** Peroxynitrite (ONOO<sup>-</sup>) synthesis was done 12 hrs before the assay, according to Beckman et al (19). Acidic solution (0.6 M HCl) of 5 ml H<sub>2</sub>O<sub>2</sub> (0.7 M) was mixed with 5 ml of 0.6 M KNO<sub>2</sub> on an ice bath for 1 s and 5 ml of ice-cold 1.2 M NaOH was added to the reaction mixture. Excess H<sub>2</sub>O<sub>2</sub> was adsorbed by granular MnO<sub>2</sub> and the reaction

mixture was left at -20°C. The concentration of the peroxy nitrite solution was measured spectrophotometrically at 302 nm ( $\epsilon = 1670 \text{ M}^{-1} \text{ cm}^{-1}$ ).

Evans blue bleaching assay was used to measure the peroxy nitrite scavenging activity (**18**). The percentage of scavenging of  $\text{ONOO}^-$  was calculated by comparing the results of the test and blank sample. All tests were performed six times. Gallic acid was used as reference compound.

**Hydrogen peroxide scavenging:** FOX-reagent method was used to determine this activity of the sample and the reference compound sodium pyruvate, by a formerly depicted method (**20**). The absorbance of the ferric-xylenol orange complex was measured at 560 nm. All tests were carried out for six times.

**Singlet oxygen scavenging:** Singlet oxygen ( $^1\text{O}_2$ ) production, and at the same time, its scavenging by the sample and the reference compound lipoic acid can be monitored by *N,N*-dimethyl-4-nitrosoaniline (RNO) bleaching, using a earlier reported method (**18**). Singlet oxygen was generated by a reaction between  $\text{NaOCl}$  and  $\text{H}_2\text{O}_2$  and the bleaching of RNO was read at 440 nm. All tests were performed six times.

**Hypochlorous acid scavenging:** According to a previously described method [18], hypochlorous acid (HOCl) was prepared just before the experiment by adjusting the pH of a 10% (v/v) solution of  $\text{NaOCl}$  to pH 6.2 with 0.6 M  $\text{H}_2\text{SO}_4$  and the concentration of HOCl was determined by taking the absorbance at 235 nm using the molar extinction coefficient of  $100 \text{ M}^{-1} \text{ cm}^{-1}$ . The scavenging activities of the plant extract and the standard, ascorbic acid, a potent HOCl scavenger (**21**) was evaluated by measuring the decrease in the absorbance of catalase at 404 nm. All tests were performed six times.

**Fe<sup>2+</sup> ion chelating:** The ability of chelating of ferrous ion by the sample in comparison to the standard EDTA was evaluated by a standard method (**18**). In a Hepes buffer (20 mM, pH 7.2) medium, the plant root extract was added to ferrous sulfate solution (12.5  $\mu\text{M}$ ) and the reaction was started by the addition of ferrozine (75  $\mu\text{M}$ ). The mixture was shaken vigorously and left standing for 20 min at room temperature. The absorbance was then taken at 562 nm. All tests were performed six times.

**Reducing power:** The  $\text{Fe}^{3+}$ -reducing power of the extract was determined by a standard method (**18**). In a phosphate buffer solution (0.2 M, pH 6.6), different concentrations (0.0-0.4 mg/ml) of the extract were mixed with potassium hexacyanoferrate (0.1%), followed by incubation. After incubation, the upper portion of the solution was diluted, and  $\text{FeCl}_3$  solution (0.01%) was added. The reaction mixture was left at room temperature for color development and the absorbance was measured at 700 nm. All tests were performed six times. A higher absorbance of the reaction mixture indicated greater reducing power. Butylated hydroxyl toluene (BHT) was used as a positive control.

**Lipid peroxidation inhibition:** The inhibition of lipid peroxidation of mice brain homogenate was assayed by measuring malondialdehyde (MDA), according to the method of Kizil et al. (**22**), with slight modification. The homogenate was prepared by centrifuging the brain ( $20 \pm 2 \text{ gm}$ ) with 50 mM phosphate buffer (pH 7.4) and 120 mM KCl, at 3000 rpm for 10 min. A 100  $\mu\text{l}$  aliquot of the supernatant homogenate was mixed with plant extract of various concentrations (2.5-25  $\mu\text{g/ml}$ ), followed by addition of 0.1 mM  $\text{FeSO}_4$  and 0.1 mM ascorbic acid, each of 100  $\mu\text{l}$  and incubated for 1 hr at 37 °C. 500  $\mu\text{l}$  28% TCA was used to stop the reaction and then 380  $\mu\text{l}$  2% TBA was added with 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 repeated 6 times. Trolox was used as the standard.

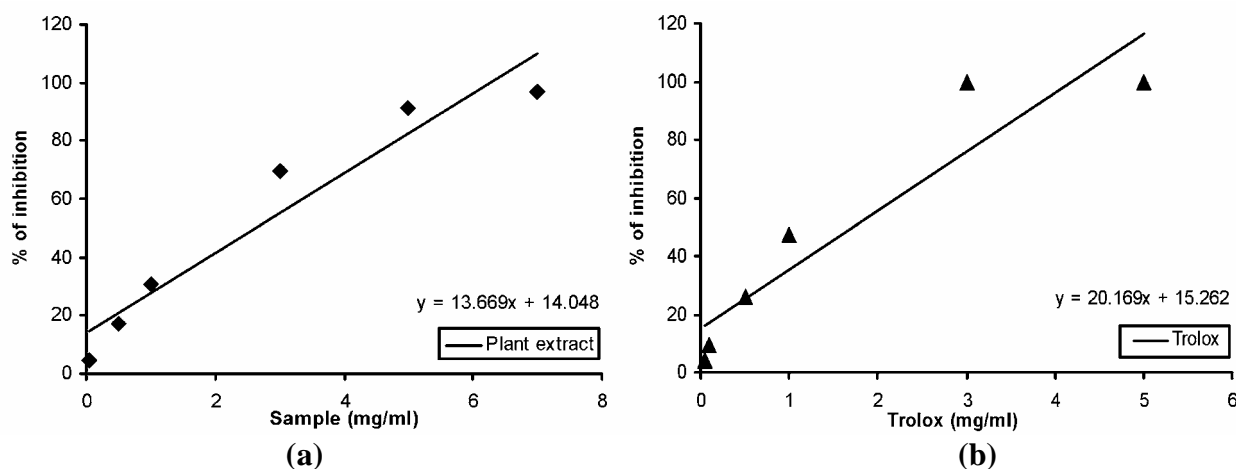
**Assay of total phenolic content:** The amount of total phenols present in the plant root extract was determined using Folin-Ciocalteu (FC) reagent by a formerly reported method (18). The phenolic content was evaluated from gallic acid standard curve.

**Assay of total flavonoid content:** The amount of total flavonoids was determined with aluminium chloride ( $\text{AlCl}_3$ ) according to a known method (18). The flavonoid content was calculated from quercetin standard curve.

**Statistical analysis:** All data were reported as the mean  $\pm$  SD of six measurements. The statistical analysis was performed by KyPlot version 2.0 beta 15 (32 bit). The  $\text{IC}_{50}$  values were calculated by the formula,  $Y = 100 \cdot A1 / (X + A1)$  where  $A1 = \text{IC}_{50}$ ,  $Y = \text{response}$  ( $Y = 100\%$  when  $X = 0$ ),  $X = \text{inhibitory concentration}$ . The  $\text{IC}_{50}$  values were compared by paired t test.  $p < 0.05$  was considered significant.

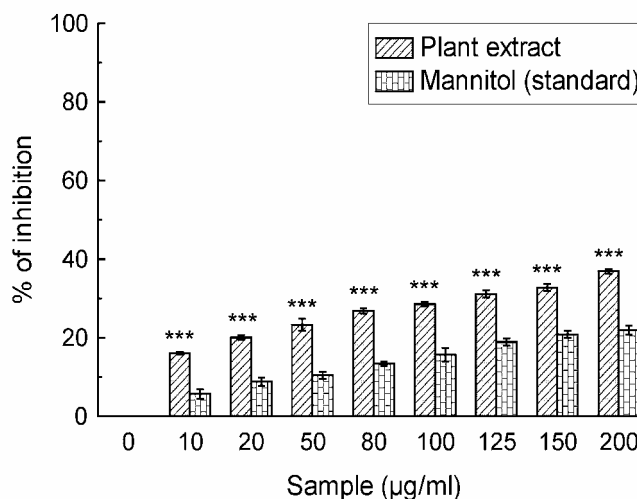
## Results

**Total antioxidant activity:** The total antioxidant activity of the extract and trolox, as shown in Figure 1(a) and (b), respectively was calculated from the spectral data of decolorization of  $\text{ABTS}^+$ , obtained at 734 nm. The TEAC value of the extract was  $0.67 \pm 0.01$ .



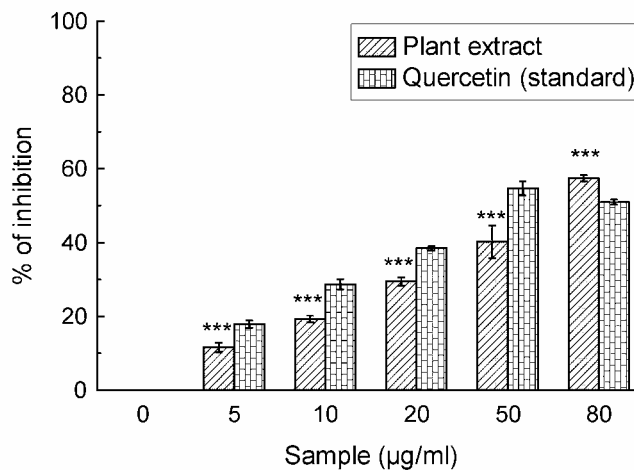
**Figure 1. Total antioxidant activity:** Total antioxidant activity of plant extract and trolox. Effect of (a) *Hemidesmus indicus* extract and (b) reference compound trolox on decolorization of  $\text{ABTS}^+$  radical cation. The percentage inhibition was plotted against the concentration of sample. All data are expressed as mean  $\pm$  S.D. ( $n=6$ ).

**Hydroxyl radical scavenging:** The abilities of the extract and the standard mannitol in scavenging hydroxyl radical generated in a  $\text{Fe}^{3+}$ -EDTA-ascorbic acid- $\text{H}_2\text{O}_2$  system are shown in Figure 2. The  $\text{IC}_{50}$  values (Table 1) of the extract and standard in this assay were  $257.78 \pm 7.22 \mu\text{g/ml}$  and  $571.45 \pm 20.12 \mu\text{g/ml}$ , respectively.



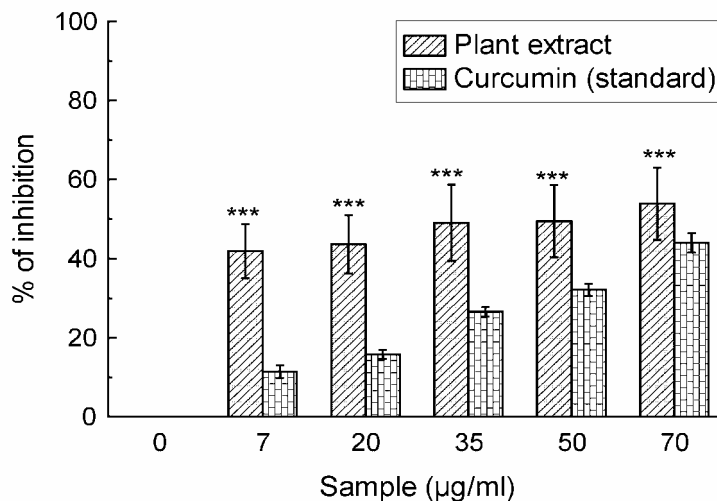
**Figure 2. Hydroxyl radical scavenging:** Hydroxyl radical scavenging activities of the *Hemidesmus indicus* extract and the reference compound mannitol. The data represent the percentage inhibition of deoxyribose degradation. The results are mean  $\pm$  S.D. of six parallel measurements. \*\*\* $p < 0.001$  vs 0  $\mu\text{g/ml}$ .

**Superoxide radical scavenging:** The PMS-NADH coupling generated superoxide scavenging efficacies of the plant extract and the standard quercetin are shown in Figure 3, also substantiated in their  $\text{IC}_{50}$  values (Table 1) which were  $58.4 \pm 3.93 \mu\text{g/ml}$  and  $42.06 \pm 1.35 \mu\text{g/ml}$ , respectively.



**Figure 3. Superoxide radical scavenging:** Scavenging effects of *Hemidesmus indicus* plant extract and the standard quercetin on superoxide radical. The data represent the percentage superoxide radical inhibition. All data are expressed as mean  $\pm$  S.D. ( $n=6$ ). \*\*\* $p < 0.001$  vs 0  $\mu\text{g/ml}$ .

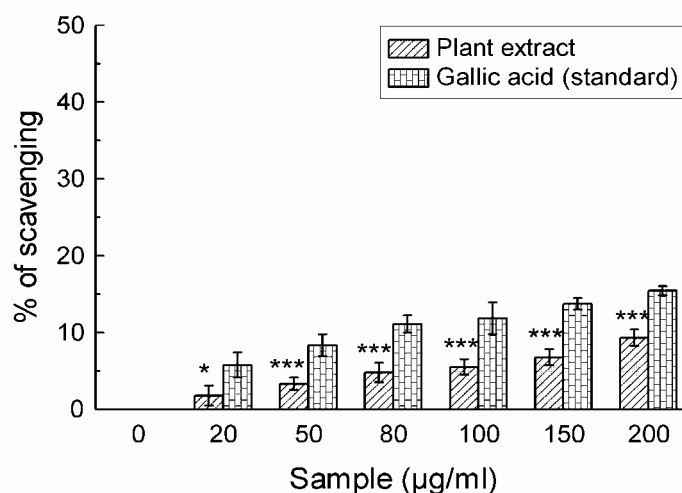
**Nitric oxide radical scavenging:** *H. indicus* root extract also caused a dose-dependent inhibition of nitric oxide with an (Table 1) of  $38.24 \pm 16.7 \mu\text{g/ml}$  (Figure 4). Curcumin was used as a reference compound and  $90.82 \pm 4.75 \mu\text{g/ml}$  curcumin was needed for 50% inhibition.



**Figure 4. Nitric oxide radical scavenging:** The nitric oxide radical scavenging activities of *Hemidesmus indicus* extract and the standard curcumin. The data represent the percentage nitric oxide inhibition. Each value represents mean  $\pm$  S.D. (n=6). \*\*\* $p < 0.001$  vs 0  $\mu\text{g/ml}$ .

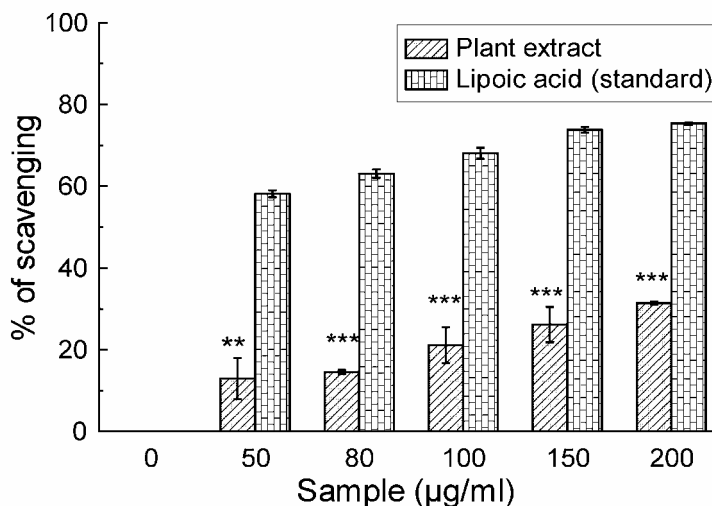
**Hydrogen peroxide scavenging:** Hydrogen peroxide scavenging was assayed by the FOX reagent method (20). The extract was found too poor to be represented. So, the Figure and the  $\text{IC}_{50}$  values were not provided.

**Peroxynitrite anion scavenging:** As illustrated in Figure 5, no significant scavenging activity of the plant root extract was observed for peroxynitrite anion in comparison to the standard gallic acid, which is also supported by the  $\text{IC}_{50}$  values (Table 1) of the extract ( $\text{IC}_{50} = 1.91 \pm 0.31 \text{ mg/ml}$ ) and gallic acid ( $\text{IC}_{50} = 0.88 \pm 0.06 \text{ mg/ml}$ ).



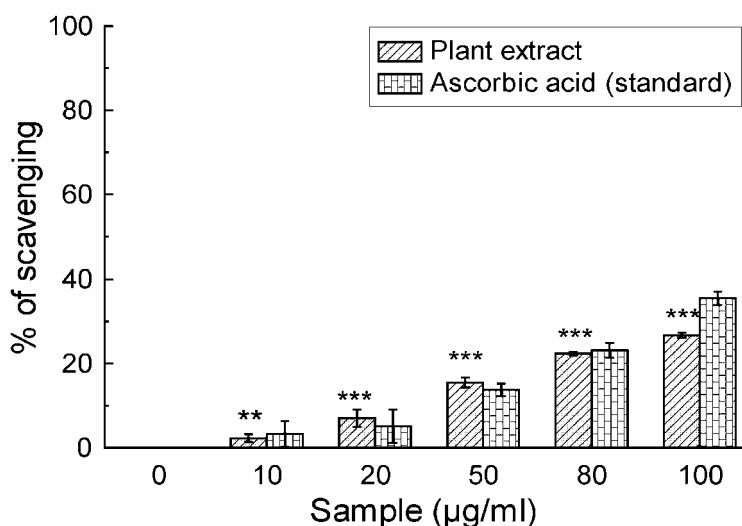
**Figure 5. Peroxynitrite anion scavenging:** The peroxynitrite anion scavenging activities of *Hemidesmus indicus* plant extract and the standard gallic acid. Each value represents mean  $\pm$  S.D. (n=6). \* $p < 0.05$  and \*\*\* $p < 0.001$  vs 0  $\mu\text{g/ml}$ .

**Singlet oxygen scavenging:** *H. indicus* extract showed a low but dose-dependent activity as a scavenger of singlet oxygen (Figure 6) and in comparison to the standard, lipoic acid. The  $IC_{50}$  value (Table 1) of the test sample was  $421.47 \pm 54.81 \mu\text{g/ml}$  whereas that of lipoic acid was  $46.15 \pm 1.16 \mu\text{g/ml}$ .



**Figure 6. Singlet oxygen scavenging:** Effects of *Hemidesmus indicus* plant extract and the standard lipoic acid on the scavenging of singlet oxygen. The results are mean  $\pm$  S.D. of six parallel measurements. \*\* $p < 0.01$  and \*\*\* $p < 0.001$  vs 0  $\mu\text{g/ml}$ .

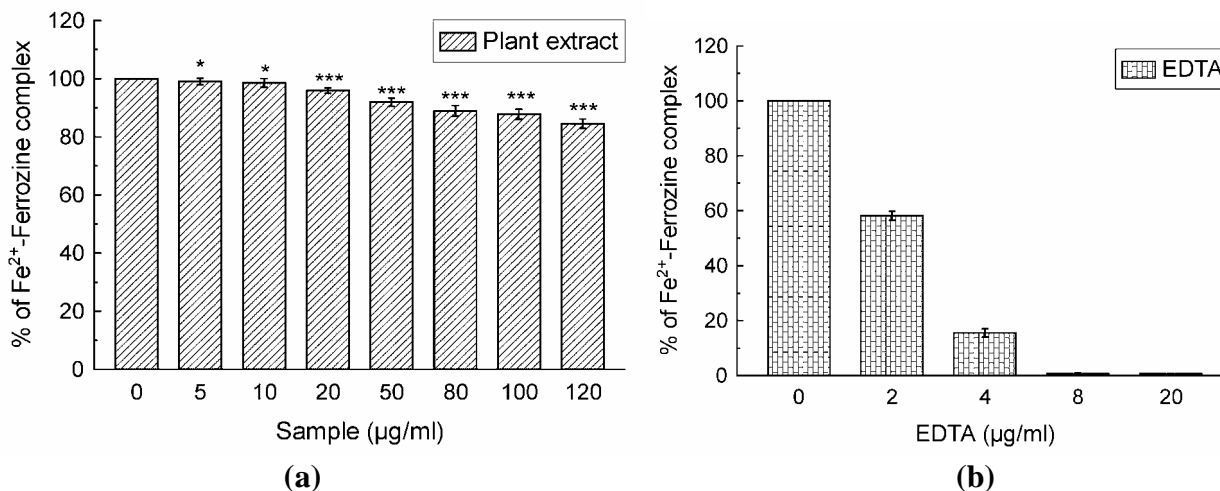
**Hypochlorous acid scavenging:** Figure 7 shows the dose-dependent hypochlorous acid scavenging activity of *H. indicus* extract compared to that of ascorbic acid. The results indicate that the extract scavenged hypochlorous acid as efficiently ( $IC_{50} = 276.45 \pm 6.49 \mu\text{g/ml}$ ) as ascorbic acid ( $IC_{50} = 235.95 \pm 5.75 \mu\text{g/ml}$ ) (Table 1).



**Figure 7. HOCl scavenging:** Hypochlorous acid scavenging activities of *Hemidesmus indicus* plant extract and the standard ascorbic acid. All data are expressed as mean  $\pm$  S.D. (n=6). \*\* $p < 0.01$  and \*\*\* $p < 0.001$  vs 0  $\mu\text{g/ml}$ .

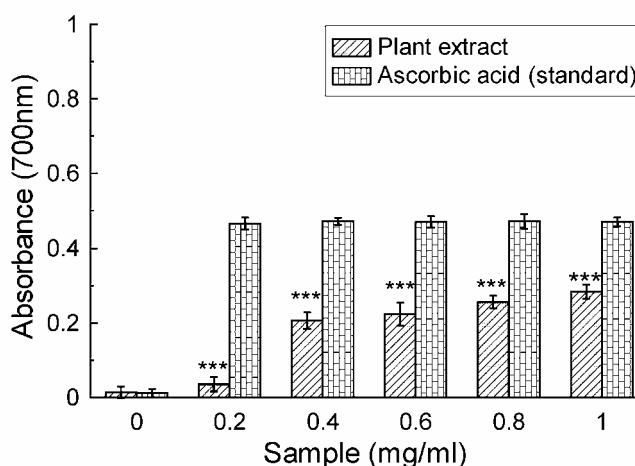


**Fe<sup>2+</sup> chelating:** The results [Figure 8(a) and (b)] demonstrated that the sample extract and the standard EDTA compete individually with ferrozine in forming the ferrozine-Fe<sup>2+</sup> complex, thus inhibiting the formation of the complex. The result is also corroborated by the IC<sub>50</sub> values (Table 1) of the plant extract and EDTA that were 655.48 ± 10.9 µg/ml and 1.27 ± 0.05 µg/ml, respectively.



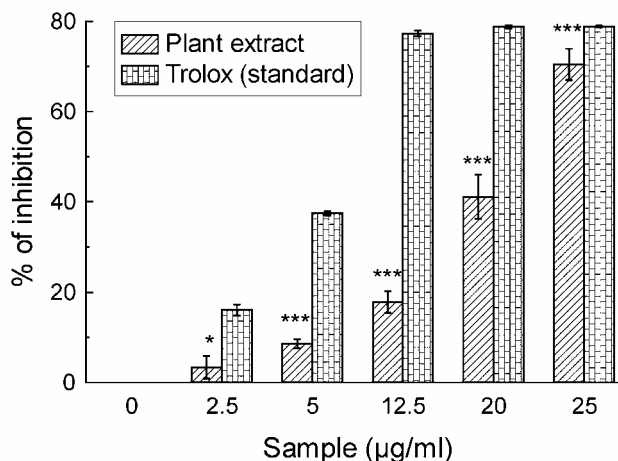
**Figure 8. Fe<sup>2+</sup> chelating:** Effects of (a) *Hemidesmus indicus* plant extract and (b) standard EDTA on ferrozine-Fe<sup>2+</sup> complex formation. The data are expressed as percentage inhibition of chromogen formation. The results are mean ± S.D. of six parallel measurements. \**p* < 0.05 and \*\*\**p* < 0.001 vs 0 µg/ml.

**Reducing power:** In the presence of *H. indicus* extract and the reference compound ascorbic acid, Fe<sup>3+</sup> was transformed to Fe<sup>2+</sup>, as illustrated in Figure 9. At 0.2 mg/ml, the absorbances of the plant extract and ascorbic acid were 0.036 and 0.466, respectively, while at 1 mg/ml, the absorbances of both extract and ascorbic acid were close.



**Figure 9. Reducing power:** The reductive abilities of *Hemidesmus indicus* extract and the standard ascorbic acid. The absorbance (A<sub>700</sub>) was plotted against concentration of sample. Each value represents mean ± S.D. (n=6). \*\*\**p* < 0.001 vs 0 mg/ml.

**Lipid peroxidation inhibition:** Figure 10 indicates the dose dependent inhibition of lipid peroxidation of the plant extract and the standard trolox. The IC<sub>50</sub> values from the Table 1 also shows that the sample (IC<sub>50</sub> = 25.93 ± 1.27 µg/ml) has got an activity merely comparable to the standard (IC<sub>50</sub> = 6.76 ± 0.17 µg/ml).



**Figure 10. Lipid peroxidation inhibition:** Lipid peroxidation inhibition activities of *Hemidesmus indicus* plant extract and the standard trolox. All data are expressed as mean  $\pm$  S.D. (n=6). \* $p < 0.05$  and \*\*\* $p < 0.001$  vs 0  $\mu\text{g/ml}$ .

**Determination of total phenolic content:** Antioxidant capacity can be associated directly to the phenolic content. The total phenolic content was  $55.7 \pm 0.001$  mg/ml gallic acid equivalent per 100 mg plant extract.

**Determination of total flavonoid content:** The total flavonoid content of the 70% methanolic extract of *H. indicus* was  $174.6 \pm 0.007$  mg/ml quercetin per 100 mg plant extract.

## Discussion

Antioxidants are compounds that hold back the oxidation of essential biological macromolecules by inhibiting the propagation of the oxidizing chain reaction. Keeping in mind the adverse side effects of synthetic antioxidants, researchers have channelized their interest in isolating natural antioxidants (23). In the living system, oxidative stress is initiated by free radicals which are generated constantly and seek stability through electron pairing with biological macromolecules such as proteins, lipids and DNA of healthy human cells and can cause various diseases. Natural antioxidants are very effective to control the oxidative stress and hence prevent the initiation of disease propagation. The present study focused on the evaluation of antioxidant and free radical scavenging activity of 70% methanol extract of the root of *H. indicus*.

The total antioxidant activity of the extract was calculated based on the decolorization of the  $\text{ABTS}^{\cdot+}$  produced by reaction of ABTS with potassium persulfate, and measured spectrophotometrically at 734 nm. The addition of the plant extract and trolox convert this pre-formed radical cation to ABTS in a concentration dependant manner. The obtained TEAC value reflects the antioxidant potency of the extract.

**Table 1** - Scavenging of reactive oxygen species and iron chelating activity (IC<sub>50</sub> values) of *Hemidesmus indicus* and reference compounds

Activity	Extract/Reference	IC <sub>50</sub> (#)
Hydroxyl radical (OH <sup>•</sup> ) scavenging	<i>Hemidesmus indicus</i>	257.78 ± 7.22 (6)
	Manitol	571.45 ± 20.12 (6) ***
Superoxide anion (O <sub>2</sub> <sup>•-</sup> ) scavenging	<i>Hemidesmus indicus</i>	58.4 ± 3.93 (6)
	Quercetin	42.06 ± 1.35 (6) ***
Nitric oxide radical (NO) scavenging	<i>Hemidesmus indicus</i>	38.24 ± 16.7 (6)
	Curcumin	90.82 ± 4.75 (6) **
Peroxynitrite (ONOO <sup>-</sup> ) scavenging	<i>Hemidesmus indicus</i>	1.91 ± 0.31 (6)
	Gallic acid	0.88 ± 0.06 (6) ***
Singlet oxygen ( <sup>1</sup> O <sub>2</sub> ) scavenging	<i>Hemidesmus indicus</i>	421.47 ± 54.81 (6)
	Lipoic acid	46.15 ± 1.16 (6) ***
Hypochlorous acid (HOCl) scavenging	<i>Hemidesmus indicus</i>	276.45 ± 6.49 (6)
	Ascorbic acid	235.95 ± 5.75 (6) ***
Iron chelating	<i>Hemidesmus indicus</i>	655.48 ± 10.9 (6)
	EDTA	1.27 ± 0.05 (6) ***
Lipid peroxidation inhibition	<i>Hemidesmus indicus</i>	25.93 ± 1.27 (6)
	Trolox	6.76 ± 0.17 (60) ***

# Units of IC<sub>50</sub> for all activities are µg/ml, except Peroxynitrite scavenging, where the units are mg/ml. Data are expressed as mean ± S.D. Data in parenthesis indicate number of independent assays. EDTA, Ethylenediamine tetraacetic acid. \*\* p< 0.01. \*\*\* p< 0.001 vs *Hemidesmus indicus*.

The most detrimental of the free radicals formed in biological systems is the hydroxyl radical that causes enormous damage on biomolecules of the living cells (24). In course of the Fenton reaction, hydroxyl radicals are formed by incubating Ferric-EDTA with ascorbic acid and H<sub>2</sub>O<sub>2</sub> at pH 7.4 that cause 2-deoxy-2-ribose damage and generate malondialdehyde (MDA) like product. This compound forms a pink chromogen upon heating with TBA at low pH. As the *H. indicus* extract or standard mannitol is added to the reaction mixture the hydroxyl radicals are scavenged and thereby sugar damage can be blocked. The results indicate that the plant extract is a better hydroxyl radical scavenger than standard mannitol.

Superoxide anion is also another harmful reactive oxygen species as it damages cellular components in biological systems. PMS-NADH coupling reaction accelerates the yield of superoxide radicals from dissolved oxygen. These superoxide radicals can be measured by its ability to reduce NBT. The ability of the plant extract and the reference compound quercetin to quench superoxide radicals from reaction mixture is reflected in the decrease of the absorbance at 560 nm. According to the Figure and Table, it can be put forward that the plant extract is not more potent than the standard, yet a scavenger of superoxide radical.

Nitric oxide radical plays an important role in various types of inflammatory processes. Persistent production of nitric oxide radical result in direct tissue toxicity and contribute to the vascular collapse associated with septic shock, whereas chronic emergence of nitric oxide radical is linked with various carcinomas and inflammatory conditions including juvenile diabetes, multiple sclerosis, arthritis and ulcerative colitis (25). The present study showed that the generation of nitrite from nitric oxide evolved from sodium nitroprusside is restrained by *H. indicus* root extract in a better way than the standard curcumin, as can be found from the Figure and Table.

Furthermore, the lethal consequence of NO increases significantly upon reaction with superoxide radical resulting the formation of highly reactive peroxy nitrite anion (ONOO<sup>-</sup>), especially its protonated form, peroxy nitrous acid (ONOOH) (26). However, as reflected in Figure 5 and Table 1, no highly considerable result was obtained for the scavenging effect of the studied extract compared to the standard gallic acid.

Hydrogen peroxide is a weak oxidizing agent that deactivates few enzymes, crosses cell membrane directly and thus many of its toxic effects are clarified. But, as compared to the standard sodium pyruvate, the activity of the plant extract was found non significant to be represented.

A high energy form of oxygen, singlet oxygen was generated in the skin upon UV-radiation and it induces hyperoxidation, oxygen cytotoxicity and decreases the antioxidant activity. The Figure 6 and the IC<sub>50</sub> values from Table 1 indicated that the *H. indicus* extract has singlet oxygen scavenging activity but poor compared to standard lipoic acid.

Hypochlorous acid is another harmful ROS. At the sites of inflammation, the oxidation of Cl<sup>-</sup> ions by the neutrophil enzyme myeloperoxidase results in the production of this ROS, which breaks down the heme prosthetic group and inactivates the antioxidant enzyme catalase. The HOCl scavenging activity of the plant extract signified by the inhibition of catalase deactivation, was found to be nearly equivalent to the standard ascorbic acid.

Iron is a major factor that invigorates lipid peroxidation by decomposing lipid hydro-peroxides into peroxy and alkoxy radicals that can also be held responsible for lipid peroxidation. The metal chelating capacity is noteworthy since it reduces the concentration of the catalyzing transition metal in lipid peroxidation. The results demonstrated that the formation of ferrozine-Fe<sup>2+</sup> complex is inhibited in the presence of test and reference compound thus intending that the extract has, but low iron chelating property, reportedly not as efficient as the standard EDTA.

As illustrated in Figure 9, Fe<sup>3+</sup> was transformed to Fe<sup>2+</sup> in the presence of *H. indicus* extract and the reference compound ascorbic acid to measure the reductive capability. At 0.2 mg/ml, the absorbances of the plant extract and ascorbic acid were 0.036 and 0.466, respectively, while the absorbance of the sample gradually increased to be close to the standard at 1.0 mg/ml. This result indicates that the activity of the extract, although quite good, still not equivalent to ascorbic acid.

Lipid peroxidation inhibition establishes the antioxidant capacity of a substrate. Through catalysis, Fe<sup>3+</sup>/Fe<sup>2+</sup> system generates ferri-perferry complex or hydroxyl radicals that decompose lipid hydro-peroxides into peroxy and alkoxy radicals. These radicals damage biological molecules, when it reacts with polyunsaturated fatty acid moieties of cell membrane phospholipids that eventually yield carbonyl products like malondialdehyde (MDA), which generate a pink chromogen with TBA. Thus, the lipo-peroxidation inhibitory effect of *H. indicus* root extract can be indirectly symbolized as the antioxidant capacity of the plant, as is also reflected in Figure 10.

Being stable metal chelators and good reducing agents through hydrogen donation, phenolic compounds are very important as plant constituent (27). They can also have scavenging ability due to their hydroxyl groups. In addition, flavonoids show their antioxidant feat through scavenging or chelating processes (28). The presence of both these compounds in human diet and their effects on human nutrition and health are major. The phytochemical analysis showed that *H. indicus* root extract has  $55.7 \pm 0.001$  mg/ml gallic acid equivalent phenolic content and  $174.6 \pm 0.007$  mg/ml quercetin equivalent flavonoid content per 100 mg plant extract. The results indicate that *H. indicus* root extract contains significant amount of flavonoids and phenolic content.

### Conclusions

On the basis of the results obtained in the present study, it is concluded that a 70% methanolic extract of *Hemidesmus indicus* root, which contains large amounts of flavonoids and phenolic compounds, exhibits reasonable antioxidant and free radical scavenging activities. It also chelates iron and has reducing power. These *in vitro* assays indicate that this plant extract contains constituents that can be a significant source of natural antioxidant, as also been reported earlier (6, 9, 17), which might be helpful in preventing the progress of various oxidative stresses. However, the possible anti-cancer and the immunomodulatory effects of the plant still require attention. Therefore, further investigation is needed to study those properties and the components of the plant responsible for it. Furthermore, the various activities of this plant, apart from those been reported (11, 12), must be assessed in *in vivo* system to facilitate it for clinical trials.

### Acknowledgements

The authors would like to thank Mr. Amartya Sen and Mr. Ranjit Das for assistance.

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