

Assessment of antioxidant potentials of free and bound phenolics of *Hemidesmus indicus* (L) R.Br against oxidative damage

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ABSTRACT

Background: *Hemidesmus indicus* R.Br. is a twining shrub commonly found in India, which was known to have wide pharmacological actions. 2-hydroxy-4-methoxy-benzoic acid (HMBA) and a number of pregnane glycosides were believed to be responsible for its various bioactivities. Until now, there are no reports regarding the antioxidant properties of phenolics from *H. indicus*. **Objective:** To establish the role of phenolics in the properties of *H. indicus*. **Materials and Methods:** Hemidesmus free phenolic fraction (HDFP) and Hemidesmus bound phenolic fraction (HDBP) have been isolated from *H. indicus*, and the antioxidant activity was evaluated for inhibition of lipid peroxidation, DNA protection, free radical scavenging (FRS), reducing power and cytoprotective activities. **Results:** HDFP and HDBP exhibited potent inhibition of lipid peroxidation (IC_{50} - 19.5 ± 0.5 and 21.7 ± 0.5 μ g gallic acid equivalent - GAE/mL), FRS (IC_{50} - 7 ± 0.2 and 8.6 ± 0.2 μ gGAE/mL), reducing power (110.3 ± 2 and 33.5 ± 1 U/g) and red blood cell protection (14.8 ± 0.4 and 14.5 ± 0.5 μ g GAE/mL). HDFP is constituted by gallic (18%), caffeic (17%), ferulic acids (16%) and HDBP by syringic acid (35%) as major phenolic acids. Besides, both HDFP and HDBP contained significant levels of HMBA; in HDFP (10%) and HDBP (57%), respectively. Results indicated a 34- and 27-folds better contribution to the antioxidant activity by HDFP and HDBP, respectively, than that of HMBA. **Conclusion:** Potent antioxidant activities of phenolics may be one of the mechanisms by which *H. indicus* is effective against several health disorders as encountered in traditional medicines.

Key words: Antioxidant activity, bound phenolics, DNA protection, free phenolics, red blood cell protection

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INTRODUCTION

The traditional Indian medicine and the use of plant drugs as alternatives against various diseases receive considerable attention in recent days. *Hemidesmus indicus* (L) R.Br. belonging to the Asclepiadaceae family, commonly known as Indian sarsaparilla, is a twining shrub that has been used as folk medicine and as an ingredient in Ayurvedic and Unani preparations against diseases of blood, inflammation, etc.^[1] Some important chemical constituents of the root include 2-hydroxy-4-methoxy-benzoic acid,

b-sitosterol, α - and β -amyrins, lupeol, tetracyclic triterpene alcohols, resin acids, fatty acids, tannins, glycosides and a ketone.^[2-6] The root has been used for treating blood diseases, diarrhea, respiratory disorders, skin diseases, syphilis, fever, bronchitis, asthma, eye diseases, epileptic fits in children, kidney and urinary disorders, loss of appetite, burning sensation, rheumatism and in gastric ailments.^[7] The root extract also has potent chemoprotective anti-inflammatory, antipyretic and antioxidant properties.^[8-10]

The role of free radicals in many disease conditions has been well established. Several biochemical reactions in our body generate reactive oxygen species and these are capable of damaging crucial biomolecules. If they are not effectively scavenged by cellular constituents, they lead to disease conditions.^[11] In recent years, one of the areas that attracted a great deal of attention is antioxidants in the

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control of degenerative diseases in which oxidative damage has been implicated.

Phenolic compounds have attracted the attention of food and medical scientists because of their strong *in vitro* and *in vivo* antioxidant activities.^[12,13] Actually, numerous reports on the antioxidant activities of *H. indicus* are found in the literature.^[6,9,14] However, there is no information on the phenolic contents of *H. indicus* and its antioxidant activities against oxidative stress. Hence, the current study investigates the role of phenolics in the antioxidant properties of *H. indicus*. We report the isolation and characterization of Hemidesmus free phenolic fraction (HDFP) and Hemidesmus bound phenolic fraction (HDBP) as well as their antioxidant potency. High-performance liquid chromatography (HPLC) analysis of the fractions were also carried out to characterize the phenolic fractions, which would facilitate the identification of phenolic acids in the free and bound phenolic fractions.

MATERIALS AND METHODS

H. indicus root

H. indicus roots were purchased from the local market (Devaraja Market, Mysore, Karnataka, India) and were identified and authenticated from the Department of Botany, University of Mysore, Mysore. The *H. indicus* roots were cleaned, separated from the root and dried in a hot air oven for 24 h at 55°C, powdered and stored in air-tight containers at 10°C till further use.

Isolation of free and bound phenolics of *H. indicus*

Free phenolics were isolated according to the method of Siddaraju and Shylaja.^[15] Ten grams of defatted *H. indicus* powder was extracted with 70% ethanol (w/v), (4 × 250 mL, 1 h each) and the supernatants were obtained by centrifugation at 600 g for 20 min at room temperature and concentrated. The pH was adjusted to 2–3 with 4 M HCl. Phenolic acids were separated by ethyl acetate phase separation (5 × 250 mL) and the pooled fractions were treated with anhydrous di-sodium sulfate to remove moisture, filtered and evaporated to dryness. The phenolics were reconstituted in methanol and designated as HDFP.

The bound phenolics of *H. indicus* were extracted according to the method of Nordkvist *et al.*^[16] Ten grams of *H. indicus* powder was extracted with 70% ethanol (w/v) (4 × 250 mL) to remove the free phenolics. The dried residue was extracted with 1 M sodium hydroxide (2 × 200 mL, 2 h each) containing 0.5% sodium borohydride under nitrogen atmosphere, and the clear supernatants were collected by centrifugation at 600 g at room temperature for 10 min. The combined supernatants were acidified with 4 M HCl

to pH 1.5 and the phenolics were extracted in the same way as free phenolics and designated as HDBP.

Determination of total phenolics

The total phenolic contents of HDFP and HDBP were determined according to Folin-Ciocalteu method.^[17] Samples (0.1 mL) were mixed with 1 mL of two-folds diluted Folin-Ciocalteu reagent and 2 mL of 10% sodium carbonate solution. The absorbance was measured at A 765 nm with a Shimadzu UV-visible spectrophotometer after incubating for 30 min at room temperature. Gallic acid was used as the reference standard and results were expressed as gallic acid equivalents (GAE) in milligrams per gram dry weight (d.w.) of sample.

Identification of phenolic acids by high-performance liquid chromatography

The phenolic acid components of the phenolic fractions were identified by HPLC as previously standardized in our laboratory.^[15] The HPLC system (Shimadzu LC-10A, Kyoto, Japan) was equipped with a dual pump LC-10AT binary system, a UV detector SPD-10A and a phenomenex ODS-2 column (4.6 × 250 mm) and the data were integrated by Shimadzu Class VP series software. A solvent system consisting of water:acetic acid:methanol (80:5:15 v:v:v) was used as the mobile phase at a flow rate of 1 mL/min. Eluants were monitored continuously in a UV detector at A 280 nm and results (mg/g d.w.) were obtained by comparison of the peak areas of the samples with those of the standards.

Determination of antioxidant activity

Inhibition of lipid peroxidation

The effect of HDFP and HDBP on FeSO₄-H₂O₂-stimulated lipid peroxidation in rat liver homogenate was determined by malondialdehyde (MDA)-thiobarbituric acid (TBA) adduct formation.^[18] Rat liver was homogenized with 50 mM sodium phosphate buffer (pH 7.4) using a homogenizer equipped with a Teflon pestle. A mixture containing 0.5 mL of 10% (w/v) liver homogenate in potassium phosphate buffer (pH 7.4), 5 nM FeSO₄, 0.3% H₂O₂ and various concentrations of HDFP and HDBP (5–25 µg/mL GAE) were incubated for 10 min at 37°C. The level of Thiobarbituric Acid Reactive Substances (TBARS) production was measured and the percent inhibition of lipid peroxidation was calculated from the following equation:

$$\% \text{ inhibition} = [(A_{\text{Control}} - A_{\text{Sample}}) / A_{\text{Control}}] \times 100$$

DNA protection assay

The DNA protective effect of the phenolic fractions was determined by electrophoretic method using calf thymus DNA.^[19] Calf thymus DNA (1 µg) was subjected

to oxidation by Fenton's reagent (30 mM H_2O_2 , 50 μM ascorbic acid and 80 μM FeCl_3) in the presence and absence of the phenolic fractions. Relative differences in the migration between the native and the oxidized DNA was ensured on 1% agarose gel electrophoresis after staining with ethidium bromide. DNA on degradation moves faster on the gel while the recovery of the same on treatment with HDFP and HDBP indicated normalized band pattern. The intensity of the bands was determined by gel documentation (Herolab, Germany) followed by the use of Easywin software. Protection to DNA was calculated based on the DNA band with the migration rate corresponding to that of the native DNA considering the extent of inhibition of migration. Slower migration results in the retention of the bands; percent retention is proportional to the ability of the extracts inhibiting migration, which is due to oxidation.

Free radical scavenging activity

The effect of HDFP and HDBP on the DPPH radical was measured according to the method of Lai *et al.*^[20] Aliquots of HDFP and HDBP (2–10 $\mu\text{g}/\text{mL}$ GAE) and standard antioxidants - BHA were taken in 100 mM Tris-HCl buffer (800 mL, pH 7.4) and 1 mL of 500 mM DPPH in methanol (final concentration 250 $\mu\text{mol}/\text{L}$) was added. The mixture was shaken vigorously and allowed to stand for 20 min at room temperature in the dark. Changes in absorbance were measured at A 517 nm. The free radical scavenging (FRS) activity was expressed as the inhibition percentage of FRS and was calculated using the following formula:

$$\text{FRS (\%)} = [1 - A_{\text{Sample (517 nm)}} / A_{\text{Control (517 nm)}}] \times 100$$

Measurement of reducing power

The reducing power of HDFP and HDBP were determined according to the method of Yen and Chen.^[21] Aliquots of HDFP and HDBP (5–25 $\mu\text{g}/\text{mL}$ GAE) were mixed with 0.2 M phosphate buffer, pH 6.6 and 1% potassium ferricyanide. This mixture was incubated at 50°C for 20 min. An equal volume of 10% Trichloroacetic acid (TCA) was added to the mixture, which was then centrifuged at 1000 g for 10 min. The supernatant was mixed with distilled water and 0.1% ferric chloride at a ratio of 1:1:2 (v:v:v) and absorbance was read at A 700 nm. Higher absorbance of the reaction mixture indicated a higher reducing power.

Inhibition of hemolysis

Red blood cells (RBC) were obtained from healthy donors after taking their consent. Heparinized blood was centrifuged at 3000 g for 10 min. After removal of plasma and buffy coat, the RBCs were washed three times with phosphate buffer containing NaCl (150 mM), NaH_2PO_4 (1.9 mM), Na_2HPO_4 (8.1 mM), pH 7.4 at room temperature and resuspended in phosphate-buffered saline four-times

its volume for subsequent analyses.^[22] The RBCs (10%, v/v) were incubated with phenolic fractions in PBS in the presence of $\text{FeSO}_4\text{-H}_2\text{O}_2$ in a shaking bath for 15 min at 37°C. After 15 min of incubation, RBC suspension was centrifuged at 3000 g for 10 min and the hemolysis was measured spectrophotometrically at A 540 nm as hemoglobin (Hb) release from cells gives colour absorbance in the supernatant.^[23]

Statistical analysis

All the experiments were carried out in triplicate ($n = 3$) and the results are expressed as mean $\pm$ standard deviation (SD). The correlation coefficient R, between phenolics and antioxidant activity, was determined using SPSS (version 10 for windows XP, SPSS Inc., Chicago, Ill., USA).

RESULTS AND DISCUSSION

Total phenolic content

H. indicus has been reported to have health beneficial effects from time immemorial and compounds such as 2-hydroxy-4-methoxybenzaldehyde and its acid derivative 2-hydroxy-4-methoxybenzoic acids (HMBA) have been reported to be the active constituents in *H. indicus*. The current study particularly addresses the role of phenolics. Data confirmed that, as indicated by earlier workers, *H. indicus* contains HMBA as a major component; besides, there were abundant contents of other phenolic acids also. Hence, free and bound phenolics were isolated and the total phenolic content was determined spectrophotometrically according to Folin-Ciocalteau method and calculated as GAE. The free phenolic content was 4.3 ± 0.8 mg GAE/g d.w., while the bound phenolic content was 1 ± 0.2 mg GAE/g d.w.

Phenolic acid composition in hemidesmus free and bound phenolic fractions

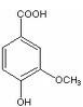
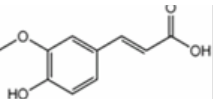
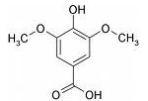
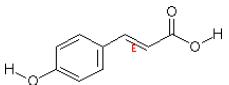
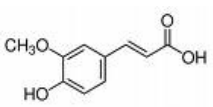
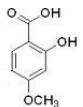
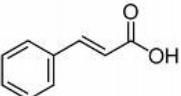
The nature of the phenolics present in the free and bound phenolic fractions was found to be responsible for their potency; hence, they were analyzed on HPLC, and the nature of the phenolic acids (mg/g) present in HDFP and HDBP are given in Table 1 and the chromatograms are represented in Figure 1a and b.

Antioxidant activity

Inhibition of lipid peroxidation

Both the phenolic fractions of *H. indicus* inhibited the $\text{OH}^\bullet$ radical-mediated lipid peroxidation in a concentration-dependent manner, which was determined by the amount of TBARS in the liver homogenate, as given in Figure 2a. The addition of 5–25 $\mu\text{g}/\text{mL}$ GAE of HDFP and HDBP to the rat liver homogenate significantly reduced TBARS formation. The half-inhibition concentration (IC_{50}) of HDFP and HDBP was 10.7 ± 0.9 and 11.6 ± 1 μg GAE/mL, respectively. The results revealed that HDFP and

Table 1: The antioxidant potency and phenolic content of hemidesmus free phenolic and bound phenolic fraction

Samples	Structures	Antioxidant activity* IC50 (µg/mL)	Phenolic content mg/g	
			HDFP	HDBP
Gallic acid (3,4,5-trihydroxybenzoic acid)		1.1 ± 0.1 ^a	0.365	0.04
Protocatechuic acid (3,4-dihydroxybenzoic acid)		1.35 ± 0.1 ^a	0.084	-
Gentisic acid (2,5-dihydroxy benzoic acid)		1.9 ± 0.1 ^a	1.35	-
Vanillic acid (3 methoxy, 4 hydroxybenzoic acid)		6.6 ± 0.3 ^b	0.094	0.04
Caffeic acid (3,4-dihydroxy cinnamic acid)		3.0 ± 0.2 ^b	0.121	0.128
Syringic acid (4-hydroxy-3,5-dimethoxybenzoic acid)		1.8 ± 0.1 ^b	0.089	-
p-Coumaric acid (p-Hydroxy cinnamic acid)		64.9 ± 1 ^a	0.025	-
Ferulic acid (4-hydroxy-3-methoxy cinnamic acid)		4.6 ± 0.2 ^a	0.25	-
HMBA (2-hydroxy 4-methoxy benzoic acid)		213.2 ± 2 ^b	0.103	0.387
Cinnamic acid (3-phenyl-2-propenoic acid)		81 ± 1.2 ^a	0.045	0.142

HDFP and HDBP containing different phenolic acids given with their yield (mg/g) and structure. IC₅₀ (concentration of sample or standard required to scavenge 50% of the DPPH free radicals) values of HDFP and HDBP were compared with the standard antioxidants. The amount of the samples is given in µg GAE of phenol content and the activity is expressed in terms of percent scavenging activity ± SD (n = 3). *P < 0.001, ^bP < 0.01, ^cP < 0.05. Samples were analyzed statistically employing Origin 6.1 SPSS (version 10 for windows XP, SPSS Inc.); *Antioxidant activity measured as free radical scavenging activity, HDFP = Hemidesmus free phenolic fraction, HDBP = Hemidesmus bound phenolic fraction

HDBP have an approximately equal capacity to prevent the oxidative deterioration of polyunsaturated lipids.

DNA protection ability of hemidesmus free and bound phenolic fraction

Oxidative stress induces several types of damage in the DNA, such as strand scissions, base damage, sugar damage, etc. Figure 2b shows the effect of the HDFP and HDBP fractions of *H. indicus* in inhibiting DNA strand cleavage by the Fenton reaction-mediated hydroxyl radical (HO[•]). The antioxidant properties of a compound may be evaluated

by monitoring the HO[•]-induced single strand breaks in DNA in the presence of that compound. Ascorbate and Fe (III) together form a H₂O₂ and HO[•] generating system that readily induces DNA strand cleavage. The HDFP and HDBP fractions of *H. indicus* (2 and 4 µg GAE) along with DNA prevented the damage of DNA as 90% of the native DNA was unaffected. Data thus demonstrated scavenging of HO[•] by *H. indicus*. In general, HO[•] is a highly reactive reactive oxygen species formed in biological systems. It can react with biological molecules such as lipids, sugars, phospholipids, proteins and nucleic

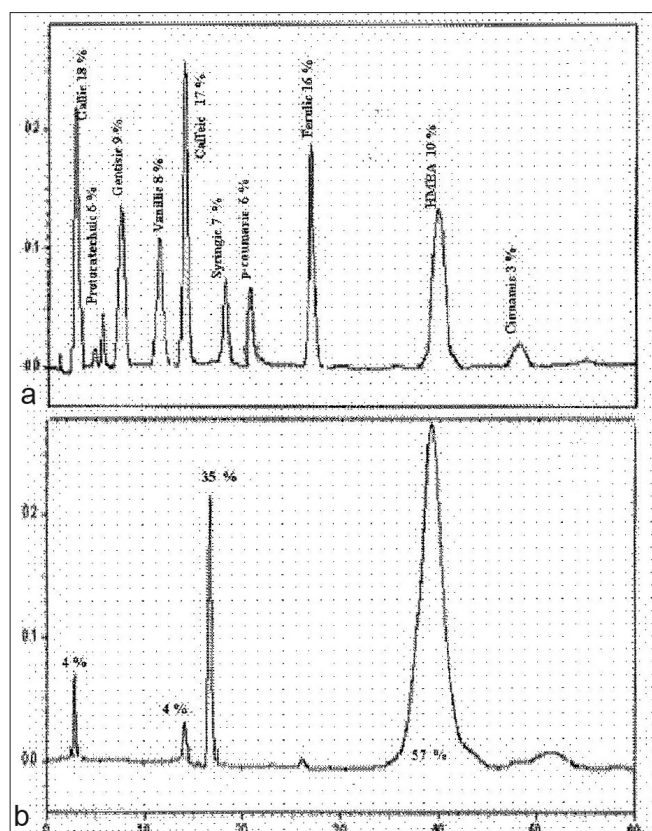


Figure 1: High-performance liquid chromatography analysis of phenolic acid constituents in (a) free (HDFP) and (b) bound (HDBP) phenolic fractions. A 20 μ L dilution of mg/mL standard phenolic acids was loaded independently and the specific retention time (min) was established. Phenolic acids in each fraction were identified comparing their retention time with that of the known standards

acids.^[24-26] The antioxidant property of these phenolic fractions had been attributed to their FRS and antioxidant activity.^[11] This would suggest that phenolic fractions could also be protecting the DNA by a similar mechanism.

FRS activity

DPPH analysis is one of the tests used to determine the ability of the phenolic fractions to act as donors of hydrogen atoms. The free and bound phenolic fractions showed a significant effect in inhibiting DPPH, reaching up to 80% and 64% at the concentration of 10 μ g/mL, with an IC_{50} of 6.2 ± 0.2 and 7.8 ± 0.2 μ g GAE/mL, respectively [Figure 2c]. Antioxidants cease the free radical chain of oxidation and donate hydrogen from the phenolic hydroxyl groups. Therefore, formed stable end-product does not permit further oxidation of the lipid. The free and bound phenolic fractions are therefore free radical inhibitors or scavengers as well as primary antioxidants that react with free radicals, which may limit the free radical damage occurring in the human body.

Reducing power ability

Figure 2d illustrates the reductive capability of extracts in a

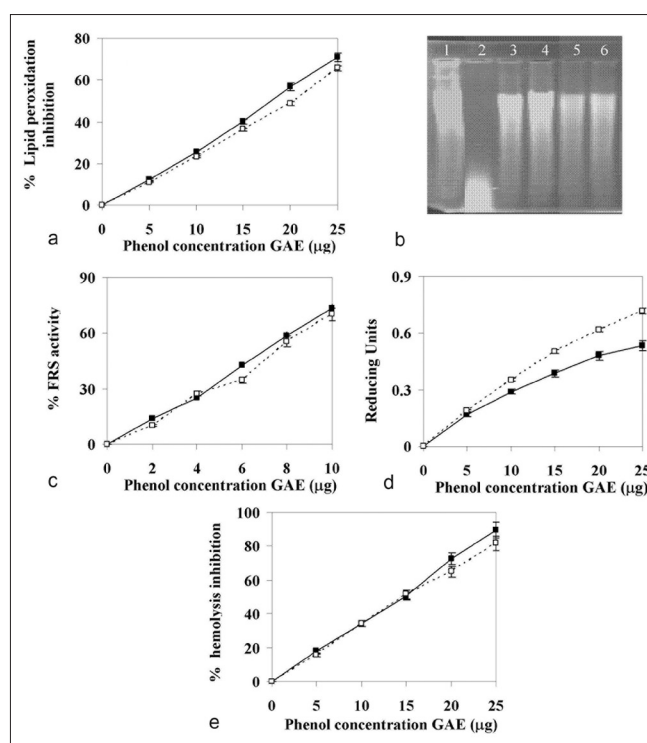


Figure 2: Antioxidant activity of HDFP (■) and HDBP (□). (a) Inhibition of TBARS formation. (b) DNA protection ability -1 μ g of native calf thymus DNA in (Lane 1); DNA treated with Fenton's reagent (Lane 2); DNA pretreated with 2 μ g of HDFP (Lane 3); 4 μ g of HDFP (Lane 4); 2 μ g of HDBP (Lane 5); 4 μ g of HDBP (Lane 6). (c) Dose-dependent DPPH scavenging activity. The IC_{50} value was calculated from this graph [Table 1]. (d) Dose-dependent reducing power activity. (e) Inhibition of red blood cell lysis. Values represent mean $\pm$ standard deviation ($n = 3$)

concentration-dependent manner. Fe^{3+} - Fe^{2+} transformation in the presence of phenolic fractions was investigated. HDFP and HDBP showed a reducing power of 215.6 ± 2.5 and 28.9 ± 1 U/g, respectively. The reducing properties are generally associated with the presence of reductones, which has the ability to break the free radical chain by donating a hydrogen atom or reacting with certain precursors of peroxide to prevent peroxide formation.^[27] It is presumed that the phenolic compounds may act in a similar fashion as reductones by donating electrons and reacting with free radicals to convert them to more stable products and terminating the free radical chain reaction.

Inhibition of hemolysis

It is known that the oxidation of polyunsaturated fatty acids in biological membranes can lead to the formation and propagation of lipid radicals, uptake of oxygen, rearrangement of the double bonds in unsaturated lipids and even destruction of membrane lipids. Many of these biochemical activities can lead to the production of breakdown products that are highly toxic to most cell types. Lipid oxidation of the erythrocyte membrane induced by

Fe^{2+} - H_2O_2 causes membrane damage and, subsequently, hemolysis.^[28] To study the antioxidant effects of phenolic fractions on RBC, we used the model of oxidative stress-induced damage of RBC by Fe^{2+} - H_2O_2 . Fe^{2+} - H_2O_2 caused considerable RBC lysis, which was inhibited significantly by both the fractions in a dose-dependent manner [Figure 2e]. The free and bound phenolic fractions showed a similar protective activity, providing about 50% inhibition of the RBC lysis at a concentration of 11.3 ± 0.3 and 17.2 ± 0.5 μg GAE/mL, respectively. Our results suggest that these fractions may inhibit oxidative damage to lipids in cellular membranes.

Phenolics and bioactivity

The antioxidant activity based on the FRS activity indicated an IC_{50} of 1.1, 1.3, 1.9, 6.6, 3, 1.8, 64.9, 4.6, 213.2 and 81 $\mu\text{g}/\text{mL}$ for gallic, protocatechuic, gentisic, vanillic, caffeic, syringic, p-coumaric, ferulic, HMBA and cinnamic acids, respectively [Table 1]. Based on the antioxidant activity of individual phenolic acids, the precise contribution from phenolic acids was calculated and compared with that of the reported HMBA. Studies for the first time revealed the significant contribution of phenolic acids to the antioxidant activity of *H. indicus*. These components may thus be responsible for the prevention of oxidative stress-induced diseases such as cancer, ulcer, etc. Considering this antioxidant activity of phenolic acids in HDBP, 26%, 5%, 56%, 1%, 3%, 4% and 4% activity in total was found to be contributed by gallic, protocatechuic, gentisic, vanillic, caffeic, syringic and ferulic acids respectively. However, in HDBP, 41%, 7%, 48%, 2% and 2% activity was contributed by gallic, vanillic, caffeic, HMBA and cinnamic acids, respectively [Figure 3].

The correlation analysis between phenolic contents and

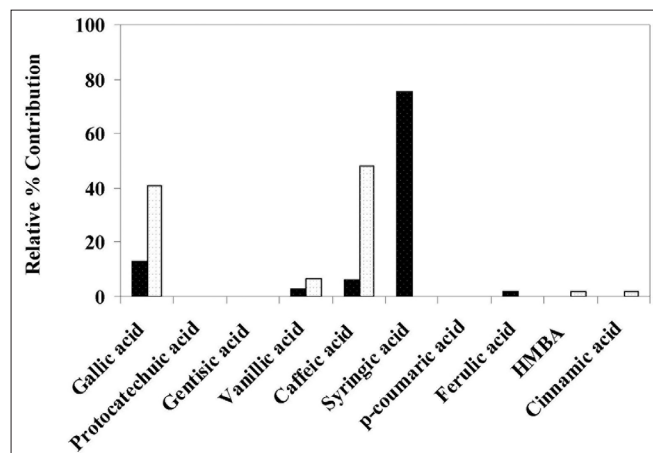


Figure 3: Relative percentage contribution of individual phenolic acid towards the antioxidant activity. Relative percent contribution of each individual phenolic acids found in HDBP (■) and HDBP (□) against the antioxidant activity. Antioxidant activity was measured as the free radical scavenging activity

results of antioxidant assays demonstrated a strong positive association. There was a direct relationship between total phenolic content and DPPH radical activity; the IC_{50} values were statistically significant $P < 0.001$ and had high regression coefficients in FRS activity ($R^2 = 0.980, 0.997$), reducing power activity ($R^2 = 0.985, 0.993$), inhibition of lipid peroxidation ($R^2 = 0.997, 0.995$) and inhibition of hemolysis ($R^2 = 0.997, 0.998$). These results suggest that phenolics play a significant role in the antioxidant property and hence can be attributed to various health beneficial properties of *H. indicus*

CONCLUSION

In conclusion, from the above investigation, using several *in vitro* models, the phenolic fractions of *H. indicus* have been known to effectively prevent lipid peroxidation, damage of calf thymus DNA and RBC membrane. The result suggests that phenolics play a significant role in the antioxidant property and hence can be attributed to various health beneficial properties. Free radical neutralizing and, mainly, antioxidant properties may be attributed to phenolic acids of *H. indicus* phenolic fractions. The role of HMBA also in health beneficial properties may not be ruled out since it can interfere at several steps of pathogenicity apart from antioxidant property. The FRS property of *H. indicus* phenolic fractions may be one of the mechanisms by which this source is effective in traditional medicines.

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