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Antioxidant, antibacterial and GC-MS analysis of ethanol root extract of *Hemidesmus indicus* (L.) R.Br.

P Boominathan, CV Chittibabu, C Sivaraj, K Saraswathi and P Arumugam

Abstract

Hemidesmus indicus (L.) R.Br.– Anantamool, is a plant species of Apocynaceae family commonly found in India, especially in different areas of West Bengal. It is a slender, laticiferous, semi-erect endangered shrub; specifically known for its immense medicinal values. In Tamil it is called Nannari. It is traditionally used in dysentery, diarhhoea, skin diseases, syphilis, dyspepsia, leucoderma, diuretic, blood purifier, burning of body, chronic fever and asthma. The aim of the present study was to evaluate the antioxidant and antibacterial activities of ethanol root extract of *H. indicus* and to identify the bioactive compounds by GC-MS analysis. Antioxidant assays such as DPPH$^{\bullet}$ radical, superoxide ($O_2^{\bullet}$-) radical, hydroxyl ($OH^{\bullet}$) radical, ABTS$^{\bullet+}$ radical cation, Fe^{3+} reducing power and phosphomolybdenum reduction assays were carried out for evaluating antioxidant activities. The maximum DPPH$^{\bullet}$ radical scavenging activity of ethanol root extract was 68.85±0.28% at 120 µg/mL concentration and the IC_{50} was 57.90 µg/mL concentration. The maximum superoxide ($O_2^{\bullet}$-) radical scavenging activity of ethanol root extract was 75.79±0.19% at 120 µg/mL concentration and the IC_{50} was 53.37 µg/mL concentration. The maximum hydroxyl ($OH^{\bullet}$) radical scavenging activity of ethanol root extract was 61.65±0.42% at 120 µg/mL concentration and the IC_{50} was 90.55 µg/mL concentration. The maximum ABTS$^{\bullet+}$ radical cation scavenging activity of ethanol root extract was 90.73±0.44% at 12 µg/mL concentration and the IC_{50} was 5.03 µg/mL concentration. The maximum of Mo^{6+} reduction and Fe^{3+} reduction were 89.73±0.39% and 53.23±0.25% at 120 µg/mL concentration and the RC_{50} values were 48.86 µg/mL and 80.58 µg/mL concentration. The antibacterial activity showed maximum zone of inhibition of 16 mm for *Bacillus subtilis* as well as *Shigella flexneri* at 625 µg/mL concentration. Antioxidant compounds such as 2,4-bis(1,1-dimethylethyl)-phenol and flavone were present in ethanol root extract of *H. indicus* which were eluted by GC-MS exhibiting therapeutic applications.

Keywords: *Hemidesmus indicus*, DPPH$^{\bullet}$, ABTS$^{\bullet+}$, superoxide ($O_2^{\bullet}$-), antibacterial activity, GC-MS

1. Introduction

Hemidesmus indicus, generally known as Indian sarasaparilla, belongs to the family Asclepiadaceae. Various pharmacological studies have shown the potential of plant as an anti-inflammatory [1], antimicrobial and anticarcinogenic agent. Roots of the plant are used in various herbal formulations that are available in market for treating various ailments. It is found all over India growing under mesophytic to semi dry plains to an altitude up to 600 m. [2, 3] It is also found in Pakistan, Srilanka, Iran and Bangaldesh. It is a slender, laticiferous twining shrub distributed to greater part of India. Leaves are opposite, shortly petioled, elliptically oblong to linear lanceolate, dark green above but paler and sometimes pubescent below. Flowers are greenish outside but purplish inside with calyx deeply five lobed. Seeds are black, flattened with a silvery white coma. The stem and branches are twine anticlockwise, and are abundantly, elongate, and narrow, having a purplish brown colour. Roots are woody, slender and aromatic. Fruits are two straight slender narrowly cylindrical widely divergent follicles. The plant has many seeds that are flat, oblong, with a long tuft of white silky hairs. In south India, it is a traditional and popular herb with a long history of use as a medicine and is one of the most wanted plant species in Ayurveda widely known as Nannari [4, 5].

Fig 1: Habit of *Hemidesmus indicus* (L.) R.Br. and its roots.

2. Materials and Methods

2.1 Collection of roots and preparation of extract

The roots of *Hemidesmus indicus* were collected from Thirukovillur, Villupuram district Tamilnadu, India. The roots were cut into small pieces and soaked in ethanol for 72 h. Then the supernatant was filtered by using filter paper and condensed by rotary evaporator at 50°C, which yields viscous mass.

2.2 Qualitative phytochemical analysis

The root extract of *H. indicus* was subjected to preliminary phytochemical screening for different classes of phytoconstituents using specific reagents as followed in standard methods [6, 7].

2.3 Determination of total phenols

Folin-Ciocalteau reagent method was used to determine the total phenolic compounds [8] with slight modifications. One hundred µL of root extract (1mg/mL) of *H. indicus* was mixed with 900 µL of methanol and 1 mL of Folin-Ciocalteau reagent (1:10 diluted with distilled water). After 5 min, 1 mL of Na_2CO_3 (20% w/v) solution was added. The mixture was then allowed to stand for 30 min incubation in dark at room temperature. The absorbance was measured by UV-Vis spectrophotometer at 765 nm. The total phenolic content was expressed in terms of gallic acid equivalent (µg/mg of extract), which is a common reference standard.

2.4. Determination of total flavonoids

The total flavonoid content of root extract of *H. indicus* was determined using aluminium chloride reagent method with slight modifications [9]. One mL of root extract (1mg/mL) was mixed with 0.5 mL of methanol and 0.5 mL of 5% (w/v) sodium nitrite solution and incubated for 5 min at room temperature. Then, 0.5 mL 10% (w/v) aluminium chloride solution was added and incubated for further 5 min. Then 1 mL of 1 M NaOH solution was added and incubated at room temperature for 30 min. Absorbance was measured at 510 nm using UV-Vis spectrophotometer and the result is expressed as (µg/mg of extract) quercetin equivalent.

2.5. In vitro antioxidant assays

2.5.1. DPPH• radical scavenging assay

The antioxidant activity of root extract of *H. indicus* was measured based on the scavenging activity of the stable 1, 1-diphenyl 2-picrylhydrazyl (DPPH) free radical [10]. One mL of 0.1 mM DPPH solution in methanol was mixed with 1 mL of various concentrations (20-120 µg/mL) of ethanol root extract. The mixture was then allowed to stand for 30 min incubation in dark. One mL methanol mixed with 1 mL DPPH solution was used as control. The decrease in absorbance was measured at 517 nm using UV-Vis Spectrophotometer. Ascorbic acid was used as the standard reference. The percentage of inhibition was calculated as:

$$\text{\% of DPPH}^{\cdot}\text{ radical inhibition} = \left[\frac{\text{Control} - \text{Sample}}{\text{Control}}\right] \times 100$$

2.5.2. Superoxide radical ($O_2^{\bullet-}$) scavenging assay

Superoxide radical scavenging activity was carried out by the method of Ravishankara *et al* [14]. Different concentrations of root extract of *H. indicus* (20-120 µg/mL) was mixed with 200 µL of 1.5 mM riboflavin, 200 µL of 12 mM EDTA and 100 µL of 50 mM NBT solution, added in that sequence. All the reagents should be prepared in 50 mM of phosphate buffer (pH 7.4). The reaction was started by illuminating the reaction mixture for 15 min. After illumination, the absorbance was measured at 590 nm in UV-Vis Spectrophotometer. Ascorbic acid was used as standard reference.

$$\text{\% of superoxide radical }(O_2^{\bullet-})\text{ inhibition} = \left[\frac{\text{Control} - \text{Sample}}{\text{Control}}\right] \times 100$$

2.5.3. Hydroxyl radical ($OH^{\bullet}$) scavenging assay

Hydroxyl radical scavenging activity was measured by the salicylic acid method (Smirnoff and cumbes 1989) with some modifications [15]. One mL of root extract (20-120 µg/mL) of *H. indicus* was mixed with 500 µL of 9 mM salicylic acid, 500 µL mL of 9 mM ferrous sulphate and 50 µL of 9 mM H_2O_2 solution. The reaction mixture was incubated for 60 min at 37ºC in water bath after incubation the absorbance of the mixtures was measured at 510 nm in UV-Vis spectrophotometer. Ascorbic acid was used as the standard reference. The % of hydroxyl radical scavenging activity was determined as follows:

$$\text{\% of hydroxyl radical }(OH^{\bullet})\text{ inhibition} = \left[\frac{\text{Control} - \text{Sample}}{\text{Control}}\right] \times 100$$

2.5.4. $ABTS^{\bullet+}$ radical cation scavenging assay

The antioxidant capacity was determined in terms of the $ABTS^{\bullet+}$ radical cation scavenging activity [11]. $ABTS^{\bullet+}$ was obtained by reacting 7 mM ABTS stock solution with 2.45 mM potassium persulfate and the mixture was left to stand in the dark at room temperature for 12-16 h before use. The $ABTS^{\bullet+}$ solution (stable for 2 days) was diluted with distilled water and set an absorbance of 0.70±0.02 at 734 nm. Then 1 mL of root extract of different concentrations (5-30 µg/mL) was mixed with 500 µL of diluted $ABTS^{\bullet+}$ solution. The mixture was then allowed to stand for 10 min incubation. Ascorbic acid was used as the standard reference. The absorbance was measured at 734 nm and the $ABTS^{\bullet+}$ radical-scavenging activity was expressed as:

$$\text{\% of ABTS}^{\bullet+}\text{ radical cation inhibition} = \left[\frac{\text{Control} - \text{Sample}}{\text{Control}}\right] \times 100$$

2.5.5 Phosphomolybdenum reduction assay
The antioxidant capacity of root extract of *H. indicus* was assessed by the method of Prieto *et al* [12]. The root extract with different concentrations, ranging from 20 to 120 µg/mL was combined with 1 mL of reagent solution containing ammonium molybdate (4 mM), sodium phosphate (28 mM) and sulphuric acid (600 mM). The reaction mixture was incubated in water bath at 95ºC for 90 min. The absorbance of the coloured complex was measured at 695 nm. Ascorbic acid was used as the standard reference. The percentage of reduction was calculated as:

$$\text{\% of phosphomolybdenum reduction} = \left[\frac{\text{Sample} - \text{Control}}{\text{Sample}} \right] \times 100$$

2.5.6 Ferric (Fe^{3+}) reducing power assay
The reducing power of root extract of *H. indicus* was determined by slightly modified method of Yen and Chen [13]. One mL of root extract of different concentrations (20 - 120 µg/mL) was mixed with 1 mL of phosphate buffer (0.2 M, pH 6.6) and 1 mL of potassium ferricyanide [$K_3Fe(CN)_6$] (1% w/v). The mixture was then incubated at 50°C for 30 min in water bath. Five hundred µL of trichloroacetic acid (10% w/v) was added and mixed well. Then 100 µL of freshly prepared $FeCl_3$ (0.1% w/v) solution was added and the absorbance was measured at 700 nm. Ascorbic acid was used as the standard reference. The percentage of Fe^{3+} reduction was calculated as:

$$\text{\% of } Fe^{3+} \text{ reduction} = \left[\frac{\text{Sample} - \text{Control}}{\text{Sample}} \right] \times 100$$

2.6 Antibacterial activity
2.6.1 Microbial strains
The microorganisms of Gram positive strains such as *Staphylococcus aureus*, *Bacillus subtilis* and *Micrococcus luteus* as well as Gram negative strains such as *Escherichia coli, Proteus vulgaris* and *Shigella flexneri* as well as were used for the evaluation of antibacterial activity.

2.6.2 Aseptic conditions
The aseptic chamber which consist of a wooden box (1.3m x 1.6m x 0.6m) with a door, was cleaned with 70% ethanol and irradiated with short wave UV light (from lamp).

2.6.3 Nutrient broth agar medium preparation
Nutrient broth agar medium was prepared according to the standard methods (peptone-5 g, yeast-3 g, NaCl-5 g, distilled water- 1000 mL, agar-20 g). Depending upon the availability of strains, the medium was calculated and suspended in 200 mL of distilled water in a 500 mL conical flask, stirred, boiled to dissolve and then autoclaved at 15 lbs and at 121ºC for 15 min [14]. The hot medium was poured in sterile petri plates which were kept in the aseptic laminar air flow chamber and allowed to solidify for 15 min.

2.6.4 Agar well Diffusion method
Antibacterial activity of root extract of *H. indicus* was carried out using agar well diffusion method [14]. The solidified nutrient agar in the petri plates was inoculated by dispensing the inoculum using sterilized cotton swabs which is previously immersed in the inoculum containing test tube and spread evenly onto the solidified agar medium. Five wells were created in each plate with the help of a sterile well-borer of 8 mm diameter. The root extract was then poured into each well containing 375, 500 and 625 µg/mL concentrations. All the plates with extract loaded wells were incubated at 37ºC for 24 h and the antibacterial activity was assessed by measuring the diameter of the inhibition zone formed around the well. Tetracycline (25 µg) was used as positive control.

2.7 Thin layer chromatography
Thin layer chromatography (TLC) was carried out for root extract of *H. indicus* in silica gel precoated TLC aluminium sheets (Merck/60 F254). The root extract was spotted at 0.3 mm above from the bottom of the TLC plate (1.5x5 cm). The spotted TLC plate was placed in a 100 mL beaker containing 2 mL of methanol and the chromatogram was developed. The spots were visualized in UV light chamber at 254 nm as well as under iodine. The R_f values of coloured spots were calculated [15].

$$R_f \text{ value} = \frac{\text{Distance travelled by the solute}}{\text{Distance travelled by the solvent}}$$

2.8. Gas chromatography–Mass Spectrometry (GC–MS)
In GC-MS analysis, the ethanol root extract of *H. indicus* was injected into a HP-5 column (30 m X 0.25 mm i.d with 0.25 µm film thickness), Agilent technologies 6890 N JEOL GC Mate II GC-MS model. Following conditions were used: Helium as carrier gas, flow rate of 1 mL/min; and the injector was operated at 200°C and column oven temperature was programmed as 50-250°C at a rate of 10°C/min injection mode. Following MS conditions were used: ionization voltage of 70 eV; ion source temperature of 250°C; interface temperature of 250°C; and mass range of 50-600 mass units [16].

2.8.1 Identification of components
The database of National Institute Standard and Technology (NIST) having more than 62,000 patterns was used for the interpretation on mass spectrum of GC-MS. The mass spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library.

3. Results and Discussion
3.1 Phytochemical analysis
The phytochemical analysis of ethanol root extract of *H. indicus* (Table 1) showed the presence (+) of alkaloids, terpenoids, phenolic compounds root, flavonoids, glycosides and saponins.

Table 1: Qualitative phytochemical analysis of ethanol root extract of *H. indicus*

S. No	Phytochemicals	Tests	Results
1	Alkaloids	(a) Mayer's test	+
		(b) Hager's test	+
2	Phenols	Ferric chloride (0.5%) test	+
3	Tannins	Ferric chloride (0.1%) test	+
4	Flavonoids	Sodium hydroxide test	+
5	Glycosides	Legal's test	+
6	Steroids	Libermann-Burchard test	+
7	Terpenoids	Salkowski test	+
8	Saponins	Foam test	+

3.2 Total phenols and flavonoids
The total phenols and flavonoids were quantified in the ethanol root extract of *H. indicus* seemed to be responsible for the antioxidant activity. Flavonoids have been associated with decreased risk of cardiovascular diseases and hydroxycinnamic acid derivatives have been linked with anti-diabetic, antioxidant and anti-cancer properties [17]. Phenols and flavonoids prevent cell damage by the free radicals associated with cancer development. Plant foods are the most significance source of natural antioxidants and its flavonoids and phenolic acids have attracted the most attention as potential therapeutic agents against cancer. The total phenol content in the ethanol root extract was 57.09±0.17 µg/mg of GAE and the total flavonoid content was 274.83±0.30 µg/mg of QE (Table 2). These results provide a comprehensive profile of the antioxidant activity of spices of *Hemidesmus indicus* with respect to their phenols and flavonoids content.

Table 2: Quantitative estimation of ethanol root extract of *H. Indicus*

S. No	Phytochemicals	Amount (µg/mg)*
1	Phenols	57.09±0.17
2	Flavonoids	274.83±0.30

(*Average value of 3 replicates)

3.3 DPPH• radical scavenging assay
The ability of ethanol root extract of *H. indicus* to scavenge free radicals was assessed by using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical. The root extract of *H. indicus* has capacity to reduce the stable purple colour DPPH (1, 1-diphenyl-2- picrylhydrazyl) radical to yellow colour 1,1-diphenyl-2-picrylhydrazine and the reducing capacity increased with increasing concentration of the extract. The maximum DPPH˙ radical scavenging activity was 68.85±0.28 at 120 µg/mL concentration (Table 3 and Fig 2). The IC_{50} was 57.90 µg/mL concentration and was compared with standard ascorbic acid (IC_{50} = 11.98 µg/mL concentration).

3.4 Superoxide ($O_2^{\bullet -}$) radical scavenging assay
Superoxide anion is also very harmful to cellular components and their effects can be magnified because it produces other kinds of free radicals and oxidizing agents. Flavonoids are effective antioxidants, mainly because they scavenge superoxide anions. Superoxide anions derived from dissolved oxygen by the riboflavin-light-NBT system will reduce NBT in this system. In this method, superoxide anion reduces the yellow dye (NBT^{2+}) to blue formazan, which is measured at 590 nm in UV-Vis spectrophotometer. Antioxidants are able to inhibit the blue NBT formation and the decrease of absorbance with antioxidants indicates the consumption of superoxide anion in the reaction mixture. The maximum superoxide radical scavenging activity of *H. indicus* was 75.79±0.19% at 120 µg/mL concentration (Table 3 and Fig 2) and the IC_{50} was 53.37 µg/mL concentration. It was compared with the standard of ascorbic acid (IC_{50} = 9.65 µg/mL concentration).

3.5 Hydroxyl radical (OH•) scavenging assay
Hydroxyl radical has a short half-life and the most reactive, known to be capable of abstracting hydrogen atoms from cell membranes and induce oxidative damage to DNA, lipids and protein. Hydroxyl radical (OH•) is the neutral form of hydroxyl ion and the most reactive free radical in biological systems generated from free metal ions (copper or iron) catalysed breakdown of H_2O_2 (Fenton reaction) [25] which was scavenged by the root extract in a concentration dependent manner. The maximum OH• radical scavenging activity was 61.65±0.42% at 120 µg/mL concentration (Table 3 and Fig 2) and the IC_{50} was 90.55 µg/mL concentration, which was compared with standard ascorbic acid (IC_{50} = 5.84 µg/mL concentration).

Table 3: DPPH• radical, superoxide ($O_2^{\bullet -}$) radical and hydroxyl (OH˙) radical scavenging assay of ethanol root extract of *H. indicus*

S. No	Concentration (µg/mL)	% of inhibition*		
		DPPH• radical	Superoxide radical ($O_2^{\bullet -}$)	Hydroxyl radical (OH˙)
1	20	35.82±0.15	22.59±0.47	17.89±0.30
2	40	42.63±0.36	27.93±0.26	19.70±0.46
3	60	51.81±0.29	56.21±0.42	25.77±0.28
4	80	58.57±0.42	58.65±0.33	44.17±0.30
5	100	64.47±0.45	67.01±0.10	55.55±0.40
6	120	68.85±0.28	75.79±0.19	61.65±0.42
		IC_{50} = 57.90 µg/mL	IC_{50} = 53.37 µg/mL	IC_{50} = 90.55 µg/mL

(* Average of three replicates)

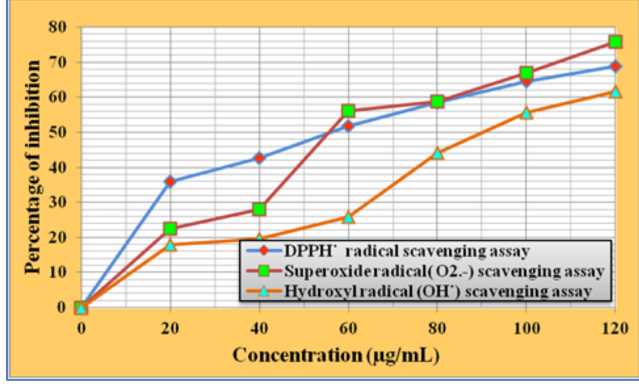

Fig 2: DPPH• radical, superoxide ($O_2^{\bullet -}$) radical and hydroxyl (OH˙) radical scavenging activity of ethanol root extract of *H. indicus*

3.6 ABTS•+ radical cation scavenging assay
ABTS•+ is a blue chromophore produced by the reaction between ABTS and potassium persulfate and ABTS•+ radical cation gets reduced in the presence of root extract and the remaining radical cation concentration was then quantified at 734 nm. It can be prepared using $K_2S_2O_8$ as an oxidant. The blue-green colour of aqueous ABTS solution is formed by the loss of an electron by the nitrogen atom of ABTS (2,2-azinobis(3-ethylbenzothiazolin-6-sulfonic acid)). The decolourization of the solution takes place in the presence of hydrogen donating antioxidant (nitrogen atom quenches the hydrogen atom). The maximum ABTS•+ radical cation scavenging activity was 90.73±0.44 at 30 µg/mL concentration (Table 4 and Fig 3) and the IC_{50} was 2.01 µg/mL concentration, which was compared with standard ascorbic acid (IC_{50} = 4.08 µg/mL concentration).

Table 4: ABTS$^{\bullet+}$ radical cation scavenging activity of ethanol root extract of *H. indicus*

S. No	Concentration (µg/mL)	% of inhibition* ABTS$^{\bullet+}$ radical cation
1	5	49.72±0.24
2	10	62.72±0.26
3	15	79.05±0.10
4	20	80.11±0.36
5	25	87.73±0.37
6	30	90.73±0.44
IC_{50} = 4.08 µg/mL		

(*Average of three replicates)

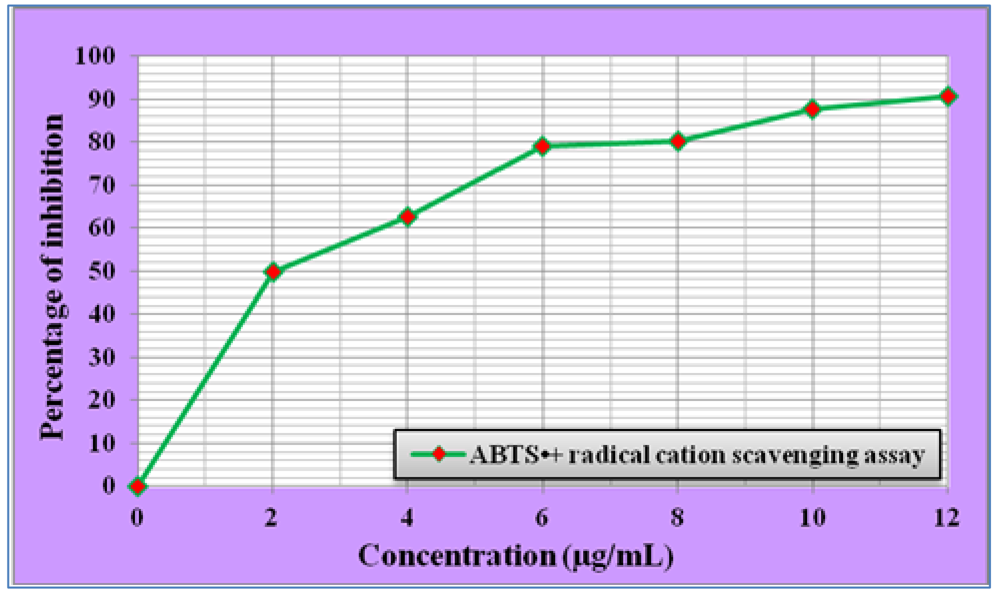

Fig 3: ABTS$^{\bullet+}$ radical cation scavenging activity of ethanol root extract of *H. indicus*

3.7 Phosphomolybdenum reduction assay

The total antioxidant activity of ethanol extract of *H. indicus* was measured by phophomolybdenum reduction method which is based on the reduction of Mo (VI) to Mo (V) by the formation of green phosphate Mo (V) complex at acidic pH, with a maximum absorption at 695 nm. The maximum phosphomolybdenum reduction was 89.73±0.39% at 120 µg/mL concentration (Table 5 and Fig 4) and the RC_{50} was 48.86 µg/mL concentration. It was compared with the standard ascorbic acid (RC_{50} = 6.34 µg/mL concentration). PM assay is a quantitative method to investigate the reduction reaction rate among antioxidant, oxidant and molybdenum ligand. It involves in thermally generating auto-oxidation during prolonged incubation period at higher temperature.

Table 5: Phosphomolybdenum reduction and Fe^{3+} reduction of ethanol root extract of *H. indicus*

S. No	Concentration (µg/mL)	% of reduction* Phosphomolybdenum reduction	Fe^{3+} reduction
1	20	23.18±0.33	18.70±0.27
2	40	40.93±0.26	23.97±0.24
3	60	77.38±0.31	28.79±0.28
4	80	84.79±0.47	49.64±0.38
5	100	89.00±0.36	49.94±0.28
6	120	89.73±0.39	53.23±0.25
Phosphomolybdenum reduction – RC_{50} = 48.86 µg/mL		Fe^{3+} reduction – RC_{50} = 80.58 µg/mL	

(*Average of three replicates)

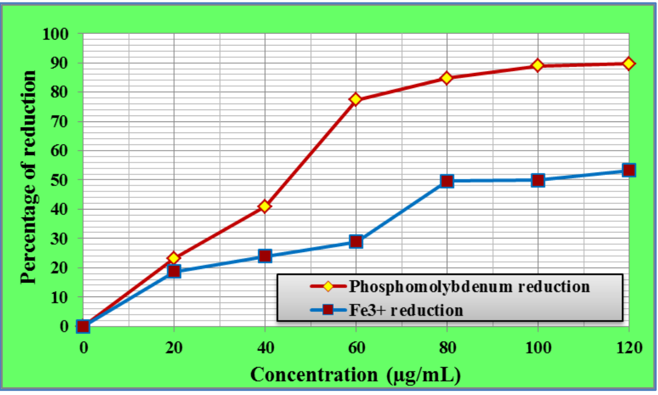

Fig 4: Phosphomolybdenum reduction and Fe^{3+} reduction of ethanol root extract of *H. indicus*

3.8 Ferric (Fe^{3+}) reducing power activity

The reducing power assay was carried out by the reduction of Fe^{3+} to Fe^{2+} by the ethanol root extract of *H. indicus* and the subsequent formation of ferro-ferric complex. The reduction ability increases with increase in concentration of the extract. The maximum Fe^{3+} reduction was 53.23 ±0.25 at 120 µg/mL concentration and the RC_{50} was 80.58 µg/mL concentration. It was compared with the standard ascorbic acid (RC_{50} =7.72 µg/mL concentration). Higher absorbance of reaction mixture indicates higher reduction potential. The reducing capacity of the extract was performed using Fe^{3+} to Fe^{2+} reduction assay as the yellow colour changes to green or blue colour depending on the concentration of antioxidants. The antioxidants such as phenolic acids sand flavonoids were present in considerable amount in the extract of *H. Indicus*

and showed reducing capacity in a concentration dependant manner (Table 5 and Fig 4). The antioxidant can donate an electron to free radicals, leads to the neutralization of the radical which was visualized by forming the intense green colour complex and then measured at 700 nm.

3.9 Antibacterial activity

The ethanol root extract of *H. indicus* were investigated for *in vitro* antibacterial activity against Gram-positive bacteria (*Bacillus subtilis*, *Micrococcus luteus*, *Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli, Proteus vulgaris, Shigella flexneri*). The antibacterial sensitivity of the crude extract and their potency were assessed quantitatively by measuring the diameter of clear zone in cultures in petriplates. The antibacterial activity of these extracts could be correlated to the presence of secondary metabolites such as flavonoids, phenolic compounds, terpenoids, tannin and alkaloids that adversely affect the growth and metabolism of microbes. The antibacterial activity showed maximum zone of inhibition (Table 6) of 16 mm for *Bacillus subtilis* as well as *Shigella flexneri* at 625 µg/mL concentration (Fig 5).

Table 6: Antibacterial activity of ethanol root extract of *H. indicus*

Bacterial pathogens	Zone of inhibition (mm)			
	375 µg/mL	500 µg/mL	625 µg/mL	Standard (Tetracycline)
Bacillus subtilis	-	13	16	20
Micrococcus luteus	11	13	15	22
Staphylococcus aureus	12	13	14	25
Escherichia coli	11	14	15	21
Proteus vulgaris	12	13	15	24
Shigella flexneri	12	14	16	24

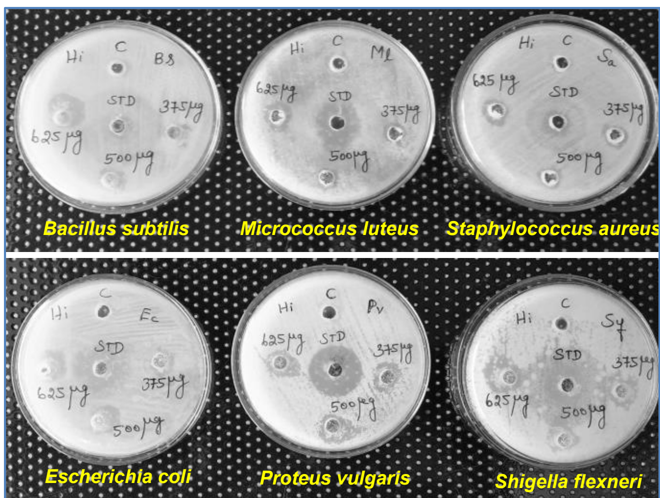

Fig 5: Antibacterial activity of ethanol root extract of *H. indicus*

Phospholipoidal cell membranes having a consequence of increasing the permeability process as well as loss of cellular constituents, enzymatic damage are involved in the cellular energy production of cellular energy, structural components synthesis and genetic material inactivation. The antibacterial effect of plant extract against the testing bacterial pathogens occurs mainly due to cytoplasmic membrane disturbance, disrupting the proton motive force, electron flow, active transport mechanisms, and coagulation of cell composition. Cationic amino acids are attracted by negative charges such as phospholipoidal groups on the surface followed by hydrophobic acid and positively charged patches of the peptides interacting with the aliphatic fatty acids and anionic components. This induces membrane destabilization and bacteria are thought to be killed by the leakage of cytoplasmic contents, loss of membrane potential, change of membrane permeability, lipid distribution, the entry of peptides and the occlusion of anionic cell components or the actuation of autolytic enzymes.

Tannins are polyphenols with significant ability to suppress bacterial cell proliferation by blocking essential enzymes of microbial metabolism such as macerating the proteolytic enzymes. Physiological activities in the human system such as phagocytic cells stimulation, host-mediated tumor activity and anti-infective actions could be possible in the presence of tannins. Saponins might act by altering the permeability of cell walls and hence exert toxicity on all organized tissues. They exert some antibacterial activity by combining with cell membranes to elicit changes in cell morphology leading to cell lysis. Phenolic acids are mainly involved in the reduction of specific adherence of organisms to the cells lining the bladder and the teeth, ultimately lowering the occurrence of urinary tract infections and dental caries. As a sequence, presence of various category of phytochemicals also induce cellular membrane perturbations, interference with certain microbial metabolic processes, modulation of signal transduction or gene expression pathways [18].

4. Thin layer chromatography

Thin layer chromatography analysis was carried out in the solvent system of 2 mL of methanol and the separated compounds in TLC (Table 7) were showed in Figure 6.

Table 7: R_f values of compounds separated by thin layer chromatography of ethanol root extract of *H. indicus*

Ethanol extract of *H. indicus*	R_f values (Under UV light and iodine)
	0.58
	0.46

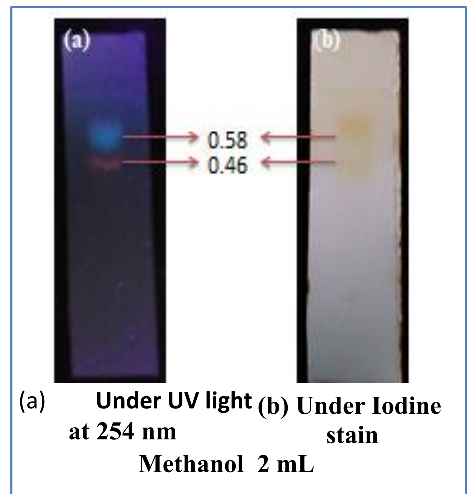

Fig 6: Compounds separated by thin layer chromatography

4.1 GC-MS analysis

GC-MS analysis was carried out for the ethanol root extract of *H. indicus* (Fig 7) and antioxidant compounds such as 2,4-bis(1,1-dimethylethyl)-phenol and flavone were eluted (Table 8 and 9).

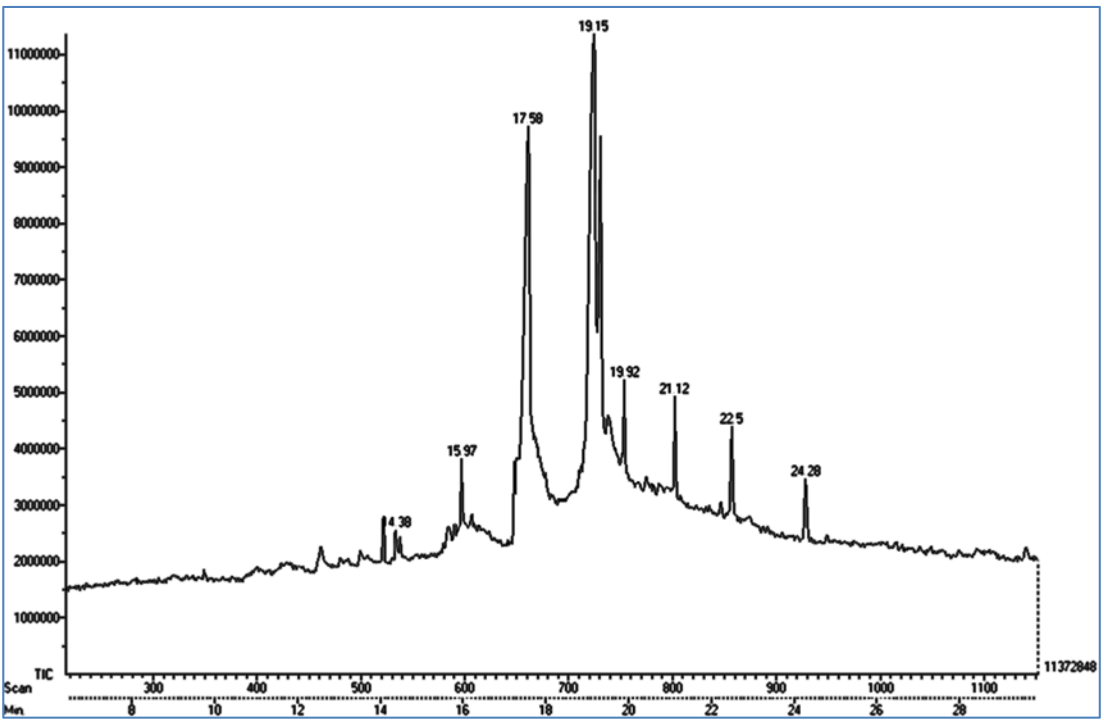

Fig 7: Gas chromatogram of ethanol root extract of *H. indicus*

Table 8: Compounds eluted and identified by GC-MS analysis of ethanol root extract of *H. indicus*

S. No	Compound Name	RT	Compound Structure	Molecular weight (g/mol)	Molecular formula
1	Trans-2-methyl-3-phenyl-2-propen-1-ol	14.38		148	$C_{10}H_{12}O$
2	2,4-bis(1,1-dimethylethyl)-phenol [19]	15.97		206	$C_{14}H_{22}O$
3	Ethanone,1-[4-methoxy-3-(4-methylphenoxy)phenyl)-	19.15		256	$C_{16}H_{16}O_3$
4	Piperazine-2,5-dione,1,4-(4-methylphenyl)-	22.5		294	$C_{18}H_{18}N_2O_2$
5	Isopropyl stearate [20]	24.28		326	$C_{21}H_{42}O_2$
6	Z,E-2-Methyl-3,13-octadecadien-1-ol	21.12		280	$C_{19}H_{36}O$

7	Butanal,3-methyl-[2,4-dinitrophenyl] hydrazone	19.92		266	$C_{11}H_{14}N_4O_4$
8	Flavone [21]	17.58		222	$C_{15}H_{10}O_2$

Table 9: Bio-activity of ethanol root extract of *H. indicus* from GCMS analysis

S. No	Compound Name	Pharmacological Activity
1	Phenol	Antioxidant activity Antimicrobial activity Anti-inflammatory activity
2	Isopropyl stearate	An emollient, skin conditioner Binder and humectant activities
3	Flavone	Reactive Oxygen Species (ROS) production can be reduced due to the presence of flavone. Formation of oxygen radicals can be prevented by flavonoids thereby inhibiting the enzyme activity,

Phenolic acids, hydrolysable tannins, and flavonoids have anti-carcinogenic and anti-mutagenic effects since they act as protective agents of DNA against free radicals, by inactivating carcinogens, inhibiting enzymes involved in pro-carcinogen activation and by activating of xenobiotics detoxification enzymes. In particular flavonoids and L-ascorbic acid have a synergistic protective effect towards oxidative damages of DNA in lymphocytes [22, 23, 24] Block *et al.* [25] stated that a diet rich in vegetables reduces the risk for colon cancer. Phenolic acids play a potential protective role against different kinds of oxidative damaged diseases through consumption of fruits and vegetables. Many studies have suggested that flavonoids like rutin, kaempferol, quercetin, apigenin are well-known for its anti-inflammatory, anti-allergic, anti-thromboic, hepatoprotective, anti-spasmodic and anticancer properties [26]. The antioxidant activity of phenols and flavonoids plays an important role in absorption or neutralization of free radicals. Polyphenols also enhance the level of cellular antioxidant system and induce the intracellular cytochrome P-450 resulting in detoxifying the activity of carcinogens.

4. Conclusion

The results of the present study indicate that ethanol root extract of spices of *Hemidesmus indicus* has significant antioxidant activities to scavenge free radicals as well as to protect cells from oxidative damage. Further studies are necessary to isolate the active compounds present in ethanol root extract of *Hemidesmus indicus* and there pharmacological activities against cancer cells. The results of various therapeutic activities conducted in the present study provide promising guideline regarding the potential uses of *Hemidesmus indicus* as an antioxidant agent.

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