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

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Abstract
Hemidesmus indicus (L.) R.Br. belongs to the family Asclepiadaceae, and is called Indian sarsaparilla, anantamool or nannari. It is a well-known indigenous medicine for antioxidant and antiinflammatory diseases. Tribal people use this plant to treat skin, colorectal, and liver cancers. The root decoction of the plant is use to treat in biliousness, respiratory disorders, eye diseases, epileptic fits in children, kidney and urinary disorders, loss of appetite and burning sensation. The aim of the present study was to evaluate the antioxidant and antibacterial activities of methanol root extract of *H. indicus* and to identify the bioactive compounds by GC-MS analysis. Antioxidant activities such as DPPH radical, $ABTS^{\cdot+}$ radical cation, Fe^{3+} reducing power and phosphomolybdenum reduction assays were carried out. The maximum DPPH radical scavenging activity of methanol root extract was 64.31±4.50% at 120 µg/mL concentration and the IC_{50} was 37.44 µg/mL concentration. The maximum $ABTS^{\bullet+}$ radical cation scavenging activity of methanol root extract was 77.37±5.41% at 30 µg/mL concentration and the IC_{50} was 12.93 µg/mL concentration. The maximum of Mo^{6+} reduction and Fe^{3+} reduction were 87.17±6.10% and 64.13±4.48% at 120 µg/mL concentration and the RC_{50} values were 35.77 µg/mL and 87.03 µg/mL concentration. The antibacterial activity showed maximum zone of inhibition of 18 mm for *Micrococcus luteus* at 625 µg/mL concentration. Antioxidant compounds such as 5,6-epoxy-5,6-dihydro-retinoic acid and oleic acid were present in methanol root extract of *H. indicus* which were eluted by GC-MS.

Keywords: *Hemidesmus indicus*, DPPH, $ABTS^{\cdot+}$, antibacterial activity, GC-MS

1. Introduction
Hemidesmus indicus, generally known as Indian sarasaparilla, belongs to the family Asclepiadaceae. It is found all over India growing under mesophytic to semi dry plains to an altitude up to 600 m. [1-3] It is also found in Pakistan, Srilanka, Iran and Bangladesh. The stem and branches of *Hemidesmus indicus* twine anticlockwise, and are abundantly laticiferous, elongate, narrow, having a purplish brown colour. Roots are woody, slender and aromatic. Since the roots have the smell like camphor, it is also known as Kapoori. Leaves are simple, petiole, exstipulate, apiculate acute or obtuse, dark green above but paler and sometimes pubescent below. Flowers may be greenish yellow to greenish purple outside, dull yellow to light purplish inside with calyx deeply five lobed. 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]

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 the traditional plant Siddha market, Broadway, Chennai, Tamil Nadu. The roots were cut into small pieces and soaked in methanol for 72h. 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 [5] as followed in standard methods [6, 7].

2.3 Determination of total phenols

Folin-Ciocalteu 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-Ciocalteu reagent (1:10 diluted with distilled water). After 5 min, 1 mL of methanol solution of Na_2CO_3 (20% w/v) 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 *Hemidesmus 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$^{\cdot}$ radical scavenging assay

The antioxidant activity of root extract of Hemidesmus *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 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. The percentage of inhibition was calculated as:

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

2.5.2 ABTS$^{\cdot+}$ 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. The absorbance was measured at 734 nm and the ABTS$^{\bullet+}$ radical-scavenging activity was expressed as:

$$\% \text{ of ABTS}^{\cdot+} \text{ radical cation inhibition} = \left(\frac{\text{Control-Sample}}{\text{Control}} \right) \times 100$$

2.5.3 Phosphomolybdenum reduction assay

The antioxidant capacity of root extract of *Hemidesmus indicus* was assessed by the method of Prieto *et al.* [12]. The root extract with different concentrations, ranging from 20 to 120µg/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 95 ^{o}C for 90 min. The absorbance of the coloured complex was measured at 695 nm. Ascorbic acid was used as standard reference. The percentage of reduction was calculated as:

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

2.5.4 Ferric (Fe $^{3+}$) reducing power assay

The reducing power of root extract of *Hemidesmus 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:

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

2.6 Antibacterial activity

2.6.1 Microbial strains

The microorganisms of Gram negative strains such as *Escherichia coli, Klebsiella pneumonia* and *Shigella flexneri* as well as Gram positive strains such as *Staphylococcus aureus*, *Bacillus subtilis* and *Micrococcus luteus* 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 121 ºC 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 *Hemidesmus 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 250, 375, 500 and 625 µg/mL concentrations. All the plates with extract loaded wells were incubated at 37 ºC 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 *Hemidesmus 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 solvent mixture of toluene: methanal (1:1) 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 methanol root extract of *Hemidesmus 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 methanol root extract of *H. indicus* showed the presence (+) of alkaloids, terpenoids, phenolic compounds root, flavonoids, glycosides and saponins.

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

S. No	Phytochemicals	Tests	Results
1	Alkaloids	Mayyor's test Hager's test	+
2.	Terpenoids	$CHCl_3$ + conc. H_2SO_4	+
3.	Steroids	Liebermann-Burchard test (acetic anhydride + Con. H_2SO_4)	+
4.	Flavanoids	NaOH solution	+
5.	Phenols	$FeCl_3$ solution	+
6.	Glycosides	Sodium nitroprusside solution + Con. H_2SO_4	+
7.	Saponins	Foam test	+

3.2 Total phenols and flavonoids

The total phenols and flavonoids were quantified in the methanol 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 prevents 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 methanol root extract was 87.43±3.65 µg/mg of GAE and the total flavonoid content was 2.88±0.50µg/mg of QE. 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 methanol root extract of *H. indicus*

S. No	Phytochemicals	Value(µg/mg)*
1.	Phenols	87.43±3.65
2.	Flavonoids	2.88±0.50

(*Average value of 3 replicates)

3.3 DPPH˙ radical scavenging assay

The ability of methanol root extract of *H. indicus* to scavenge free radicals was assessed by using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical. The maximum DPPH˙ radical scavenging activity was 64.31±4.50 at 120µg/mL concentration. The root extract of *H. indicus* demonstrated high capacity for scavenging free radicals by reducing 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 IC_{50} was 37.44 µg/mL concentration and was compared with standard ascorbic acid (IC_{50} = 11.98 µg/mL concentration).

Table 3: DPPH radical scavenging assay of methanol root extract of *H. indicus*

S. No.	Concentration (µg/mL)	% of inhibition*
1	20	46.80±3.27
2	40	53.42±3.73
3	60	56.55±3.95
4	80	58.37±4.08
5	100	61.51±4.30
6	120	64.31±4.50

(*Average value of 3 replicates)

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

ABTS$^{\bullet+}$ is a blue chromophore produced by the reaction between ABTS and potassium persulfate and ABTS$^{\bullet+}$ 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$^{\bullet+}$ radical cation scavenging activity was 77.37±5.41 at 30 µg/mL concentration and the IC_{50} was 12.93 µg/mL concentration, which was compared with standard ascorbic acid (IC_{50} = 4.21 µg/mL concentration).

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

S. No.	Concentration (µg/mL)	% of inhibition*
1	5	11.67±0.81
2	10	38.68±2.70
3	15	78.10±5.46
4	20	79.19±5.54
5	25	82.11±5.74
6	30	77.37±5.41

(*Average value of 3 replicates)

3.5 Phosphomolybdenum reduction assay activity

The total antioxidant activity of methanol extract of *Hemidesmus indicus* was measured by phosphomolybdenum 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 87.17 ±6.10% at 120 µg/mL concentration and the RC_{50} was 35.77 µg/mL concentration. It was compared with the standard ascorbic acid. 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+} reducing power assay of methanol root extract *H. indicus*

S. No	Concentration (µg/mL)	% of reduction*	
		Phosphomolybdenum reduction at 695nm	Fe^{3+} reducing power at 700 nm
1	20	26.77 ±1.87 3.033	16.01 ±1.12 3.033
2	40	55.92 ±3.91	31.32±2.19
3	60	67.93±4.75	34.31±2.40
4	80	75.78 ±5.30	45.96 ±3.21
5	100	81.97 ±5.73	58.58±4.10
6	120	87.17 ±6.10	64.13 ±4.48

(*Average value of 3 replicates)

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

The reducing power assay was carried out by the reduction of Fe^{3+} to Fe^{2+} by the methanol 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 64.13 ±4.48 at 120 µg/mL concentration. Higher absorbance of the reaction mixture indicates higher reductive potential. The reducing capacity of a compound may serve as a significant indicator of its potenti al antioxidant activity. 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. 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 λ 700 nm.

3.7 Antibacterial activity

The methanol 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 petri plates. 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.

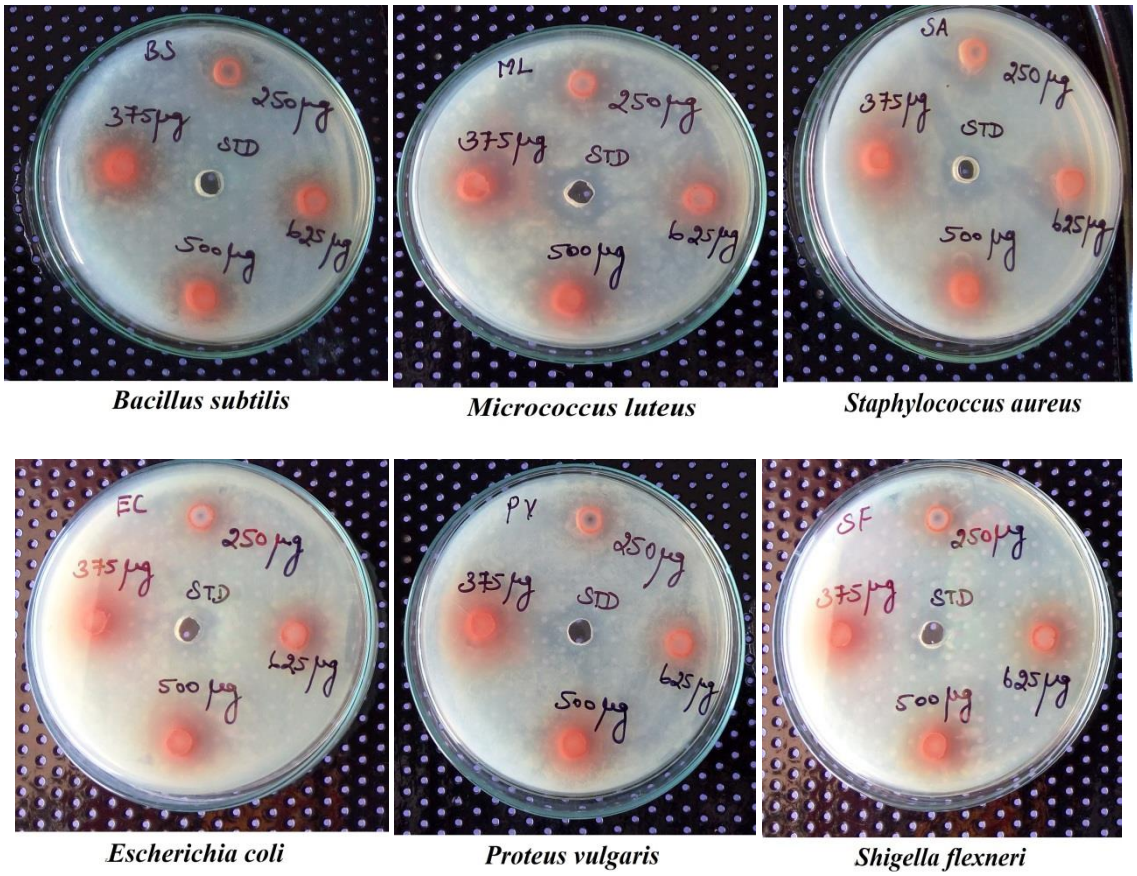


Fig 2: Antibacterial activity of methanol root extract of *H. indicus* on various pathogenic organisms

Table 7: Antimicrobial activity of methanol root extract of *H. indicus* on various pathogenic organism

S. No	Organisms	Zone of inhibition, (mm)				
		250 µg	375 µg	500 µg	600 µg	Standard (Tetracycline)
1	*Bacillus subtilis*	10	14	15	17	10
2	*Micrococcus luteus*	13	14	16	18	18
3	*Staphylococcus aureus*	10	11	12	16	16
4	*Escherichia coli*	12	13	14	15	14
4	*Proteus vulgaris*	11	12	14	15	12
5	*Shigella flexneri*	10	11	12	14	12

3.8 Thin Layer Chromatography

Thin layer chromatography analysis was carried out in the solvent system of Methanol: isopropyl alcohol with the ratio of 1:1. The separated compounds in TLC were showed in Figure.

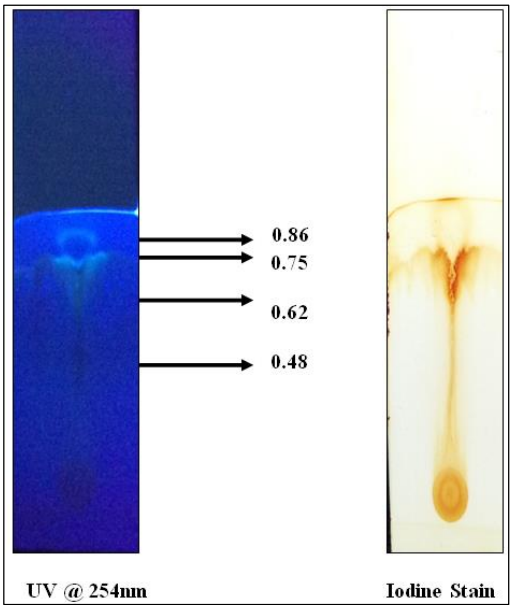


Fig 3: Compounds separated by Thin Layer Chromatography

3.9 GC-MS analysis

GC-MS analysis was carried out for the methanol root extract of *H. indicus* and antioxidant compounds such as 5, 6-epoxy-5,6-dihydro-retinoic acid and oleic acid with more RT value were eluted (Table 9).

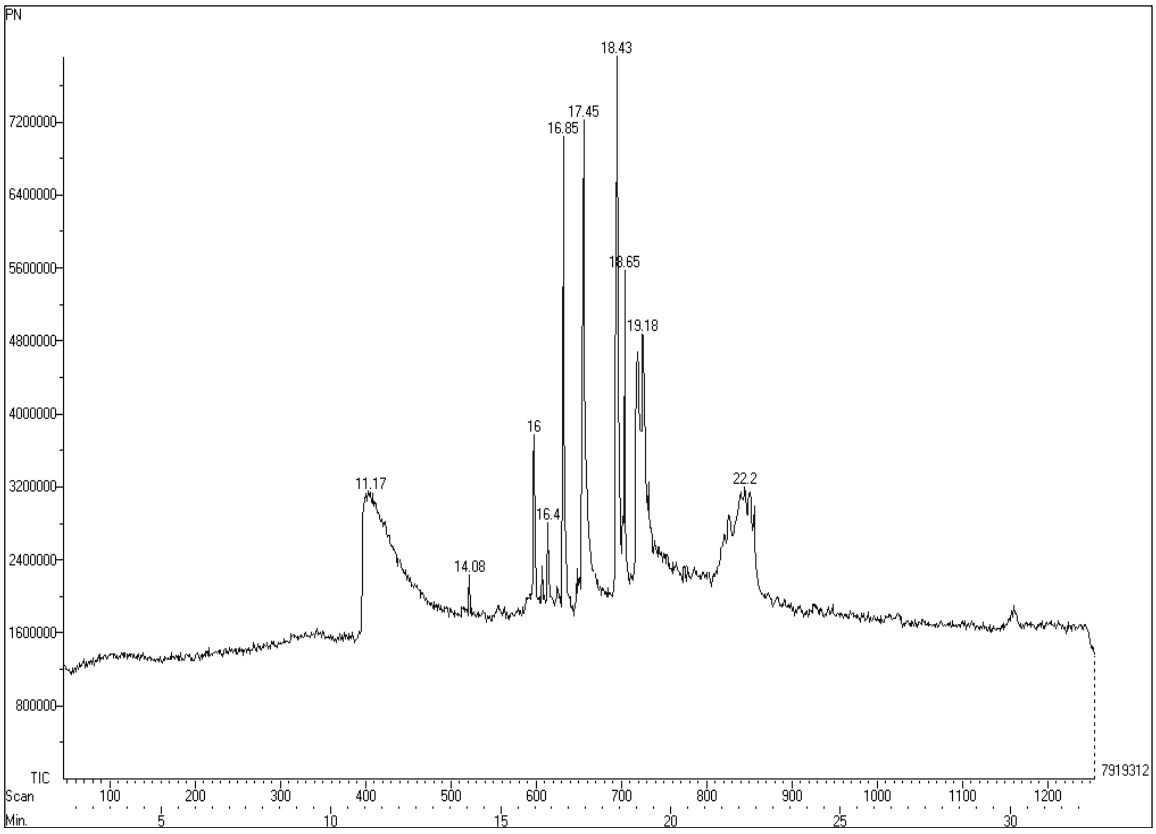
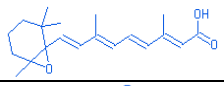
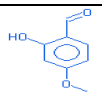
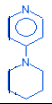
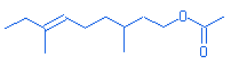
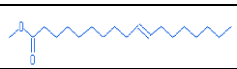


Fig 4: Gas chromatogram of methanol root extract of *H. indicus*

Table 9: Active compounds identified in methanol root extract of *H. indicus* in GC-MS

S. No	Compound Name	RT	Compound Structure	Molecular Weight	Molecular Formula g/mol
1	Octadecanoic acid, methyl ester	18.65		298.51	$C_{19}H_{38}O_2$
2	Retinoic acid,5,6-epoxy-5,6-dihydro-	22.2		316.44	$C_{20}H_{28}O_3$
3	Benzaldehyde,2-hydroxy-4-methoxy-	11.17		152.14	$C_8H_8O_3$
4	4-Piperidino pyridine	14.08		162.23	$C_{10}H_{14}N_2$
5	1,11-Tridecadiene	16		180.33	$C_{13}H_{24}$
6	3,7-Dimethyl-6-nonen-1-ol acetate	16.4		212.32	$C_{13}H_{24}O_2$
7	Hexadecanoic acid, methyl ester	16.85		270.45	$C_{17}H_{34}O_2$
8	Cetylic acid	17.45		256.43	$C_{16}H_{32}O_2$
9	10-Octadecenoic acid, methyl ester	18.43		296.49	$C_{19}H_{36}O_2$
10	Oleic acid	19.18		282.46	$C_{18}H_{34}O_2$

Flavonoids and phenolic acids are the main types of phenolic compounds present in plants [18]. They have been studied due to their therapeutic potential, which has been partly attributed to their antioxidant properties [19]. Flavonoids and phenolic acids are molecules characterized by having at least one aromatic ring with one or more hydroxyl groups and have been linked with many pharmacological properties such as neuroprotective, anti-inflammatory, antioxidant, antiasthmatic, antiulcer, decreased risk of cardiovascular diseases, anti-diabetic, anti-cancer [20-21].

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, antithrombotic, hepatoprotective, anti-spasmodic and anticancer properties. The antioxidant activity of phenols and flavonoids plays an important role in absorption or neutralization of free radicals. Mutations of p53 are among the most common genetic abnormalities in human cancers. The inhibition of expression of p53 may lead to arrest the cancer cells in the G2-M phase of the cell cycle. Flavonoids are found to down regulate expression of mutant p53 protein to nearly undetectable levels in human breast cancer cell lines [22]. The anti-mutagenic and anti-carcinogenic activity of polyphenols is mostly due to their antioxidant activity, which inactivates direct mutagens/carcinogens and inhibits the activation of indirect mutagens/carcinogens extracellularly. Polyphenols also enhance the level of cellular antioxidant system and induce the intracellular cytochrome P-450 resulting in detoxifying the activity of carcinogens [23].

4. Conclusion

The results of the present study indicate that methanol root extract of spices of *Hemidesmus indicus* has significant antioxidant activities to reduce harmful effect of radicals. Further molecular studies are required to find out the mechanism of action of bioactive compounds present in *Hemidesmus indicus* before it can be recommended for any practical widespread use of the plant. The results of various experiments conducted in the present study provide promising guideline regarding the potential uses of *Hemidesmus indicus* as an antioxidant agent.

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