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In-vitro antioxidant activity of methanolic leaf and root extracts of *Elephantopus scaber*

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Abstract
Natural antioxidants have an important role in the prevention of many diseases and promotion of health. Among natural antioxidants, flavonoids and other phenolic compounds are powerful antioxidants. The antioxidant activities of the methanol extracts from the leaf and root extracts of *Elephantopus scaber* (*E. scaber*) were assessed by using 1,1-diphenyl-2-picryl hydroxyl (DPPH) quenching assay, 2,2'-azinobis-3-ethylbenzothiozoline-6-sulfonic acid (ABTS) decolorization test, reducing power activity and ferric reducing antioxidant power (FRAP) and superoxide anion, hydrogen peroxide (H_2O_2) radical, hydroxy radical scavenging assay. In the present study, reducing power, FRAP and the free radical scavenging activity were increased in a dose depending manner. The antioxidant activity of the samples was comparable with that of the standard antioxidant butylated hydroxyl toluene (BHT). The results indicate that both the leaf and root extracts of *E. scaber* displayed antioxidant activity, which may be attributed to the presence of adequate phenolic and flavonoid compounds. This study suggests that *E. scaber* extracts exhibit great potential for antioxidant activity and may be useful for the nutritional and medicinal purpose.

Keywords: Antioxidants, phytochemicals, DPPH, *Elephantopus scaber*

Introduction
Antioxidant compounds in food play an important role in human health, which delays or inhibits oxidative damage of the biomolecules [1]. The main characteristic of an antioxidant is its ability to trap free radicals. Antioxidant compounds like phenolic acids, polyphenols and flavonoids scavenge free radicals such as peroxide, hydroperoxide or lipid peroxyl [2]
Free radicals which have one or more unpaired electrons are produced in physiological or pathological cell metabolism. The most biologically significant free radicals are reactive oxygen species (ROS). ROS are various forms of activated oxygen, which include free radicals such as superoxide anion radicals ($O_2^{\bullet}$) and hydroxyl radicals ($OH^{\bullet}$), as well as non-free radical species (H_2O_2) and the singlet oxygen ($^1O_2$) [3- 6]. Excessive generation of ROS, induced by various stimuli reduces the antioxidant capacity of the organism and initiate the peroxidation of membrane lipids, leading to the accumulation of lipid peroxidation products. The peroxidation products and their secondary oxidation products such as malondialdehyde and 4-hydroxyinonenal can react with biological substrates such as protein, amines, and deoxyribonucleic acid leads to a diversity of pathophysiological processes such as inflammation, genotoxicity, cancer and diabetes [7-9].
Herbal plants considered as a good antioxidant since ancient times. A great number of aromatic and other medicinal plants contain chemical compounds exhibit antioxidant properties. Sources of natural antioxidants are primarily the plant phenolics that may occur in all parts of plants such as fruits, vegetables, nuts, seeds, leaves, roots and barks [10].
Therefore, this study was aimed to resolve the antioxidant activity of *Elephatopus scaber* by using *in vitro* analysis. Synthetic antioxidants have many adverse effects. So we need natural antioxidants to; prevent and cure the diseases. The genus *Elephantopus scaber* (*E. scaber*) is a tropical species of flowering plant in the sunflower family, is the largest genus of medicinal plants widely distributed in most part of the India. The whole plant has been reported to possess anti-bacterial, anti-amoebic, anti-fungal, antiviral, anti-diarrheal, sedative, anxiolytic, analgesic, anti-pyretic and anti-malarial properties [11].

Fig 1: *Elephantopus scaber*

Plant material

E. scaber Linn leaves and roots were collected in and around Ullannoor, Pathanamthitta District, Kerala, in the month of January-February. Authentication of the plant was done by the Department of Botany, Annamalai University, Chidambaram.

Reagents

DPPH was purchased from Sigma-Aldrich Chemical Co., St. Louis. Methanol, chloroform, ethylacetate, BHT, potassium persulphate, ABTS(2,2'–azinobis-(3-ethylbenzothiazoline-6-sulfonic acid), hydrogenperoxide, sodium nitroprusside and all other chemicals used were of analytical grade, purchased from E. Merck, Mumbai, India.

Preparation of plant extracts

The shade dried leaves of plant (at 20º) were powdered in mechanical grinder. Solvent 150 ml of was added to the 20 grams of leaf and stem powder and kept for 3 days. The extracts were filtered using Whaatman No.1 filter paper and the supernatants were collected. The residue was again extracted two times (with 3 days of interval for each extraction) and supernatants were collected. The supernatants were pooled and evaporated (at room temperature, 28 ±1C) until the volume was reduced to 150 ml. Extracts of the leaves and stems were stored in the freezer till the use.

Assessment of antioxidant activity

DPPH radical scavenging activity

The free radical scavenging activity of methanolic extract of *E. scaber* was determined spectrophotometrically in a dark room by using DPPH• method of Brand Williams *et al.*, (1995) [12]. DPPH• is a stable free radical and reacts with an antioxidant compound that can donate hydrogen and gets reduced. The colour changes from deep violet to blue were measured. The intensity of the colour developed was depends on the amount and nature of radical scavengers present in the sample. 1ml of various concentrations leaf and root extract of *E. scaber* was taken, added 1ml of DPPH and this was made up to 3ml with water. The blue colour developed was read at 517nm and BHT was used as a standard. Radical scavenging activity was expressed as the inhibition percentage of free radical by the samples and was calculated using the following formula:

2.2' Azinobis-(3- Ethylbenzothiazoline - 6 - Sulfonic Acid) ($ABTS^{+}$) Assay

The total antioxidant activity of the samples was measured by [2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)] ABTS•+ radical cation decolorization assay according to the method of Re *et al.* (1999) [13]. ABTS•+ was produced by reacting 2.4mM potassium persulfate with 7mM ABTS+ aqueous solution in the dark for 12-16 hours at room temperature. Prior to assay, this solution was diluted in ethanol (about 1:89 v/v) and equilibrated at 300C to give an absorbance of 0.7000±0.02 at 734 nm. Then, 2ml of diluted ABTS•+ solution was added to the sample. After 30 minutes of incubation at room temperature, the absorbance was recorded at 734nm and percentage of inhibition was calculated as described earlier. BHT was used as a reference standard. Triplicates were performed

Ferric reducing antioxidant power (FRAP) assay:

The FRAP (Ferric reducing antioxidant power assay) procedure described by Benzie and Strain (1999) [14].The principle of this method is based on the reduction of a ferric-tripyridyl triazine complex to its ferrous colored form in the presence of antioxidants. The FRAP reagent was produced by mixing 300 mM sodium acetate buffer with pH 3.6, 10mM TPTZ (tripyridyl triazine) solution and 20mM FeCl3 solution in a ratio of 10:1:1 in volume. 3 ml of FRAP reagent was added to the different concentrations (25, 50, 100 and 200 µg/mL) of samples and the reaction mixture was incubated at 37 °C for 30 min. The increase in absorbance at 593 nm was measured. Percentage of inhibition was calculated as described earlier.

Superoxide anion radical scavenging assay:

Superoxide anion scavenging activity was measured based on the method described by Liu *et al.,* (1997) [15]. 0.1ml of sample solution was mixed with 1ml of NBT and 1ml of NADH solution. This mixture was incubated at 25ºC for 5 minutes. A control was performed with reagent mixture without the sample. Absorbance was measured spectrophotometrically at 560nm. BHT was used as reference standard. Percentage of inhibition was calculated as described earlier.

Hydroxyl radical scavenging activity:

The scavenging activity of the extract on hydroxyl radical was measured according to the method of Klein *et al.,* (1991) [16]. Various concentrations of extracts were added with 1.0ml of Ferrous ammonium sulphate - EDTA solution, 0.5ml of EDTA solution (0.018%), and 1.0ml of dimethyl sulphoxide (DMSO).The reaction was initiated by adding 0.5ml of ascorbic acid and incubated at 80–90°C for 15 min in a water bath. After incubation the reaction was terminated by the addition of1.0 ml of ice - cold TCA. 3 ml of Nash reagent was added and left at room temperature for 15min. The reaction mixture without sample was used as control. The intensity of

the color formed was measured spectrophotometrically at 412 nm against reagent blank. BHT was used as reference standard. Percentage of inhibition was calculated as described earlier.

Scavenging activity against hydrogen peroxide

The scavenging capacity of extracts on hydrogen peroxide was determined according to the method of Nabavi *et al.*, (2009) [17].Test samples were prepared with 2.0 ml of various concentrations of extracts (25 to 200 µg/ml) and a solution of H_2O_2 (1.2 ml, 40 mM) in phosphate buffer (pH 7.4). A blank solution was prepared in the same way without H_2O_2. After 10 min incubation of the mixture, the absorbance was recorded at 230 nm. BHT was used as reference standard. Radical scavenging activity was expressed as the inhibition percentage of free radical by the sample and the percentage of inhibition was calculated as described earlier.

Reducing power assay

The reducing power of the extracts was determined by the method of Oyaizu 1986 [18]. Substances, which have reductions potential, react with potassium ferricyanide (Fe3+) to form potassium ferrocyanide (Fe2+),which then reacts with ferric chloride to form ferric ferrous complex that as an absorption maximum at 700nm. Various concentrations of samples (25-200 µg/ml) were mixed with of phosphate buffer and 5mL of 0.2 M phosphate buffer at pH 6.6. To this, 5 mL of 1% potassium ferricyanide solution was added. The mixture was incubated at 50°C in water bath for 20 min. After cooling, 5mL of 10% TCA was added and the content was centrifuged at 1,000 rpm for 10 min. The upper layer of the supernatant (5mL) was mixed with 5mL of distilled water. To this, 1mL of ferric chloride (0.1%) was added and vortexed. Then, the absorbance of the reaction mixture was read spectrophotometrically at 700 nm against water blank. BHT used as a standard.

Metal ion chelating activity

The chelation of ferrous ions by extracts was estimated by method of Dinis *et al.*, 1994 [19].The sample solution at different concentrations was added to 0.05 ml of 2mM $Fecl_2$.The reaction was initiated by the addition of 5mM ferrozine (0.2mL) and the mixture was mixed well and stands for 10 min at room temperature. The absorbance was measured at 562 nm spectrophotometrically. The Fe^{2+}chelating activity (%) was calculated as described earlier

Phosphomolybdenum assay

The total antioxidant activity of the extracts was evaluated by the phosphomolybdenum assay [20]. This assay is based on the reduction of M_0 (VI)–M_0 (V) by the antioxidants and subsequent formation of a green phosphate/M0 (V) complex at acidic pH. 0.3 ml of BCA sample is taken in a tube and mixed with 3 ml of reagent containing 0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate and it was incubated at 95 °C for 90 min. The absorbance of the mixture was measured at 695 nm with methanol blank. The percentage of inhibition was calculated as described earlier.

Fourier Transform Infrared Spectrophotometer (FT-IR):

Fourier Transform Infrared Spectrophotometer (FT-IR) is perhaps the most powerful tool for identifying the types of chemical bonds (functional groups) present in the compounds. The wavelength of light absorbed is characteristic of the chemical bond as can be seen in the annotated spectrum. By interpreting the infrared absorption spectrum, the chemical bonds in a molecule can be determined. Dried powder of the stems and leaves methanolic extracts were used for FT-IR analysis.10 mg of the dried extract powder was encapsulated in 100 mg of KBr pellet, in order to prepare translucent sample discs. The powdered sample of each plant specimen was loaded in FT-IR spectroscope, with a scan range from 400 to 4000 cm^{-1} with a resolution of 4 cm^{-1}.

Statistical Analysis

All the above experimental assays were performed and carried out in triplicate. Experimental assays results are expressed as mean ± standard deviation. The results were analyzed using one way analysis of variance and the group means were compared using Duncan's multiple range tests using SPSS version 17.

Results and Discussion

Effect of Methanol extracts of *E. scaber* root and leaf extracts on DPPH

DPPH is a stable free radical, which has been widely accepted as a tool for estimating free radical scavenging activities of plant extracts. One of the reasons is that this method is simple and highly sensitive. The antioxidant effect is proportional to the disappearance of $DPPH^{\cdot}$ in test sample. $DPPH^{\cdot}$ accepts an electron or hydrogen radical to become a stable diamagnetic molecule. This reaction is stoichiometric with respect to the number of hydrogen atoms absorbed, or to the number of electrons captured. Then, colour changing from purple to yellow is the consequence of the reducing ability of antioxidant toward DPPH stable free radical [21].The reduction capability of DPPH radical is determined by the decrease in absorbance at 517 nm by the antioxidants present in the extracts.

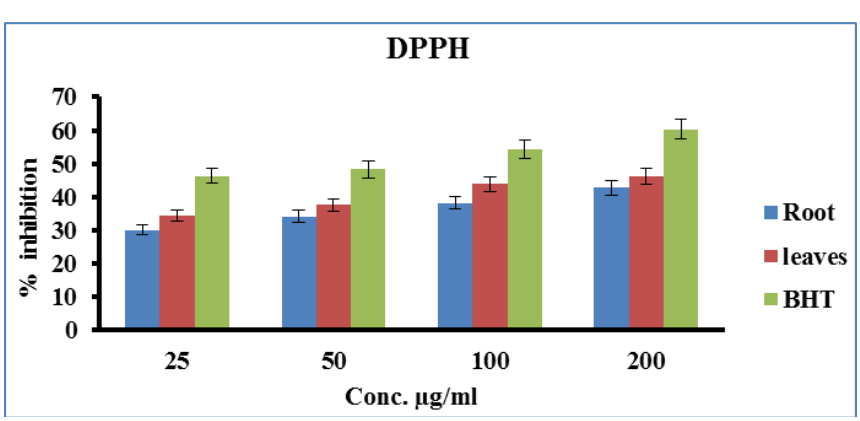

Fig 2: DPPH scavenging activity of *E. scaber* root and leaf extracts.

The percentage inhibition of methanol extracts of roots and leaves were 38.25 and 43.85 % respectively. BHT was used as a standard antioxidant with 56.36 % inhibition. All the test samples exhibited potent scavenging activities in a concentration dependent manner and maximum scavenging activity was observed at a concentration of 200µg/mL of both root and leaf extracts. The higher the discoloration of the DPPH solution, the lower the absorbance of the reaction mixture indicating the significant free radical scavenging capacity of root and leaf extracts of *E. scaber*.

Effect of Methanol extracts of *E. scaber* root and leaves on ABTS$^{\bullet+}$

ABTS$^{\bullet+}$ assay is a simple and indirect method for determining the antioxidant activity of natural compounds. ABTS$^{\bullet+}$ radical is rather stable, but it reacts energetically with an H-atom donor, such as phenolics, been converted into a non-colored form of ABTS$^{\bullet+}$ [22].The decolorization of the ABTS$^{\bullet+}$ are used to measure the reduction of the radical cation as the percentage inhibition of absorbance at 734 nm [23].

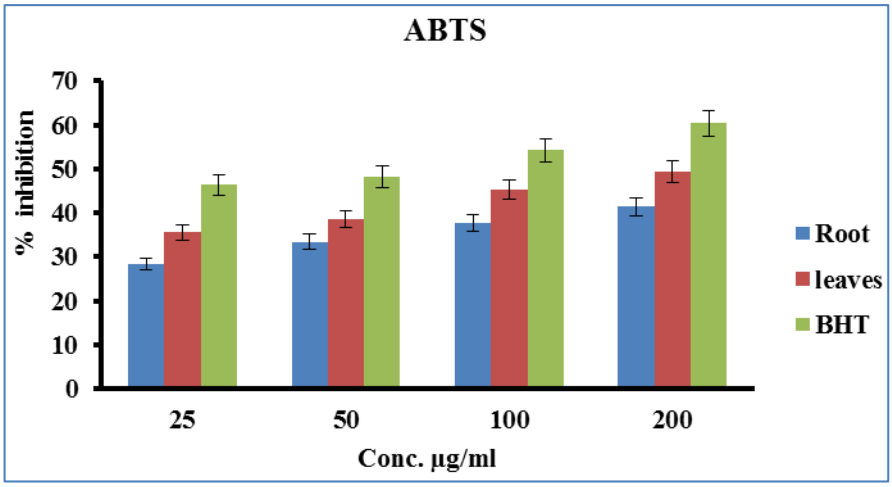

Fig 3: Activity of *E. scaber* leaves and root extracts on ABTS.

The percentage inhibition of methanol extracts of root and leaves were exhibited maximum at 200µg/mL with 37.83 and 45.23 % respectively. BHT was used as standard antioxidants with 56.36 %. All test samples exhibited potent scavenging activities in a dose dependent manner. The 200µg/ml extract showed the best antioxidant activity, where among them, the leaves extract was the highest (45.23%).This study described that the scavenging activity was increased with the increasing concentrations of the extracts of roots and leaves of *E. scaber*.

Effect of methanol extracts of *E. scaber root and leaves* on FRAP

FRAP assay measures the reducing potential of an extract by reacting with a ferrictripyridyltriazine (Fe^{3}+-TPTZ) complex and producing a coloured ferrous tripyridyltriazine (Fe^{2}+-TPTZ) [24]. Generally, the reducing properties of extracts are associated with the presence of antioxidants which exert their action by breaking the free radical chain by donating a hydrogen atom. The ferric ion reducing activities of methanol extracts of root and leaves of *E. scaber* and BHT standard are shown in Fig.8. The absorbance *E. scaber* leaves and stem extracts were increased in dose dependent manner due to the formation of the Fe^{2+}-TPTZ complex. The methanol extracts of *E. scaber* leaves showed increased ferric reducing power with the increased concentration as standard antioxidants. Hence, they should be able to donate electrons to free radicals stable in the actual biological and food system.

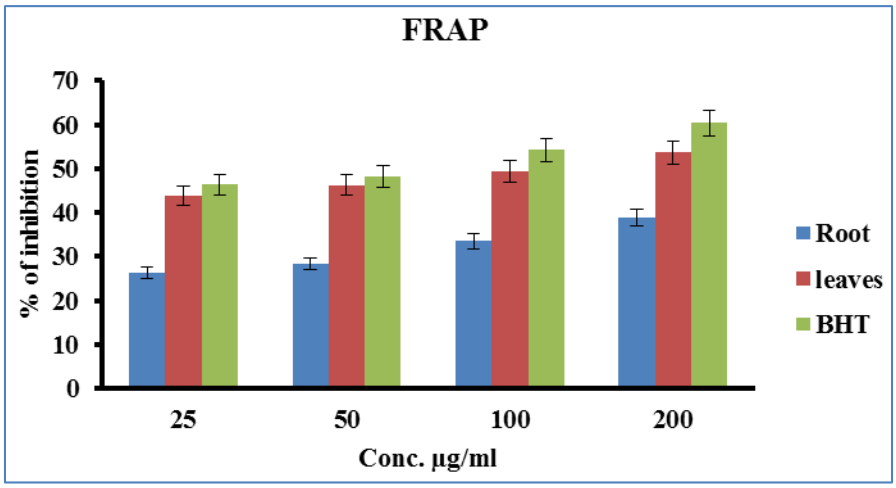

Fig 4: FRAP activity of *E. scaber* root and leaves extracts.

Fig.4 illustrated the percentage inhibition of methanol extracts of root and leaves were maximum at 33.53 and 49.36 % respectively. BHT was used as standard antioxidants with maximum percentage of inhibition in high dose at 56.36 %. Both the extracts exhibited the potent scavenging activities in a concentration dependent manner. The result shows that the

test samples were exhibited potent antioxidant activities in a concentration dependent manner, which indicates the antioxidant activities of the extracts.

Effect of methanol extracts *E. scaber root* and leaves on Superoxide anion

The superoxide anion is known to be very harmful to cellular components as they serve as precursors of more ROS [26].In the present study, superoxide radical reduces NBT to blue coloured formazan that is measured at 560 nm [25]. Methanol leaves extract of *E. scaber* was found to be a good scavenger of superoxide radicals as the absorbance was decreased with the increase in concentration of the extract. The probable mechanism of scavenging the superoxide may be due to the inhibitory effect of the methanol extract of *E. scaber* towards generation of superoxides in the *in vitro* reaction mixture. This inhibitory effect of extracts may be due to the removal of oxygen from the reaction mixture. The effect may also be mediated by trapping the electron released from EDTA for the generation of superoxide anion or by reducing superoxide anion to a non- radical.

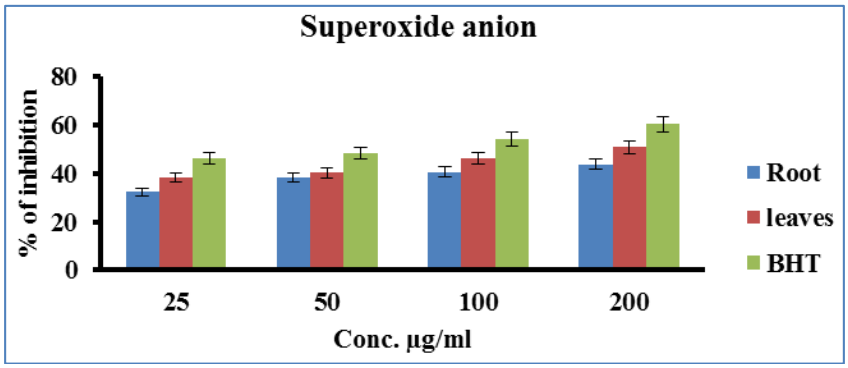

Fig 5: Superoxide anion scavenging activity of *E. scaber* rot and leaves extracts.

The superoxide anion scavenging activity of the methanol extracts of the root and leaves of *E. scaber* plant were also occurs in dose–response manner, as BHT. The extract of *E. scaber* leaves scavenge superoxide anion efficiently than the stem extract. At a concentration of 200 µg/mL, the scavenging activity of the root and leaves reached 43.72 % and 50.82 % respectively. BHT was used as standard antioxidant and inhibition of superoxide anion formation was 60.36 % in 200 µg. The increase in concentration of extracts and BHT increases the scavenging activity.

Effect of Methanol extracts of *E. scaber* root and leaves on Hydroxyl radical scavenging activity

Hydroxyl radicals can be generated by biochemical reaction. Superoxide radical is converted by superoxide dismutase to hydrogen peroxide, which can subsequently produce extremely reactive hydroxyl radicals in the presence of divalent metal ions, such as iron and copper [26]. The results demonstrated that the methanolic extract of *E. scaber* on leaves and stem had appreciable hydroxyl radical scavenging activity when compared with standard antioxidant BHT.

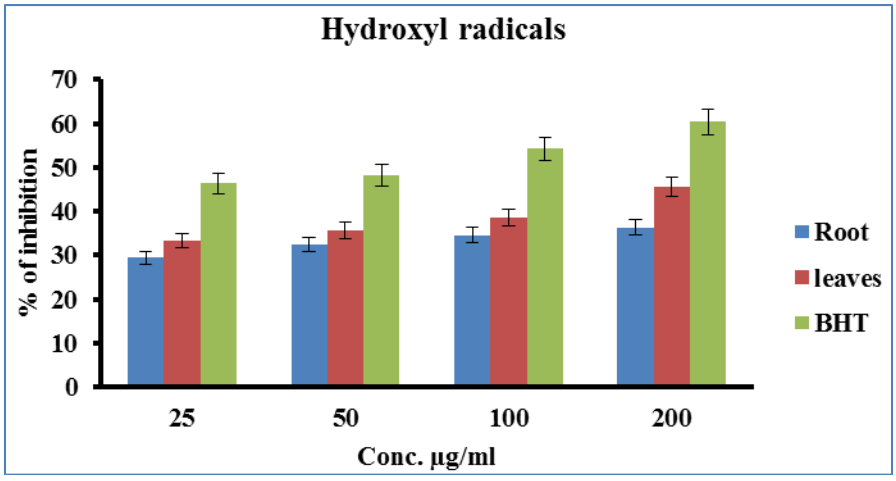

Fig 6: Hydroxy radical scavenging activity of *E. scaber* root and leaves extracts

The hydroxyl radical scavenging activity of the methanol extracts of the root and leaves of *E. scaber* plant was displayed in figure 6. The results shows that the methanol extract of the *E. scaber* leaves have higher activity than that of the stem extract of *E. scaber*. At a concentration of 100 µg/mL, the scavenging activity of the root and leaves reached 34.53 and 38.66 %, respectively. BHT was used as standard antioxidants exhibit maximum inhibition at 56.36 %. The scavenging activities of both the extracts were increased in dose dependent manner.

Effect of methanolic extracts of *E. scaber* root and leaves on H_2O_2 activity

Hydrogen peroxide is a weak oxidizing agent that inactivates a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. It can cross cell membranes rapidly; once inside the cell, it can probably react with Fe^{2+} and possibly Cu^{2+} ions to form hydroxyl radicals and this may be the origin of many of its toxic effects [27]. From the results, it appeared that the H_2O_2 scavenging activity of the plant extracts are comparable with the standard.

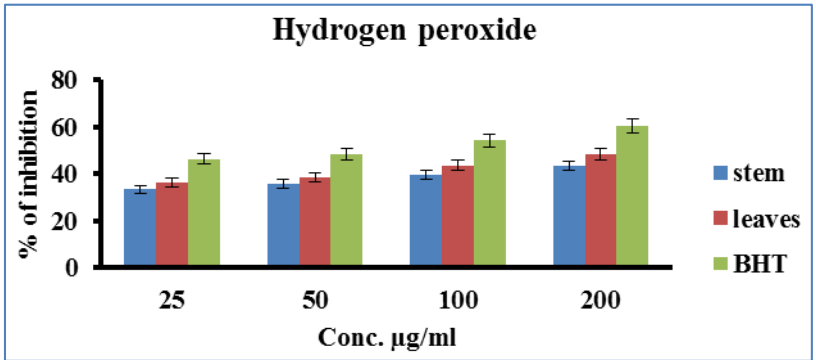

Fig 7: H_2O_2 scavenging activity of *E. scaber* root and leaves extracts.

The percentage inhibition of methanol extracts of root and leaves were 39.56 and 43.65 % respectively. BHT was used as standard antioxidant and exhibit percentage inhibition of 56.36 %. Hydrogen peroxides were scavenged by the extracts of *E. scaber* in efficient manner as the standard. The maximum concentration shows the maximum activity. In this study, 200 µg/ml was the maximum dose and both the extracts at this concentration shows highest scavenging activity.

Effect of Methanol extracts of *E. scaber* root and leaves on reducing activity

This assay is used to evaluate the ability of natural antioxidant to donate electron. In a redox reaction, antioxidants act as 'reductants'. In this context, the antioxidant power can be referred to as 'reducing ability'. A reducing power is an indicative of reducing agent having the availability of atoms which can donate electron and react with free radicals and then convert them into more stable metabolites and terminate the radical chain reaction [28]. Reduction power, is widely used in evaluating antioxidant activity of plants, was determined by the potassium hexacyanoferrate (III) reduction method. In this assay, the presence of reductans in the antioxidant sample causes the reduction of the Fe^{+3}/Hexacyanoferrate (III) complex to Fe^{+2}/Hexacyanoferrate (III) complex, so the reducing power of sample can be monitored by measuring the formation Perl's Prussian Blue complex at 700 nm. Increased absorbance indicated increase antioxidant activity.

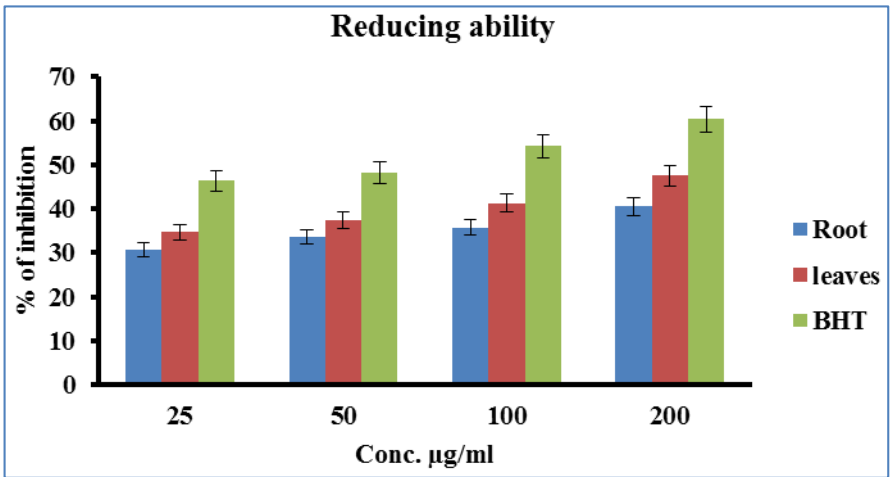

Fig 8: Reducing ability of *E. scaber* root and leaves extracts.

The reducing activity of the methanol extracts of the root and leaves on *E. scaber plant* possess in dose–response manner as BHT. It was observed that the extract of the *E. scaber* leaves had higher activity than that of the root extract. At a concentration of 200 µg/mL, the reducing activity of the root and leaves reached 40.52 and 47.53 % respectively. The percentage inhibition of standard BHT was 60.36 %. Both the test samples exhibited potent reducing activity in a concentration dependent manner.

Effect of methanolic extracts of *E. scaber* root and leaves on metal ion activity

Ion is essential for oxygen transport, respiration and activity of many enzymes. However, iron is an extremely reactive metal and catalyzes oxidative changes in lipids, proteins and other cellular components [29]. It causes lipid peroxidation through the Fenton and Haber-weiss reaction ($H_2O_2 + Fe^{2+}$ ---> Fe^{3+} OH- +OH-), and decomposes the lipid hydroxide into peroxyl and alkoxyl radicals that can perpetuate the chain reactions.

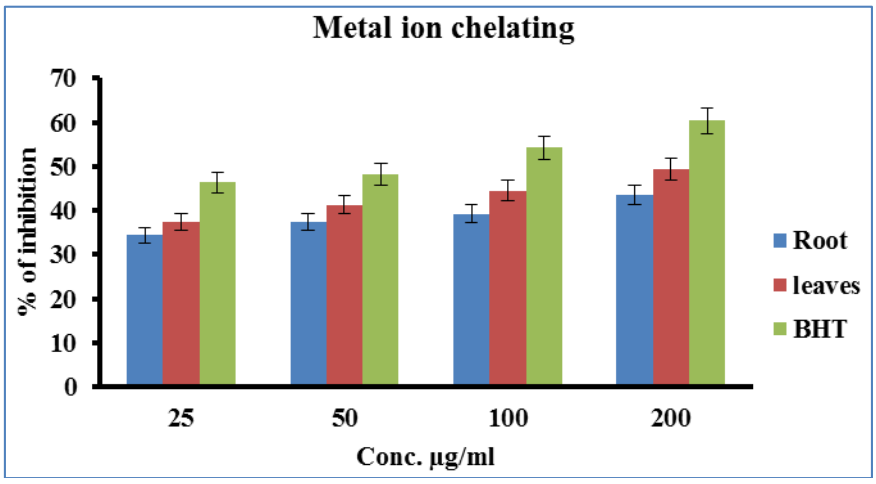

Fig 9: Ion chelating activity of *E. scaber* root and leaves extracts.

The ion chelating activity of the methanolic extracts of the root and leaves of *E. scaber* was shown in fig. 9. The result shows that the extract of the *E. scaber* leaves had more activity than that of the extract of root. At a concentration of 200 µg/mL, the scavenging activity of the root and leaves reached 43.45 and 46.35 %, respectively. BHT was used as standard antioxidants with 60.36 %. All test samples exhibited ion chelating activities in a dose dependent manner.

Effect of methanolic extracts of *E. scaber* root and leaves on phosphomolybdenum activity.

The total antioxidant potential of *E. scaber* medicinalis plant extracts of root and leaves fractions was estimated from their ability to reduce the Mo (VI) to Mo (V) and resulting in the formation of green phosphate/Mo (V) complex with maximum absorption at 695 nm. Increased Absorbance of the reaction mixture indicated increased total antioxidant capacity [30]. In the present assay all the different fractions showed good total antioxidant index, which was concentration-dependent manner [31]. The results of reducing power of methanol extracts of root and leaves of *E. scaber* are illustrated in fig.10.

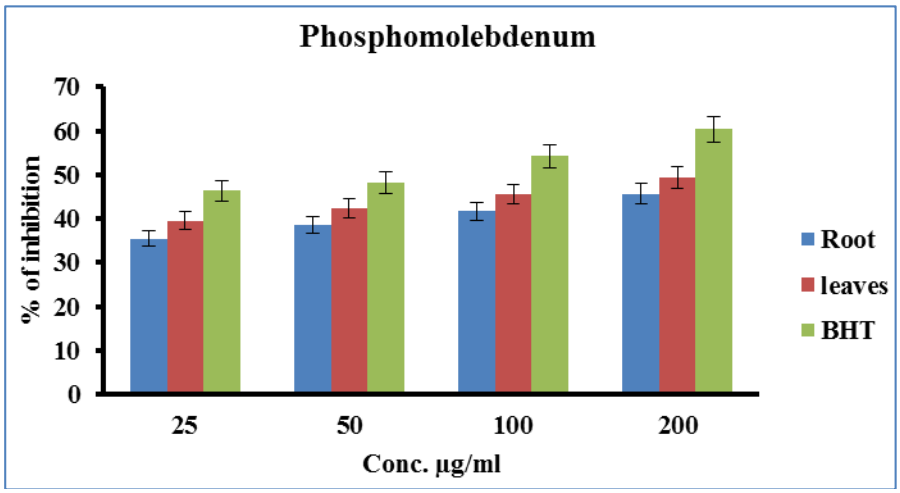

Fig 10: Phosphomolybdenum activity of *E. scaber* root and leaves extracts

The maximum percentage inhibition of methanol extracts of root, leaves and BHT were 45.66 %, 49.26 % and 60.36 % respectively at 200µg/ml. All test samples exhibited potent scavenging activities in a concentration dependent manner. In the present study, root and leaves extracts of *E. scaber* showed that 200 µg/ml of methanolic extracts of leaves was more effective in reduction of Mo(VI) to Mo(V) while the lowest effect was shown root extract of 25 µg/ml concentration. The reduction of Mo (VI) to Mo (V) by the extracts suggested the presence of effective antioxidants in various fractions of *E. scaber*

Fourier Transform Infrared Spectrophotometer (FT-IR)

FT-IR spectral analysis was used to characterise the functional groups of plant parts like leaf and stem of the medicinal plant of *E. scaber*. Ragavendran *et al.* (2011) [32]have reported the presence of characteristic functional groups of *E. scaber* such as carboxylic acids, amines, amides, sulphur derivatives, polysaccharides, nitrates, chlorates, and carbohydrate, which are responsible for various medicinal properties. The functional groups of *E. scaber* were analyses by FT-IR displayed in fig. 11 and table 1. All fractions were rich in phenols, carbonyl compounds, and other compounds.

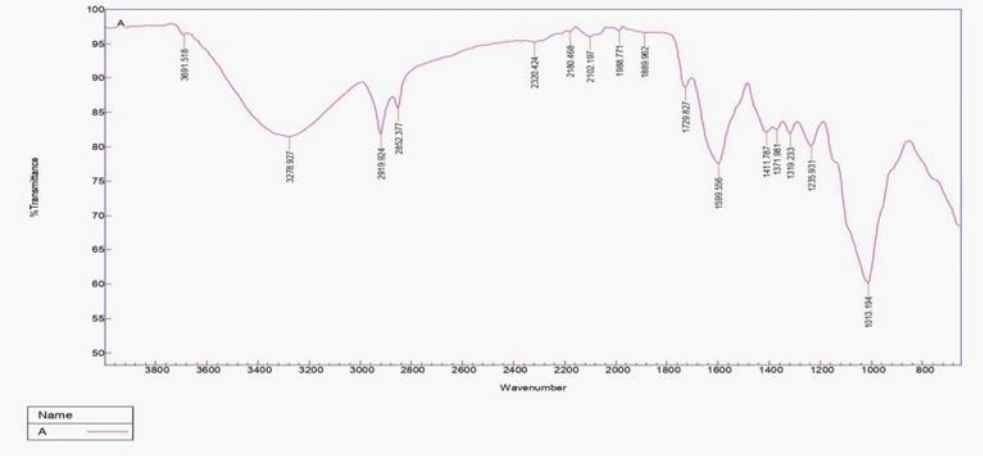

Fig 11: Fourier Transform Infrared Spectrophotometer (FT-IR) *E. scaber*

Table 1: FT-IR peak values of methanolic extract of *E. scaber*

S. No	Peak values	Functional groups
1	3691.927	-OHgroup and C-H group
2	3278.927	C-H stretching and Alkanes
3	2919.924	C-O group
4	2852.377	Alphatic amines
5	1899.194	Carboxylic acid

Conclusion

In the present investigation, root and leaves extracts of *E. scaber* with various groups of phytochemicals exhibited potential scavenging effects on DPPH, ABTS, FRAP, NO, H_2O_2, hydroxy radical, superoxide anion and also exhibit potent ion chelating, phosphomolybdenum and reducing ability. The results of this study shows that the leaves extracts of *E. scaber* contained high level of phenolic and flavonoid content that might have accounted for the strong antioxidant activity against the free radicals and for many pharmacological properties of *E. scaber*. Since this investigation is a preliminary study, a detailed study of the antioxidant mechanisms of specific phenolic components has an absolute necessity.

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