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EVALUATION OF *IN VITRO* ANTIOXIDANT ACTIVITY IN EDIBLE FRUITS OF *EHRETIA LAEVIS* ROXB.

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ABSTRACT

Four successive extracts of the fruits of *Ehretia laevis* were subjected to preliminary phytochemical analysis, total phenols, tannins, flavonoid and Vitamin C content analysis and subjected to *in vitro* antioxidant activity using eight different test methods including Reducing ability, Ferrous chelating and metal chelating ability, DPPH (1, 1-diphenyl-2-picrylhydrazyl), Hydroxyl radical, Hydrogen peroxide, Nitric oxide radical scavenging abilities. The total phenol and flavonoid contents in the methanol extract were found to be higher than that of other extracts. The reducing power ability of the methanol extract was commendable with IC_{50} value of 27.33 ± 0.45µg/ml. The DPPH radical scavenging activity of the hexane extract was richer than that of the methanol extract. Overall, methanol extract was found to have high antioxidant potential. This antioxidant potential along with good phenolic content tends to pave this wild plant fruit suggested for domesticate and health supplement which will definitely fragile the disease prone.

KEYWORDS: ***Ehretia laevis*, Antioxidant activity, DPPH, phenolics, tannins, vitamin C**

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INTRODUCTION

Free radicals contribute to more than one hundred disorders in humans including atherosclerosis, arthritis, Ischemia and reperfusion injury of many tissues, central nervous system injury, gastritis, cancer and AIDS. Free radicals due to environmental pollutants, radiation, chemicals, toxins, deep fried and spicy foods as well as physical stress, cause depletion of immune system, change in gene expression and induced abnormal proteins. Oxidation process is also one of the most important routes for producing free radicals in food, drugs and even living systems.[1, 2]. It is possible to reduce the risks of chronic diseases and prevent disease progression by either enhancing the body's natural antioxidant defenses or by supplementing with proven dietary antioxidants[3]. Because synthetic antioxidants showing low solubility and moderate antioxidant activity[4]. Therefore, a practical way to control these diseases is to increase the dietary intake of fruits and vegetables, which are rich sources of antioxidants[5, 6].Antioxidant compounds in food play an important role as a health-protecting factor. Natural antioxidant substances are accepted to be secure and sound from the time when they occur in plant foods and more enviable than synthetic one[7]. *Ehretia laevis* is a small tree of Boraginaceae family commonly found in Asia and Australian tropics. The plant has significant medicinal importance and finds uses in traditional medicine as a remedy for the treatments of diarrhea, cough, cachexia, syphilis, toothache, stomach and veneral disease and as an antidote to vegetable poison[8].In connection to the above, all parts of the *Ehretia laevis* plant are used for different medicinal purposes. Decoction of the fresh root is used in the treatment of syphilis and that of the stem bark for the treatment of diphtheria. The paste of tender leaves is used externally to cure eczema, and the powder of flowers with milk is employed as an aphrodisiac (http://www.indianetzone.com/38ehretia -laevis-roxb-plant.htm). The leaves are used as cattle fodder. It has many uses such as ornaments, pot herbs, dye for wood, stone, medicines, wines, cosmetics etc. the inner bark of the tree and the insipid fruit of *Ehretia laevis* are eaten in times of scarcity (S. J. Ali, J. Nasir Flora of Pakistan No.191). Leaves are applied to ulcers and in headache. The quantitative estimation of phytoconstituents of trace elements of the leaves study establishes the nutritional value to contribute as the resources of fats, proteins, carbohydrates, vitamin-C, E, A, Riboflavin and Thiamine[9].Fruit is astringent, anthelmintic, diuretic, demulcent, expectorant and is used in affections of urinary passages, disease of lungs and spleen. Powdered kernel mixed with oil is a remedy in ringworm. Seeds are anthelmintic[10]. These reported activities and many of the ethno medical uses of the plant should related to its antioxidant activity. Besides being an edible fruit, there is no previous antioxidant evaluation, hence, the present study is to analyze the total phenols, tannins and flavonoids and to study the *in vitro* antioxidant potential of various extracts of *Ehretia laevis*.

MATERIALS AND METHODS

Plant Collection

The fruits of *Ehretia laevis* Roxb. were collected during the month of January 2012 from Krishnagiri District, Tamil Nadu, India. The Botanical Survey of India, Southern circle, Coimbatore, authenticated the plant ((Ref.no.BSI/SC/5/23/11-12/Tech.552).

Extraction and Phytochemical Analysis

The air-dried and pulverized materials were successively extracted (200g) with hexane, chloroform, ethyl acetate and methanol in Soxhlet's apparatus for 24 hours individually and filtered. The extracts were concentrated to dryness under reduced pressure and controlled temperature (45-60º C) in vacuum in a rotary evaporator. The percentage yield was expressed in terms of air- dried weight of the plant material which was calculated by

comparing the fruit powder (in g) taken for the extraction and yield (in mg). All the extracts were subjected to qualitative chemical tests for identification of various phytochemicals[11]. The total phenol content was determined by Folin-Ciocalteu's reagent[12] and the total flavonoid content was determined by the aluminium chloride method[13]. Tannins were estimated by modified Prussian blue method and calculated as tannic acid equivalents. Vitamin C content was determined using the Dichloroindophenols (DIP) method with a modification by Yen and Chen, 1996[14] and calculated using standard Ascorbic acid.

Preparation of test and standard solutions

Four successive extracts of *E. laevis* and the standard antioxidants [ascorbic acid, rutin, butylated hydroxyanisole(BHA) and α-tocopherol] were dissolved in DMSO individually and used for *in vitro* antioxidant assay with eight different methods. For, hydrogen peroxide method alone, the extracts and the standards were dissolved in methanol just before use. More than one standard have been used here for better understand of the extract ability.

IN VITRO ANTIOXIDANT ANALYSIS

Reducing Power Ability

The reducing power was assayed as described by Fejes *et al*., 2000[15]. One ml of the extract (100-500 µg/ml), 2.5 ml of phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide were incubated at 50°C for 30 min and 2.5 ml of 10% trichloroacetic acid was added to the mixture and centrifuged for 10 min at 3000 g. About 2.5 ml of the supernatant was diluted with 2.5 ml of water and shaken with 0.5 ml of freshly prepared 0.1% ferric chloride. The absorbance was measured at 700 nm. Butylated Hydroxyl Anisole (BHA) (50-800 µg/ml) was used as the standard. All tests were performed in triplicate and the graph was plotted with the average of the three determinations. Increased absorbance values indicate a higher reducing power.

DPPH Radical Scavenging Assay

The free radical scavenging activity of the fractions was measured *in vitro* by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) assay[16]. About 0.3 mM solution of DPPH in 100% ethanol was prepared and 1ml of this solution was added to 3 ml of the extracts dissolved in ethanol at different concentrations (25-400 µg/ml). The mixture was shaken and allowed to stand at room temperature for 30 min and the absorbance was measured at 517 nm using a spectrophotometer. The percentage scavenging activity at different concentrations was determined and was compared with that of ascorbic acid, which was used as the standard.

Hydroxyl Radical Scavenging Assay

Hydroxyl radical scavenging assay was carried out using iron-EDTA solution[17]. Extracts of different concentrations were placed in a test tube and evaporated to dryness. 1 ml of iron-EDTA solution [0.13% ferrous ammonium sulfate and 0.26% EDTA], 0.5ml of 0.22% ascorbic acid was added to each tube. The tubes were capped tightly and heated in a water bath at 80-90°C for 15 min. The reaction was terminated by adding 1ml of ice-cold trichloroacetic acid (17.5% m/v). 3ml of Nash reagent (75.0 g ammonium acetate, 3ml glacial acetic acid and 2ml acetyl acetone were mixed and water was added to a total volume of 1L distilled water) was added to each tube. The tubes were left at room temperature for 15min for colour development. The intensity of the yellow colour formed was measured at 412nm against a blank of the reagent. Percentage of inhibition was determined by comparing the results of the test and standard compounds of Butylated Hydroxyl Anisole.

Hydrogen Peroxide Scavenging Assay

Hydrogen peroxide scavenging activity was performed by the method following Guddadarangavvanahally *et al*., 2004[18]. Hydrogen peroxide solution (20 mM/L) was prepared with standard phosphate buffer (pH 7.4). Different concentration of the extracts (50-400 µg/ml) in distilled water was added to 0.6 ml of hydrogen peroxide solution. Absorbance was

determined at 230 nm after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage scavenging activity at different concentrations of the extracts was determined and the IC_{50} values were compared with the standard, ά-tocopherol.

Nitric Oxide Radical Inhibition Assay

The reaction mixture (6 ml) containing sodium nitroprusside (10 mM, 4 ml), phosphate buffered saline (1ml) and extract (1ml) was incubated at 25°C for 15 min. After incubation 0.5ml of the reaction mixture was removed 1ml of sulphanilic acid reagent (0.33% in 20% glacial acetic acid) was mixed and allowed to stand for 5 min for completion of the diazotization reaction. 1 ml of naphthyl ethylene di amine dihydrochloride was added, and the mixture was allowed to stand for 30 min in diffused light. The absorbance was measured at 540 nm. Ascorbic acid was used as the standard[19].

Thiocyanate Method

The peroxy radical scavenging activity was determined by thiocyanate method. Increasing concentration of the extracts (50-800 µg/ml) in 0.5 ml of distilled water was mixed with 2.5 ml of 0.02 M linoleic acid emulsion (in 0.04 M phosphate buffer pH 7.0) and 2ml phosphate buffer (0.04 M, pH 7) in a test tube and incubated in darkness at 37°C. At intervals during incubation, the amount of peroxide formed was determined by reading the absorbance of the red colour developed at 500 nm by the addition of 0.1 ml of 30% ammonium thiocyanate solution and 0.1ml of 20mM ferrous chloride in 3.5% hydrochloric acid to the reaction mixture[20]. The percentage scavenging activity was compared with the standard, ά-tocopherol.

Ferrous Chelating Ability

The ferrous chelating ability was assayed as described by Huang and Kuo, 2000[21]. The reaction mixture containing 1.0 ml of different concentrations of the fractions (50-800 µg/ml) was mixed with 3.7 ml of methanol, 0.1 ml of 2mM ferrous chloride and 0.2 ml of 5 mM ferrozine to initiate the reaction and the mixture was shaken vigorously and left to stand at room temperature for 10 min. The absorbance of the solution was measured at 562 nm[21]. The percentage chelating effect on ferrozine-ferrous complex was calculated. The percentage scavenging activity was compared with ascorbic acid.

Evaluation of Total Antioxidant Activity

The total antioxidant capacity of the extracts was determined by phosphomolybdate method[22]. An aliquot of 0.1ml of the extracts (100 µg) solution was combined with 1.0 ml of reagent (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated in a boiling water bath at 95°C for 90 min. After the samples had cooled to room temperature, the absorbance was measured at 695nm against the blank using an UV spectrophotometer. The blank solution contained 1.0 ml of reagent solution and the appropriate volume of the same solvent used for the sample and it was incubated under same conditions as rest of the sample. The total antioxidant capacity was compared with Ascorbic acid.

RESULTS AND DISCUSSION

Yield Percentage

The methanol extract of *E. laevis* showed highest yield recovery (6%) and it was a brown coloured residue. The yields of extracts vary in quantity due to their polarity. The yield of extract in this study gradually rises as the extraction proceeded from non polar solvent to polar solvent. Hence, logically, the *E. leavis* plant fruit is found to have rich quantity of polar compounds than non polar compounds.

Preliminary Phytochemical Estimation

The preliminary phytochemical analysis indicated the presence of alkaloids, flavonoids, tannins and phenols in all the four extracts which is shown in table 1. Steroids were present in hexane and methanol extract. Steroids were found to be present in all the plants. The present investigated plants contained steroidal compounds. It should be noted that steroidal

compounds are of importance and interest in pharmacy due to their relationship with such compounds as sex hormones[23]. Except for ethyl acetate extract, triterpenoids were present in other three extracts. Saponins were absent only in hexane extract. Oils and fats were found only in hexane extract. Diversity of medicinal plants and herbs containing various phytochemicals with biological activity can be of valuable therapeutic key. Different phytochemicals have been found to have a broad range of activities, which may help in protection against chronic diseases[9].

Table 1
Characteristics and phytochemical content of the different extracts of the fruit of E. laevis

Extract	Colour	Yield Percentage	Phytochemical content (Qualitative)
Hexane	Reddish brown sticky residue	2.6%	Alkaloids, flavonoids, steroids, phenols, tannins, triterpenoids, oils, fats
Chloroform	Deep brown sticky residue	3.8%	Alkaloids, flavonoids, saponins, phenols, tannins, triterpenoids
Ethyl acetate	Brown semisolid residue	4.4%	Alkaloids, flavonoids, saponins, glycosides, tannins, phenols.
Methanol	Brown semisolid residue	6 %	Alkaloids, flavonoids, phenols, tannins, steroids, saponins, triterpenoids, glycosides.

Quantification of Phenols, Flavonoids, Tannins and Vitamin C

Phenolic compounds are commonly found in both edible and non-edible plants and they have multiple biological effects. Tannins were identified as another class of phenolics in the tested medicinal herbs[24]. The total phenol, flavonoid, tannins and vitamin C contents in the methanol extract of the fruit of *E.laevis* were found to be higher than that of other extracts. The total phenolics of methanol is 92.56mg GAE/g and flavonoids as 57.23 mg Rutin equivalence per gram(mg RE/g) and Vitamin C as Acetic acid equivalence such as 56.09 mg AAE/g and lowest quantity is reported in hexane extract (23.12 mg AAE/g) The results of total phenolics representing in table 2 as Gallic acid equivalence in gram. The presence of phenolic compounds (phenolic acids, polyphenols and flavonoids) in plant, herbs and spices is gaining increasing attention because of their various functions, such as antioxidant activity and flavouring properties[25]. According to Ferguson et al. (2004)[26], several plant species rich in flavonoids were reported having disease prevention and therapeutic properties.Ethyl acetate extract is next to the methanol in all the tabulated content except tannin. Tannin is mentioning as tannic acid equivalence per gram. Chloroform extract is yielding well next to the methanol in this case. Overall the results were shown in table 2. Primary constituents comprise common sugars, amino acid, proteins and chlorophyll while secondary constituents consist of alkaloids, terpenoids, saponins, phenolic compounds and so on. These natural compounds formed the foundations of modern prescription drugs, as we know today[27].

Table 2
Quantification of phenols, flavonoids, tannins and Vitamin C

Extract	Total phenols (mgGAE/g) [a]	Flavonoids (mg RE/g) [a]	Tannins (mgTAE/g) [a]	Vitamin C (mgAAE/g)[a]
Hexane	40.00±0.23	12.83±0.63	34.54±0.43	23.12±0.02
Chloroform	76.45±0.78	23.07±0.21	56.09±1.12	36.07±0.00
Ethyl acetate	84.32±0.13	46.22±0.28	43.03±0.12	46.01±0.52
Methanol	**92.56±0.38**	**57.23±0.12**	**63.21±0.70**	**56.09±0.21**

[a] ***Average of three determinations, mean±SEM***

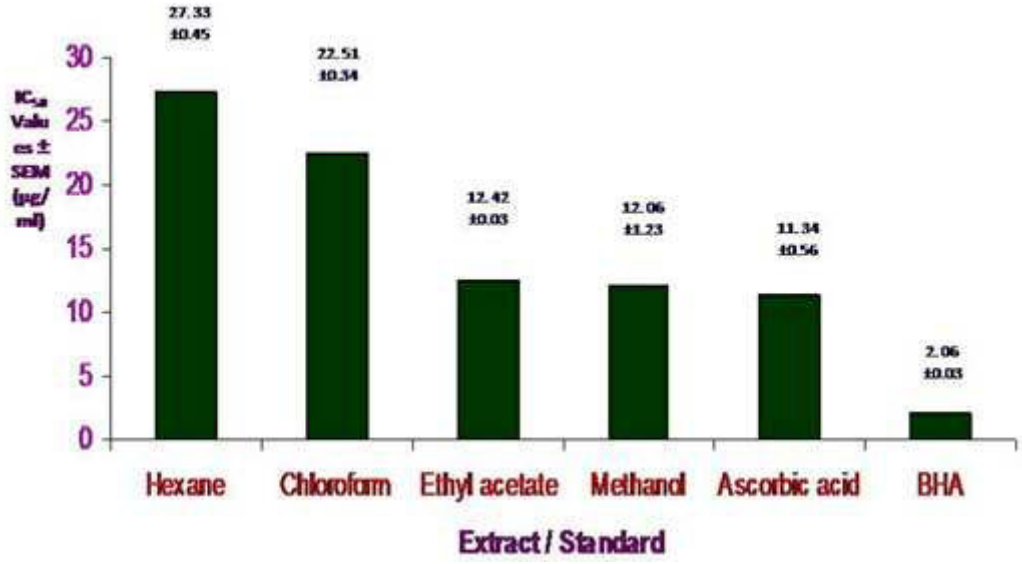
Antioxidant Activity

Many antioxidant compounds, naturally occurring from plant sources, have been identified as free radical or active oxygen scavengers[28]. Recently, interest has increased considerably in finding naturally occurring antioxidant for use in foods or medicinal materials to replace synthetic antioxidants, which are being restricted due to their side effects such as carcinogenicity. Natural antioxidants can protect the human body from free radicals and retard the progress of many chronic diseases as well as retard lipid oxidative rancidity in foods[29, 30].

Reducing power Ability

The presence of reductants (antioxidants) in the extracts would result in the reduction of Fe^{3+}/ ferricyanide complex to the ferrous form. The Fe^{2+} can therefore be monitored by measuring the formation of Perl's Prussian Blue at 700 nm[31]. The reducing power ability of the various solvent extracts of the sample studied is shown in Fig. 1. The reducing power ability of the methanol extract was the efficient with IC_{50} value of 12.06 ±1.23 µg/ml, followed by ethyl acetate and then chloroform extract, where the efficiency was approximately equivalent to the standard ascorbic acid (11.34 ±0.56).

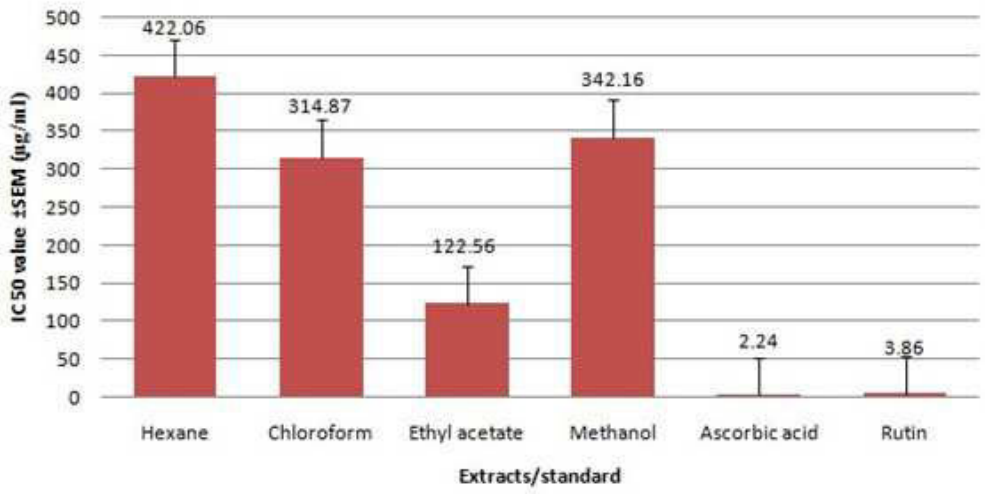
Figure 1
IC 50 values (µg/ml) of the reducing power ability of the different solvent extracts of E. laevis with the standards



DPPH radical Scavenging Activity

The free radical scavenging activity of different parts of the different solvent extracts of the fruit of *E. laevis* was determined DPPH radical scavenging activity along with the standards Ascorbic acid, rutin and BHA and the results are shown in Fig. 2 and are expressed as IC_{50} values. This activity occurs due to the scavenging of radical by hydrogen ion donation. It causes a visually noticeable colour change of the test solution from purple to yellow. The least IC_{50} value indicates a higher antioxidant activity. The DPPH radical scavenging activity of the ethyl acetate extract was richer (122.56 ±1.27) followed by chloroform extract, methanol extract and the lesser activity is that of hexane extract. The variations in activity may be due to the fact that diversity in the basic chemical structure of phytoconstituents possesses different degree of antioxidant activity against different free radicals[32].

Figure 2
IC $_{50}$ values (µg/ml) of DPPH radical scavenging ability of the different solvent extracts of E. laevis with the standards



Hydroxyl ion Scavenging Activity

Hydroxyl radical is the most deleterious and reactive among the ROS and it bears the shortest half-life compared with other free radicals. Hydroxyl radicals react with lipid, polypeptides, proteins and DNA[33]. Hydroxyl ion scavenging activity was determined by generating the hydroxyl radicals using Ascorbic acid – iron EDTA complex. The ions formed by the oxidation will react with dimethyl sulfoxide (DMSO) to form formaldehyde, which provides a convenient method to detect hydroxyl radicals, by treatment with Nash reagent[34]. As shown in table 3, the hydroxyl ion scavenging activity of the methanol extract (112.02 ±0.26) was the best followed by the ethyl acetate extract (202.66 ±0.33).

Hydrogen Peroxide Scavenging Activity

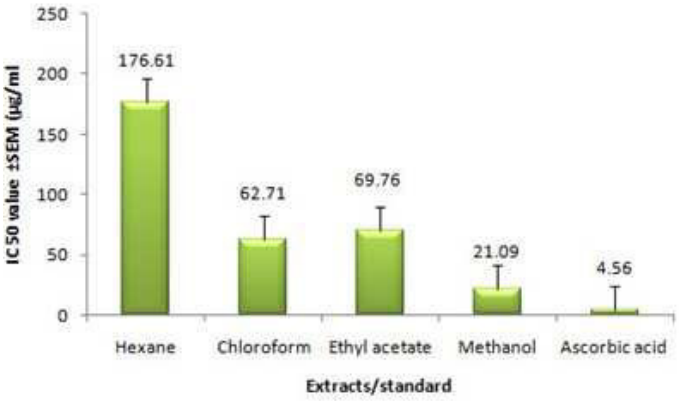
Hydrogen peroxide itself is not particularly reactive with most biologically important molecules, but is an intracellular precursor of hydroxyl radicals, which is very toxic to the cell[35]. Thus, scavenging of hydrogen peroxide is a measure of the antioxidant activity of the extracts. All the different solvent extracts analysed in this study scavenged hydrogen peroxide, which may be attributed to the presence of phenolic groups that could donate electrons to hydrogen peroxide, thereby neutralising it into water. Out of all the four extracts studied, hydrogen peroxide scavenging effect of methanol extract was good with (IC_{50} is 14.86 ±0.28 µg/ml) but poorer than the standards for example ά-tocopherol (3.46 ±0.43 µg/ml).

Table 3
IC_{50} values of Hydroxyl and Hydrogen peroxide antioxidant assays in different extracts of E. leavis fruit

Extract	IC $_{50}$ values (Mean ± SEM)	
	Hydroxyl radical scavenging assay	Hydrogen peroxide assay
Hexane	787.96 ±0.09	72.82 ±0.21
Chloroform	517.26 ±0.32	37.26 ±0.22
Ethylacetate	202.66 ±0.33	22.03 ±2.76
Methanol	**112.02 ±0.26**	**14.86 ±0.28**
Ascorbic acid	>1000	8.32 ±0.12
Rutin	>1000	-
BHA	>1000	4.66 ±0.14
α-Tocopherol	-	3.46 ±0.43

Nitric Oxide Radical Inhibition Assay

Figure 3.
Nitric oxide inhibition ability of the different solvent extracts of E. laevis with the standards



Nitric oxide is a very unstable species, so under aerobic condition, it can react with O_2 to produce stable products such as nitrate and nitrite through intermediates NO_2, N_2O_4. In the presence of a scavenging test compound, the amount of nitrous acid will decrease and can be measured at 546nm. The nitric oxide scavenging effect of the extracts studied is shown in Fig. 3. The higher nitric oxide radical scavenging activity was expressed by methanol extract as 21.09 µg/ml followed by chloroform and the lesser activity was given by hexane extract. This may be due to the antioxidant principles in the extracts, which compete with oxygen to react with NO thereby inhibiting the generation of nitrite.

Thiocyanate method and Ferrous chelating activity

The amount of formed peroxides was measured by the thiocyanate method. A decrease in absorbance indicated the antioxidant activity of the extracts, which might be due to the inactivation of the free radicals and the presence of flavonoid like phytochemicals. As shown in the table 4, methanol extract was found to be good at scavenging activity.The metal chelating ability of the extracts studied is presented in table which was measured by the formation of ferrous ion- ferrozine complex[35]. The methanol extract have an effective capacity of iron binding, suggesting its antioxidant potential when compare to the other extracts (table 4). The metal chelating ability of the extracts demonstrated that they reduce the

concentration of the catalyzing iron metal involved in the peroxidation of lipids.

Table 4
Results shows IC_{50} values of Thiocyanate and Ferrous chelating Ability assays in different extracts of E. leavis fruit

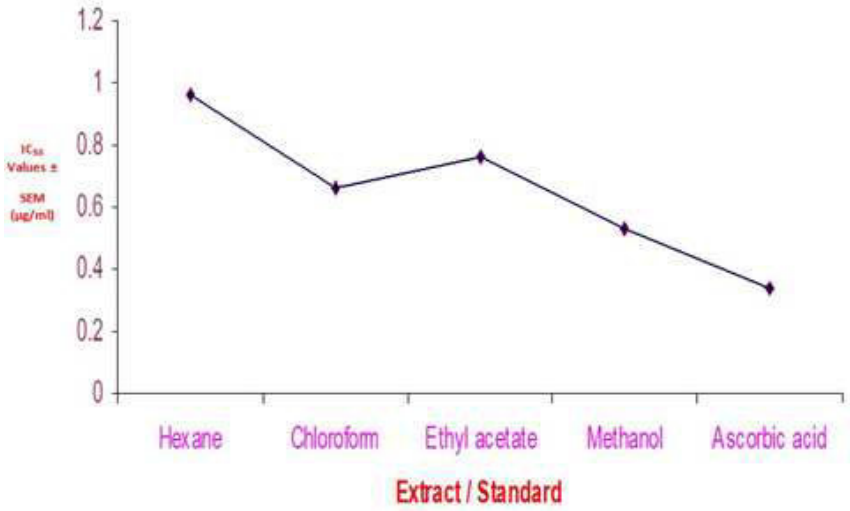
Extract	IC $_{50}$ values	
	Thiocyanate method	Ferrous chelating ability
Hexane	512.32 ± 1.03	392.98± 0.27
Chloroform	212.21 ± 0.89	316.66± 0.89
Ethylacetate	92.32± 0.88	172.36 ± 9.01
Methanol	**72.02 ± 1.54**	**74.01 ± 0.22**
Ascorbic acid	82.76 ± 0.65	42.46 ± 0.61
α- tocopherol	12.45±0.65	-

Total Antioxidant Activity

The phosphomolybdenum method is merely quantitative and collectively, the phosphomolybdenum method usually detects antioxidants such as ascorbic acid, some phenolics, α- tocopherol, and carotenoids[36]. It is also one of the methods to measure the reducing capability and in some studies it is also known as total antioxidant power assay. Since the total antioxidant capacity is expressed as Ascorbic acid equivalents. In this study, the methanol extract is found to effective (Fig.4).

Figure4
Total antioxidant activity of the different solvent extracts of E. laevis with the standards by phosphomolybdenum method.

Phosphomolybdenum Method



CONCLUSION

On the whole, methanol extract was found to have high antioxidant potential. This may be attributed to the presence of high quantity of total phenols and flavonoids in it. The antioxidant capacity of the ethanol extract of stem and leaves of this plant also was found to be high as it was also rich in phenolics and flavonoids[37]. Phenolic compounds are widely

distributed in plants, which have gained much attention, due to their antimutagenic, antitumor, antioxidant activities and free radical scavenging abilities, which potentially have beneficial implication for human health[38]. Plant flavonoids are known to exhibit potent antioxidant activity. Hence, the observed antioxidant activity of the extracts may be due to the presence of these constituents.The antioxidant activity of the fruit of *E.laevis* can be correlated to its phytochemical constituents is wrapping up the present study. This antioxidant potential along with good phenolic content tends to pave this wild plant fruit suggested for domesticate and health supplement which will definitely fragile the disease prone. However, further investigation in animal models and isolation and characterization of the active principle can only establish the nutritional and medicinal potent of the fruit of *E. laevis*.

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