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Original Article

EVALUATION OF *IN VITRO* FREE RADICAL SCAVENGING ACTIVITY OF LEAF EXTRACT FRACTIONS OF *MANILKARA HEXANDRA* (ROXB) DUBARD IN RELATION TO TOTAL PHENOLIC CONTENTS

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ABSTRACT

Objective: To evaluate *in vitro* free radical scavenging, reducing power and total antioxidant activity of leaf extract fractions of *Manilkara hexandra* (Roxb.) Dubard in relation to their total phenolic contents.

Methods: 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging, Fe^{3+}reducing power and the total antioxidant activities of the five extract fractions (petroleum ether extract fraction, PEF; chloroform extract fraction, CHF; ethyl acetate extract fraction, EAF; methanolic extract fraction, MEF; aqueous extract fraction, AQF) were performed and the results were expressed as ascorbic acid equivalent and the total phenolic content was also measured and the data expressed as tannic acid equivalent.

Results: Different extract fractions show the varied degree of free radical scavenging and reducing potentials and the order determined as MEF>AQF>EAF>CHF>PEF. The highest total antioxidant activity, 60.37±6.24 mg of ascorbic acid equivalent, and also the highest total phenolic content, 89.91±8.72 mg of tannic acid equivalent, were estimated in per gram of the leaf MEF. The MEF showed the maximum DPPH free radical scavenging activity, Fe^{3+}reducing power and the total antioxidant activities than all the other extract fractions tested.

Conclusion: *In vitro* free radical scavenging and reducing potentials of the leaf extract fractions of *M. hexandra* were explored here and the leaf MEF identified as the most potential extract fraction contained the highest quantities of antioxidants and that positively correlate with the presence of relatively higher quantities of total phenolic contents.

Keywords: Antioxidant activity, Phenolics, DPPH, Total antioxidant.

INTRODUCTION

Manilkara hexandra (Roxb.) Dubard (Family: Sapotacae; common name in Hindi: Khirni) is a small to medium sized evergreen tree. It is native to South Asia and Tropical Countries and widely distributed in South, North and Central India-mainly in Rajasthan, Gujrat, Madhya Pradesh and Maharashtra [1]. Leaves, fruits, roots and barks of *M. hexandra* are well known for their medicinal values. Its bark is used to treat a wide range of gastrointestinal symptoms like ulcers, dyspepsia, opacity of cornea, bronchitis, urethrorrhea, leprosy, astringent, refrigerant, aphrodisiac, alexipharmic, stomachic, anthelminthic, ulorrhagia, ulitis, odontopathy, fever, flatulence, colic, helminthiasis, hyperdipsia, burning sensation and vitiated conditions of pitta [2] and it contains procyanidins, flavonoids, terpenoids and saponins [3]. Fruits of *M. hexandra* are a good source of minerals, sugars, proteins, carbohydrates and Vitamin A and its consumption is also useful in hallucinations, loss of consciousness, anorexia, dipsia, bronchitis, urethrorrhea, leprosy and antimicrobial property against different bacterial strains [4]. The leaf alcoholic and aqueous extracts were having antimicrobial property [5] and the stem bark methanolic extract has been shown to have antioxidant activity [6].

The majority of pathological conditions like hyperglycemia, cancer, atherosclerosis, rheumatism, cataracts, acquired immune deficiency syndrome, and many other old age and auto-immune diseases are associated with oxidative stress [7]. There are various groups of free radical scavengers, reducing agents/antioxidants, like vitamins C and E, thiols, polyphenols, tri-peptide like glutathione, enzymes such as peroxidase, catalase and superoxide dismutase that act to prevent oxidative damages to deoxyribo nucleic acid (DNA), proteins and lipids [8-10]. Antioxidants can delay, prevent or inhibit the oxidation of other oxidisable substances by scavenging free radicals, reactive oxygen species (ROS) and retreating oxidative stress [11]. Antioxidants are considered as important components of nutraceuticals because these have many health benefits and are extensively used in the foodstuff industry as inhibitors of lipid peroxidation. The interest on the protective biochemical functions of natural antioxidants became increased as they have lesser side effects conversely synthetic antioxidants cause liver damage and carcinogenesis [12]. Vegetables, fruits, seeds, woods, barks, roots, leaf spices and herbs are the potential source of antioxidants [13].

Phenolics are one of the major aromatic secondary plant metabolites with phenol group. They are responsible for the sensory qualities, colour, nutritional and antioxidant properties of foods [14, 15]. Plant food products are rich in phenolics like phenols, phenolic acids, flavonoids, tannins, lignans etc. play vital role in the maintenance of human health [16]. Phenolics are the major groups of phyto constituents with free radical scavenging potentials and associated with the diverse pharmacological activities like antiinflammatory, antimicrobial, antiviral, antiallergic, and anticarcinogenic [17-21]. Moreover, they can diminish oxidative modification of lipid peroxidation and low density lipoprotein by means of their antioxidant activities [22]. Flavonoids are the major group of phenolic compounds which derive from phenylalanine, tyrosine and malonate. Well studied bio flavonoids are flavans, flavones, flavanonols, flavonols, flavanones, flavanols, catechins, anthocyanidins and isoflavones [23]. These are found in vegetables, fruits, grains, barks, roots, stems, flowers, tea and wine [24]. Flavonoids function primarily as antioxidant, anti-inflammatory, anti allergic, anticancer, Antiaggregational, cardio protective [25] anti atherosclerotic and detoxification activities as their hydroxyl groups having free radical scavenging ability [26]. Many studies have correlated antioxidant activity with high phenolic contents such as in oregano, clove, peppermint, garden thyme, sage, all spice and cinnamon [27]. The renewed interest on antioxidants is mainly due to the fact that the major pathological conditions are linked with the oxidative stress and reactive oxygen species. At the present state of knowledge the free radical scavenging and reducing potentials of leaf extracts of *M. hexandra* are not well studied. Therefore, this study was performed to evaluate *in vitro* free radical scavenging and reducing potentials of leaf extract fractions of *M. hexandra* in relation to their total phenolics contents. In addition, the relative abundance of the other phytochemicals presence in all the extract fractions was also detected.

MATERIALS AND METHODS

Materials

Quercetin was purchased from Sigma-Aldrich, St Louis, MO, USA. Tannic acid powder was obtained from Himedia Laboratories Pvt. Ltd., Mumbai, India. Aluminium chloride was obtained from Merck Specialities Pvt. Ltd., Mumbai, India. Ammonium molybdate and sulphuric acid were obtained from Qualigens, Sodium phosphate was obtained from Merck Specialities Pvt. Ltd., Mumbai, India. Folin-Ciocalteu and Sodium citrate were obtained from BDH Chemicals Ltd., Poole Dorset, UK. Benzene and Ethyl acetate were obtained from SRL, Pvt. Ltd., Mumbai, India. Other chemicals used in this study were of analytical grade and obtained from the reputed manufacturers.

Plant products collection, storage and extract preparation

Fresh leaves of *M. hexandra* were collected from the Golapbag campus of The University of Burdwan, West Bengal, India. This plant species was identified by Professor Ambarish Mukherjee, Taxonomist, Department of Botany, The University of Burdwan. The voucher specimen (No**.** BUGBSD015) is maintained in the Department of Zoology for future reference.

Collected leaves were washed in running tap water, shade dried, directly crushed into small pieces and followed to pulverize using electric grinder (Philips Mixer Grinder HL1605). Leaf powder was stored in air tight container for future use. Using soxhlet apparatus and different organic solvents successive five extract fractions of the dried leaf powder were prepared.

DPPH free radical scavenging assay

The antioxidant capacity of the leaf extracts fractions of *M. hexandra* was determined by DPPH free radical scavenging assay [28, 29]. It has an advantage than the other such assays as it is not affected by the side reactions like metal chelating and enzyme inhibition [30]. DPPH, a free radical, becomes colorless when it accepts an electron or hydrogen radical. Ascorbic acid (5-100 µg/ml) was used as a standard antioxidant. DPPH methanolic stock solution (0.002%) was freshly prepared. 1 ml of different concentrations (5, 10, 15, 20, 25, 50 and 100 µg/ml) of ascorbic acid and all the leaf extract fractions of *M. hexandra* were taken in the respective test tubes. Then in each test tube 3 ml of methanol and followed by 0.5 ml of 1 mM DPPH solution was added. The test tubes were then incubated in darkness for 35 min at room temperature (25±2 °C) and the optical density was measured at 517 nm using spectrophotometer (UV-1800 Series, Shimadzu, Japan). Percentage of free radical scavenging activity was calculated by the following equation—

$$\frac{\text{Absorbance of Control- Absorbance of Sample}}{\text{Absorbance of Control}} \times 100$$

Reducing power assay

Reducing power assay is widely used as an indicator of antioxidant activity. Reducing power of the different leaf extract fractions of *M. hexandra* was determined according to the method as described by Oyaizu [31] with little modification. Briefly, 0.5 ml of ascorbic acid and the different extract fractions (10, 25, 50, 75 and 100 µg/ml) were taken in the respective test tubes and then 1 ml of phosphate buffer (0.2 M) and 1 ml of $K_3Fe(CN)_6$ (1%) were added and the mixtures were incubated at 50º C for 20 min. Then 1 ml 10% TCA was added to each of the test tubes and centrifuged at 3000 rpm for 10 min. 1.5 ml of the supernatant was mixed with 1.5 ml distilled water and 0.1 ml $FeCl_3$ (0.1%) solution. The absorbance was measured at 700 nm on spectrophotometer. Higher absorbance indicates the higher reducing power and that was compared with the standard ascorbic acid. The effective extract concentration providing the half maximal absorbance (EC_{50}) was calculated from the standard ascorbic acid graph.

Total antioxidant capacity

This assay is based on the reduction of Mo (VI) to Mo (V) and the development of green phosphate Mo (V) complex at acidic pH. Total antioxidant potentials of the different extract fractions were measured by their ability to reduce Mo (VI) to Mo (V) and the ultimate formation of green Mo complex that gives the highest absorbance at 695 nm. In each test tube, 0.3 ml of sample (100µg/ml) was taken and then 3 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphates and 4 mM ammonium molybdate) was added. The reaction mixtures were incubated at 95 °C for 90 min. Using spectrophotometer the absorbance of the solutions were recorded at 695 nm in room temperature. Ascorbic acid was used as a standard reducing agent [32] and data presented in terms of mg/g extract and dry leaf powder (dlp).

Phyto chemical detection

All the extract fractions were tested to detect alkaloids, triterpenoids, anthraquinones, flavonoids, phlobatannins, tannins, steroids, saponins, glycosides and carbohydrates following the procedures [33-35] as described earlier in details [36].

Estimation of total phenolics

The total phenolic content was measured by using Folin-Ciocalteu reagent [37]. 10µl of each sample was taken in the respective test tubes and the volume was made up to 1 ml by adding distilled water. To each test tube 0.5 ml of Folin-Ciocalteu (1N) and 2.5 ml 20% sodium carbonate (25 g of Na_2CO_3, 10 H_2O was dissolved in 125 ml distilled water) solution were added and the test tubes were kept in dark for 40 min at room temperature. The optical density was measured at 725 nm using spectrophotometer. The total phenolic content was estimated using the tannic acid standard curve. Standard tannic acid solution (0.5 mg/ml) was prepared by dissolving 5 mg tannic acids in 10 ml distilled waters. From this stock solution the different standard concentrations (2.5-25 µg/ml) were prepared by serial dilution method and the data presented in terms of mg/g extract and dry leaf powder.

Statistical analysis

All the assays were performed at least in triplicate and all the data points were expressed as mean**±**SEM. Correlation of coefficient (r) and coefficient of determination (r^2) were calculated using Microsoft Excel.

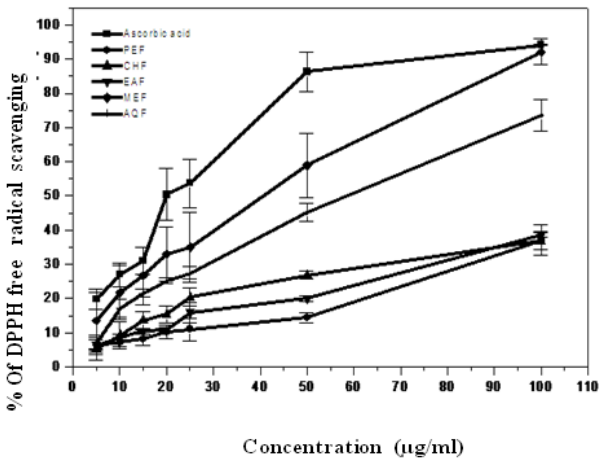

Fig. 1: Showing DPPH free radical scavenging potentials of ascorbic acid and five different solvents extract fractions of *M. hexandra* leaf. Data expressed as mean±SEM of triplicate set of experiments

RESULTS

DPPH free radical scavenging assay

Data indicate the leaf extracts fractions of *M. hexandra* have the differential capacity to neutralize the DPPH free radicals (fig.1) and the variation in the IC_{50} values (Table.1). Here, the leaf MEF showed the maximum free radical scavenging activity than the other extract fractions. Leaf MEF neutralizes 50% DPPH free radicals (IC_{50}) at a concentration of 44.25±11.4 µg/ml. DPPH free radical IC_{50} values were determined for leaf PEF, CHF, EAF and AQF respectively as199.52±25.94, 139.23±9.9, 129.5±2.33 and 59±5.8 µg/ml (table 1). Here in this study, the MEF showed least IC_{50} value than that of

the other extract fractions. The potency to scavenge 50% DPPH free radicals by the ascorbic acid is almost double than that of the methanolic extract fraction. Amongst the entire extract fractions, the leaf MEF of *M. hexandra* proved to be the most effective DPPH free radical scavenger containing fraction and it is comparable with the standard reducing agent-ascorbic acid.

Reducing power assay

Data indicate the differential reduction of potassium ferricyanide, $K_3Fe(CN)_6$, by leaf extract fractions of *M. hexandra*. All the extract fractions showed its concentration dependent reducing capacity. Amongst the entire leaf extract fractions, the methanolic extract fraction showed the maximum reducing property. Ascorbic acid was used as a standard reducing agent (fig. 2). The EC_{50} values of ascorbic acid was calculated as 54.06±5 µg/ml and for the different solvent extract fractions, PEF, CHF, EAF, MEF and AQF were respectively as1521.43±67.85,731.21±60.20, 479.13±18.43, 104.59± 15.40, and 111.10±3.60 µg/ml (table1).

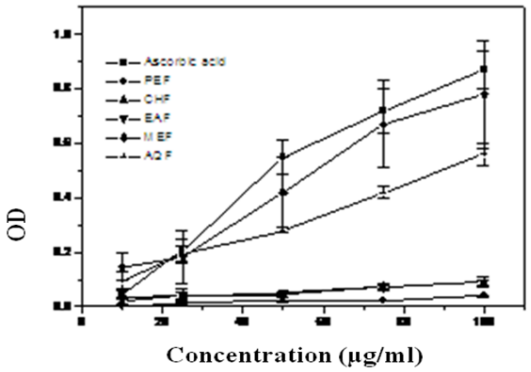

Fig. 2: Showing ferric reducing potency of ascorbic acid and the five different solvents extract fractions of *M. hexandra* leaf. Data were expressed as mean±SEM of triplicate set of experiments

Table 1: DPPH free radical scavenging IC_{50} and reducing power EC_{50} values of ascorbic acid and five different solvents extract fractions of *M. hexandra* leaf

Extract fractions	DPPH assay IC_{50} (µg/ml) mean±SEM	Reducing power EC_{50} (µg/ml) mean±SEM
Ascorbic acid	19.06±02.2	54.06±5.00
PEF	199.52±25.94	1521.43±67.85
CHF	139.23±09.9	731.21±60.20
EAF	129.50±02.3	479.13±18.43
MEF	44.25±11.4	104.59±15.40
AQF	59.00±05.8	111.10±3.60

Data were expressed as mean±SEM of triplicate set of experiments.

Table 2: Phytochemical analysis of different extract fractions of *M. hexandra*

S. No.	Phytochemicals	Tests performed	Results				
			PEF	CHF	EAF	MEF	AQF
1.	Steroids	Kantamreddi *et al.*, 2010 [38]	++++	++++	++++	-	-
2.	Alkaloids	Wagner's test	+	+	+	++	++
3.	Flavonoids	Zinc hydrochloride test	+	+	+	+++	++
4.	Terpenoids	Kantamreddi *et al.*, 2010	+	-	-	+++++	++
5.	Tannins	Ferric chloride test	-	-	-	+++++	++++
6.	Phlobatannins	HCl test	-	-	-	+++	++
7.	Saponins	Froth test	-	-	-	+	+++
8.	Carbohydrates	Benedict's test	-	-	-	++++	++++
9.	Reducing sugars	Fehling's test	-	-	-	+++	+++
10.	Glycosides	Alkaline reagent test	+	+	+	-	+
11.	Anthraquinones	Borntrager's test	-	-	-	-	-

Symbols "+"and "-" indicate presence and absence of corresponding phyto chemicals respectively and repetition of symbols indicates relative abundance.

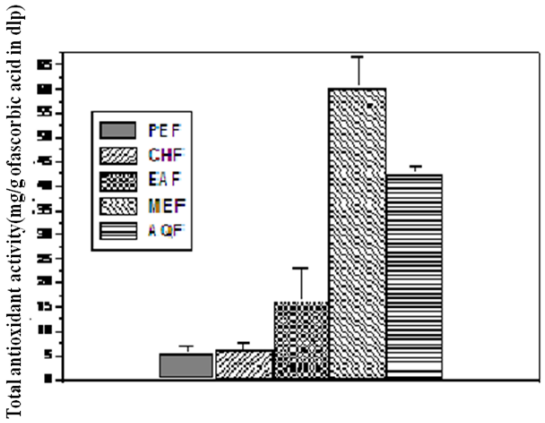

Fig. 3: Total antioxidant capacity of different extract fractions of *M. hexandra* leaf. Data were expressed as mean±SEM of triplicate set of experiments

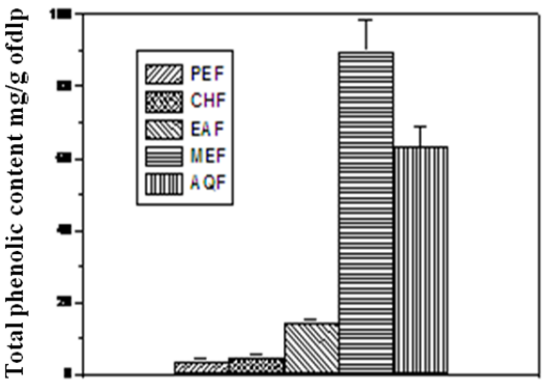

Fig. 4: Showing the total phenolic contents of the five sequentially extracted leaf extract fractions of *M. hexandra* with organic solvents. Data expressed as mean±SEM. dlp; dry leaf powder

Total antioxidant capacity

The MEF showed the highest antioxidant activity (60.37±6.24 mg ascorbic acid equivalents per gram of extract) than the other solvents extract fractions. Other extract fractions like AQF, EAF, CHF and PEF showed total antioxidant activity respectively as 42.53±1.8, 16.35±1.11, 6.2±1.4 and 5.6±1.12 mg ascorbic acid equivalent per gram of extract (fig. 3).

Phytochemical detection

The preliminary chemical analyses indicate the presence of relatively higher quantities of tannins, carbohydrates and phlobatanins in MEF and AQF, steroids in CHF and EAF, terpenoids and flavonoids in MEF, saponins in AQF and glycosides in CHF (table 2).

Estimation of total phenolics

M. hexandra leaf MEF contains 89.91±8.72 mg tannic acid equivalent phenolics per gram of dry leaf powder which is slightly more than that of the AQF, 63.32±5.8 mg. PEF contained least phenolics, 3.65±0.51 mg, while EAF and CHF contain moderate amounts respectively 14.4±0.84 and 4.72±0.55 mg of tannic acid equivalent per gram of dry leaf powder (fig. 4).

Table 3: Total phenol and ascorbic content of different extract fractions of *M. hexandra*

Extract fractions	Yield%	Total phenol content in dried extract (mg/g) mean±SEM	Total antioxidant ascorbic acid equivalent in dried extract (mg/g) mean±SEM
PEF	3.25	3112.3±15.6	174.66±34.50
CHF	2.18	214.66±25.2	232.66±12.25
EAF	4.43	307.21±23.8	368.94±25.16
MEF	12.0	749.2±72.7	503.09±52.03
AQF	9.70	652.75±59.8	438.50±18.56

Table 4: Correlations (r and r^2) between different antioxidant capacity parameters (by DPPH, and Reducing property assays) and total phenolic contents of different leaf extract fractions of *M. hexandra*

Fractions	DPPH r (r^2)	Reducing property r (r^2)
PEF	0.976(0.952)	0.956(0.914)
CHF	0.977(0.954)	0.971(0.943)
EAF	0.998(0.995)	0.990(0.980)
MEF	0.998(0.995)	0.903(0.816)
AQF	0.990(0.98)	0.997(0.994)

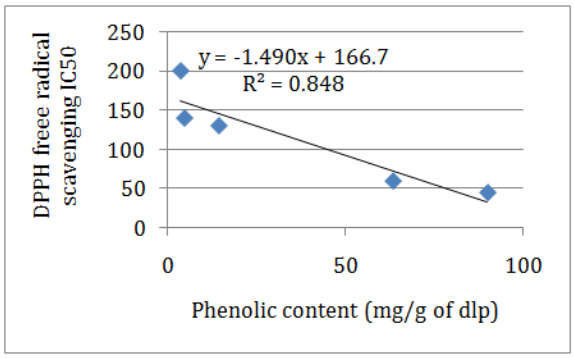

Fig. 5: Showing a linear negative correlation between total phenolic content (X) and DPPH free radical scavenging IC_{50} value (Y) of the extract fractions of *M. hexandra*

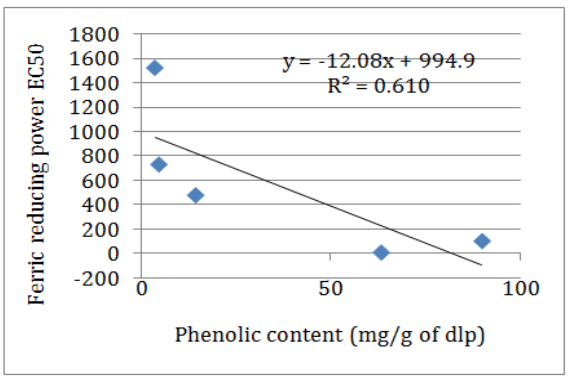

Fig. 6: Showing a linear negative correlation between total phenolic content (X) and ferric reducing power EC_{50} value (Y) of the extract fractions of *M. hexandra*

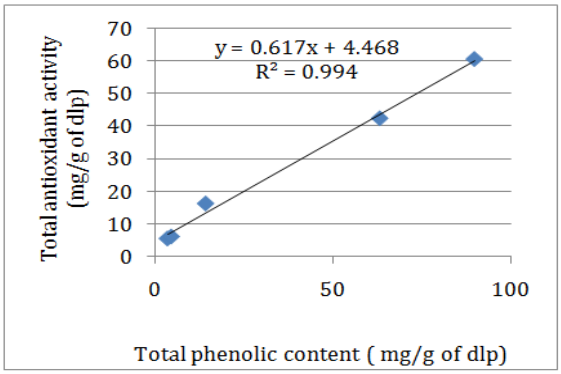

Fig. 7: Showing a positive linear correlation between total antioxidant capacity (Y) and total phenolic contents (X) of five different extract fractions of *M. hexandra*

Correlation between total phenolic and antioxidant activities

The phenolic compounds are the major contributors for the antioxidant activity. The correlation of coefficient (r) and coefficient of determination (r^2) of the different extract fractions and their phenolic contents was determined (table 4). Our results indicate a linear negative correlation between the total phenolic content and DPPH free radical scavenging IC_{50} values (fig. 5) and Fe^{+3} reducing power EC_{50} (fig.6), and there a linear positive correlation exists between total antioxidant activities and phenolic contents (fig. 7).

DISCUSSION

Many chronic diseases are related to free radical-induced oxidative stress and cellular damage. Phenolics are the major group of plant secondary metabolites which are responsible for the primary antioxidant property of plant extracts [39]. *M. hexandra* leaves are frequently used in herbal medicine [2]. Here, *in vitro* antioxidant potentials of the five different solvents extract fractions of *M.*

hexandra leaves were studied in relation to their total phenolic contents. Data indicate the leaf extracts fractions of *M. hexandra* have the differential capacity to neutralize the DPPH free radicals (fig.1) and the variation in the IC_{50} values (table.1). Here, the leaf MEF showed the maximum DPPH free radical scavenging activity and least IC_{50} value than the other extract fractions. Amongst the entire extract fractions, the leaf MEF of *M. hexandra* proved to be the most effective DPPH free radical scavenger containing fraction and it is comparable with the standard reducing agent-ascorbic acid. Therefore, different solvent extract fractions of *M. hexandra* leaves showed the differential DPPH free radical scavenging activity and thus indicate their differential hydrogen donating ability. The percentage of free radical scavenging activity may be related to phenolics content of the different extract fractions as they have high redox potentials and are good reducing agents, hydrogen donors and singlet oxygen quenchers [40]. Ferric reducing power assay indicates the electron donating capacity of an antioxidant. The reduction of Fe^{+3} ion is the indicator of reducing activity of the extract and it has an important correlation with the antioxidant activity [41]. Like DPPH assay, ferric reducing power assay also indicate differential electron donating capacity of the different solvents extracts fractions of leaves of *M. hexandra*. The leaf MEF of *M. hexandra* has also shown the most potency in ferric reducing power and which positively correlate with their relatively higher abundance of total phenolics.

Total antioxidant assay was performed to determine the presence of ascorbic acid equivalent reducing agent in the leaf extracts fractions of *M. hexandra*. Total antioxidant activity of the different extract fractions was estimated and expressed as ascorbic acid equivalent. The MEF showed the highest antioxidant activity than with the other solvents extract fractions. The basic principle of the total antioxidant assay includes the reduction of Mo (VI) to Mo (V) by the plant extract possessing antioxidant compounds [42]. In the present study, various solvent extract fractions showed the varied degree of reduction of Mo (VI) to Mo (V) may be due to the presence of different amounts of effective antioxidants.

Free radical scavenging and stabilizing activities are an important feature of phenolics. These compounds are with reducing potentials and can check the oxidation of organic molecules. *M. hexandra* leaf MEF contains slightly more phenolic content than that of the AQF. PEF contained least phenolics, while EAF and CHF contain moderate amounts of phenolic content respectively. Several extensive studies have documented earlier those antioxidant activities of phyto chemicals are mainly due to the phenolic components [18-20]. Natural phenolic content in plants are attribute enormously to their pharmacological values.

The phenolic compounds are the major contributors for the antioxidant activity. The coefficient of determination (r^2) and correlation of coefficient (r) of the different extract fractions and their phenolic contents was determined (table 4). Our results indicate a linear negative correlation between the total phenolic content and DPPH free radical scavenging IC_{50} values (fig. 5) and Fe^{+3} reducing power EC_{50} (fig. 6), and there a linear positive correlation exists between total antioxidant activities and phenolic contents (fig. 7). There are reports of positive correlation between the antioxidant activities of phytochemicals and the total phenolic content [43]. The highest total antioxidant activity of the leaf MEF of *M. hexandra* has further correlated with the highest total phenolics content. This observation led to the conclusion that the most of the antioxidant active compounds dissolve in higher polar solvents. Methanol is reported to be a good solvent for the phenolics extraction than that of the water and other solvents [44]. Methanolic extract fraction was with potent antioxidant active secondary metabolites. Secondary metabolites have good antioxidant activity and their effects are significant on the human health and disease prevention.

These antioxidant properties may be due to the synergistic action of the phenolics. There is a positive correlation between consumption of polyphenolic containing food and the lower incidence of degenerative diseases including cancer, arthritis, heart disease, inflammation, brain dysfunction and cataracts [45, 46]. Therefore, the novelty of the present study is that *M. hexandra* leaf aqueous and methanolic extract fractions are identified as potent extract fractions with free radical scavenging and reducing property. It may be due to the presence of phenolics. At this point, further work is necessary to isolate and identify the active compounds.

CONCLUSION

The leaf MEF of *M. hexandra* showed the most effective DPPH free radical scavenging, ferric reducing power and total antioxidant activity in terms of ascorbic acid equivalent and correlated with its total phenolic contents and the relative abundance of flavonoids. Therefore, the MEF of *M. hexandra* seems to be the most promising extract fraction as the free radical scavenger and may be considered as a natural source of antioxidants though there is need for further *in vivo* antioxidant *an*d toxicity assessments.

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CONFLICT OF INTERESTS

All authors have none to declare

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