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Antioxidant and Free Radical Scavenging Activity of Trigonella foenum-graecum L, *Murraya koenigii* , *Coriandrum sativum* and *Centella asiatica*

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A B S T R A C T

Antioxidants are naturally occurring substances that combat oxidative damage in biological entities. An antioxidant achieves this by slowing or preventing the oxidation process that can damage cells in the body. It does this by getting oxidized itself in place of the cells. The aim of the present study was to evaluate the *in vitro* antioxidant and free radical scavenging activities of aqueous and 95% methanol leaf extracts of four herbs viz. *Trigonella foenum-graecum L*, *Murraya koenigii*, *Coriandrum sativum* and *Centella asiatica* which have frequent use in Indian cuisine. Both aqueous and 95% methanol leaf extracts have shown significant amount reducing power. Both aqueous and 95% methanol leaf extracts of *Coriandrum sativum* had significant DPPH radical scavenging activity with IC_{50} value of 0.21± 0.3 mg/L and 0.176 ± 0.008 mg/L respectively. The aqueous leaf extract of *Trigonella foenum-graecum L* showed low scavenging activity. Among all the leaf extracts, the aqueous leaf extract of *Centella asiatica* has exhibited significantly high NO radical scavenging activity (80%) with IC_{50} value of 0.11 ± 0.17 mg/L. The aqueous leaf extracts of the samples have showed significantly high superoxide radical scavenging activity. The activity was maximum for the aqueous leaf extract of *Centella asiatica*, IC_{50} value is 4.36 ± 0.41 mg/L. anti lipid peroxide activities were very high (> 90 %) for aqueous leaf extracts of *Coriandrum sativum* (IC_{50} = 0.064 ± 0.85 mg/L) and *Centella asiatica* (IC_{50} = 0.066 ± 0.9mg/L) at a concentration of 0.16 mg/L. The aqueous leaf extracts of the samples were found to contain large amounts of flavonoids and phenolic compounds and exhibited high antioxidant and free radical scavenging activities. These in vitro assays indicate that these plant extracts are significant source of natural antioxidants which might be helpful in preventing the progress of various oxidative stresses.

Introduction

Reactive oxygen species (ROS) which consist of free radicals such as hydroxyl (OH^-) , superoxide (O^{2-}), nitric oxide (NO), peroxyl (RO^{2-}), Lipid peroxyl (LOO^-) radicals and non- free radical species such as hydrogen peroxide (H_2O_2), singlet oxygen (O_2^{-1}), ozone (O_3), lipid peroxide (LOOH), are different forms of activated oxygen (Halliwell et al., 1999; Yildirim et al., 2000; Gulcin et al., 2002a). ROS are produced by all aerobic organisms and can easily react with most biological molecules like proteins, lipid, lipoproteins, DNA etc.ROS can generate oxidative stress and many pathophysiological disorders such as arthritis, diabetes, inflammation, cancer and genotoxicity (Kourounakis et al., 1999; Gulcin et al., 2002b). Antioxidants are naturally occurring substances that combat oxidative damage in biological entities. An antioxidant achieves this by slowing or preventing the oxidation process that can damage cells in the body. It does this by getting oxidized itself in place of the cells. Antioxidants prevent the human system by neutralizing the free radicals interactively and synergistically. Recently, suppression of active oxygen species by natural antioxidants from teas spices and herbs have been studied intensively. The most commonly used antioxidants at the present time are butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG) and tert-butylhydroquinone (TBHQ). However they are suspected of being responsible for liver damage and carcinogenesis (Grice, 1986; Wichi, 1988). Therefore, the development and utilization of more effective antioxidants of natural origin are desired (Gulcin et al. 2002a, Oktay et al., 2003). The aim of the present study was to evaluate the *in vitro* antioxidant and free radical scavenging activities of aqueous and 95% methanol leaf extracts of four herbs viz. *Trigonella foenum-graecum L*, *Murraya koenigii*, *Coriandrum sativum* and *Centella asiatica* which have frequent use in Indian cuisine.

Materials and Methods

Materials

Methanol (M), 2.2 -diphenyl -1 - picryl hydrazyl (DPPH), nitro blue tetrazolium, reduced nicotinamide adenine dinucleotide phosphate sodium salt monohydrate (NADPH), phenazine methosulphate (PMS), trichloro

acetic acid (TCA), thiobarbituric acid (TBA), $FeSO_4$. 7 H_2O, KOH, KH_2PO_4, ethylene diamine tetra acetic acid, EDTA, ascorbic acid, ferric chloride ($FeCl_3$), sodium nitropruside, sodium carbonate (Na_2CO_3) were purchased from either SRL (India) or Himedia (India), or Merck (India).

Methods

Plant material: The herbs were collected from local supplier and authenticated through J.B.Roy State Ayurvedic MedicalCollege and Hospital, Kolkata, India.

Extraction: The leaves of *Trigonella foenum-graecum L*, *Murraya koenigii* , *Coriandrum sativum* and *Centella asiatica* were shed dried for 5 days, finely powdered and used for extraction. The powder (10 g) of each sample was mixed with 100 ml water for aqueous extract and with 95% methanol for methanol extract and then agitated at 120 rpm in a rotary shaker for 5 hours. The mixtures were then centrifuged at 5000rpm and the supernatants were decanted. The supernatants were concentrated in a rotary vacuum evaporator. The concentrated extracts were then lyophilized. The residues were kept at -20°C for future use.

Measurement of reducing power: The reducing power of extracts can be determined by slight modification of method of Oyaizu, (1986). Plant extract (0.02-0.16 mg/L) in water/methanol was mixed with phosphate buffer (2.5ml) and potassium ferricyanide (2.5ml). This mixture was kept at 50°C in water bath for 20 mins. After cooling 2.5 ml of 10% trichloro acetic acid was added and centrifuged at 3000 rpm for 10 min whenever necessary. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and a freshly prepared ferric chloride solution (0.5 ml). The absorbance was measured at 700nm. Control was prepared in similar manner excluding samples. Ascorbic acid was used as standard compound. Increased in absorbance of the reaction means increase in reducing power.

DPPH based free radical scavenging assay: The free radical scavenging activities of each fraction was assayed using stable DPPH, following standard method (Blois, 1958). The reaction mixture containing 1.8 ml of 0.1 mM DPPH and 0.2 ml of each serial dilution (0.02-0.16 mg/L) of plant extracts. Simultaneously a control was prepared without plant extracts. The reaction mixture was allowed to incubate for five minutes at room temperature in the dark and scavenging activity of each fraction was quantified by decolourization at 515 nm. Ascorbic acid was used as standard compound. Percentage of free radical scavenging activity was expressed as percentage inhibition from the given formula:

$$\% \text{ DPPH radical} = \frac{AC-AS}{AC} \times 100 \quad \text{(Eq 1)}$$

Where;
AC=Abs of control,
AS=Abs of sample

Nitric oxide radical scavenging assay: Nitric oxide radical scavenging activity was measured spectrophotometrically .When sodium nitroprusside was mixed with aqueous solution at physiological pH; suddenly it generates nitric oxide, which reacts with oxygen to produce nitrite ions that can be estimated using Greiss reagent. Nitric oxide scavengers compete with oxygen leading to reduced production of nitrite ions. About 1 ml of Sodium nitroprusside (5 mM) in phosphate buffer (pH 7.4, 0.1 M) was mixed with different concentrations of extracts (0.02-0.16mg/L) (both aqueous and methanolic) in phosphate buffer saline (pH 7.4, 0.1 M). The tubes were then incubated at 25°C for 2 h. After incubation the extracts were diluted with 1.5 ml of Greiss reagent [1% sulphanilamide, 2% O-phosphoric acid and 0.1%of N-(1- naphthyl) ethylenediamine dihydrochloride] and centrifuged at 3000 rpm for 5 mins. The absorbance of the chromophore formed during diazotization of the nitrite with sulphanilamide and subsequent coupling with N-(1-naphthyl) ethylenediamine dihydrochloride) was measured spectrophotometrically at 546 nm using the following equation. Control tube was maintained with all chemicals excluding extract. Percentage inhibition was calculated using the following the equation. Ascorbic acid was used as standard.

$$\% \text{ Inhibition} = \frac{AC-AS}{AC} \times 100 \quad \text{(Eq 2)}$$

Where;
AC=Abs of control,
AS=Abs of sample

Superoxide radical scavenging assay: The measurement of radical scavenging activity was done by using standard method (Nishikimi et. al., 1972) with slight modification. The reaction mixture containing 1 ml of Nitro blue tetrazolium (NBT) solution (312 µM prepared in phosphate buffer, pH 7.4) and 1 ml of NADH solution (936 µM prepared in phosphate buffer, pH 7.4). To this reaction mixture plant extract (0.8-5.6 mg/L) was added and volume was adjusted to 1ml. Finally the reaction was accelerated by adding 100 µl Phenazine methosulphate (PMS) solution (120 µM prepared in phosphate buffer, pH 7.4) to it. The final reaction mixture was incubated at 25°C for 5 minutes and absorbance was measured at 560 nm against control. Percentage inhibition was calculated using equation 2. Quercetin was used as standard.

Anti-lipid peroxide (ALP) assay: The anti-lipid peroxidation in the goat liver homogenate was measured by the standard method (Dhalwal et. al., 2005) followed by slight modification. 2.8 ml of 10% goat liver homogenate, 0.1 ml of 50 mM $FeSO_4$ and 0.1 ml plant extract (0.04 -1.6 mg/L) was mixed. The reaction mixture was incubated for 30 min at 37^{o}C. 1 ml of reaction mixture was mixed with 2ml 10% Trichloro acetic acid (TCA) - 0.67% Thio Barbituric acid (TBA) in acetic acid (50%) to stop the reaction. Then the mixture was boiled for 1hr at 100^{o}C and centrifuged for 10,000 rpm for 5 min. Supernatant was taken for absorbance at 535 nm against a blank. Blank contains all reagents except for liver homogenate and extract. Identical experiments were performed to determine the control (without extract and $FeSO_4$) and induce (without extract). Quercetin was used

as standard anti-lipid peroxidant. ALP percentage was calculated using equation 3.

$$\%ALP = \frac{AFI + IP - AS}{AFI + IP - AC} \times 100 \quad \text{(Eq 3)}$$

Where;
AFI = Abs of Ferrous ion
IP = Induced peroxidation
AS = Abs of control
AC = Abs of control

Estimation of total phenol content

The total phenol compounds were determined according to the method described by Slinkerd et al, 1977. Plant extract 0.1ml was mixed separately with 1 ml 2%Folin's reagent and 2ml of 20% Na_2CO_3. Subsequently, the mixture was incubated in a shaking incubator at 45°C for 15 min and its absorbance was measured at 760 nm. Results were expressed as gallic acid equivalents.

Estimation of flavonols content: The total flavonoid contents were measured with the aluminium chloride colorimetric assay. The test solution standard as well as sample of 1 ml was mixed with 0.3 ml of $NaNO_2$ (5% w/v) and after 5 mins, 0.5 ml of $AlCl_3$ (2% w/v) was added. After 6 mins later each mixture was neutralized with 0.5 mL of 1 M NaOH solution. The mixture was left for 10 min at room temperature and then absorbance was measured against a freshly prepared reagent blank at 510 nm. Results were as quercetin equivalents.

Statistical Analysis

Results were expressed as mean value ± SD (n = 5). Statistical significance was evaluated employing t-test and $P<0.05$ which were considered to be significant.

Results

Reducing Power

Fig. 1(a) showed the reducing power of aqueous leaf extracts of *Trigonella foenum-graecum L* (IC_{50}= 3.885± 0.002mg/L), *Murraya koenigii* (IC_{50} = 6.175 ± 0.002 mg/L), *Coriandrum sativum* (IC_{50}= 3.669 ± 0.004 mg/L) and *Centella asiatica* (IC_{50} =5.263 ± 0.0052 mg/L).

Fig. 1(b) showed the reducing power of 95% methanol leaf extracts of *Trigonella foenum-graecum L* (IC_{50} = 5.638 ± 0.01 mg/L), *Murraya koenigii* (IC_{50} = 1.654 ± 0.009 mg/L), Coriandrum *sativum* (IC_{50} = 7.668 ± 0.007 mg/L) and *Centella asiatica* (5.526 mg/L ± 0.01 mg/L). In both cases Ascorbic acid (IC_{50} = 1.133 mg/L) was used as standard. 95% methanol leaf extract of *Murraya koenigii* (IC_{50} = 1.654 ± 0.009 mg/L) showed maximum reducing power compared to others. As prevalent from the graph that reducing power of both aqueous and 95% methanol leaf extracts of *Centella asiatica* showed less activity than the others. The reducing power of the reference compound, however, (Ascorbic Acid) was found to be higher than all the tested compounds.

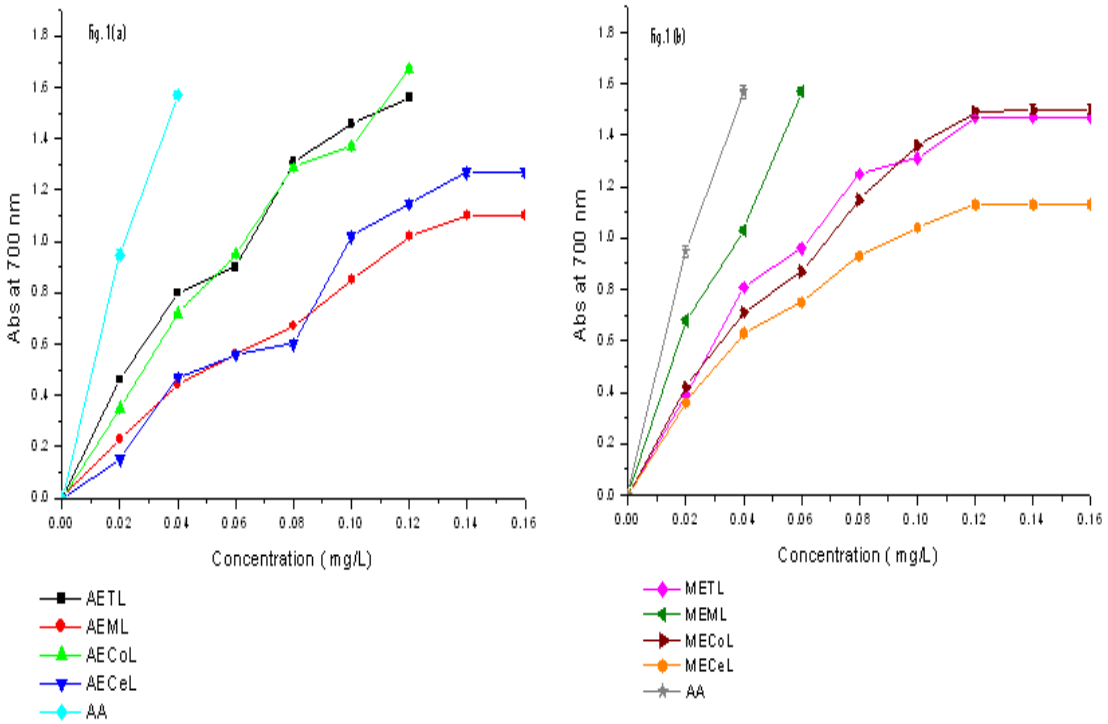

Fig. 1 (a): Reducing power of aqueous leaf extracts of *Trigonella foenum-graecum L* (AETL), *Murraya koenigii* (AEML), *Coriandrum sativum* (AECoL), *Centella asiatica* (AECeL) with Ascorbic acid (AA) as standard. 1(b): Reducing power of 95% methanol leaf extracts of *Trigonella foenum-graecum L* (AETL), *Murraya koenigii* (AEML), *Coriandrum sativum* (AECoL), *Centella asiatica* (AECeL) with Ascorbic acid (AA) as standard.

DPPH radical scavenging activity: From the graphs 2(a) and 2(b) it is evident that both aqueous and methanolic leaf extracts of *Coriandrum sativum* had significant DPPH radical scavenging activity with IC_{50} value of 0.21± 0.3 mg/L and 0.176 ± 0.008 mg/L respectively. The aqueous leaf extract of *Trigonella foenum-graecum L* showed low scavenging activity, but its 95% methanol leaf extract did not show significant antioxidant activity. The aqueous and 95% methanol leaf extracts of both *Murraya koenigii* and *Centella asiatica*, however, did not show any DPPH scavenging activity. Ascorbic acid which was used as standard compound showed maximum DPPH scavenging activity with IC_{50} value of 0.074 ± 0.01 mg/L.

NO radical scavenging activity: NO radical scavenging activity was performed with both the aqueous and methanolic leaf extracts of all the four samples as well as that of ascorbic acid as standard. Among the leaf extracts of the samples, both aqueous and methanol, only the aqeous leaf extract of *Centella asiatica* has exhibited significantly high NO radical scavenging activity (80%) with IC_{50} value of 0.11 ± 0.17 mg/L, even slightly higher than standard compound Ascorbic acid (75%) at the same concentration (0.16 mg/L) with IC_{50} value of 0.16 ±0.19 mg/L.

Superoxide radical scavenging assay: The superoxide radicals generated from dissolved oxygen by PMS-NADH coupling can be measured by their ability to reduce NBT. The decrease in absorbance at 560 nm with the plant extract and the reference compound quercetin indicate their abilities to quench superoxide radicals in the reaction mixture. As shown in fig. 4, the aqueous leaf extracts of the samples have showed significantly high superoxide radical scavenging activity. The activity was maximum for the aqueous leaf extract of *Centella asiatica*, IC_{50} value is 4.36 ± 0.41 mg/L. The aqueous leaf extracts of *Murraya koenigii* (IC_{50} = 4.82± 0.4 mg/l) and *Trigonella foenum-graecum L* (IC_{50} = 5.30 ± 0.54 mg/L) also showed significantly high scavenging activity, higher than those of the standard compound Quercetin (IC_{50} = 7.416 ± 0.25mg/L).The superoxide scavenging activity is however very low for aqueous leaf extract of *Coriandrum sativum* (IC_{50} = 37.51 ± 0.1 mg/L).

Anti-lipid peroxide (ALP) assay: The figure 5(a) revealed that anti lipid peroxide activities were very high (>90%) and at par with Quercetin for aqueous leaf extracts of *Coriandrum sativum* (IC_{50} = 0.064 ± 0.85 mg/L) and *Centella asiatica* (IC_{50} = 0.066 ± 0.9mg/L) at a concentration of 0.16 mg/L. The aqueous leaf extracts of *Trigonella foenum-graecum L* (IC_{50} = 0.1 2± 0.4 mg/L) and *Murraya koenigii* (IC_{50} = 0.1 ± 0.45 mg/L) were also showing reasonably good ALP activity, 66% and 62% respectively at a concentration of 0.16 mg/L.

The fig. 5(b) represented the anti lipid peroxide activities of the 95% methanol leaf extracts of all the herbs. The ALP activity of the methanolic leaf extract of *Centella asiatica* showed very high ALP activity (99% inhibition) with IC_{50} = 0.053± 0.01mg/L. Other methonolic leaf extracts however exhibited diminished ALP activities compared to their aqueous extracts.

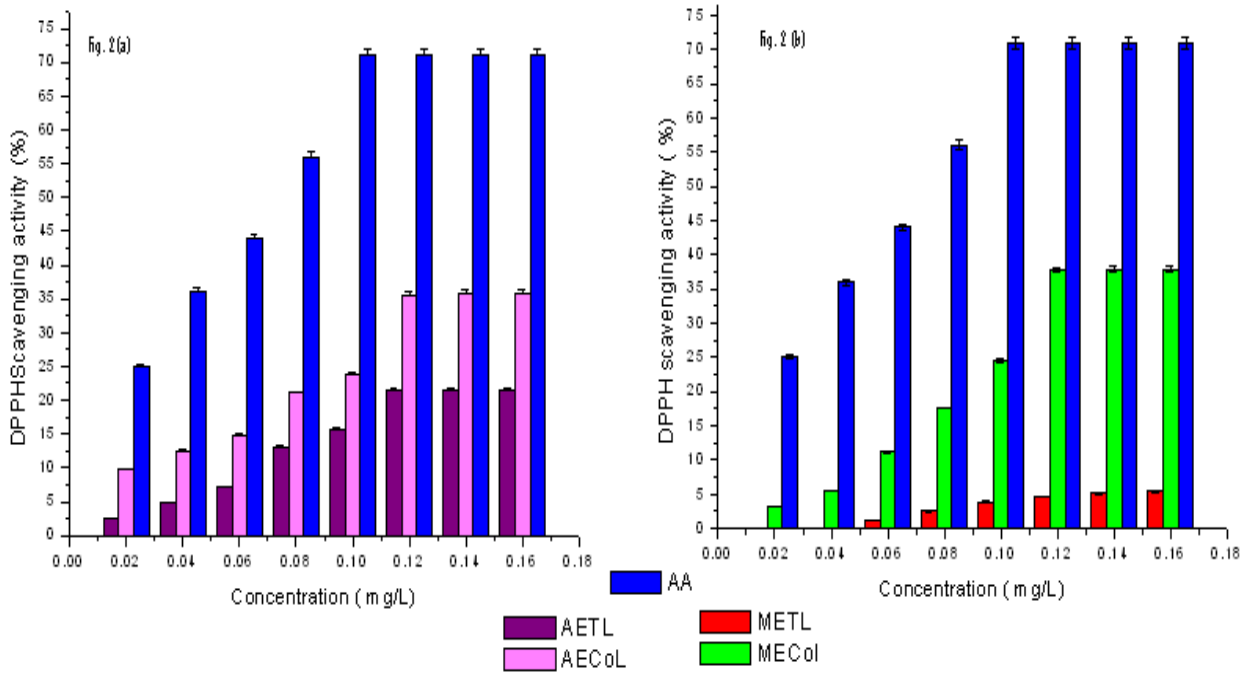

Fig. 2(a) DPPH scavenging activity of aqueous leaf extracts of *Trigonella foenum-graecum L* (AETL), *Coriandrum sativum* (AECoL) with Ascorbic acid (AA) as standard. Fig. 2(b): DPPH scavenging activity of 95%methanol leaf extracts of *Trigonella foenum-graecum L* (AETL), *Coriandrum sativum* (AECoL) with Ascorbic acid (AA) as standard.

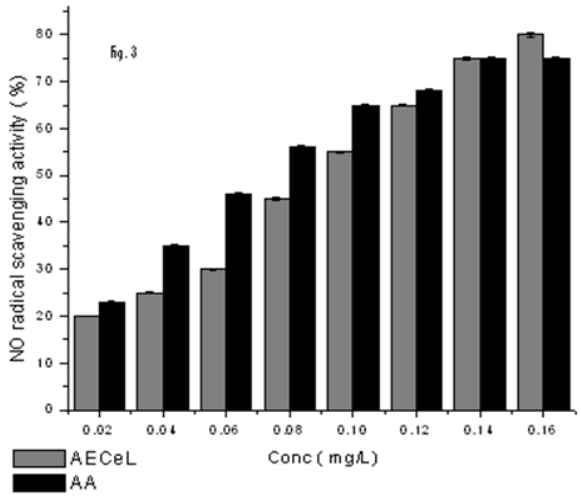

Fig. 3 NO radical scavenging activity of the aqueous extract of *Centella asiatica* with Ascorbic acid as standard.

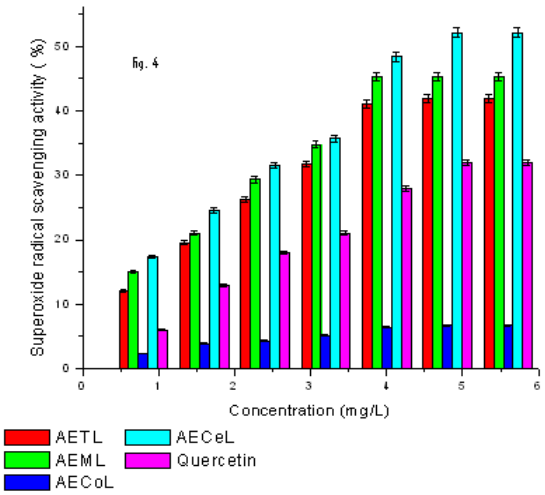

Fig. 4 Superoxide scavenging activity of aqueous extracts of *Trigonella foenum-graecum L* (AETL), *Murraya koenigii* (AEML), *Coriandrum sativum* (AECoL), *Centella asiatica* (AECeL) with Quercetin as standard.

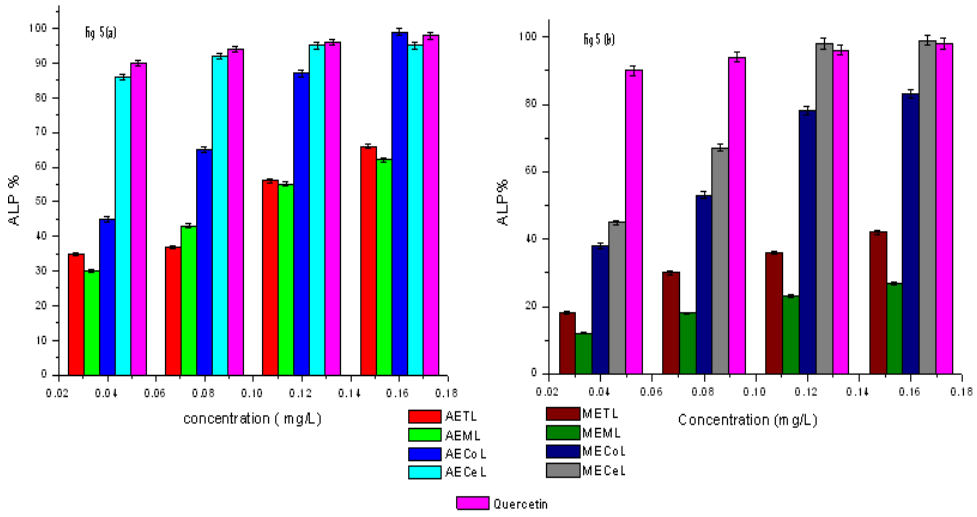

Fig. 5(a) Antilipid peroxidation assay of aqueous leaf extracts of *Trigonella foenum-graecum L* (AETL), *Murraya koenigii* (AEML), *Coriandrum sativum* (AECoL), *Centella asiatica* (AECeL) with Quercetin as standard. Fig. 5(b) Antilipid peroxidation assay of 95% methanol leaf extracts of *Trigonella foenum-graecum L* (AETL), *Murraya koenigii* (AEML), *Coriandrum sativum* (AECoL), *Centella asiatica* (AECeL) with Quercetin as standard.

Phenol content: The total phenol contents of the aqueous and 95% methanol leaf extracts of the samples were given below:

Total flavonoid content: Table 2 showed the total flavonoid contents of both aqueous and 95% methanol leaf extracts of the samples.

Table 1 Determination of the total phenolic content of the aqueous and 95% methanol leaf extracts

Sample	Total phenol content (mg/L in Gallic acid equivalent per 100mg plant extract)	
	Aqueous extract	95% Methanol extract
Trigonella foenum-graecum L	153.37±0.004	87.56±0.004
Murraya koenigii	162.01±0.01	42.7±0.01
Coriandrum sativum	149.8±0.005	56.7±0.003
Centella asiatica	160.50±0.02	134.65±0.02

Table 2 Determination of the total flavonoid content of the aqueous and 95%methanol leaf extracts.

Sample	Total flavonoid content (mg/L in Quercetin equivalent per 100mg plant extract)	
	Aqueous extract	95% Methanol extract
Trigonella foenum-graecum L	347.76±0.03	86.53±0.06
Murraya koenigii	306.42±0.02	75.42±0.01
Coriandrum sativum	402.23±0.05	92.06±0.03
Centella asiatica	413.27±0.02	109.88±0.02

Discussion

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. However, the activities of antioxidants have been attributed to various mechanisms such as prevention of chain initiation, decomposition of peroxides, reducing capacity and radical scavenging (Yildrim et al, 2000). From the results of the reducing power it is clear that both aqueous and methanolic leaf extracts of all the four herbs may act as electron donors. Reducing power of different leaf extracts have increased with the concentration of the extract. They could react with free radicals to convert them into more stable products and then terminate the free radical chain reactions.

DPPH base free radical scavenging activity is the most frequently employed method for evaluating antioxidant activity. Phenolic compounds and flavonoids have been reported to be associated with this type of antioxidative action in biological systems, acting as scavengers of singlet oxygen and free radicals (Rice-Evans et al, 1997; Jorgensen et al, 1999).

Bioorganic macromolecules like DNA or proteins, are not affected directly by NO because in aerobic condition NO is very unstable and produces intermediates like NO_2, N_2O_4, N_3O_4 which can react with oxygen. In this reaction the stable products nitrite and nitrate are also produced (Marcocci et al, 1994a, b). Peroxynitrite can also be produced by reacting with superoxide (Wink et al, 1991). These products are genotoxic. Incubation of solutions of sodium nitroprusside in phosphate buffer saline at 25°C for 2 h results in linear time-dependent nitrite production, which is probably reduced by the tested aqueous extract of *Centella asiatica.* Due to the antioxidant property of the extract it competes with oxygen to react with nitric oxide thereby inhibiting the generation of nitrite.

Superoxide anion is very harmful to cellular components (Korycka-Dahl et al, 1978). Robak et al in 1988 reported that flavonoids are effective antioxidants mainly because they scavenge superoxide anions. Fig. 4 revealed that except for the aqueous leaf extract of *Coriandrum sativum*, the superoxide radical scavenging activities of the other aqueous leaf extracts have increased with increasing concentrations and are more potent scavengers of superoxide radical than Quercetin.

All the aqueous leaf extracts exhibited reasonably good amount of anti lipid peroxide activity. Lipid peroxides may be pro -inflammatory and can damage tissues directly. Protection against free radical - induced lipid peroxidation by plant extracts is of great importance for their traditional use against inflammatory disorders, many of which are associated with membrane damage and tissue recovery (Halliwell, 1990).

Phenolic compounds are very important plant constituents because their hydroxyl groups confer scavenging ability (Yildrim et al, 2000). Table 1 indicates that aqueous leaf extracts contain significant amounts of phenolic compounds.

All the extracts have shown significant amount of flavonoid contents, but the aqueous leaf extracts of the samples contain maximum flavonoids. Flavonoid compounds have good antioxidant potential and their effects on human nutrition and health are considerable. The mechanism of action of flavonoids is through scavenging and chelation (Cook et al, 1996).

Conclusion

On the basis of the results obtained in the present study, it is concluded that aqueous leaf extracts of the samples which contain large amounts of flavonoids and phenolic compounds, exhibit high antioxidant and free radical scavenging activities. These in vitro assays indicate that these plant extracts are significant source of natural antioxidants which might be helpful in preventing the progress of various oxidative stresses. However, the components responsible for the antioxidant activity are currently unclear. Therefore, further investigations are needed to isolate and identify the antioxidant compounds present in these plant extracts.

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