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Antioxidant and free radical scavenging activity of *Sida rhomboidea.* Roxb methanolic extract determined using different *in vitro* models

[Actividades antioxidante y neutralizante de radicales libres del extracto metanólico de *Sida rhomboidea.* Roxb determinadas por diferentes modelos *in vitro*]

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Abstract

Antioxidant potential of *Sida rhomboidea*.Roxb methanol extract (MESR) was evaluated using *in vitro* models such as lipid peroxidation, metal chelation and reducing potential. Its free radical scavenging ability was also examined using *in vitro* assays for 1, 1-diphenyl-2-picryl-hydrazil (DPPH,) superoxide ($^{\cdot}O_2$), hydrogen peroxide (H_2O_2), nitric oxide ($NO^{\cdot}$) and hydroxyl radical ($HO^{\cdot}$) radical scavenging. MESR recorded dose dependent effect on inhibition of lipid perodixation (IC_{50}= 92.15±1.21µg/ml), effective metal chelation (IC_{50}=65.69±1.22µg/ml) and higher reducing potential (OD_{max}=1.20±0.27). MESR could efficiently scavenged DPPH (IC_{50}=63.23±1.59µg/ml), $^{\cdot}O_2$ (IC_{50}=142.36±2.59µg/ml), H_2O_2 (IC_{50}=125.96±3.00 µg/ml), $NO^{\cdot}$ (IC_{50}=85.36±2.01 µg/ml) and $HO^{\cdot}$ (IC_{50}=90.45±1.88 µg/ml) radicals in a dose dependent manner. Results obtained herein can be attributed to the presence of polyphenols (35.60±1.20 mg/ml gallic acid equivalent-polyphenols), flavanoids (26.94±0.94 mg/ml quercetin equivalent-flavanoids) and ascorbic acid (28.71±1.14 mg/ml ascorbic acid) in MESR. These observations suggest antioxidant and free radical scavenging potentials of MESR that adds a new dimension to its already established therapeutic uses.

Keywords: antioxidant, free radical, *Sida rhomboidea* Roxb.

Resumen

El potencial antioxidante del extracto metanólico de *Sida rhomboidea*.Roxb (MESR) fue evaluada por modelos *in vitro* tales como peroxidación lipídica, quelación de metales y potencial reductor. Su capacidad de neutralizar radicales libres fue determinada *in vitro* en ensayos de DPPH, superóxido ($^{\cdot}O_2$), peróxido de hidrógeno (H_2O_2), óxido nítrico ($NO^{\cdot}$) and el radical hidroxil ($HO^{\cdot}$). MESR mostró una inhibición dosis dependiente de la peroxidación lipídica, (IC_{50}= 92.15±1.21 µg/ml), una quelación efectiva de metales (IC_{50}=65.69±1.22 µg/ml) y un alto potencial reductor (OD_{max}=1.20±0.27). MESR neutralizó eficientemente el DPPH (IC_{50}=63.23±1.59 µg/ml), $^{\cdot}O_2$ (IC_{50}=142.36±2.59 µg/ml), H_2O_2 (IC_{50}=125.96±3.00 µg/ml), $NO^{\cdot}$ (IC_{50}=85.36±2.01 µg/ml) y $HO^{\cdot}$ (IC_{50}=90.45±1.88 µg/ml) de una manera dosis dependiente. Los resultados pueden ser atriuidos a la presencia en el MESR de polifenoles (35.60±1.20 mg/ml polifenoles equivalentes al ácido gálico), flavanoides (26.94±0.94 mg/ml flavanoides equivalentes a la quercetina) y ácido ascórbico (28.71±1.14 mg/ml). Estas observaciones sugieren actividades antioxidante y neutralizante de radicales libres en MESR que añaden una nueva dimensión a sus conocidas aplicaciones terapéuticas.

Palabras Clave: antioxidante, radical libre, *Sida rhomboidea* Roxb.

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INTRODUCTION

Reactive oxygen species (ROS) or free radicals (FR) are formed as an essential part of aerobic life and metabolism in any living system. These FR are however scavenged by inherent enzymatic and non-enzymatic antioxidant defense systems in the body to keep oxidative damage under check. But, excessive production of FR induces damage to biomolecules such as carbohydrates, protein, lipids and DNA (Halliwell, 1994) thus inducing the onset of diseases. Increased intracellular generation of ROS has been proposed as a mechanism of tissue injury associated with a variety of pathological manifestations like diabetes, cancer, neurodegenerative disease, inflammation, atherosclerosis and thrombosis (Jadeja *et al*., 2009). Many synthetic antioxidants like butyrated hydroxyanisole, butylated hydroxytoluene (BHT) and *tert*-butylhydroquinone (TBHQ) are commercially available but studies have shown that these synthetic antioxidants are prone to cause negative health effects (Stich, 1991). Hence, an alternative antioxidant with less toxic effects is desirable.

Sida rhomboidea. Roxb. (SR; *fam*. Malvaceae) is a weed found in marshy places throughout India. In Ayurveda it is known as "Mahabala" and is useful against fever, heart diseases, burning sensations, urinary disorders, piles and all kinds of inflammation (Puri, 2002). In North Eastern parts of India, decoctions prepared from leaves of SR are consumed for its therapeutic potential against various ailments including obesity and diabetes. It has been shown to have significant analgesic activity (Venkatesh *et al*., 1999). Recent studies from our laboratory have documented its hypolipidemic (Thounaojam *et al*., 2009a,b), antidiabetic (Thounaojam *et al*., 2010a) and cardioprotective (Thounaojam *et al*., 2010b) properties. Chronic oral administration of SR in mice is non toxic up to 3000mg/kg bodyweight dose (Thounaojam *et al*., 2010c).

There are no pharmacological studies highlighting the role of SR extract as a natural antioxidant. Large diversity of analytical methods is now available to determine antioxidant capacity of various herbs, spices, fruits etc. These assays differ from each other in terms of their mechanisms and expression of results for specific free radicals and their reaction conditions (Visavadiya *et al*., 2010). Since, these methods are widely used in physiology, pharmacology, nutrition and agro chemistry, it is difficult to select the most appropriate method(s) so as to avoid inappropriate applications and misinterpretation of the results (Cao and Prior, 1998; Magalhaes *et al*., 2008). In this context, it is advantageous to choose the most appropriate and commonly employed assay for the assessment of antioxidant property of a phytochemical extract in question. Hence, the present inventory encompasses an array of analytical methods to understand the biological activity of SR as a natural antioxidant.

MATERIALS AND METHODS

Plant material

Fresh leaves of SR were collected from the natural habitats of North East India, identified by Dr. Hemchand Singh, a taxonomist in the Department of Botany, D.M college of Science, Manipur University, Imphal and herbarium (voucher specimen No-216) was deposited at Department of Botany, D.M college of Science, Manipur University, Imphal.

Extraction

Leaves of SR were collected in the month of June, shade-dried and grinded to fine powder. Hundred grams of this powder was subjected to extraction using methanol in a Soxhlet apparatus and the resultant filtrate was concentrated under reduced pressure by rotary evaporator (Buchi, Germany). A semi solid paste obtained was later stored at 0^0C. The extractive value of MESR was 21 % w/w, which was later dissolved in Milli Q water (Millipore India Pvt Ltd) and subjected to antioxidant and free radical scavenging assays.

Total polyphenol estimation

Total polyphenolic compounds were determined as per Chandler and Dodds (1993). MESR (1 ml) was mixed with 1 ml of 95% ethanol, 5 ml of distilled water and 0.5 ml of 50% Folin Ciocalteu reagent. This mixture was allowed to react for 5 min and later 1 ml of 5% Na_2CO_3 was added. Thereafter, it was thoroughly mixed and placed in dark for 1 h and the absorbance was determined at 725 nm. The calibration curve was prepared using gallic acid solution at various concentrations in methanol. Concentration of polyphenols was expressed in terms of mg/ml equivalent-gallic acid/100 mg plant extract.

Total ascorbic acid estimation

SR powder (5 gm) was taken into an extraction tube and 100 ml of EDTA: TCA (2:1) extracting

solution was added and the mixture was shaken for 30 min on a shaking platform, and then centrifuged at 800 X g for 20 min. Its volume was adjusted to 100 ml in a volumetric flask with EDTA: TCA (2:1) solution. 20 ml of this solution was taken and 1% starch indicator (2-3 drops) was added and titrated against 20% $CuSO_4$ solution with appearance of dark brown colour indicating the end point (Barakat *et al*., 1973). The amount of ascorbic acid was expressed as mg/ ml/100 mg plant extract.

Total flavanoids estimation

Total flavanoid content was determined using aluminum chloride colorimetric method (Chang *et al*., 2002). 1 ml of MESR was mixed with 3 ml of methanol, 0.2 ml of 10% aluminum chloride, 0.2ml of 1 M potassium acetate and 5.6 ml of distilled water. The reaction mixture was allowed to stand at room temperature for 30 min and later absorbance was obtained at 415 nm. Calibration curve was prepared using quercetin solution at various concentrations in methanol. The concentration of flavanoids was expressed in terms of mg/ml equivalent- quercetin/100 mg plant extract.

Lipid peroxidation assay

A modified thiobarbituric acid reactive species (TBARS) assay was performed to determine the amount of lipid peroxide formed (Ruberto *et al*., 2000). Egg yolk homogenate (0.5 ml of 10% v/v) and 0.1 ml of MESR or ascorbic acid (AA) were added to a test tube and made up to 1 ml with distilled water. 0.05 ml of FeSO4 (0.07 M) was added to induce lipid peroxidation and the mixture was then incubated for 30 min. 1.5 ml acetic acid (20%) and 1.5 ml thiobarbituric acid (0.8% w/v in 1.1% sodium dodecyl sulphate) were added and the resultant mixture was vortexed and heated at 95 °C for 60 min. 5.0 ml of butanol were added to each tube and centrifuged at 1000 X g for 10 min. Absorbance of the upper layer was obtained at 532 nm.

Metal iron chelating activity

The metal chelating activity of MESR and AA were estimated as per the method of Dinis *et al*. (1994). MESR extract (0.94 ml) or EDTA (at varying concentrations) was added to 0.02 ml $FeCl_2$ (2 mM) and adding 0.04 ml ferrozine started the reaction. The contents were mixed thoroughly and allowed to stand for 10 min and later absorbance was obtained at 562 nm.

Assay of total reducing power

The antioxidant activity was evaluated in terms of reducing power as per the method of Oyaizu (1986). MESR (2.5 ml) or AA (at varying concentration) was mixed with 2.5 ml of phosphate buffer (200mM, pH 6.6) and 2.5ml of 1% potassium ferricyanide. This mixture was placed in a water bath at 50°C for 20 min. The resulting solution was cooled rapidly on ice, mixed with 2.5 ml of 10% trichloroacetic acid and centrifuged at 1000 X g for 10 min. 5.0 ml supernatant was mixed with 5 ml of distilled water and 1ml of 1% ferric chloride. Absorbance of the resultant mixture was obtained after 10 min at 700 nm. Increased absorbance is indicative of increased reducing power.

DPPH radical scavenging activity

Free radical scavenging activity of MESR or AA was measured by DPPH (1, 1-diphenyl-2-picryl-hydrazil) as per Yokozawa *et al*. (1998). 1 ml of DPPH (0.08mM) was added to 0.3 ml of MESR or AA (at varying concentrations). The reaction mixture was mixed thoroughly and allowed to stand at room temperature for 30 min, and later absorbance was obtained at 517 nm.

Superoxide ($^{\cdot}O_2$) radical scavenging activity

The assay was based on the capacity of the sample to inhibit blue formazan formation by scavenging the superoxide radicals generated in riboflavin-light-NBT system (Beauchamp and Fridovich, 1971). 50 mM phosphate buffer (pH 7.6), 20 µg riboflavin, 12 mM EDTA, and NBT (0.1 mg/3 ml) were mixed and the resultant mixture was exposed to direct sunlight for 150 sec and later, absorbance was obtained at 590 nm. This process was repeated with various concentrations of MESR and AA.

Hydrogen peroxide (H_2O_2) radical scavenging activity

The ability of MESR or AA to scavenge hydrogen peroxide was determined (Ruch *et al*., 1989). 40 mM solution of hydrogen peroxide (H_2O_2) was prepared in phosphate buffer solution (PBS, pH 7.4). Various concentrations of 0.5 ml of the MESR or AA were added to 1ml of H_2O_2 solutions in PBS. After 10 min, the absorbance was obtained at 230 nm.

Nitric oxide ($NO^{\cdot}$) radical scavenging activity

Nitric oxide generated from sodium nitroprusside was measured by the Griess reagent (Green *et al*., 1982). Various concentrations of MESR or AA were mixed with sodium nitroprusside (1mM in PBS) and incubated at 25°C for 150 min. 0.5ml of this solution was mixed with equal volume of Griess reagent (1% sulfanilamide, 2% orthophosphoric acid, and 0.1% naphthalene ethylene diamine dihydrochloride) and absorbance was obtained at 546 nm.

Hydroxyl radical ($HO\cdot$) scavenging activity

Hydroxyl radicals generated by the Fenton reaction were measured as per Chung *et al*. (1997). The Fenton reaction mixture constituted of 0.2ml $FeSO_4 \cdot 7H_2O$ (10mM), 0.2ml EDTA (10mM) and 0.2ml 2-deoxyribose (10mM) mixed with 1.2 ml phosphate buffer (0.1 M, pH 7.4). MESR (0.2ml) or AA (at varying concentrations) was added to Fenton reaction mixture followed by 0.2ml H_2O_2 (10 mM) and incubation at 37 °C for 4h. Later, 1ml TCA (2.8%) and 1ml TBA (1%) were added in reaction mixture and placed in a boiling water bath for10 min. The resultant mixture was brought to room temperature and centrifuged at 395 X *g* for 5min and absorbance was obtained at 532 nm.

Statistical analysis

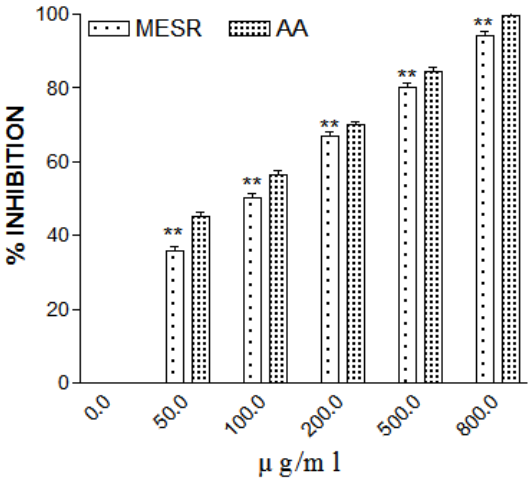
Data were expressed as mean ± S.D for three parallel measurements using Graph Pad Prism version 3.0 for windows, Graph Pad Software, San Diego, California, USA. Statistical analysis was done by student's *t* test and $p<0.05$ considered as significant. The 50 % inhibitory concentration (IC_{50}) was calculated from the dose response curve (Graph Pad Prism Version 3.0) obtained by plotting percentage inhibition *versus* concentrations. Linear regression analysis was done for total reducing power assay. Percentage (%) inhibition was calculated as: -[(absorbance of control – absorbance of test sample) ÷ absorbance of control] × 100.

RESULTS

Quantitative phytochemical analysis revealed the presence of 35.60±1.20 mg/ml gallic acid equivalent-polyphenols, 26.94±0.94 mg/ml quercetin equivalent-flavanoids and 28.71±1.14 mg/ml ascorbic acid per 100 mg MESR.

Lipid peroxidation assay showed a dose dependent (50-600 µg/ml) response of MESR ($p<0.05$) with 600 µg/ml being the optimal concentration (Figure.1).

Figure 1. Effect of MESR and AA on inhibition of lipid peroxidation



Data expressed as mean± S.D for three measurements. **$p<0.001$ compared to control

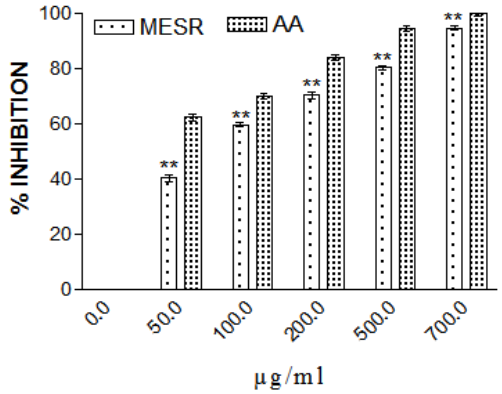
IC_{50} value for MESR was 92.15±1.21µg/ml whereas that of AA was 62.36±1.09 µg/ml indicating that the lipid peroxidation inhibitory activity of MESR was less than AA (Table. 1).

Table 1. Fifty percent inhibitory concentration (IC_{50}) of MESR and AA.

Assay	IC_{50} (µg/ml)	
	MESR	AA
Inhibition of lipid peroxidation	92.15±1.21	62.36±1.09
Metal chalation	65.69±1.22	43.26±0.98
DPPH radical scavenging	63.23±1.59	60.47±1.42
Superoxide radical scavenging	142.36±2.59	40.23±1.00
Hydrogen peroxide radical scavenging	125.96±3.00	50.19±1.37
Nitric oxide radical scavenging	85.36±2.01	39.09±1.21
Hydroxyl radical scavenging	90.45±1.88	90.45±1.88

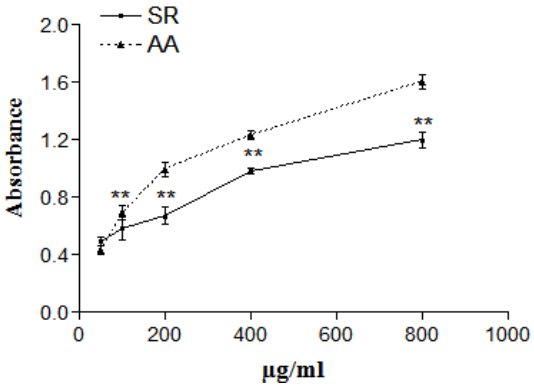
Ferrozine induced metal chelating assay revealed that MESR showed a dose dependent (50-500 µg/ml) metal chelating property ($p<0.05$) (Figure.2).

Figure 2. Effect of MESR and AA on metal chelation.



Data expressed as mean ± S.D for three measurements. **p<0.001 compared to control.

Figure 3. Reducing potential of MESR and AA

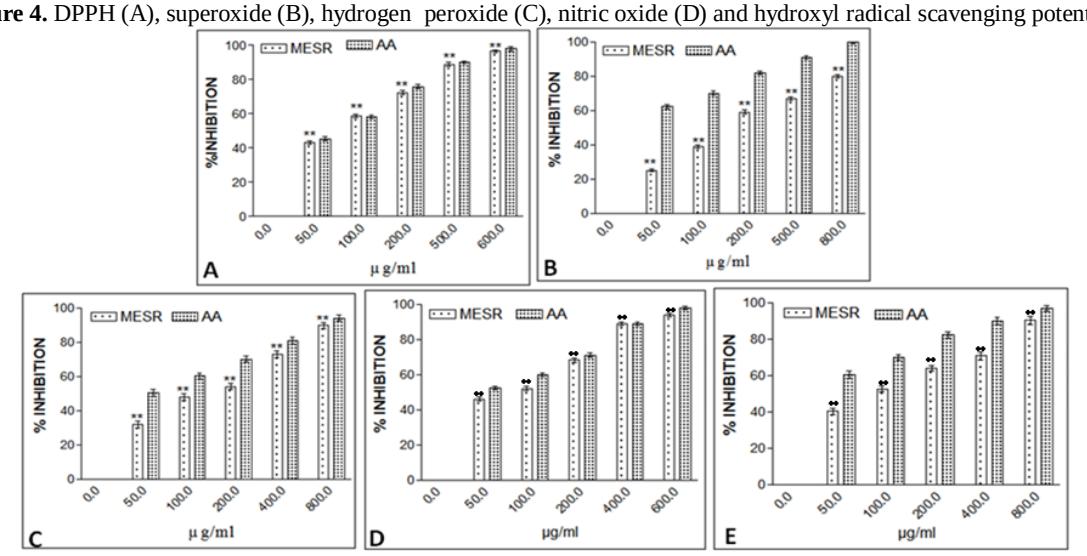


Data expressed as mean ± S.D for three measurements. **p<0.001 compared to control.

IC_{50} values of MESR (65.69±1.22 µg/ml) and that of AA (43.26±0.98 µg/ml) were comparable (Table.1). Antioxidant potential in form of inhibition of metal chelation was superior to inhibition of LPO (65.69±1.22 µg/ml vs. 92.15±1.21µg/ml). MESR recorded linear increase in optical density (r^2=0.95) with increase in concentration (50-800 µg/ml) with maximum absorbance being 1.20 (800 µg/ml). Ascorbic acid recorded relatively higher absorbance values (Figure.3).

The DPPH free radical scavenging assay revealed a dose dependent potential (p<0.05) of MESR (50-500 µg/ml) (Figure.4A), with IC_{50} value being 63.23±1.59 µg/ml whereas, that of AA was 60.47±1.42 µg/ml (Table.1). Similarly, scavenging assays for $^{\cdot}O_2$, H_2O_2 and $NO^{\cdot}$ radicals were performed in which MESR recorded a dose dependent response (p<0.05) (Figure.4B,4C and 4D). The IC_{50} values of MESR for O_2, H_2O_2 and $NO^{\cdot}$ radicals vs. IC_{50} values of AA were recorded to be 142.36±2.59 µg/ml vs. 40.23±1.00 µg/ml, 125.96±3.00 µg/ml vs. 50.19±1.37 µg/ml and 85.36±2.01 µg/ml vs. 39.09±1.21 µg/ml respectively (Table.1). $HO^{\cdot}$ scavenging assay also recorded a dose dependent effect (p<0.05) of MESR (50-600 µg/ml) (Figure.4E), with an IC_{50} value of 90.45±1.88 µg/ml that was comparable with IC_{50} value of 90.45±1.88 µg/ml observed in AA (Table.1).

Figure 4. DPPH (A), superoxide (B), hydrogen peroxide (C), nitric oxide (D) and hydroxyl radical scavenging potential of MESR and AA.



Data expressed as mean± S.D for three measurements. **p<0.001 compared to control

DISCUSSION

Present study investigates antioxidant and free radical scavenging potential of MESR based on its ability to (i) inhibit/promote various biological processes such as lipid peroxidation, metal chelation and reducing potential (ii) scavenge biologically important oxidants such as NO, $^{\cdot}O_2$, $HO^{\cdot}$ and H_2O_2 and (iii) scavenge non biological stable free radicals (DPPH).

Lipid peroxidation is initiated by the abstraction of a hydrogen atom in an unsaturated fatty acyl chain and later propagated as a chain reaction (Droge, 2002; Kohen and Nyska, 2002). Lipid peroxides are involved in numerous pathological events, including inflammation, metabolic disorders and cellular aging (Oyaizu, 1986). Therefore, inhibition of lipid peroxidation is of great importance in the induction/onset of diseases involving free radicals. Egg yolk lipids undergo a non-enzymatic lipid peroxidation when incubated with ferrous sulphate yielding a carbonyl product such as malonaldehyde (MDA). We recorded dose dependent inhibition of MDA formation by MESR. Metal chelating capacity is an important antioxidant mechanism because it reduces concentration of catalyzing transition metal during lipid peroxidation (Diplock, 1997). In the present study, we recorded significant dose dependent metal chelating potential of MESR. Hence, results obtained from metal chelating capacity assay using MESR further validate results obtained in lipid peroxidation assay. Previous studies have reported a direct correlation between antioxidant activities and reducing power of certain plant extracts (Jadeja *et al*., 2009). In the reducing power assay, the presence of antioxidants in the sample reduced Fe3+/ferricyanide complex to the ferrous form. Inhibitory effect on lipid peroxidation, effective metal chelation and higher reducing potential of MESR recorded in this study suggests that MESR has a strong antioxidant property. Previous studies have correlated the reducing capacity of phytocompounds to its electron-donating ability (Aruoma, 2003; Roginsky and Lissi, 2005). Hence it can be assumed that effective electron donating ability attributed to the observed overall antioxidant property of MESR.

Free radicals are produced in living system during normal metabolic process or in presence of xenobiotics that initiates oxidative damage. MESR could quench DPPH free radicals in a dose dependent manner and the IC_{50} value was also comparable to that of AA. These observations suggest that MESR has free radical scavenging ability. DPPH radical is a stable FR that has been widely used to evaluate the FR scavenging ability of natural antioxidant (Ruch *et al*., 1989). Antioxidants, on interaction with DPPH, either transfer an electron or hydrogen atom to DPPH, thus neutralizing its FR character (Oyaizu, 1986). In keeping with our previous assumption of effective electron donating ability of MESR, observations on stabilizing DPPH free radicals observed herein are further validated.

$NO^{\cdot}$ radical plays multiple roles in a variety of biological processes viz. as an effector molecule, neuronal messanger, vasodilator, antimicrobial agent, etc (Hagerman *et al*., 1998). It has been reported to react with $^{\cdot}O_2$ radical to form peroxynitrite radicals ($ONOO^{-}$) that cause toxicity to biomolecules such as proteins, lipids and nucleic acids (Yermilov *et al*., 1995). During the process of inflammation, cells of the immune system generate superoxide radicals in which NADPH oxidase plays an important role in induction of vascular complications (Droge, 2002; Kohen and Nyska, 2002). $^{\cdot}O_2$ further decomposes into singlet oxygen and $HO^{\cdot}$ that results in massive mitochondrial damage. MESR significantly inhibits generation of $NO^{\cdot}$ and $HO^{\cdot}$ radicals in a dose dependent manner. These observations further highlight the importance of MESR in preventing physiological deletoriation caused by $NO^{\cdot}$ and $^{\cdot}O_2$ radicals.

$HO^{\cdot}$ radical is the most reactive among ROS that causes peroxidation of membranes lipids and is known to be highly mutagenic and carcinogenic (Miyake and Shibamoto, 1997). H_2O_2 radical is also known to produce $OH^{\cdot}$ radical by crossing cell membrane rapidly and by reacting with Fe^{2+} and Cu^{2+} ions. The same has been related to the initiation of many toxic effects (Miller *et al*., 1993). It is therefore biologically advantageous for cells to control the accumulation of H_2O_2. MESR was found to be highly potent in scavenging H_2O_2 radical thus validating previous observations pertaining to its antioxidant potential. These results suggest that *in vitro* FR scavenging activity of MESR was evident from the results obtained in DPPH, $NO^{\cdot}$, $^{\cdot}O_2$, $OH^{\cdot}$ and H_2O_2 scavenging assays.

Quantitative assay of phytocompounds of MESR revealed high contents of total polyphenols, flavanoids and ascorbic acid. Plant polyphenols are

multifunctional and act as hydrogen-donating antioxidants and metal chelators. Velioglue *et al*. (1998) reported a high correlation between total phenolic content and antioxidant potential in some fruits, vegetables and grain products. Hence, high phenolic content observed in MESR can be attributed to its FR scavenging ability. Also, the presence of flavanoids and ascorbic acid in the MESR could be a possible reason for its significant antioxidant property (Jadeja *et al*., 2009).

CONCLUSIONS

It can be concluded from the present study that MESR possesses potent antioxidant and FR scavenging properties that have been demonstrated using a variety of *in vitro* experimental models. Hence, this study makes valuable addition to the existing wealth of information on this indigenous natural antioxidant.

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