Cellular and Molecular Biology ™ **55**, OL1083-OL1095
DOI 10.1170/124

ISSN 1165-158X
2009 Cell. Mol. Biol.™

SUPPRESSION OF REACTIVE OXYGEN SPECIES AND NITRIC OXIDE BY *ASPARAGUS RACEMOSUS* ROOT EXTRACT USING IN VITRO STUDIES

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Received September 1st, 2008; Accepted December 29th, 2008; Published February 25th, 2009

Abstract – Recent clinical and experimental data showed the involvement of reactive oxygen species/nitrogen species (ROS/RNS) in many human pathophysiolgocial conditions. Antioxidant activity of the aqueous (ARA) and ethanolic extracts (ARE) of *Asparagus racemosus* (AR) root were evaluated in a series of *in vitro* assays including ROS generation in chemicals and biological model systems. The dose-dependant ARA and ARE extracts showed the scavenging activity against DPPH (IC_{50} = 60.7 and 52.5 µg/ml), nitric oxide (IC_{50} = 141.9 and 63.4 µg/ml), superoxide (IC_{50} = 221 and 89.4 µg/ml), hydroxyl (IC_{50} = 318.7 and 208.8 µg/ml) and $ABTS^{\cdot+}$ (IC_{50} = 134.5 and 71.9 µg/ml) radicals. The antioxidant capacity of ARA and ARE were assessed for their reducing power using FRAP (Ferric Reducing antioxidant power) and potassium ferricyanide reducing methods as well as free radical scavenging capacity by TEAC (Trolox Equivalent Antioxdaint Capacity) method. ARA and ARE extracts were also found to be effective at suppressing lipid peroxidation induced by Fe^{2+}/ascorbate system in rat liver mitochondrial preparation (IC_{50} = 511.7 and 309.2 µg/ml, respectively). Further, ARA and ARE root extracts significantly decreased ($P < 0.05$) copper-mediated human LDL oxidation by prolongation of lag phase time with decline in oxidation rate, maximal yield of conjugated dienes, lipid hydroperoxides and malondialdehyde concentrations. The addition of ARA and ARE root extracts to human serum significantly reduced ($P < 0.05$) the formation of lipid peroxidation in medium. Trolox, α-tocopherol and mannitol were tested similarly to compare their antioxidant activities. In conclusion, antioxidant activity of ARE as compared to ARA extract is more effective which act as hydrogen donors, metal ion chelators, reducing agents, radical scavengers and anti-lipid peroxidative. These effects are attributed to the high amount of lipophilic phenolics content of ARE root extract.

Key words: Antioxidant, *Asparagus racemosus*, LDL oxidation, lipid peroxidation

INTRODUCTION

Free radicals are fundamental to any biochemical process and represent an essential part of cellular metabolism. Naturally, there is a dynamic balance between the amount of free radicals generated and antioxidants in the body, protecting body against the deleterious effects of free radicals. However, the imbalance between prooxidant and antioxidant homeostasis potentially leads to oxidative stress causing majority of abnormalities such as atherosclerosis, hypertension, ischaemic diseases, alzhemier's disease, parkinsonism, cancer, diabetes mellitus and inflammatory conditions (29,14). Oxidative stress is initiated by reactive oxygen species/nitrogen species (ROS/RNS) such as superoxide ion radical ($O^{\cdot}_2{}^{-}$), hydroxyl radical ($OH^{\cdot}$), peroxyl ($ROO^{\cdot}$) and alkoxyl radicals ($RO^{\cdot}$), nitric oxide radical ($NO^{\cdot}$) and peroxynitrite (ONOOH) which can cause damage to biological targets such as lipids, DNA, and proteins by propagating chain reaction cycle. Antioxidants defense systems have coevolved with aerobic metabolism to counteract oxidative damage from ROS and RNS (29,18). The inhibition of such free radical-mediated pathophysiology has become a central focus for research efforts designed to prevent or ameliorate tissue injury.

Numbers of studies have been performed to discover antioxidants from natural products or medicinal plants. In this respect flavonoids and other polyphenolic compounds have received the greatest attention (26).

Asparagus racemosus willd. (Family: Liliaceae), commonly known as shatavari, satawar or satmuli, is found at low altitudes throughout India. The main medicinal /pharmacological value of *A. racemosus* root is attributed by the presence of steroids viz. saponins and sapogenins. The dried root of the plant is used as ayurvedic drug, tonic, galactogogue, aphrodisiac, rejuvenator, anti-spasmodic, anti-ulcerous and anti-inflammatory (28). In Ayurveda, *A. racemosus* has been described as a rasayana herb and has been used extensively as an adaptogen to increase the non-specific resistance of organisms against a variety of stresses (5). The root of *A. racemosus* is also used in the treatment of nervous disorders, dyspepsia, diarrhea, dysentery, tumors, hyperdipsia, neuropathy and hepatopathy (16). This plant is reported to have immunostimulant, antihepatotoxic, antioxytocic and antioxidant activities (3,16,37). Our previous work on *A. racemosus* showed dose-dependent (5 and 10 g% of dried roots powder) favorable effects on hypercholesteremic rats by modulating cholesterol metabolism and antioxidant enzyme activities (46). However, no studies have explored the complete report of antioxidant activity exerted by *A.racemosus* root extracts using wide-range of in vitro assays. The present study, therefore, has been designed to investigate potential antioxidant effects of two *A. racemosus* root extracts (i.e., aqueous and ethanol) on a series of *in vitro* assays including ROS/RNS generation in chemicals and biological model systems. Furthermore, human low-density lipoprotein (LDL) was isolated and the resistance of LDL oxidation was evaluated by monitoring copper-induced lipid peroxidation, which is usually driven by catalytic transition metals, especially copper, and represents a focal aspect of atherogenesis (12).

MATERIALS AND METHODS

Chemicals

The vitamin-E (α-tocopherol), 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (*R*-Trolox), 2,4,6-tripyridyl-*s*-triazine (TPTZ) were from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA); 2-deoxyribose, mannitol, phenazine methosulfate (PMS), nitroblue tetrazolium (NBT), nicotinamide adenine dinucleotide (NADH), ethylene diamine tetra acetic acid (EDTA), 2-thiobarbituric acid (TBA), Xylenol Orange [*o*-cresolsulphonphthalein-3,3-bis (methyliminodiacetic acid sodium salt)], butylated hydroxytoluene (BHT), naphthyl ethelene diamine dihydrochloride, heparin, gallic acid, quercetine were purchased from SRL Chemical Co. (Mumbai, India). All other chemicals used were of analytical grade.

Plant Material and Extraction

Roots of *A. racemosus* (AR) were collected from university botanical garden, Sardar Patel University, Vallabh Vidyanagar, India. The roots were washed thoroughly with tap water and dried at 37 °C in an incubator, sliced into small pieces, powdered in a mixer grinder and used for the extraction. The powder (25 g) was successively extracted with 250 ml of distilled water (at 80 ^{o}C) and ethanol (at 40 ^{o}C) using soxhlet extractor for 8 h to obtain water and lipid-soluble components. The aqueous and ethanol extracts were filtered through Whatman filter paper (No. 1). Aqueous and ethanol filtrates were evaporated in vacuum oven at 100 mm of hg at 60 and 40 ^{o}C respectively. The percentage yield was 5.7 % (w/w) for aqueous extract (ARA) and 7.5 % (w/w) for ethanol extract (ARE). The dried powder was stored at -20 ^{o}C in a desiccant until required.

Preparation of standard, ARA and ARE solutions

Vitamin E (α-tocopherol) was used as a positive standard, unless specifically stated in the text. Vitamin E and ARE stock solution were prepared at the concentration of 1000 µg/ml in ethanol and subsequently diluted with 10 mM phosphate buffer (PB, pH 7.4) to obtain working solution of 25, 50, 100, 250, 500 and 1000 µg/ml (final ethanol concentration, 10%). Aqueous extract of ARA root was prepared in PB with concentration of 1000 µg/ml as stock solution. From the stock solution different concentrations *viz*. 25, 50, 100, 250, 500 and 1000 µg/ml was used for antioxidant studies.

Determination of Total Polyphenolic Compounds

The concentration of phenolic compounds was measured according to the method of Taga *et al.* (43). Both ARA and ARE dried power (0.1 g) was dissolved in 5 ml solution of 0.3% HCl in methanol/water (60:40, v/v). The resulting solution (100 µl) was added to 2 ml of 2% Na_2CO_3. After 2 min incubation at room temperature, 50% (v/v) Folin-Ciocalteu reagent (100 µl) was added to the mixture, which was then left for 30 min. Absorbance was measured at 750 nm. Results were expressed as mg per gram of gallic acid equivalents. All measurements were carried out in triplicate.

Determination of Total Flavonoids

Flavones and flavonols in the water and ethanol extracts of AR were estimated according to the method of Kosalec *et al.* (30). One gram of dried powder was mixed with 1.5 ml ethanol (95% v/v) and added to 100 µl aluminum chloride (10% w/v), 100 µl sodium acetate (1 M) and 2.8 ml water. The volume of 10% aluminum chloride was substituted by the same volume of distilled water in blank. After incubation at room temperature for 30 min, absorbance of the reaction mixture was measured at 415 nm. The mean of three readings was used and the total flavonoid content was expressed in mg of quercetin equivalents/g extract.

DPPH Radical Scavenging Activity

This was carried out according to the Blois method with a stable radical DPPH (4). For experiment, DPPH in ethanol (0.5 mM, 1 ml) was added to 1 ml of various concentrations of test compounds as ARA and ARE extracts. Alpha-tocopherol was used as standard. Mixture was then shaken vigorously and held for 30 min at room temperature in dark. The decrease in absorbance of DPPH was measured at 517 nm. Phosphate buffer was used as blank while DPPH in PB served as control. Inhibition of DPPH radical was calculated using the equation: I (%) =100 × (A_0–A_1)/A0, where A_0 is the absorbance of the control, and A_1 is the absorbance of the standard or test compounds. The inhibition curve was plotted and IC_{50} values obtained. The IC_{50} value represented the concentration of the extract or standard that caused 50% inhibition of DPPH radicals. The value was used in all other in vitro assays, unless otherwise mentioned.

Nitric Oxide Radical Scavenging activity

Nitric oxide radical inhibition can be estimated by the use of Griess Illosvoy reaction (32). The reaction mixture containing sodium nitroprusside (10 mM, 2 ml), phosphate buffer saline (0.5 ml) and standard or test compounds (0.5 ml) was incubated at 25 °C for 15 min. After incubation, 0.5 ml of the reaction mixture was mixed with 1.5 ml of Griess reagent (1% (w/v) sulphanilamide, 2% (v/v) phosphoric acid and 0.1% (w/v) naphthyl ethelene diamine dihydrochloride). The absorbance of the chromophore formed during diazotization of the nitrite with sulphanilamide and subsequent coupling with naphthylethelene diamine was measured at 546 nm along with a control.

Superoxide Anion Scavenging Activity

Superoxide radicals were generated by the PMS/NADH system according to the method of Kakker *et al*. (25). The reaction mixture was composed of 1 ml of NBT (156 µM), 1 ml NADH (468 µM) and 100 µl either of test or standard compounds. The reaction was started by addition of 100 µl of PMS (60 µM) to the mixture. All components were dissolved in 100 mM phosphate buffer (pH 7.4). The reaction mixture was incubated at 25 ºC for 5 min, and the absorbance was measured at 560 nm against sample blank.

Hydroxyl Radical Scavenging Activity

The ability of AR root to scavenge $OH^{\cdot}$ radical was assessed using the classic deoxyribose degradation assay described by Halliwell *et al*. (19). One ml of the reaction mixture contained 100 µl of 2.8 mM 2-deoxy-2-ribose (dissolved in phosphate buffer, pH 7.4), 200 µl of 20 µM $FeCl_3$ and 100 µM EDTA (1:1 v/v – freshly prepared) and 100 µl H_2O_2 (1 mM), with absence (control) or presence of standard/test compounds. The reaction was commenced by addition of 100 µl ascorbic acid (100 µM). All components were dissolved in KH_2PO_4–KOH buffer (10mM, pH 7.4). The samples were incubated for 60 min at 37 ºC in shaking water bath followed by the TBARS (thiobarbituric acid reactive substances) assay as malondialdehyde (MDA) content was measured by modified method of Buege and Aust (7). One ml each of TBA (0.7% in 0.05 mM NaOH) and TCA (2.5%) were added to above 1 ml reaction mixture; test tubes were heated at 95 °C for 15 min to develop the color. After a cooling period, TBARS formation was measured spectrophotometrically at 532 nm against an appropriate blank. The effect of the test compounds on hydroxyl radical-induced deoxyribose degradation was also examined in the absence of EDTA. The extent of inhibition was compared with mannitol, the well-known $OH^{\cdot}$ radical scavenger.

Reducing Power

The reducing power of standard and test compounds was evaluated by the method of Oyaizu (35). Briefly, one ml of various concentrations of standard or test compound was mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml potassium ferricyanide (1%), the mixture was incubated at 50 °C for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. Finally, 2.5 ml of the supernatant solution was mixed with 2.5 ml distilled water and 0.5 ml $FeCl_3$ (0.1%). The absorbance was measured at 700 nm. Increase in absorbance of the reaction mixture indicated stronger reducing power.

TEAC (Trolox Equivalent Antioxidant Capacity) with $ABTS^{\cdot+}$

The TEAC was determined according to the method of Re *et al*. (39). The method is based on the ability of antioxidant molecules to quench the long-lived $ABTS^{\cdot+}$, a blue-green chromophore with characteristic absorption at 734 nm, compared with that of Trolox (1 mg/ml) - a water-soluble vitamin-E analog. Five ml of solution of ABTS (7 mM) was added along with 88 µl of potassium persulfate (2.45 mM) solution and left at room temperature in dark for 12-16 h. Before use, the solution was diluted (1:88) with 10 mM sodium phosphate buffer (pH 7.4) to an absorbance of 0.700 ± 0.05 at 734 nm. The final reaction mixture contained 0.9 ml of ABTS solution added to 0.1 ml of standard/test compounds/control, mixed for 45 sec. Absorbance was recorded after 15 sec at 734 nm. TEAC values express the µg of Trolox having the antioxidant capacity equivalent to a 1 mg of the test sample. The TEAC values of test compounds were calculated by dividing the slope of its concentration-response curve by the slope for the concentration-response curve corresponding to Trolox.

Ferric-Reducing Antioxidant Power (FRAP)

FRAP assay measures the antioxidant potentials of compounds to reduce the Fe^{3+}/TPTZ complex present in a stoichiometric excess to the blue colored Fe^{2+} form and method is according to the procedure described by Benzie and Strain (2). The FRAP reagent contained 2.5 ml of a 10 mM TPTZ solution in 40 mM HCl plus 2.5 ml of 20 mM $FeCl_3 \cdot 6H_2O$, plus 25 ml of 0.3 mM acetate buffer (pH 3.6). Briefly, 900 µl of FRAP reagent, freshly prepared and warmed at 37 °C, was mixed with 60 µl of distilled water and 30 µl either of test sample, standard or appropriate reagent blank. The absorbance was read at 595 nm after 5 min. Calibration curve of ferrous sulfate (100-1000 µM) was used, and results were expressed in µM Fe^{2+}/mg dry weight extract. The relative activity of the samples was compared with that of α-tocopherol.

Assay of Fe^{+2}/ascorbate induced lipid peroxidation (LPO)

The antioxidant activity of the extract was evaluated by quantifying the ability of different concentrations of plant extract to suppress iron (Fe^{+2})-induced LPO in rat liver mitochondrial fractions. Liver homogenate was prepared from male albino rats weighing between 250 and 300 g scheduled to be killed by light (diethyl ether) anesthesia as healthy untreated controls. The Institutional Animal Ethics Committee approved the present investigation. Briefly, liver was quickly removed and washed with ice-cold normal saline and homogenized in homogenization buffer (0.25 M

sucrose in 5 mM Tris-HCl buffer pH 7.4) at 4 ^{o}C. The homogenate was centrifuged at 800 *g* for 5 minutes at 4 °C to separate the nuclear debris and the supernatant was recentrifuged at 10,500 *g* for 20 minutes at 4 °C. The resulting pellet was washed three times with homogenization buffer, and the final pellet was resuspended in 0.15 M KCl in 20 mM Tris-HCl buffer (pH 7.4) to get the mitochondrial fractions (MF). The protein concentration in MF was determined according to the method of Lowry *et al.* (31) using the bovine serum albumin as a standard. Oxidation of rat liver MF was carried out as described by Demopoulos and Rekka (9). A reaction mixture containing 200 µl of MF (equivalent to 0.5 mg of protein), 200 µl of vehicle (control) or standard/test compounds and 200 µl of $FeSO_4$ (10 µM), followed by induction of lipid peroxidation using 200 µl ascorbate (0.2 mM). The samples were incubated at 37 °C for 40 min. At the end of this incubation period, 10 µl of 5% (w/v) butylated hydroxytoluene in ethanol was added to each mixture followed by the addition of 0.8 ml each of 20% (w/v) trichloroacetic acid (TCA) and 0.4% (w/v) TBA. The solutions were heated in a water bath at 80 °C for 20 min. The chromogen was extracted in butanol (2 ml) and the extent of lipid peroxidation (TBARS) measured in the organic layer as absorbance at 532 nm.

Copper-induced Human Low-Density Lipoprotein (LDL) Oxidation

Isolation of LDL

Venous blood was obtained from fasting, five healthy adult human volunteers. The informed consent was obtained from all participants according to ethical guidelines and work was carried out by approval of local ethical committee of the university. Blood samples were kept at room temperature for 1 h, and centrifuged at 1000 *g* for 10 min to obtain serum. The serum was pooled and stored at -20 °C and used for all subsequent experiments. LDL was isolated by heparin-citrate buffer precipitation method (43) from the serum sample. The precipitation buffer consisted of 0.064 M trisodium citrate adjusted to pH 5.05 with 5 M HCl, and contained 50,000 IU/l heparin. One hundred µl of the serum sample was added to 1 ml of the heparin-citrate buffer. After mixing with a Vortex mixer, the suspension was allowed to stand for 10 min at room temperature. The insoluble lipoproteins were sedimented by centrifugation at 1000 *g* for 10 min. The pellet was resuspended in 1 ml of 0.1 M phosphate buffer saline (PBS), pH 7.4, containing 0.9% NaCl and used as a source of LDL-fraction. The protein concentration of the LDL was measured with a Lowry method (31) using bovine serum albumin as standard.

Copper-induced LDL oxidation in vitro

For the oxidation experiments, LDL protein (100 µg) was diluted to 900 µl with PBS and incubated with (100 µl) or without standard/test compounds at 37 ^{0}C for 30 min. After incubation period, oxidation was initiated by adding 10 µl of freshly prepared 0.167 mM $CuSO_4$ (final concentration of $CuSO_4$ was 1.67 µm) in 1-cm quartz cuvette. Alpha-tocopherol and ARE extracts was dissolved in ethanol sample and, ethanol was evaporated at lower temperature under reduced pressure prior to the addition of LDL. Oxidation method was essentially the same as described by Palomäki *et al.* (36). Oxidation kinetics were determined as the production of conjugated dienes by continuously monitoring the change in absorbance at 234 nm as described by Esterbauer *et al.* (13), using a diode array UV-visible spectrophotometer (model 8452-A, Hewlett Packard Instruments Inc, USA) with absorbance readings made every 10 min at 37 ^{o}C for 3 h. Lag time (min) was determined from the intercept of lines drawn through the linear portions of the lag phase and propagation phase. The rate of oxidation (expressed as nmol/min.) was determined from the slope of the propagation phase. Maximum concentration of dienes formed (CD max. = expressed as nmol/mg protein) was calculated from the difference in absorbance at zero time and at diene peak. The concentrations of conjugated dienes in the samples were calculated by using a molar extinction coefficient of 2.95×10^4 $M^{-1.}$ cm^{-1} (13). The more indices of oxidized-LDL were measured including MDA and lipid hydroperoxides (LHP). Two set of 100 µl aliquot was prepared according to above mentioned reaction mixture and was added to 10 µl of 10 mM EDTA to stop oxidation after 3 h. of incubation and each one was used for measurement of MDA and LHP. For MDA estimation, 100 µl aliquot was treated with mixed with 2 ml TBA-TCA-HCl (1:1:1) reagent (0.37% thiobarbituric acid, 15% TCA, 0.25 N HCl) and placed in water bath for 20 min. The absorbance of clear supernatant was measured at 532 nm with using a molar absorption coefficient of 1.56×10^5 M^{-1} cm^{-1} (7). For LHP estimation, 100 µl aliquot was mixed with 0.9 ml of Fox reagent (88 mg butylated hydroxytoluene (BHT), 7.6 mg xylenol orange and 9.8 mg ammonium ion sulphate dissolved in 90 ml methanol and 10 ml 250 mM sulphuric acid) and incubated at 37 °C for 30 min. The color developed was read at 560 nm and LHP content was determined using the molar absorption coefficient of 4.3×10^4 M^{-1} cm^{1} (23).

Copper-induced serum oxidation in vitro

One ml of diluted human serum sample (a concentration of 0.67% in PBS) (40) was added to the presence and absence of standard/test compounds and incubated at 37 °C for 30 min. Oxidation reaction was initiated by adding $CuSO_4$ as previously done and CD max., LHP and MDA were estimated with similar incubation period as mentioned above.

Statistical analysis

Data were calculated as means ± S.D. of three different experiments. Specific AR extracts effect was evaluated by the one-way analysis of variance (ANOVA) using 10th version of SPSS plus Duncan's test as post hoc analysis. $P < 0.05$ was regarded as statistically significant.

RESULTS

Concentrations of total polyphenol, flavonoid and FRAP value

Table-1 indicates higher concentrations of total polyphenol and flavonoid compounds in the ethanolic extract than aqueous extract of AR root. The extracts of ARA and ARE were showed the concentration of polyphenols as 2.371 % and 3.515 %, respectively and flavonoids as 0.85 % and 1.281 %, respectively. It is evident from the table that the highest FRAP value of ARE extract (0.025 µmol Fe^{2+}/mg), followed by standard, α-tocopherol (0.023 µmol Fe^{2+}/mg) and ARA extract (0.018 µmol Fe^{2+}/mg) reflecting the total antioxidative capacity of plant root.

Table 1. Concentrations of total polyphenol, flavonoid, FRAP and TEAC value in *A. racemosus* root extracts.

Parameters	ARA	ARE	Standard
Polyphenols[a]	23.71±1.78 (2.371 %)	35.15±2.85 (3.515 %)	---
Flavonoids[b]	8.50±0.56 (0.85 %)	12.81±0.85 (1.281 %)	---
FRAP[c]	0.018±0.001	0.025±0.002	0.023±0.002[*]
TEAC[d]	1270	1105	1000[**]

Values = mean ± SD

a) Expressed as mg gallic acid equivalent/g dry weigh extract.

b) Expressed as mg of quercetin equivalents/g dry weigh extract.

c) Expressed as µmol Fe^{2+}/mg of dry weigh extract, [*]α-tocopherol (1000 µg/ml).

d) Expressed as µg of Trolox having the antioxidant capacity equivalent to a 1 mg of the test sample, [**]Trolox.

DPPH radical scavenging activity

The extracts of ARA and ARE exhibited a DPPH radical-scavenging effect (Fig. 1). In this test, ARE (IC_{50} 52.5 µg/ml) was more efficient than ARA (IC_{50} 60.7 µg/ml). The IC_{50} values of the both extracts were found to be lesser than the standard, α-tocopherol (IC_{50} 29.8 µg/ml).

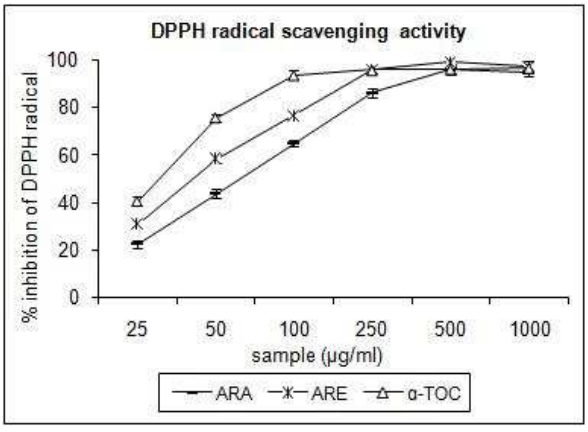


Figure 1. Effect of *A. racemosus* root extracts and α-tocopherol on DPPH radical scavenging assay.

Nitric Oxide Radical Scavenging activity

The scavenging of $NO^{\cdot}$ by plant extracts were increased in a dose-dependent manner as illustrated in figure 2. The IC_{50} value of ARE (63.4 µg/ml) was closer to the standard (57 µg/ml) as compared to ARA (141.9 µg/ml) in nitric oxide assay.

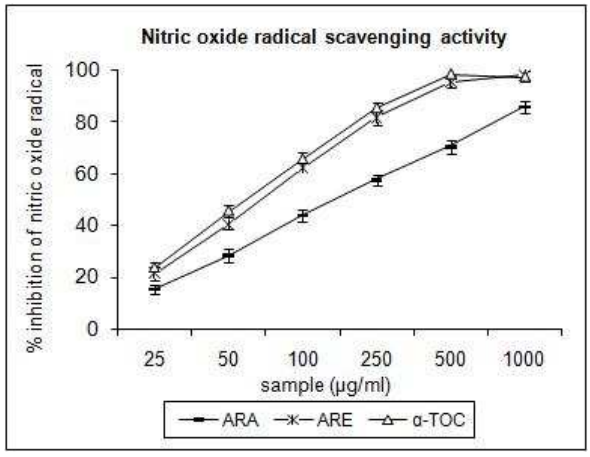


Figure.2. Effect of *A. racemosus* root extracts and α-tocopherol on nitric oxide radical scavenging assay.

Superoxide anion scavenging activity

Figure-3 shows the result obtained in the $O_2^{\cdot -}$ scavenging assay. It was observed that ARA, ARE and α-tocopherol prevented the $O_2^{\cdot -}$-induced reduction of NBT in a concentration dependent manner. The IC_{50} values of the ARA, ARE and α-tocopherol were 221 µg/ml, 89.4 µg/ml and 43.2 µg/ml, respectively.

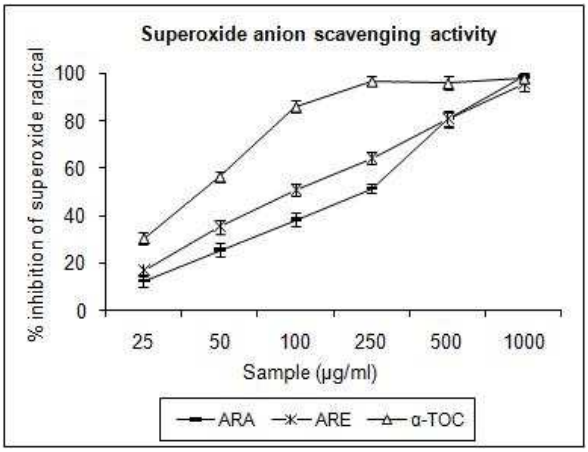


Figure 3. Effect of *A. racemosus* root extracts and α-tocopherol on superoxide radical scavenging assay.

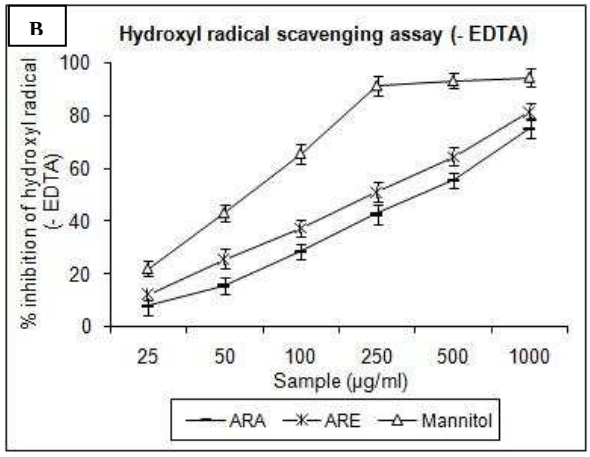
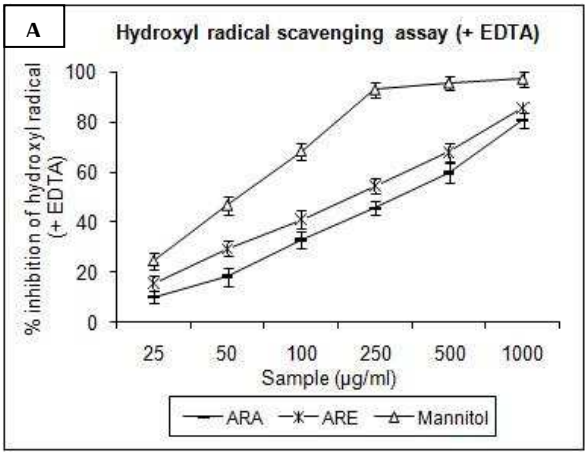


Figure 4. Effect of *A. racemosus* root extracts and α-tocopherol on hydroxyl radical scavenging assay with (+) EDTA (A) and without (-) EDTA (B).

Hydroxyl radical scavenging activity

This assay shows the ability of AR root extracts and mannitol compound to inhibit $OH^{\cdot}$ radical-mediated deoxyribose degradation in a reaction mixture with or without EDTA. In the presence of EDTA, mannitol, a classical $OH^{\cdot}$ scavenger, significantly inhibited deoxyribose degradation in a concentration-dependent manner with an IC_{50} value of 56.5 µg/ml followed by ARE (208.8 µg/ml) and ARA (318.7 µg/ml) (Fig. 4A). When we used the deoxyribose degradation assay in the absence of EDTA to assess the ability of the standard/extracts to bind iron, mannitol still exhibited suppressive action against deoxyribose degradation with an IC_{50} value of 61.9 µg/ml, followed by ARE (230.4 µg/ml) and ARA (346.7 µg/ml) (Fig. 4B).



Reducing power

Figure 5 shows the reductive capabilities of the plant extracts compared to α-tocopherol at an absorbance of 700 nm. The reducing power of ARA and ARE were increased in concentration dependent manner. The ARE extract could reduce the most Fe^{3+} ions, which had a greater reductive activity than the standard of α-tocopherol and ARA extract.

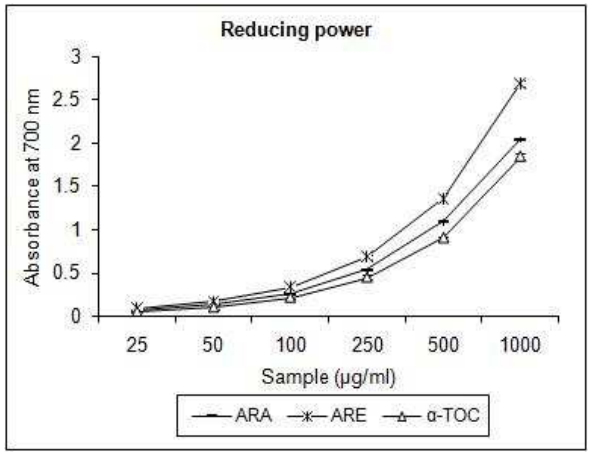


Figure 5. Effect of *A. racemosus* root extracts and α-tocopherol on reducing power assay.

TEAC (Trolox Equivalent Antioxidant Capacity) with ABTS

Figure 6 shows the result of the assessment of the scavenging effects of AR root extract and Trolox on the $ABTS^{\cdot+}$ cation. The best scavenging activity obtained from $ABTS^{\cdot+}$ assay was found in Trolox (IC_{50} = 59 µg/ml), followed by ARE (71.9µg/ml) and ARA (134.5 µg/ml).

The antioxidant responses of both extracts were compared with the antioxidant action exerted by Trolox, as a TEAC value indicated significant differences among their scores. The concentrations of Trolox, ARA and ARE required to trap ABTS are observed to be 1000 µg/ml, 1270 µg/ml and 1105 µg/ml, respectively (Table-1).

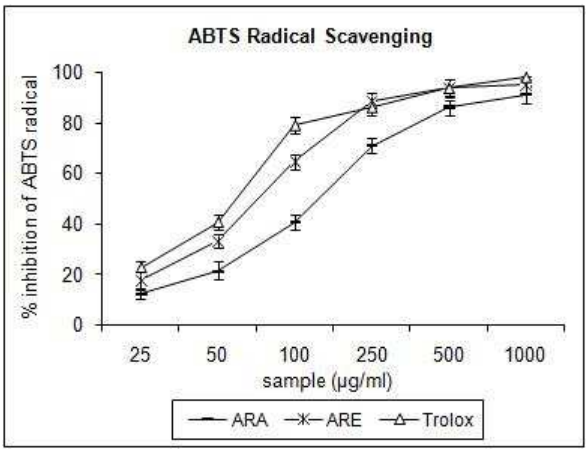


Figure 6. Effect of *A. racemosus* root extracts and α-tocopherol on $ABTS^{\cdot+}$ cation assay.

Fe^{+2}/ascorbate induced lipid peroxidation

Addition of AR root extracts (25 to 1000 µg/ml) to isolate rat liver mitochondrial fraction in the presence of Fe^{+2}/ascorbate resulted in a concentration dependent decrease in lipid peroxidation. The IC_{50}, as shown in figure 7, were found to be 511.7 µg/ml, 309.2 µg/ml and 178.3 µg/ml for ARA, ARE and α-tocopherol, respectively.

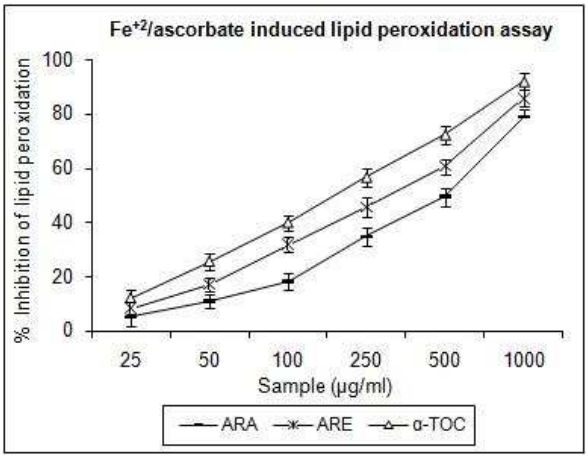
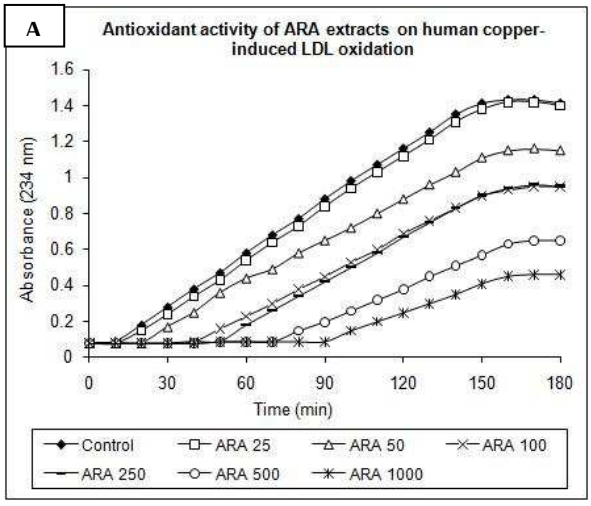
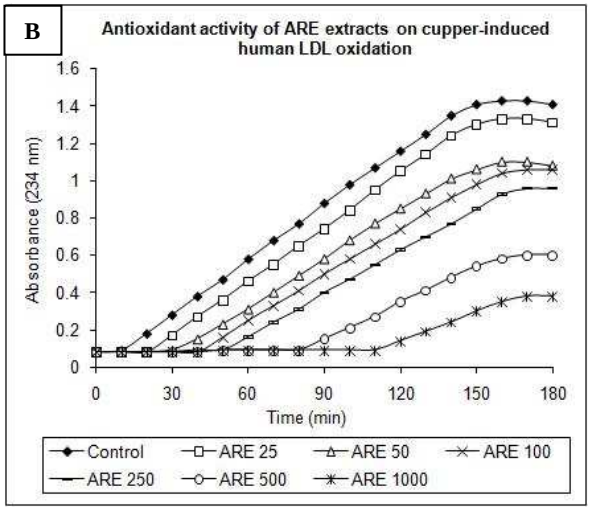


Figure 7. Effect of *A. racemosus* root extracts and α-tocopherol on Fe^{+2}/ascorbate induced mitochondrial lipid peroxidation assay.

Copper-induced LDL and serum oxidation in vitro:

Incubation of AR root extracts or α-tocopherol at dose-dependent fashion (25 to 1000 µg/ml) with isolated human LDL, followed by copper-mediated oxidation, resulted in significantly ($P< 0.05$) prolonged lag time along with decreased in oxidation rate and concentrations of CD max., LHP and MDA as compared to without incubation of test compounds (Fig. 8A to 8C and Table-2). The α-tocopherol was found to be most potent with an IC_{50} (48.4, 88 and 72.7 µg/ml for CD max., LHP and MDA, respectively), followed by ARE (397.8, 519.8 and 276.2 µg/ml for CD max., LHP and MDA, respectively) and ARA (426.9, 763.4 and 684.3 µg/ml for CD max., LHP and MDA, respectively) root extract. For the serum oxidation, AR root extracts and α-tocopherol also showed antioxidant effect on *in vitro* copper-induced serum oxidation by significantly decreased ($P< 0.05$) concentrations of CD max., LHP and MDA in test/standard compounds incubated medium compared to controls, where devoid of test/standard compounds incubation (Table-3). The calculated IC_{50} value was observed in the following order: α-tocopherol (102.6, 130.3 and 108.5 µg/ml for CD max., LHP and MDA, respectively), followed by ARE (467.9, 258.3 and 277 µg/ml for CD max., LHP and MDA, respectively) and ARA (748.4, 560 and 459.1 µg/ml for CD max., LHP and MDA, respectively) root extract.





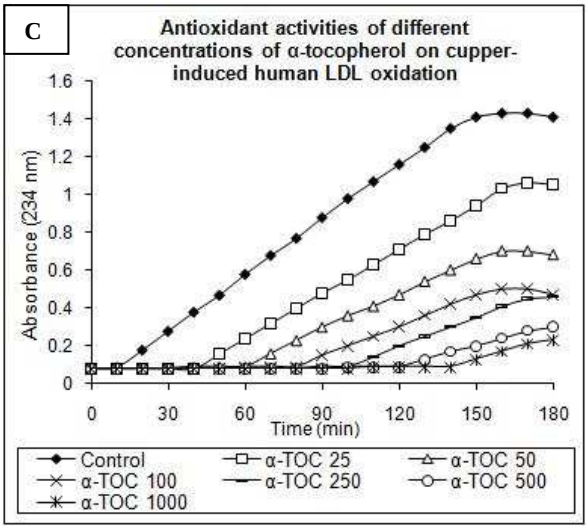


Figure 8. Antioxidant activity of different concentrations of ARA (Fig. 8A), ARE (Fig. 8B) and α-tocopherol (Fig. 8C) on copper-induced LDL oxidation in vitro. Figure represents results from a LDL oxidation kinetics experiment. Incubation of isolated human LDL was at 37 ^{o}C in PBS, pH 7.4 with 0.167 µm $CuSO_4$ in the absence (control) or presence of different concentrations of test/standard compounds.

DISCUSSION

Herbs have played a significant role in maintaining human health and improving the quality of life for thousands of years. Many active phytochemicals, including flavonoids, terpenoids, lignans, sulfides, polyphenolics, carotenoids, coumarins, saponins, plant sterols, curcumins, and phthalides, have been identified (28, 45). Numerous in vitro studies have shown that some of the phytochemicals are potent antioxidants, metal chelators, or free radical scavengers, which may account for their health promoting properties (20,41,44,45). This is the first study which identifies the dose-responsive antioxidant and scavenging activities of aqueous and ethanolic *A. racemosus* root extracts (i.e., ARA and ARE) through wide range of established *in vitro* assays.

In this study, the ethanol-soluble components of ARE root extract had profound effects on various free radical generated systems than water-soluble components of ARA root extract. As shown in Table-1, ARE extract contained higher level of phenolic compounds than ARA extract, indicated that the total phenolic content of AR root extracts were solvent dependent. The DPPH radical-scavenging activity of AR extracts could be related to the nature of phenolics, thus contributing to their electron transfer/hydrogen donating ability. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule (4). Wiboonpun et al. (47) identified that the roots of *Asparagus racemosus* were found to possess *in vitro* antioxidant property against DPPH medium. These antioxidant compounds are racemofuran along with two known compounds asparagamine A and racemosol. Nitric oxide, a short-lived free radical generated endogenously and associated with chronic inflammation. Overproduction of $NO^{\cdot}$ can mediate toxic effects, *e.g.* DNA fragmentation, cell damage and neuronal cell death (29,38). Both AR root extracts showed a potent scavenger of $NO^{\cdot}$ with dose-dependant manner, although effect was less than standard α-tocopherol compound. The presence of phenolic compounds is an important part of AR root extracts, which leads to the formation of phenoxyl radicals from the reaction of $NO^{\cdot}$ with a phenol moiety (21, 49). The superoxide is precursor of many other toxic ROS. During inflammation, the activation of mast cells, macrophages, eosinophils, and neutrophils generate $O_2^{\cdot -}$, with NADPH oxidase playing an important role (1, 10). In the PMS/NADH -NBT system, superoxide anion derived from dissolved oxygen by PMS/NADH coupling reaction reduces NBT. The decrease of absorbance at 560 nm with antioxidants thus indicates the consumption of superoxide anion in the reaction mixture. The inhibitory effects of ARE extract on superoxide radicals was superior than ARA extract, however, standard antioxidant α-tocopherol was found to be highest superoxide scavenger. The marked superoxide scavenging activity of AR root extracts may be related to its polyphenol and flavonoid compounds, which

Table 2. Effect of ARA, ARE and α-tocopherol on the kinetics study of copper-induced LDL oxidation

Groups	Lag Time (min.)	Oxidation Rate nmol CD/min/ mg LDL protein	CD max. nmol/mg LDL protein	LDL-LHP nmol/mg protein	LDL –MDA nmol/mg LDL protein
Control	12.43±2.68^{k}	3.04±0.24^{ab}	484.17±15.75^{a}	829.30±31.14^{a}	80.17±7.75^{ab}
ARA 25	12.33±2.52^{k}	3.08±0.27^{a}	480.33±18.01^{a}	828.70±29.27^{a}	82.47±7.31^{a}
ARA 50	17.17±2.75^{k}	2.49±0.26^{c}	392.67±12.50^{c}	817.00±28.05^{ab}	75.30±6.56^{abc}
ARA 100	39.67±4.04^{hi}	2.24±0.26^{cd}	322.33±12.50^{e}	748.60±20.15^{c}	72.33±7.09^{bcd}
ARA 250	48.33±3.51^{g}	2.54±0.30^{c}	325.10±10.15^{e}	673.50±21.29^{d}	62.13±6.02^{ef}
ARA 500	72.93±5.00^{e}	1.86±0.18^{def}	220.33±11.50^{fg}	546.80±20.24^{f}	49.97±4.95^{hi}
ARA 1000	88.07±4.10^{d}	1.42±0.20^{fgh}	155.23±8.01^{h}	301.23±13.89^{i}	23.40±3.42^{jk}
ARE 25	18.83±3.75^{k}	3.03±0.35^{ab}	450.33±20.50^{b}	812.67±23.59^{ab}	76.33±6.51^{abc}
ARE 50	33.67±4.04^{j}	2.68±0.33^{abc}	374.00±18.33^{cd}	790.50±19.25^{b}	70.10±7.39^{cde}
ARE 100	41.33±4.04^{h}	2.52±0.20^{c}	360.07±16.90^{d}	722.07±21.97^{c}	59.77±6.35^{fg}
ARE 250	48.33±3.51^{g}	2.41±0.31^{c}	325.33±14.50^{e}	602.00±15.10^{e}	42.83±4.25^{i}
ARE 500	79.33±4.04^{e}	1.84±0.16^{def}	203.00±10.00^{g}	426.27±15.14^{g}	18.73±3.95^{kl}
ARE 1000	108.27±5.02^{c}	1.46±0.23^{fgh}	128.10±7.85^{i}	149.33±11.02^{k}	14.83±2.84^{klm}
α-TOC 25	37.47±3.72^{hi}	2.61±0.31^{bc}	359.30±13.25^{d}	680.80±18.81^{d}	64.70±5.56^{def}
α-TOC 50	58.57±3.19^{f}	1.93±0.27^{de}	237.00±12.53^{f}	578.73±17.93^{ef}	52.27±4.61^{gh}
α-TOC 100	78.40±3.50^{e}	1.61±0.20^{efg}	169.83±9.78^{h}	381.77±16.35^{h}	29.93±4.10^{j}
α-TOC 250	102.67±4.04^{c}	1.62±0.18^{efg}	155.00±10.00^{h}	199.80±13.30^{j}	10.77±2.93^{lm}
α-TOC 500	117.67±3.51^{b}	1.17±0.13^{gh}	100.67±9.50^{j}	131.23±10.21^{k}	8.70±3.01^{m}
α-TOC 1000	138.63±4.98^{a}	1.15±0.22^{h}	77.37±7.46^{k}	89.97±8.05^{l}	8.70±3.01^{m}

Values = mean ± SD
Within a column, values with different superscripts are significantly different from each other ($P < 0.05$)

Table-3. Effect of ARA, ARE and α-tocopherol on the copper-induced serum oxidation

Groups	Serum-CD max. nmol/ml	Serum-LHP nmol/ml	Serum–MDA nmol/ml
Control	320.33 ± 15.50^{ab}	35.43 ± 2.38^{a}	22.73 ± 2.41^{ab}
ARA 25	327.50 ± 19.37^{a}	36.00 ± 3.00^{a}	23.30 ± 2.14^{a}
ARA 50	302.10 ± 14.96^{bc}	32.67 ± 2.52^{ab}	20.67 ± 3.06^{abc}
ARA 100	284.50 ± 13.03^{cd}	30.73 ± 2.41^{bc}	17.77 ± 2.54^{cde}
ARA 250	259.90 ± 12.75^{e}	26.37 ± 2.47^{cd}	15.03 ± 2.00^{ef}
ARA 500	210.93 ± 10.63^{f}	19.17 ± 1.76^{e}	10.97 ± 2.05^{gh}
ARA 1000	110.07 ± 7.90^{h}	7.27 ± 1.62^{f}	8.23 ± 1.66^{hi}
ARE 25	305.17 ± 15.28^{bc}	33.00 ± 3.00^{ab}	21.33 ± 2.52^{abc}
ARE 50	292.90 ± 12.15^{c}	30.07 ± 2.90^{bc}	19.37 ± 2.47^{bcd}
ARE 100	269.37 ± 11.06^{de}	26.33 ± 2.31^{cd}	15.90 ± 2.26^{de}
ARE 250	230.10 ± 12.05^{f}	18.10 ± 2.85^{e}	12.10 ± 2.15^{fg}
ARE 500	150.43 ± 10.36^{g}	6.17 ± 1.76^{f}	7.53 ± 1.45^{hij}
ARE 1000	49.87 ± 7.53^{jk}	5.27 ± 1.62^{f}	5.47 ± 1.15^{ijk}
α-TOC 25	291.83 ± 13.25^{c}	30.33 ± 3.51^{bc}	18.07 ± 2.10^{cde}
α-TOC 50	268.80 ± 11.85^{de}	25.43 ± 2.38^{d}	15.07 ± 2.20^{ef}
α-TOC 100	162.40 ± 13.16^{g}	20.33 ± 2.52^{e}	11.95 ± 1.93^{fg}
α-TOC 250	83.63 ± 7.27^{i}	9.67 ± 2.52^{f}	4.53 ± 1.05^{jk}
α-TOC 500	51.73 ± 8.40^{j}	8.17 ± 1.76^{f}	2.53 ± 0.55^{k}
α-TOC 1000	30.47 ± 5.32^{k}	5.37 ± 1.58^{f}	1.94 ± 0.39^{k}

Values = mean ± SD
Within a column, values with different superscripts are significantly different from each other ($P < 0.05$)

have been considered as potent superoxide scavenger (44). In the deoxyribose degradation assay, we found that the both extracts were capable of scavenging $OH^{\cdot}$ and binding free iron. The $OH^{\cdot}$ is highly reactive oxygen centered radical, which attacks proteins, DNA and capable of abstracting hydrogen atoms from membrane lipids and bring about peroxidic reactions of lipids (10,29). When this assay is performed in the presence of EDTA, Fe^{3+} ions are complexed with EDTA and the $OH^{\cdot}$ can attack the deoxyribose. Nevertheless, if an $OH^{\cdot}$ scavenger is added to the reaction, it competes with deoxyribose for the availability of hydroxyl radicals, thus reducing the amount of MDA formation. On the contrary, when the assay is carried out without EDTA, Fe^{3+} added as $FeCl_3$ can bind to the deoxyribose molecule to produce hydroxyl radicals via a site-specific mechanism. In such conditions, the generation of MDA can be inhibited due to the competition between the tested compound and deoxyribose for capturing Fe^{3+} ions (17). Both AR root extracts exhibited an inhibitory effect on hydroxyl radical-induced deoxyribose degradation in both the presence and absence of EDTA. Thus our results suggest that AR root extracts are not only effective scavengers of $OH^{\cdot}$ in this system but also iron chelators.

$ABTS^{\cdot+}$ radical cation model was also used to evaluate the free radical scavenging effect of AR root extracts, where ARE showed a higher $ABTS^{\cdot+}$ radical scavenging effect than ARA. The ABTS assay is closely correlated with DPPH because both are responsible for the same chemical property of hydrogen or electron donation (41). Further, the antioxidant responses of both extracts were compared with the antioxidant action exerted by Trolox, as a TEAC value. The TEAC value of each test compound was calculated by dividing the slope of its concentration–response curve and expressed as μg of Trolox having the antioxidant capacity equivalent to a 1 mg of the test sample. Results indicated that the root extract of ARE showed a more correlated score with that of standard Trolox, while ARA extract required more concentration to achieve Trolox equivalent activity (Table-1). The FRAP assay was evaluated to determine the presence of total antioxidant or reductant (2). The TEAC assay is based on the antioxidant's ability to react with or neutralize free radicals generated in the assay system, whereas the FRAP assay measures the reduction of Fe^{+3} (ferric iron) to Fe^{+2} (ferrous iron) in the presence of antioxidants (20). Thus, FRAP assay expressed as the combined concentrations of all electron-donating reductants or antioxidants present in the AR extracts. Result demonstrated that ARE extract has more electron-donating reductants than the standard α-tocopherol and ARA extract. This result also supported by potassium ferricyanide reduction method, where reducing power of the AR extracts were evaluated. The reducing power of a compound is related to its electron donating ability and may, therefore, serve as an indicator of its potential antioxidant activity (35). Moreover, extracts with phenolic substance-mediated antioxidant activity were shown to be concomitant with the development of reducing power (33). Thus as compared to α-tocopherol, both ARA and ARE root extracts contain higher amounts of reducing compounds which serve as electron donors and reacts with free radicals and convert them to more stable products and terminate radical chain reaction.

Second set of study was to evaluate antioxidant activity of AR root extracts on biological model systems. According to the results obtained, both AR root extracts significantly inhibited the Fe^{+2}/ascorbate induced lipid peroxidation in the mitochondrial fraction may be due to their free radical-scavenging activities. Kamat *et al.* (27) demonstrated that the possible antioxidant effects of crude extract and a purified aqueous fraction consisting of polysaccharides of *A. racemosus* against mitochondrial membrane damage induced by γ-radiation and effectively reduced formation of TBARS and lipid hydroperoxides. Presently, we have used mitochondrial fractions as a source of ROS generation, because as a by-product of electron flow through the mitochondrial electron transport chain, 1–2% of the molecular oxygen consumed may be converted to $O^{\cdot}_2{}^{-}$, this possibility has been represented as a major intracellular source of ROS (42). Iron catalyses the generation of $O^{\cdot}_2{}^{-}$, which initiates lipid peroxidation because superoxide anion acts as a precursor of singlet oxygen and $OH^{\cdot}$. Hydroxyl radicals eliminate hydrogen atoms from the membrane lipid, which results in lipid peroxidation (10). In this experiment, AR root extracts could inhibit lipid peroxidation by scavenging the $OH^{\cdot}$ radical or the superoxide radicals or by chelating the iron itself. The oxidative modification of LDL is apparently a

contributing factor to the pathogenesis of atherosclerosis as it is internalized *via* a scavenging receptor on macrophage, leading to lipid accumulation and foam cell formation (22). Therefore, increasing the resistance of LDL to oxidation should mitigate or even prevent atherosclerosis. In present study, dose-dependent administrations of AR root extracts have ability to protect against *in vitro* copper-mediated human LDL and serum oxidation modifications. As demonstrated by significant prolongation of lag time along with decline in oxidation rate during kinetic study of LDL oxidation and, maximal yield of CD production, MDA and LHP concentrations of LDL and serum oxidation. Further, ARE exhibited much higher potency to reduce susceptibility to undergo oxidative modification as compared to ARA. Copper-mediated LDL oxidation begins first with a lag phase during which protective endogenous anti-oxidants are consumed by initiating free radical species. After the consumption of all endogenous anti-oxidants, a lipid radical-propagated peroxidation chain reaction begins in which the polyunsaturated fatty acids contained in the LDL are rapidly oxidized to lipid hydroperoxides (15). Three mechanisms should be basically considered by AR root extracts as in the pharmacological antagonism of copper-mediated LDL oxidation: (i) metal complexation-inactivation; (ii) scavenging of radical species involved in LDL oxidation, i.e., propensities for hydrogen or electron donation, or their redox properties,; (iii) stabilization of the lipid moiety of LDL, conceivably via chemical interactions between AR root extract containing hydrophobic groups and polyunsaturated residues of LDL phospholipids. First two possibilities have already been tested through absence of EDTA in deoxyribose assay for iron chelating ability and scavenging of radical species with various cell-free models. While considering the third possibility also given the answer that difference between the ARA and ARE antioxidant potency, because the hydrophobic phenolic compounds is higher than hydrophilic compounds in AR root extract, as seen concentration of phenolic compounds of ARE and ARA root extracts (Table-1). Therefore, antioxidants potency of AR root extracts could be different and depending on their chemical nature, particular their partitioning abilities between the aqueous compartment and the lipophilic environment within the LDL particle. Lipophilic substances, such as α-tocopherol, carotenoids or luteolin, tend to accumulate in the plasma lipoproteins and thereby, inhibit the free radicals and may sequest metal ions, such as iron and copper (6,11). Hydrophilic antioxidants, which cannot enter the lipid moiety of LDL, would thus be less efficient as they are principially unable to encounter most of these lipophilic radicals. Such compounds, however, may act in a synergistic manner with the lipophilic antioxidants by regenerating them. For α-tocopheroxyl radical it has been reported that, for example, ascorbate and also hydrophilic phenolic substances are able to reduce it to α-tocopherol, thus increasing the oxidation resistance of LDL considerably (24, 34).

In conclusion, present study demonstrated potent antioxidant activity of ethanolic root extract than aqueous root extract and this is mediated though various mechanisms, such as prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity, radical scavenging and chemical nature, which are at least partly due to the phenolic containt in AR root.

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