International Research Journal of Biological Sciences _____E-ISSN 2278-3202
Vol. **5(5),** 35-43, May **(2016)** *Int. Res. J. Biological Sci.*

Antioxidant Activities of Phytonutrient of Amaranthus Palmeri

Rajeshwari A.[1*], Thammanna Gowda S.S.[2] and Somashekar G.N.[3]
[1]Department of Biochemistry, Adichunchanagiri Institute of Medical Sciences, B.G. Nagar, Mandya District, Karnataka. India
[2]Central Research Lab., Adichunchanagiri Institute of Medical Sciences., B.G. Nagar, Mandya District, Karnataka. India. B.G. Nagar, Mandya District, Karnataka. India
[3]Department of Biochemistry, Adichunchanagiri Institute of Medical Sciences, B.G. Nagar, Mandya District, Karnataka. India
rajeshwariarasegowda@gmail.com

Available online at: www.isca.in, www.isca.me
Received 31st March 2016, revised 04th April 2016, accepted 10th May 2016

Abstract

Amaranthus is commonly consumed green leafy vegetable in India. It is bushy perennial herb available throughout the year in India. Animal experiments and human studies have shown that, it is an effective hypoglycemic and hypocholesterolaemic agent. Amaranthus leaves rich in carbohydrates, proteins and vitamin A, vit K and vit C. It also contains minerals such as iron, magnesium, calcium and phosphorous. It reduces bad cholesterol and anemia. In the current study, we evaluated the water, methanol, and chloroform and hexane extract of Amaranthus in-terms of their antioxidant activity in-vitro models. Ours studies proved that, compared to all the other extracts, methanol and water extracts showed more activity. Among these two extracts, water extract possess highest antioxidant activity in all the model systems. Lipid peroxidation activity of the was 75 and 66% in both linoleic and RBC membrane model system. Both the extracts were capable of scavenging peroxy and superoxide, hydroxylradicals and possessed strong reducing powers and ferrous iron chelating capabilities and their activity is comparable with that of standard antioxidants BHA and α-tocopherol. The proximate analysis revealed the presence of high concentration of phenolic compounds (180-210µg) which may be the fact for maximum antioxidant activity.

Keywords: Amaranthus, Hydroxyl Radicals, Superoxide, BHA, Methanol.

Introduction

Antioxidants eradicate harmful Reactive Oxygen Species (ROS), free radicals and peroxides in our body by scavenging or reducing their activity, otherwise they could damage DNA, cell membranes and other cell components. Free radicals are the natural by-products produced during many physiological activities in our body[1]. Damages caused due to these ROS and other free radicals to various tissues can be taken care by internal body antioxidants. When the damage is much severe, there must be need of external synthetic or natural antioxidants. There is much interest among food manufacturers in natural antioxidants to act as replacement for synthetic antioxidants such as Butylated Hydroxy Anisole (BHA). Butylated Hydroxyl Toluene is commonly used by the industry to combat formation of off-flavors and rancidity, particularly in fat based products[2]. There is also an emerging consensus that the micronutrients and non nutrient components such as various phytochemicals of fruits and vegetables play a preventive role in the development of chronic diseases[3-6].

Dark green leafy vegetables are the sources of vitamins, minerals and carotenoids which act as antioxidants in the body. Compounds present in dark green leafy vegetables can inhibit the growth of certain types of cancer[7]. Epidemiological studies show that consumption of fruits and vegetables with high phenolic content correlate with reduced blood pressure, cardio and cerebrovascular disease and cancer mortality[8-10]. Sources of natural antioxidants are primarily plant phenolics that may occur in all parts of the plant[11]. Green leafy vegetables offer a cheap but rich source of a number of micronutrients and other phytochemicals having antioxidant properties. A lot of studies have been analyzed the antioxidant potential of a wide variety of vegetables including Cacao beans, potato, spinach and legumen[12,13].

Plant derived natural products such as flavonoids, terpens and alkaloids have received considerable attention in recent years due to their pharmacological properties including cytotoxic hepatoprotective and cancer chemopreventive effects. The growing interest in the substitution of synthetic food antioxidants by natural antioxidant and in health implications of antioxidants in nutraceuticals has hastened the research on vegetable sources and screening of raw materials for identifying antioxidants. The antioxidants could attenuate this oxidative damage of the tissue in directly by enhancing natural defenses of cell/or directly by scavenging the free radicals spices

Amaranth is a leaf vegetable and grain being consumed for centuries all over the world. It belongs to the family Amaranthaceae and the genus Amaranthus. All parts of the body are edible which are highly nutritious. Amaranth seeds are used since ancient time in Central and Latin America amaranth leaves are used across Asia. Amaranth grain is emerging into

the forefront among grains because of its remarkable nutritional values. It is rich in considerable amount of calcium, iron, phosphorus and carotenoids, vitamins A, C and E. Oil is rich in unsaturated fatty acids, squalene, polyphenols, flavonoids and high in fiber three time that of wheat.

The protein content of amaranth is 28.1 gms for a cup also rich in essential amino acids lysine and methionine. The leaves, seeds and flowers of the plant are responsible for all of the health benefits[14]. Amaranth seeds and leaves have been used to treat hypercholesterolemia in animals studies as on astringent for stopping diarrhea bloody stools and urine. The medicinal uses of the leaves flowers and seeds of amaranth are leaves are used to neutralize acidity leaf poultice is used to treat snake and insect bites and to remove stings of bee wasp hornet scorpion. Strong leaf decoction is used to treat diarrhea dysentery mouth irritations to remove worms and other parasites from GIT and throat irritation, wound.

Amaranthus palmeri is an edible flowering plant in the amaranth genus also known as palmeri amaranth palmeris pigweed and careless weed. The leaves, stems and seeds of palmeri amaranth are edible and highly nutritious[15]. Palmeri amaranth is widely used for thousands of years in mexico north and south. However there is lack of scientific report regarding antioxidant properties of Amaranthus palmeri, thus the present study provides the scientific validation for the traditional uses and antioxidant properties of the same.

Materials and Methods

Materials: Amaranthus leaves were purchased from local farms in Mandya, Karnataka. They were washed, dried at ambient temperature for a week with frequent dispersion under sterile conditions. The dried leaves were sieve powdered, sealed in plastic bags and stored at 4^0C until use.

Chemicals: All chemicals and reagents used were of analytical grade, purchased from Merck. Pvt. Ltd., Bombay and Sigma-Aldrich. Co. (St. Louis, USA), unless otherwise indicates all of the reagents were prepared in deionized water to eliminate the contamination of metal ions.

Methods: Preparation of extracts: Dry Amaranthus (10 gm) was weighed and extracted with 100 ml of double distilled water, methanol chloroform and hexane separately, vortexed for 5 hr at room temperature, then centrifuged at 10,000 rpm at 20^0C for 10 minutes. The residue obtained after extraction was dried and weighed to calculate the solubility. The supernatants were filtered and concentrated in vacuum evaporator to get the dry powder. Then the dried materials were weighed, dissolved in respective extracting solvents and used for further analysis.

Antioxidant activity in Linoleic acid and Erythrocyte ghost model systems: The antioxidant activities of water, methanol extracts of Amaranthus were determined according to the procedure of Shimazaki et al and an assessment of oxidation was achieved by measurement of TBARS according to Dahle. et al[16]. 6µmoles of linoleic acid (solubilized in Hexane) or 100µl of erythrocyte ghost suspension (300µg membrane proteins) were subjected to peroxidation by ferrous sulphate-ascorbate (10:100µmole) in 0.5 ml of Tris buffered saline TBS (10mM, pH 7.4, 0.15M NaCl). With or without extracts of Amaranthus (0.05 to 0.25 mg/0.5ml of TBS) 400µM BHA/α-tocopherol serving as positive controls and contents were incubated for 1 hr, at 37^0C. The reaction was terminated by addition of 500µl alcohol. The extent of lipid per oxidation was assayed in triplicates by malondialdehyde formation using 1ml of 1% thiobarbituric acid. The contents were kept in boiling water bath for 15 min, cooled then 2 ml of acetone is added to stabilize the color which is measured at 535 nm.

Reducing power activity of Amaranthus: Reducing activity of Amaranthus was tested according to the method of Oyaizu[17]. Various concentrations of the water and methanol extracts of Amaranthus (0 to 500µg) were mixed with 200µl of 0.2M phosphate buffer, pH 6.5 and 200 µl of 1% potassium ferricyanide, and then incubated at 50^0C for 20 min. 10% trichloroacetic acid (250µl) was added to the reaction mixture and centrifuged at 3000 g for 10 min at room temperature. The resulting supernatant was taken and mixed with 500 µl of double distilled water and 100µl of 0.1% ferric chloride then incubated at 37^0C for 10 mins. The absorbance was measured at 700nm. Increased absorbance indicated increased reducing power.

1, 1-Diphenyl-2- picryl hydrazyl (DPPH) radical Scavenging Effect: DPPH radical scavenging activity was determined according to the method of Schimada et al. Reactions were performed in 1ml of freshly prepared 1mM DPPH ethanolic solutions and various concentrations of water and methanol extracts of Amaranthus (0-500µg). Reaction mixtures were incubated at 37^0C for 30 minutes and the absorbance at 517 nm was measured. This assay was done in triplicates.

Hydroxyl Radicals scavenging activity: Deoxy ribose assay is used to determine the hydroxyl radical scavenging activity according to the method of Halliwell et al[19]. Reaction mixtures containing various concentrations of water and methanol extracts of Amaranthus,0.02M phosphate buffer, pH 7.4, 2mM H_2O_2, 0.05mM Ferric chloride, 0.05mM ascorbate , 6mM deoxy ribose and 0.05 mM EDTA were incubated at 37^oC for 30 minutes. The degree of deoxyribose oxidation was analyzed as thiobarbituric acid reactive material.

Ferrous ion chelating activity: Ferrous ion chelating effect was measured according to the method of Dinis et al[20]. Known concentration of the water and methanol extracts of Amaranthus and standard chelator EDTA, 200µl of 0.5 mM ferrous chloride and 200µl of 5mM ferrozine were incubated at 37^0C for 10 minutes. After adding 1.5 ml of double distilled water to the mixture, the absorbance at 562 nm was measured. The lower

absorbance at 562 nm indicated stronger chelating power.

Super oxide Scavenging Activity: Superoxide radical (O_2^{-}) scavenging activity was assessed by the method of Lee et al.,[21] with a slight modification. Briefly reaction mixture containing 100µl of 30mM EDTA (PH 7.4) and10 ml of 30 mM hypoxanthine in 50 mM NaOH, and 200µl of 1.42mM Nitro blue tetrazolium (NBT) was pre incubated with various concentrations of water and methanol extracts of Amaranthus at ambient temperatures for 3 min. After this 100µl of 0.5U/ml xanthine oxidase was added to the mixture, incubated for an hr at 37^0C and the volume was brought up to 3ml with 50mM phosphate buffer. After the solution was incubated at room temperature for 20min, absorbance was measured at 560nm. SOD wasused as positive control.

Determination of Total phenolics: Total content of phenolics was determined according to the method[22] of Kujala et al 2000, using gallic acid as standard. The Amaranthus extract (5mg) was dissolved in 5.0ml of methanol: water (50:50 v/v). The solution (0-500µl) containing various concentrations of extracts ranging from 0 – 500µg were added to series of tubes and the volume was made up to 500µl with methanol: water mixture (50:50 v/v). 500µl of 50% folin Ciocalteu reagent was added. The mixture was then allowed to stand for 10mins, then 1.0ml of sodium carbonate (20%) was added. Incubated for 10 min at 37^0C and centrifuged at 10000g for 5 min, collected the supernatant separately and its absorbance was read at 730nm.

Results and Discussion

To begin with, the Antioxidant ability of Amaranthus was evaluated using linoleic acid and RBC membrane model systems. Wherein, lipid peroxidation induced by the action of ferrous sulphate and ascorbate (10:100µM) which is the iron dependent Fenton reaction. Figure-1 and 2 shows the prevention of lipid peroxidation by water and methanol extracts of Amaranthus in linoleic and RBC membrane model system at different doses. In both the model systems, the % of inhibition was 75% and 63% for water (100µg) and methanol extracts (125µg) respectively. These lipid peroxidation activity of both the extracts are comparable with the inhibition offered by the standard antioxidants, α-tocopherol and BHA. These results indicated that, compared to the methanol extract, water extract of Amaranthus exhibits high percentage of inhibition of lipid peroxidation. A substantial portion of ROS lethality involves membrane damage by oxidants generated from iron mediated Fenton reaction[23]. We employed ferrous sulphate: ascorbate system to induce oxidative damage of RBC membrane.

The extracts of spices and herbs may well act as electron donors and can react with free radicals to convert them to more stable products and terminate chain reaction and it has been shown that the antioxidant activities of natural components may have a reciprocal correlation with their reducing power[24,25]. The reducing powers of the methanol and water extracts of Amaranthus leaves are shown in Figure-3. The concentration to attain maximum absorbance unit at 700nm were 100µg for α-tochopherol, 200µg for water and methanol extracts. This result indicated that the reducing power of water extracts of Amaranthus leaves was slightly higher than methanol extracts but less than that of α-tochopherol. Similar studies have been reported that the antioxidant activity of natural antioxidants are involved in termination of free radical reaction and exhibit reducing power[26].

1,1-Diphenyle-2-picrylhydrazyl(DPPH) is a stable free radical has been extensively used to evaluate reducing activity of substances[27]. It is well known that antioxidants can seize the free radical chain oxidation and form stable radicals, which would not initiate or propagate further oxidation. As shown in Figure 4, water and methanol extracts of Amaranthus leaves exhibited powerful DPPH radicals scavenging activity by 74.50% and 55.1% at 100µg and 140µg respectively whereas BHA and α – tocopherol showed 92.3% and 94.1% scavenging activity at four fold higher concentration than Amaranthus. These results demonstrate that water and methanol extracts of Amaranthus leaves are comprised of multiple antioxidants, which scavenges the DPPH free radical.

Hydroxyl radicals are highly reactive which causes cell damage in-vivo. In deoxyribose assay, the rate constant was determined for hydroxyl radicals. Here, the hydroxyl radicals are generated by Fe^{3+} and they reacts with thiobarbuteric acid gives pink colour. The presence of water and methanol extracts of amaranthus reduced the pink colour by preventing the degradation of deoxyribose. The maximum radical scavenging activity was shown by both water and methanol extract. We found that maximum scavenging capacity on hydroxyl radicals (80.3%) could be achieved when the concentration in water extract of Amaranthus leaves was 60µg. Similarly, at the same concentration, methanol extracts showed maximum inhibition of 64%. The standard Antioxidants BHA and α-tocopherol exhibited 60.2 and 65% at the concentration of 120 µg. Hence it is very clear that, water extract of Amaranthus is an effective radical scavenger even compared to the standard radical scavenger antioxidants.

Iron is an essential mineral for normal physiology but excess can result in cellular injury. The good chelating effect would be beneficial and removal of free iron ion from the circulation .When iron ion is chelated, it may lose pro oxidant properties. Hence, we further tested the ferrous iron chelating activity of water and methanol extracts of Amaranthus leaves. Interestingly, as seen in the Figure-6, the water extract of Amaranthus leaves (100µg/ml) were found have highest (80.3%) ferrous ion chelating ability, while methanol extract of Amaranthus leaves (200µg/ml) showed 55.6% chelating ability. The chelating efficiency of water extract of Amaranthus was almost equal to the standard iron chelator EDTA (200µg) at 50% less concentration than the EDTA. The Synergetic activities of water and methanol soluble antioxidants of

Amaranthus leaves might account for higher chelating ability.

During oxidation process as well as by the enzymes action in the body, superoxide radicals are produced and their concentration increases during oxidative stress. In addition, superoxide anions generate other types of free radicals and damaging agents (20). Therefore NBT assay system was used to assess the superoxide scavenging activity of water and methanol extracts of amaranthus leaves. In NBT assay, xanthine oxidase generated superoxide radicals, which subsequently reduced NBT to produce blue formazon. As shown in Figure-7 water extracts of Amaranthus leaves inhibited NBT reduction by 72.5% dose dependent at 200µg, followed by Methanol extracts which showed 60.4% of NBT reduction at 300ug concentration. The NBT reduction activity of water extract of Amaranthus is higher than that of standard α-tocopherol (68%/200µg). The inhibition of NBT reduction effectively by Amaranthus leaves extracts suggests that, water extract of Amaranthus leaves is potential scavenger of super oxide anions as well as hydroxyl radicals.

Plants are rich with phenolic compounds with various biological activities with antioxidant activity[29]. Moreover phenolic constituents of green leafy vegetables contribute more to the antioxidant properties than ascorbic acid. The total phenolic content of methanol and water extracts were determined spectrophotometrically using Folin-Ciocalteu method as described in materials and methods (Table-1). As shown in the table, the water extract of Amaranthus leaves contained 158.mg/g total phenolic compounds, The total phenolic content of water and methanol extracts of amaranthus was estimated (Table-1). The results indicated the phenolic content is more in water extract (158 mg/g), which is higher than that of the methanol extracts (98 mg/g) of Amaranthus leaves. Presence of high phenolic content in water extracts correlates with the high antioxidant activity when compared to the methanol extract.

Table-1
Total content of poly phenols in Anethum sowa leaves (Values are represented in mg /g dry weight of extracts)

Extracts	Polyphenols (mg/g dry weight)
Water extract of Amaranthus piperitum	158±5.6
Methanol extract of Amaranthus piperitum	98± 7.2

Our study demonstrated that methanol and water extracts of Amaranthus leaves exhibit excellent antioxidant properties. Therefore, it is worthy to explore the potentiality of Amaranthus in checking oxidative stress mediated diseases further.

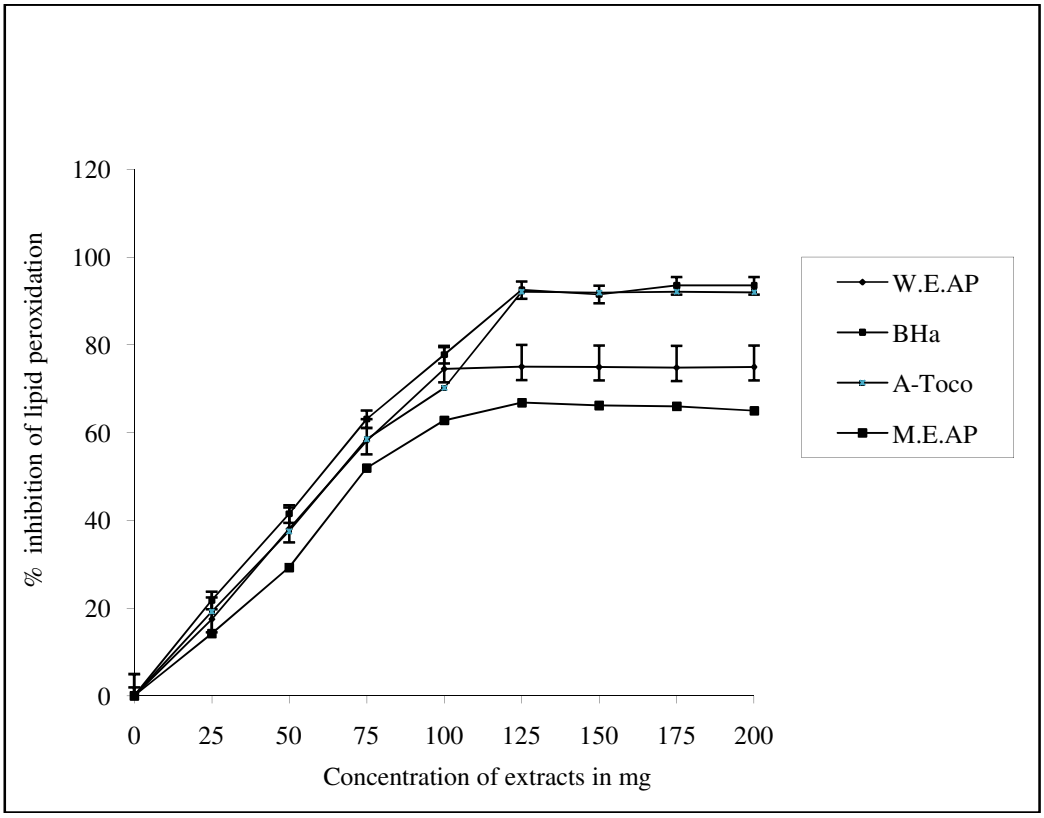

Figure-1
Inhibition of lipid peroxidation in linoleic model system (TBARS Assay)

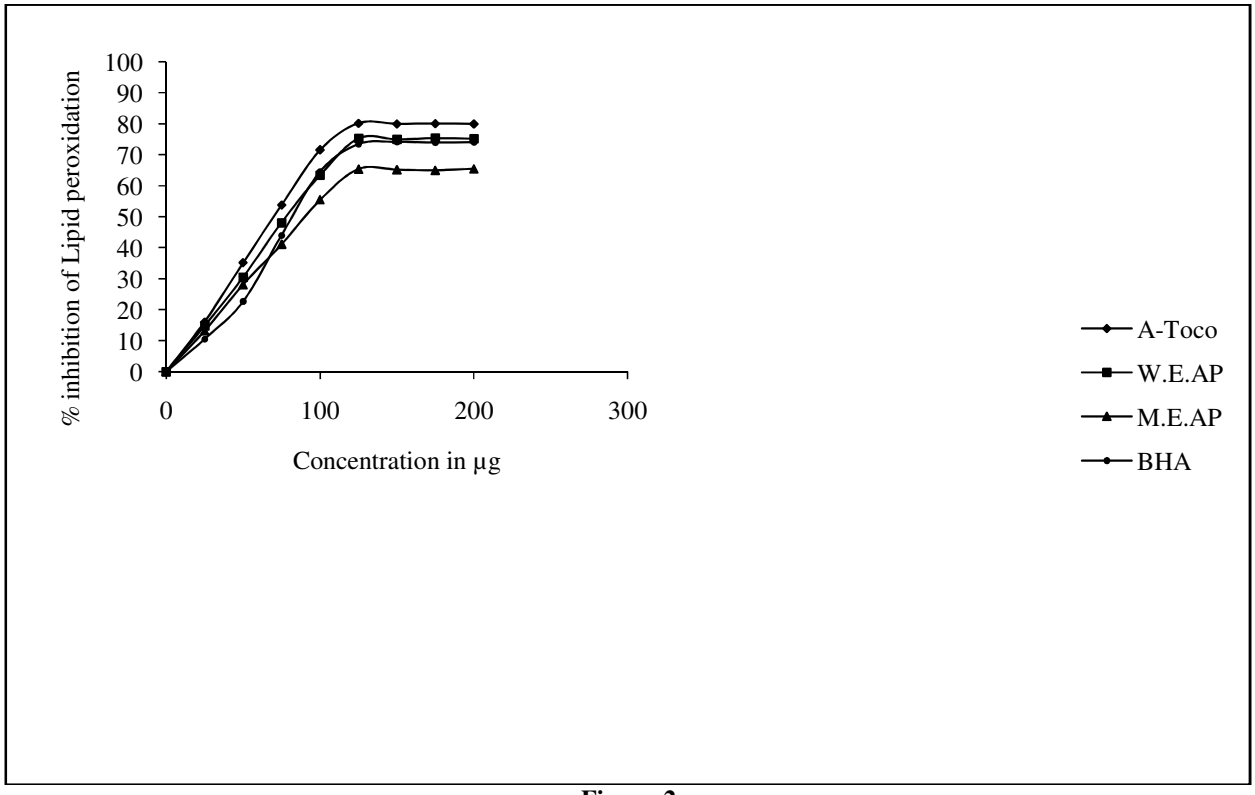

Figure-2
Inhibition of lipid peroxidation in RBC membrane model system (TBARS Assay)

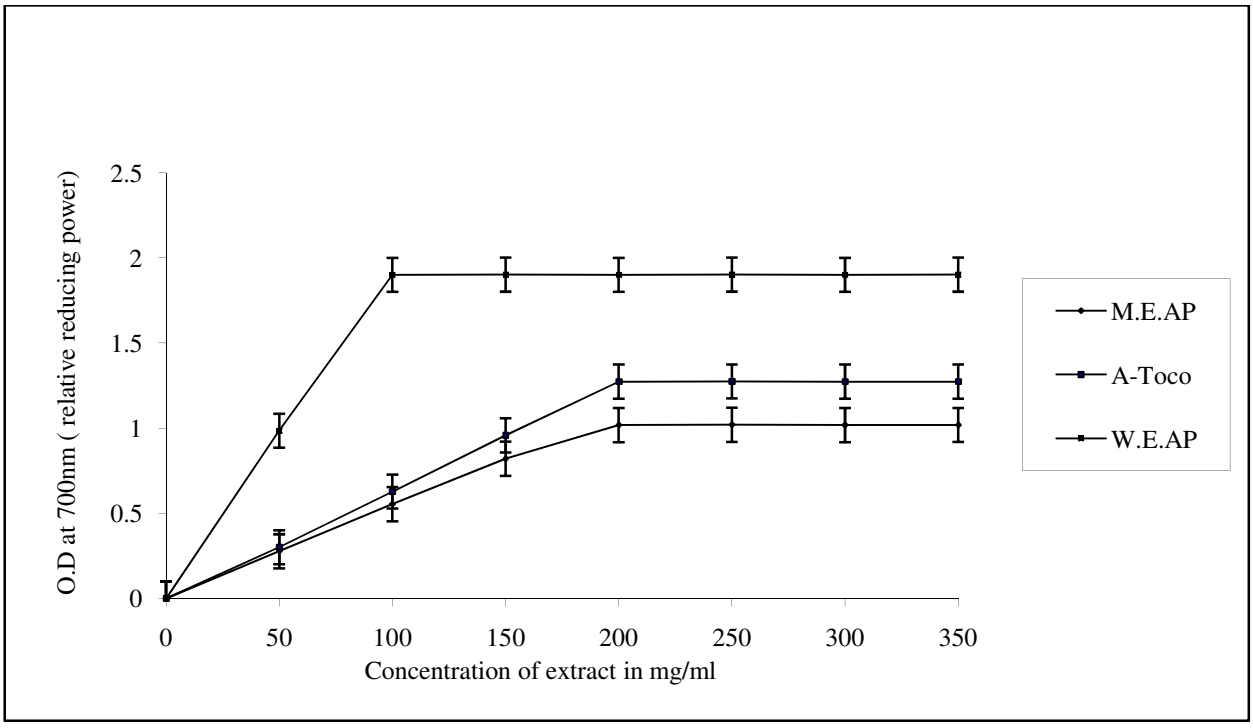

Figure-3
Reducing power of Amaranthus (dose dependent)

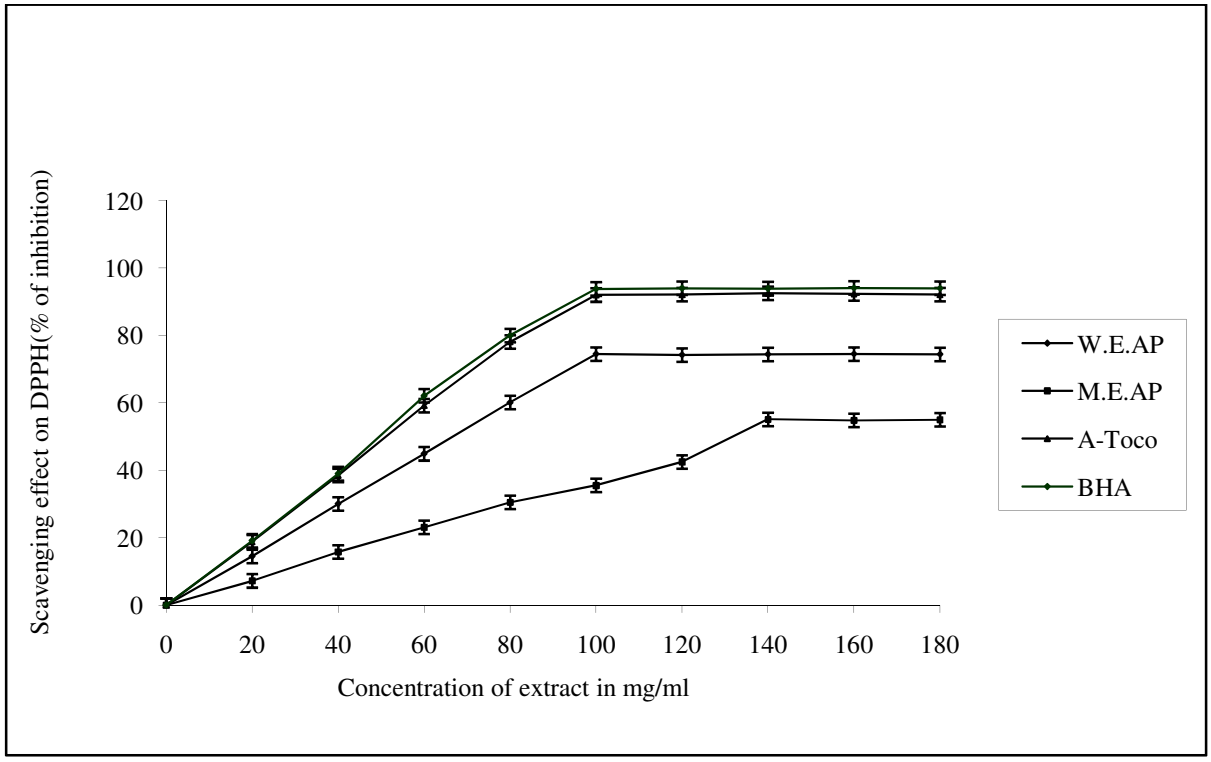

Figure-4
DPPH radical scavenging activity of Amaranthus

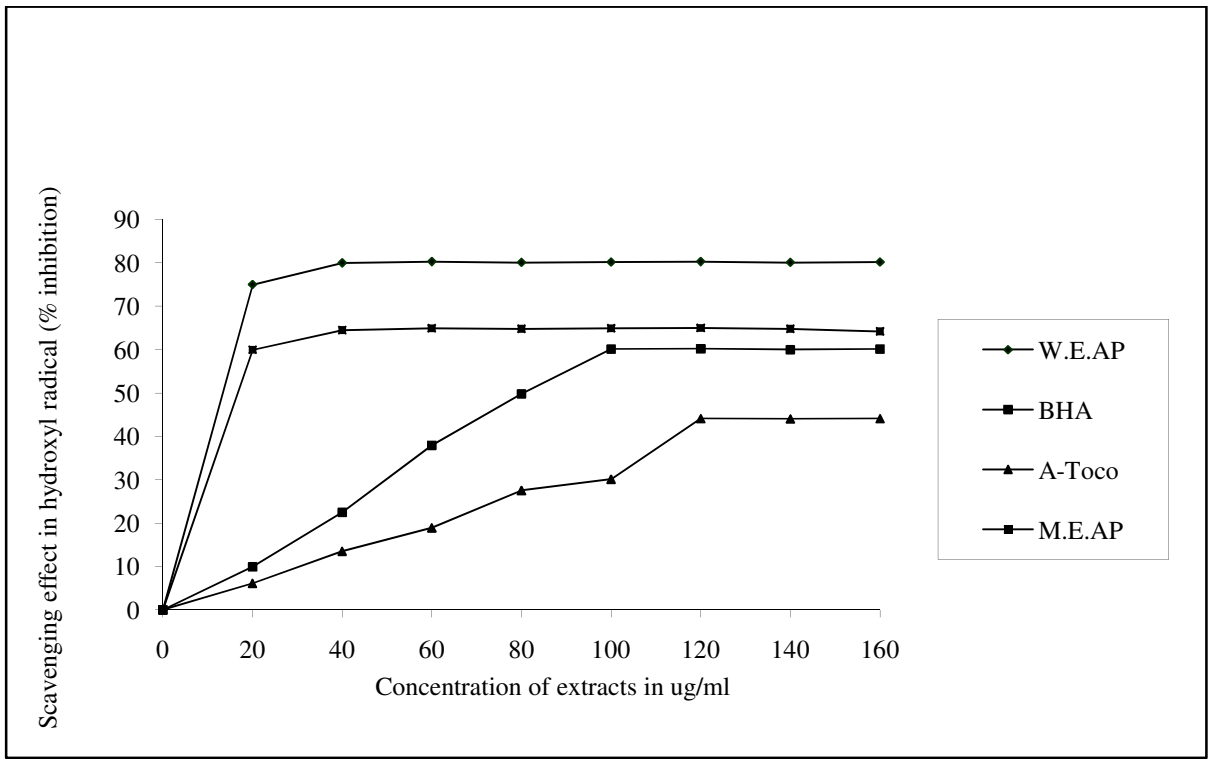

Figure-5
Hydroxyl radical scavenging activity of Amaranthus

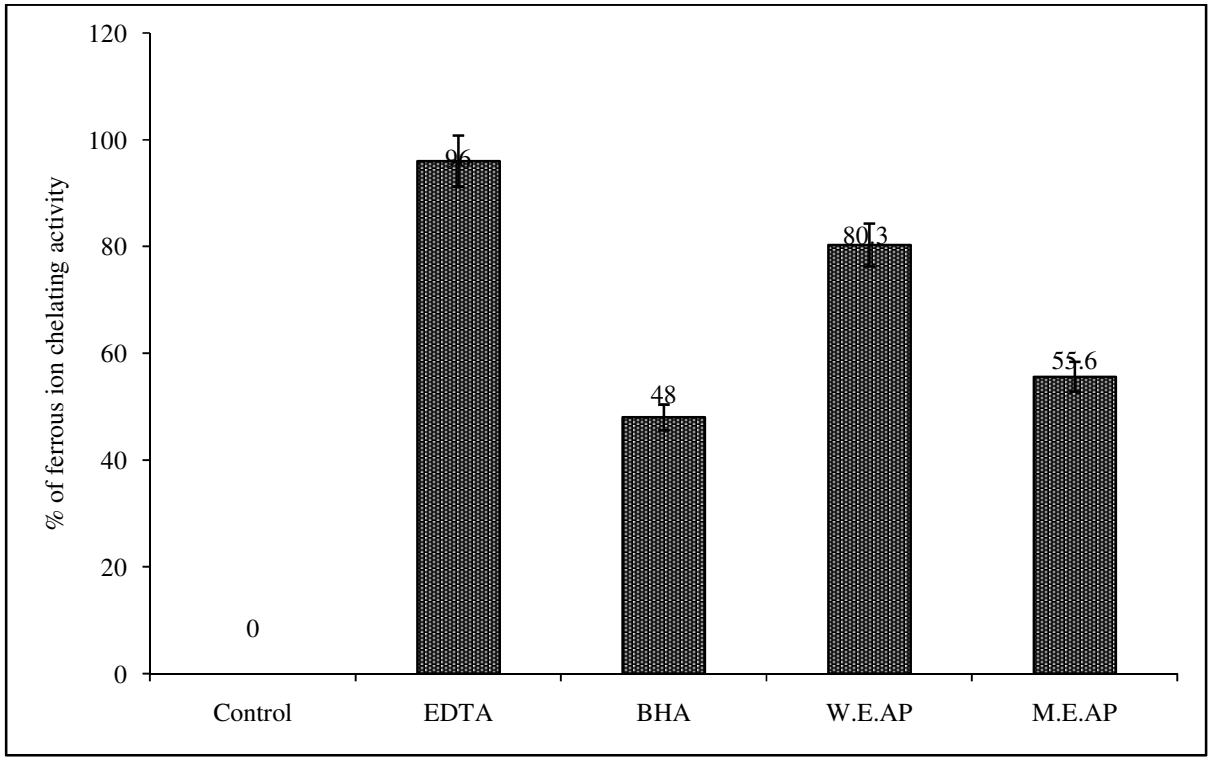

Figure-6
Ferrous ion chelating effect of Amaranthus

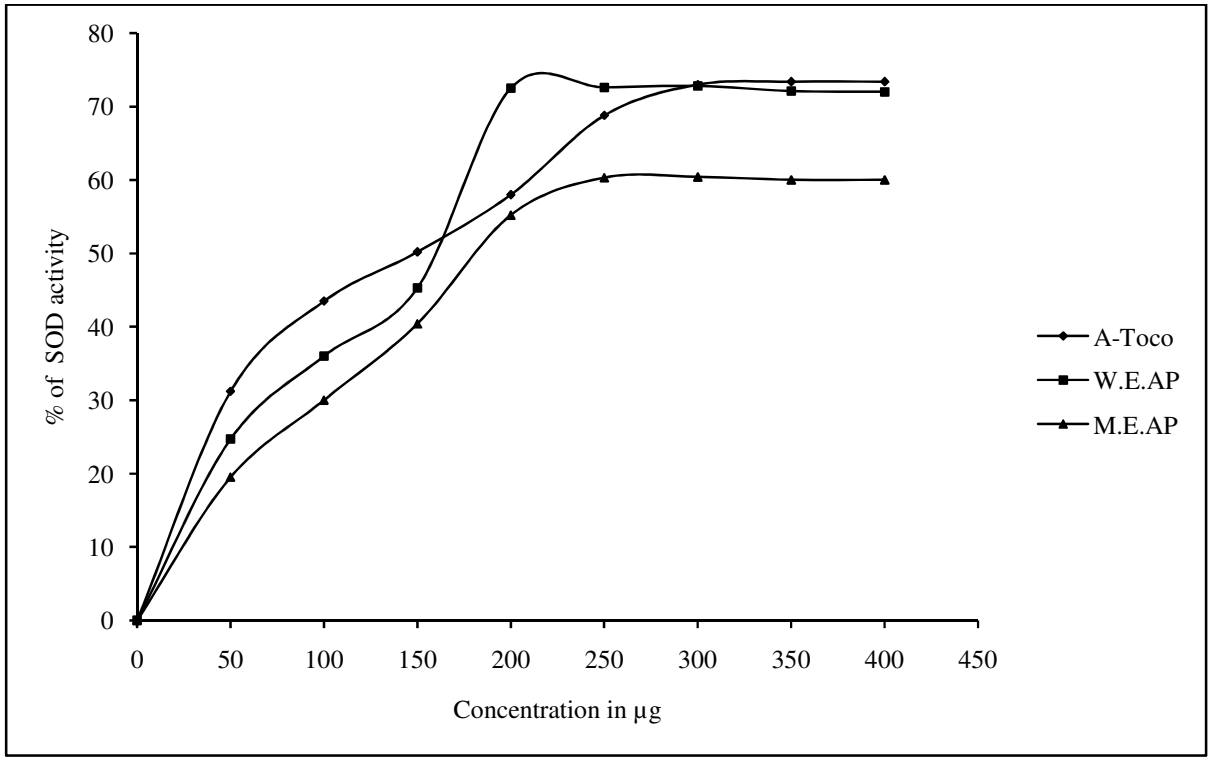

Figure-7
SOD activity of Amaranthus – Dose dependent

Conclusion

In summary, considering the results obtained it may be anticipated that Amaranthus leaves has potent antioxidant activity achieved by the scavenging abilities observed against superoxide, hydroxyl radicals, reducing power and metal chelating abilities. Regarding its phenolic composition, the protective effects observed in this study are due, most probably to the presence of phenolic compounds.

It is very clear that, if the ROS generation is beyond the level in biological system, causes oxidative stress. This stress has been implicated in several diseases such as cancer, cardio and cerebrovascular diseases, degenerative neuronal diseases. Furthermore, biochemical and clinical studies suggested that consumption of fruits and green leafy rich in polyphenolic compounds provide protection against diseases including cancers, cardio-cerebrovascular diseases, ageing and so on.

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