FREE RADICAL SCAVENGING ACTIVITY OF *AMARANTHUS BLITUM*

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Summary

Plants serve as a rich source of many novel biologically active compounds. Free radical stress leads to tissue injury and progression of disease conditions such as arthritis, hemorrhagic shock, diabetes, hepatic injury, aging and ischemia, reperfusion injury of many tissues, gastritis, tumour promotion, neurodegenerative diseases and carcinogenesis. Safer antioxidants suitable for long term use are needed to prevent or stop the progression of free radical mediated disorders. Many plants possess antioxidant ingredients that provided efficacy by additive or synergistic activities. *Amaranthus blitum* is used as a medicine against lung disorders. The antioxidant activity of ethanolic extract of *Amaranthus blitum* was assessed by using DPPH assay, FRAP assay, Reducing power assay, Superoxide, Nitricoxide, Hydroxyl, H_2O_2 radical scavenging activity and Lipid peroxidation inhibition assay. The ethanolic extract showed inhibition in a dose dependant manner and the results were expressed as IC_{50}. The antioxidant components of the natural products constitute the major source of human health promotion and maintenance.

Key words: Amaranthus blitum, Free radicals, Antioxidant activity

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Introduction

Plants produce a diverse range of bioactive molecules, making them a rich source of different types of medicines [1]. Plants have formed the basis of sophisticated traditional medicine systems that have been in existence for thousands of years and continue to provide mankind with new remedies. Medicinal plant therapy is based on the empirical findings of hundreds and possibly thousands of years of use [2].

Plants are potent biochemical factories and components of phytomedicine since times immemorial; man is able to obtain from them a wondrous assortment of industrial chemicals. Plants based natural constituents can be derived from any part of plant like bark, leaves, flowers, roots, fruits, seeds, etc (i.e.) any part of the plant may contain active components [3]. The beneficial medicinal effects of plant materials typically result from the combinations of secondary products present in the plant. The medicinal actions of plants are unique to particular plant species or groups are consistent with this concept as the combination of secondary products in a particular plant is taxonomically distinct [4].

Free radicals are fundamental to biochemical processes and represent an essential part of aerobic life and metabolism. The most common ROS include superoxide anion, hydrogen peroxide (H_2O_2), peroxyl radical (ROO^-) and reactive hydroxyl radicals (OH^-). The nitrogen derived free radicals are nitric oxide (NO_2), peroxy nitric anion ($ONOO^-$). ROS have implicated in over a hundred of disease states which range from arthritis and connective tissue disorders to carcinogenesis, aging and physical injury. In treatment of these diseases, antioxidant therapy has gained a prime position [5].

Amaranthus blitum spreads over the world from the tropics to temperate areas such as Japan and Western Europe. The cultivated type probably originates from India where, still it is an important vegetable. The main use of *Amaranthus blitum* is as a cooked leaf vegetable**.** Vegetable *Amaranthus* in general are recommended as a good food with medicinal properties for young children, lactating mothers and for patients with fever, haemorrhage, anaemia and kidney complaint. The leaves are used to treat inflammations and boils. *Amaranthus blitum* is used as a medicine against lung disorders. This plant is said to be laxative and used in hepatic disorder and enlarge spleen. The current paper deals with the antioxidant activity of ethanolic extract of *Amaranthus blitum* using various *in vitro* models.

Materials and Methods

Plant material

The leaves of *Amaranthus blitum* was used for the studies. The plant was collected in the month of January from the surroundings of Tamilnadu, India. They were identified and authenticated by a Taxonomist. The leaf was washed, shade dried, powdered and stored in air tight containers separately under refrigeration.

Extraction of the plant material

50g of powdered leaves of *Amaranthus blitum* was taken in a conical flask. To this 250ml of 95% ethanol was added. The contents of the flask were soaked overnight. This suspension was filtered and residue was resuspended in an equal volume of 95% ethanol for 48hr and filtered again. The two filtrates were pooled and the solvents were evaporated in a rotatory evaporator at temperature below 50°C and the extracts were freeze-dried. The residue was used to analyse the various *in vitro* free radical scavenging parameters.

Drugs

Chemicals used in this study were 1, 1-diphenyl-2-picrylhydrazyl (DPPH), Potassium ferricyanide, Trichloroaceticacid, Butylated Hydroxyl Anisole (BHA), Ascorbic acid, Phosphoric acid, Nitro blue tetrazolinium (NBT). All reagents used for the study were of analytical grade.

Antioxidant assay

DPPH radical scavenging assay

The free radical scavenging activity of the fractions was measured *in vitro* by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) assay [6]. About 0.3 mM solution of DPPH in100% ethanol was prepared and 1 ml of this solution was added to 3.0 ml of the fraction dissolved in ethanol at different concentrations. The mixture was shaken and allowed to stand at room temperature for 30 min and the absorbance was measured at 517nm. The percentage scavenging inhibition was determined and was compared with that of ascorbic acid which was used as the standard. The percentage inhibition was calculated by

$$Inhibition\ (\%) = \frac{(A_{control} - A_{test})}{A_{control}} x\ 100$$

Where $A_{control}$ is the absorbance of the control reaction and A_{test} is the absorbance in the presence of the sample of the extract. The antioxidant activity of the extract was expressed as IC_{50}. The IC_{50} value was defined as the concentration (in µg/ml) of extract that inhibits the formation of radicals by 50%.

FRAP (Ferric reducing antioxidant power) assay

The FRAP assay was done by the method of Pulido [7]. The assay mixture contained 2.5ml of 300mM acetate buffer at pH 3.6, 0.25ml of 10mM TPTZ solution in 40mM HCl, 0.25ml of 20mM FeCl3 and test substances in 0.1ml ethanol. The absorbance was measured after 30 min incubation at 593nm. All tests were run on triplicate and mean values were used to calculate EC_1 values. EC_1 is defined as concentration of an antioxidant having a ferric reducing ability equivalent to that of 1mM ferrous salt.

Nitric oxide radical scavenging activity

Nitric oxide was generated from sodium nitroprusside and measured by the Griess reaction [8]. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitricoxide which interacts with oxygen to produce nitrite ions that can be estimated by use of Griess reagent. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitric oxide. Nitric oxide scavenging activity was measured spectrophotometrically. Sodium nitroprusside (5 mM) in phosphate buffered saline was mixed with different concentration of the extract (20-100µg/ml) dissolved in ethanol and incubated at 25°C for 30min. A control without the test compound but with an equivalent amount of ethanol was taken. After 30 min, 1.5ml of the incubated solution was removed and diluted with 1.5ml of Griess Reagent (1% Sulphanilamide, 2% Phosphoric acid and 0.1% N-1-naphthylethylenediamine dihydrochloride). The absorbance of the chromophore formed during diazotization of the nitrite with sulphanilamide and subsequent coupling with N-1-naphtylethylenediamine dihydrochloride was measured at 546nm and percentage scavenging activity was measured with reference to standard. The percentage inhibition was calculated as in DPPH radical scavenging activity.

Superoxide radical scavenging activity

Superoxide activity of the extract was determined by McCord and Fridovich method [9] which depends on light induced superoxide generation by riboflavin and the corresponding reduction of NBT. The assay mixture contained 0.3ml of different concentrations of the extract and 0.2ml ethylene diamine tetra acetic acid (6µM containing 3µg NaCN), 0.1ml nitro blue tetrazolinium (NBT) (50µM), 0.05ml riboflavin (2µM) and 2.35ml phosphate buffer (58mM, pH 7.8) to give a total volume of 3.0ml. The tubes were uniformly illuminated with an incandescent light for 15 min and the optical density was measured at 560nm. The percentage inhibition by the extract of superoxide production was evaluated by comparing the absorbance values of control and experimental tubes. The percentage inhibition was calculated as in DPPH radical scavenging activity.

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity was measured according to the modified method of Halliwell [10]. The scavenging activity was measured by studying the competition between deoxyribose and the extract for hydroxyl radical generated from the Fe^{3+}/ascorbate/H_2O_2 system. Stock solution of EDTA (1mM), $FeCl_3$ (10mM), Ascorbic acid (1mM), H_2O_2 (10mM) and deoxyribose (10mM) were prepared in distilled deionised water.

The assay was performed by adding 0.1ml EDTA , 0.01ml of $FeCl_3$, 0.1ml of H_2O_2, 0.36ml of deoxyribose, 1.0ml of the extract (10-100µg/ml) dissolved in phosphate buffer (50mM pH 7.4) and 0.1ml of ascorbic acid in sequence. The mixture was then incubated at 37°C for 1hr. A 1.0ml portion of the incubated mixture was mixed with 1.0ml of 10%TCA and 1.0ml of 0.5% TBA to develop the pink chromogen measured at 532nm. The hydroxyl radical scavenging activity of the extract is reported as % inhibition of deoxyribose degradation.

Hydrogen peroxide scavenging activity [11]

A solution of hydrogen peroxide (40mM) was prepared in phosphate buffer (pH 7.4). Different concentrations (20- 100µg/ml) of the extract were added to a hydrogen peroxide solution (0.6ml, 40mM). Absorbance of hydrogen peroxide at 230nm was determined after 10min against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage scavenging of hydrogen peroxide of the extract and standard compounds was calculated as in DPPH radical scavenging activity.

Determination of reducing power [12]

The reducing power of the extracts was determined according to the method of Oyaizu (1986). Various concentrations of the extracts (100-500µg/ml) in 1.0ml of deionised water were mixed with phosphate buffer (2.5ml, 0.2M, pH 6.6) and 1% Potassium ferricyanide (2.5ml). The mixture was incubated at 50°C for 20min. Aliquots of trichloroaceticacid (2.5ml) was added to the mixture, which was then centrifuged at 1000 rpm for 10min. The upper layer of solution (2.5ml) was mixed with distilled water (2.5ml) and a freshly prepared $Fecl_3$ solution (0.5ml, 0.1%). The absorbance was measured at 700nm. Increased absorbance of the reaction mixture indicated increased reducing power.

Determination of lipid peroxidation inhibition activity [13]

Inhibition of lipid Peroxidation was determined by the thiobarbituric acid method. 0.1ml of different concentrations of the extract was incubated at 37°C with 25% (w/v) rat liver homogenate (0.1ml) containing Tris-HCl buffer (40mM, pH 7.0), 0.1ml KCl (30mM), 0.1ml ascorbic acid (0.06mM) and 0.1ml ferrous iron (0.16mM) in a total volume of 0.5ml for 1 hr. At the end of the incubation period , 0.4ml of the reaction mixture was treated with 0.2ml sodium dodecyl sulphate (8.1%), 1.5ml thiobarbituric acid (0.8%) and 1.5ml acetic acid (20%. pH 3.5). The total volume was then made up to 4.0ml by adding distilled water and kept in boiling water bath at 95°C for 1 hr. After the mixture had been cooled, 1.0ml distilled water and 5.0ml of butanol-pyridine mixture (15:1 v/v) were added. Following vigorous shaking, the tubes were centrifuged and absorbance of the upper layer containing the chromophore was read at 532nm. The percentage inhibition of lipid Peroxidation by the extract was determined by comparing the absorbance values of the control and experimental tubes.

Statistical analysis

Tests were carried out in triplicates. Statistical analysis was carried out using the statistical package SPSS version (10). Differences among the tested antioxidants were analysed by using one-way ANOVA. Values are expressed as the mean ± SD and differences between groups were considered to be significant if $p<0.05$.

Results and Discussion

ROS are responsible for the damage of cellular biomolecules such as proteins, enzymes, nucleic acids, lipids and carbohydrates and may adversely affect immune function [14]. Antioxidants interrupt the production of ROS and also play a key role to inactivate them [15].

1, 1-Diphenyl-2-picryl-hydrazyl (DPPH•) radical scavenging assay

DPPH is a stable free radical at room temperature and accept an electron or hydrogen radical to become stable diamagnetic molecule. The reduction capability of DPPH radicals was determined by the decrease in its absorbance at 517nm [16]. DPPH radicals react with suitable reducing agents, the electrons become paired off and the solution loses colour stochiometrically depending on the number of electrons taken up.

Table 1: Free radical scavenging activity of *Amaranthus blitum* by DPPH radical inhibition

Sample Concentration (µg/ml)	Absorbance (517nm)	
	Ascorbic acid	*Amaranthus blitum*
20	48.1±0.31[a]	47.3±0.69[a]
40	61.7±0.61[a]	58.3±0.59[a]
60	89.7±0.42[a]	72.1±0.72[a]
80	94.1±0.72[a]	80.3±0.46[a]
100	95.8±0.65[a]	92.2±0.53[a]

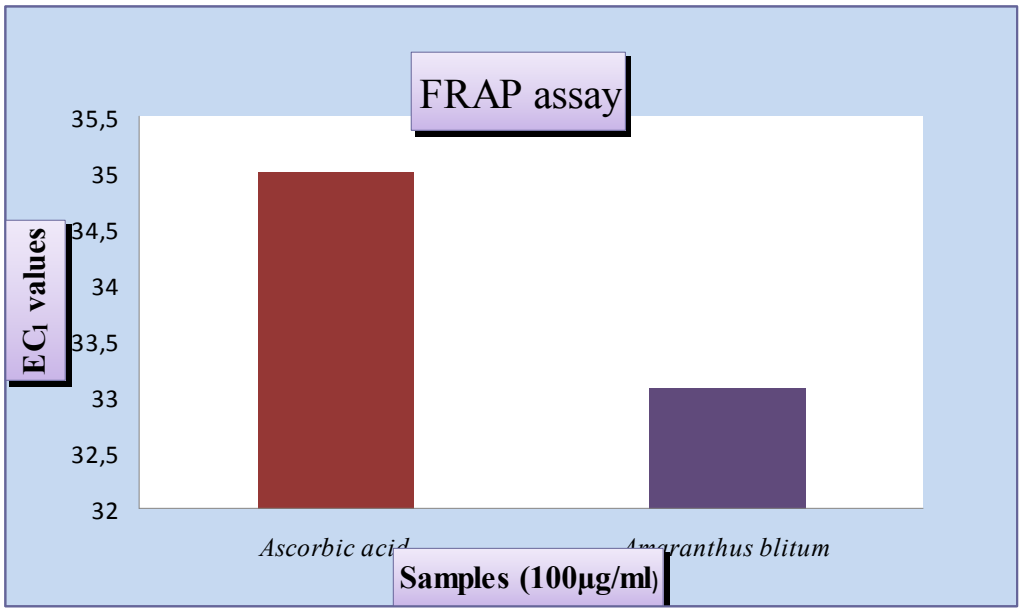
Results are expressed as mean ± SD of the three parallel measurements.
SD values followed by common superscript letter (a) are significant at 5% level when compared to control.

The IC_{50} values of *Amaranthus blitum* were found to be 37.5µg/ml when compared to the standard ascorbic acid whose IC_{50} value were found to 25.0µg/ml.

FRAP (Ferric Reducing Antioxidant Power) Assay

Ferric reducing antioxidant power (FRAP) measures the ability of antioxidants to reduce ferric 2, 4, 6-tripyridyl-s-triazine complex to intensively blue colored ferrous complex in acidic medium [17]. Hence any compound which is having redox potential lower than that of redox pair Fe (III)/Fe (II) can theoretically reduce Fe (III) to Fe (II).

Fig 1: Ferric reducing ability of *Amaranthus blitum* and standard ascorbic acid



The tests were run in triplicate and mean values were used to calculate EC_1 values. EC_1 is defined as concentration of an antioxidant having a ferric reducing ability equivalent to that of 1mM ferrous salt.

The FRAP EC_1 value of *Amaranthus blitum* was found to be 33.08µg/ml whereas the standard vitamin C was found to be 35.00 µg/ml respectively.

Superoxide radical scavenging activity

Superoxide is a highly reactive molecule that can react with many substances, produced in various metabolic processes, including phagocytosis. It causes oxidation or reduction of solutes depending on their reduction potential. Both aerobic and anaerobic organisms possess superoxide dismutase enzyme, which catalyses the breakdown of superoxide radical [18].

Table 2: Superoxide radical scavenging activity of *Amaranthus blitum* and standard ascorbic acid

Sample Concentration (µg/ml)	% of Inhibition	
	Ascorbic acid	*Amaranthus blitum*
10	34.01 ±1.05[a]	32.45 ±0.51[a]
20	47.20 ±0.06[a]	47.18 ±1.06[a]
30	65.36 ±0.09[a]	64.10 ±2.05[a]
40	82.30 ±0.10[a]	77.43 ±1.35[a]
50	93.41 ±1.62[a]	91.62 ±1.56[a]

Results are expressed as mean ± SD of the three parallel measurements.
SD values followed by common superscript letter (a) are significant at 5% level when compared to control.

The scavenging effects of the ethanolic extract on the superoxide radical of *Amaranthus blitum* were found to be 91.62%. Increasing the sample concentration range from 10 to 50µg/ml, the scavenging effect also increased in the dose dependant manner.

The IC_{50} value of ethanolic extracts of *Amaranthus blitum* were found to be 25µg/ml when compared to the standard ascorbic acid whose IC_{50} value were found to 21.5µg/ml at a concentration of 50µg/ml.

Nitric oxide radical scavenging activity

Nitric oxide (NO) is a potent pleiotropic mediator of physiological processes such as smooth muscle relaxation, neuronal signalling, inhibition of platelet aggregation and regulation of cell mediated toxicity. It is a diffusible free radical which plays major roles as an effector molecule in diverse biological systems including neuronal messenger, vasodilation, antimicrobial and antitumor activities [19].

Nitric oxide is generated from the amino acid L-arginine by vascular endothelial cells, phagocytes and certain cells in the brain. Nitric oxide reacts with superoxide and forms peroxynitrite radicals and is responsible for the inflammatory response by the release of prostaglandin. Some scientists believe that repeated infections throughout life cause an excessive production of NO, which over time, diseases such as heart disease, Alzheimer's disease and diabetes. In the present study, nitricoxide was generated from sodium nitroprusside, at physiological pH (7.4) liberates nitric acid. This nitric acid gets converted to nitrous acid and further forms nitrite ions on contact with air. The nitrite ions diazotize with sulphanilic acid and couple with naphthylethylenediamine forming pink colour complex, which was measured at 546 nm [20].

Table 3: Nitricoxide radical scavenging activity of *Amaranthus blitum* and standard ascorbic acid

Sample Concentration (µg/ml)	% of inhibition	
	Ascorbic acid	*Amaranthus blitum*
20	36.86 ±0.50[a]	34.09 ±2.31[a]
40	59.76 ±2.10[a]	56.15 ±0.50[a]
60	69.52 ±2.54[a]	65.23 ±2.80[a]
80	76.59 ±0.02[a]	74.16 ±2.87[a]
100	90.40 ±2.60[a]	89.89 ±3.03[a]

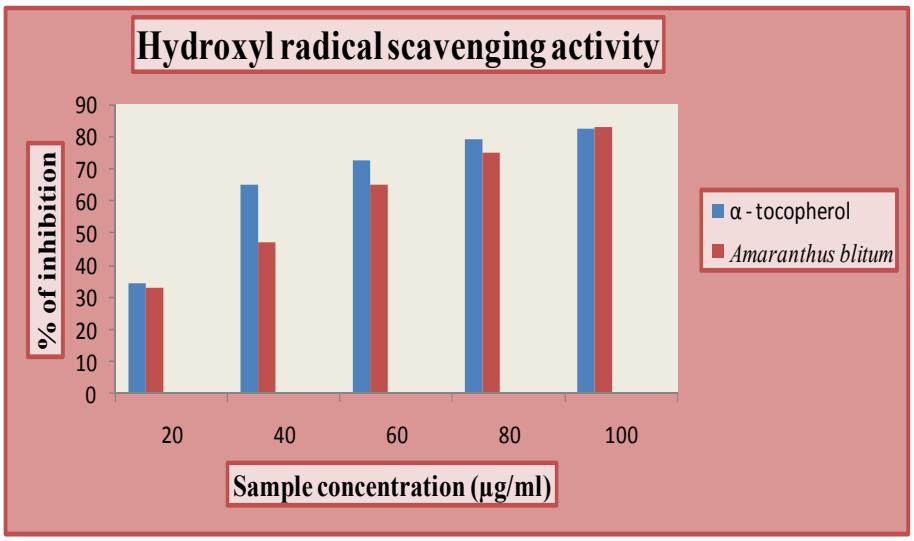
Results are expressed as mean ± SD of the three parallel measurements.
SD values followed by common superscript letter (a) are significant at 5% level when compared to control.

Amaranthus blitum (89.89%) showed nitricoxide radical scavenging activity at the concentration of 100µg/ml. The IC_{50} value of *Amaranthus blitum* was 36µg/ml whereas standard ascorbic acid showed an IC_{50} value of 32µg/ml as a positive control.

Hydroxyl radical scavenging activity

The most reactive of the ROS that attacks almost every molecule in the body is the hydroxyl radical. It initiates the peroxidation of cell membrane lipids [21] yielding malondialdehyde, which is mutagenic and carcinogenic [22]. Hydroxyl radicals are formed *in vivo* from water by high-energy irradiation or from H_2O_2 in a metal-catalyzed process [23].

Fig. 2: Hydroxyl radical scavenging activity of *Amaranthus blitum* and standard α - tocopherol.

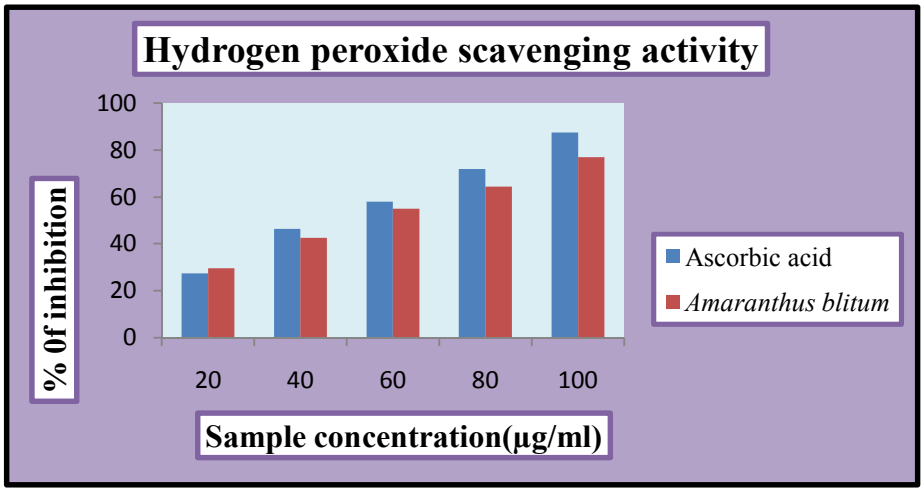


Amaranthus blitum showed inhibition with a value of 83.20% at concentration of 100µg/ml when compared to α - tocopherol with the value of 82.61%. The IC_{50} values showed an inhibition percentage of 36µg/ml for *Amaranthus blitum* at a concentration of 100µg/ml.

H_2O_2 scavenging activity assay

Hydrogen peroxide is a weak oxidizing agent and can inactivate a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. Hydrogen peroxide can cross cell membranes rapidly, once inside the cell, H_2O_2 can probably react with Fe^{2+} and possibly Cu^{2+} ions to form hydroxyl radical and this may be the origin of many of its toxic effects [24]. The decomposition of H_2O_2 by *Amaranthus blitum* may result from its antioxidant and free radical scavenging activity.

Fig. 3: Hydrogen peroxide scavenging activity of *Amaranthus blitum* and standard ascorbic acid.



The plant extract and standard ascorbic acid exhibited hydrogen peroxide in a dose dependant manner. The IC_{50} value of *Amaranthus blitum* was found to be 54µg/ml at concentration of 100µg/ml.

Lipid Peroxidation inhibition activity

In order to determine if the extract were capable of reducing *in vitro* oxidative stress, lipid peroxidation was assessed by means of an assay that determines the production of malondialdehyde and related compounds in rat liver homogenates. Malondialdehyde is one of the major degradation products of lipid peroxidation, which has been extensively studied and measured as a marker for oxidative stress [25].

Thiobarbituricacid reactive species (TBARS) are produced as by-products of lipid peroxidation that occurs in the hydrophobic core of biological membranes [26]. Therefore lipid Peroxidation has been identified as one of the basic reactions involved in oxygen free radical induced cellular damages [27] and plays an important role in the pathogenesis of several diseases.

The lipid peroxidation inhibition activity of ethanolic extract of *Amaranthus blitum* and standard ascorbic acid are illustrated in table 4.

Table 4. Lipid peroxidation inhibition activity of ethanolic extract of *Amaranthus blitum* and standard ascorbic acid.

Sample Concentration (µg/ml)	% of inhibition	
	Ascorbic acid	*Amaranthus blitum*
200	25.89 ±0.94[a]	23.28 ±1.03[a]
400	45.28 ±1.12[a]	43.72 ±1.55[a]
600	68.36 ±1.34[a]	64.48 ±2.70[a]
800	79.60 ±1.66[a]	78.90 ±4.12[a]
1000	89.12 ±1.82[a]	85.03 ±3.58[a]

Results are expressed as mean ± SD of the three parallel measurements.
SD values followed by common superscript letter (a) are significant at 5% level when compared to control.

The ethanolic extract of *Amaranthus blitum* prominently inhibited the formation of MDA and exhibited significant antilipid peroxidative effect. The percentage inhibition of lipid peroxidation of *Amaranthus blitum* is 85.03±3.58 at a concentration of 1000µg/ml were observed. The IC_{50} value of standard ascorbic acid as positive control was 420µg/ml.

Reducing power assay

The reducing ability of a compound generally depends on the presence of reductones [28] which exhibit antioxidative potential by donating a hydrogen atom for breaking the free radical chain [29]. The reducing ability of ethanolic extract of *Amaranthus blitum* is illustrated in the table 5.

Table 5: Reductive ability of *Amaranthus blitum* and standard ascorbic acid.

Sample Concentration (µg/ml)	Absorbance (700nm)	
	Ascorbic acid	*Amaranthus blitum*
10	0.15 ±0.01[a]	0.12 ±0.06[a]
20	0.30 ±0.03[a]	0.23 ±0.03[a]
30	0.45 ±0.02[a]	0.32 ±0.02[a]
40	0.60 ±0.04[a]	0.42 ±0.05[a]
50	0.75 ± 0.05[a]	0.59 ±0.04[a]

Results are expressed as mean ± SD of the three parallel measurements.
SD values followed by common superscript letter (a) are significant at 5% level when compared to control.

The ethanolic extract of *Amaranthus blitum* confirmed the presence of reductones with increase in the absorbance as the amount of the extract increases.

Conclusion

The Reactive Oxygen species or oxidants, which are formed in the human body due to exogenous and endogenous factors, are found to be responsible for many diseases. Day by day, a lot of research have shown the potential of phytochemical antioxidants as health benefactors because of their ability to neutralize free radicals, reactive oxygen species and oxidants responsible for the onset of cell damage.

The present study was undertaken to establish the free radical scavenging activity of the *Amaranthus blitum*. The ethanolic extract showed significant free radical scavenging activity when compared to other well characterized, standard antioxidant systems in vitro. However, the components responsible for antioxidant activity of *Amaranthus blitum* are currently unclear. Therefore, it is suggested that further work be performed for isolation and identification of the antioxidant components of *Amaranthus blitum*.

Interest in the search for new natural antioxidants has grown dramatically over the past years because reactive oxygen species (ROS) production and oxidative stress have been shown too linked to ageing related illnesses [30] and a large number of other illnesses.

References

1. Hennebelle T, Sahpaz HS, Joseph F, et al. Phenolics and iridoids of *Lippia alb*a. Natural Product Sciences 2006; 1:727-730.
2. Heinrich M, Barnes J, Gibbons S. Fundamentals of Pharmacognosy and Phytotheraphy, Elsevier science Ltd, 2009:107-15.
3. Zétola M, Lima TC, Sonaglio CS. Activities of liquid and spray-dried extracts from *Lippia alba* Verbenaceae (Brazilian false Melissa). Journal of Ethnopharmacology 2002; 82:207-215.
4. Naznin A, Hasan N. (2009). *In vitro* Antioxidant Activity of Methanolic Leaves and Flowers Extracts of *Lippia alba*. Research Journal of Medicine and Sciences 2009; 4(1):107-110.
5. Sangameswaran B, Balakrishnan BR, Deshiraj C, et al. *In vitro* antioxidant activity of roots of Thespesia lampas dalz and Gibs. Pak. J. Pharm. Sci. 2009; 22(4):368-372.
6. Yaushisakono A. DPPH free radical scavenging activity of medicinal plant extract. Biochemistry and Biophysics 1978; 136(1):189-195.
7. Pulido R, Bravi L, Saara-Calixto F. Antioxidant activity of dietary polyphenols as determined by a modified Ferric Reducing / Antioxidant power assay. J. Agric. Food. Chem. 2000; 48:3396-3402.
8. Govindarajan R, Rastogi S, Vijayakumar M. Bio. Pharm. Bull. 2003; 26:1424-1427.
9. McCord and Fridovich I. Superoxide dismutase: An enzymatic function for erythrocuprein. Journal of biological chemistry 1969; 244: 6049-55.
10. Halliwell B, Murcia MA, Chirico S, et al. Free radicals and antioxidants in food and in vivo: what they do and how they work. Crit. Rev. Food Sci. Nutr. 1995; 35: 7-20.
11. Ilhami GI, Haci AA, Mehmet C. Chem. Pharm. Bull. 2005; 53(3):281-285.
12. Oyaizu M. Studies on product of browning reaction prepared from glucose amine. Jpn. J. Nutr. 1986; 44:307-315.
13. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Analytical Biochem. 1979; 95:351-58.

14. Ganapathy S, Chandrasekhar VM, Chitme HR, Lakshmi Narsu M. Free radical scavenging activity of gossypin and nevadensin: An in vitro relation. Indian journal of pharmacology 2007; 39: 281-283.
15. Nilsson J, Stegmark R, Akesson B. Total antioxidant capacity in different pea (Pisum sativum) varieties after blanching and freezing. Food chem. 2004; 86:501-7.
16. Liao H, Banbury LK, Leach DN. Antioxidant activity of 45 Chinese herbs and the relationship with their TCM characteristics. eCAM 2008; 5:429-34.
17. Huang D, Ou B, Prior RL. The Chemistry behind Antioxidant Capacity Assays. J. Agric. Food Chem. 2005; 53:1841-1856.
18. Agbora R, Gupta D, Chawla R, et al. Radioprotection by plant products: Present status and future prospects. Phytotheraphy. Res. 2005; 19:1-22.
19. Hagerman AE, Riedl KM, Jones GA. High molecular weight plant polyphenolics (tannins) as biological antioxidants. J. Agric. And Food Chem. 1998; 46:1887-1892.
20. Re R, Pellegrini N, Proteggente A, et al. Antioxidant activity applying an improved ABTS radical cation decolourization assay. Free Radical Bio Med. 1999; 26:1231–7.
21. Halliwell B. Reactive oxygen species in living systems: source, biochemistry and role in human disease. Am. J. Med. 1991; 91:14 - 22.
22. Miyake T, Shibamoto T. Antioxidant activities of natural compounds found in plants. J Agric Food Chem. 1997; 45:1819- 22.
23. Cheeseman KH, Slater TF. An introduction to free radical biochemistry. Brit Med Bull. 1993; 49:481- 93.
24. Halliwell B. The role of oxygen radicals in human disease, with particular reference to the vascular system. Haemostasis 1993; 23(1):118-126.
25. Janero DR. Malondialdehyde and thiobarbituric acid reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. Free Radic Biol Med. 1990; 9:515–540.
26. Fraga C, Leibovitz B, Tappel A. Halogenated compounds as inducers of lipid peroxidation in tissue slices. Free Radical Res. 1987; 3:119–123.
27. Halliwell B. The chemistry of free radicals. Toxicology & Industrial Health 1993; 9:1-21.
28. Badmus M. Phytochemicals and *in vitro* antioxidant potentials of defatted Methanolic extract of *Holarrhena floribunda* leaves. African Journal of Biotechnology 2010; 9(3):340-346.
29. Gordon MH. The mechanism of the antioxidant action *in vitro*. In: Hudson BJF. (ed), Food Antioxidant. Elsevier, 1990; 1-18.
30. Finkel T, Holbrook NJ. Oxidants, oxidative stress and the biology of ageing. Nature 2000; 408: 224-39.