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Research Article

Antioxidant - Phytochemical Studies in *Amaranthus* varieties

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ABSTRACT

The levels of various antioxidant- phytochemical work was carried out in green leafy vegetable varieties such as *Amaranthus giganticus* L. (red) Arka suguna and *Amaranthus Palme* L. (green) RNA 1 (Family: Amaranthaceae) and *Amaranthus viridis* (Red), and the leaves posses majority of phytochemical substances that protect our cells against oxidative damage and reduces the risk of developing chronic degenerating diseases. Leafy vegetables are rich sources of natural antioxidants. The present study assessed the enzymatic, non-enzymatic and free radical scavenging activity, antioxidant activity in two different varieties of unexploited leafy vegetables (Arka suguna and RNA 1), such as levels of enzymatic antioxidants such as Catalase, Peroxidase, Polyphenol Oxidase, Glutathione Reductase and nonenzymatic antioxidants such as Total Phenols, Vitamin C, Total Carotenoids, and Flavonoids. The present study revealed possess high antioxidant activity and could be used as a dietary antioxidant.

Keywords: leafy vegetable, *in vitro* antioxidant activity, (FRAP, ABTS, DPPH) free radical scavenging activity.

INTRODUCTION

Amaranthus, known as in (English - Amaranth, Telugu – thota kura, Hindi - chavleri sag) and the varieties used for the present study is Arka suguna and RNA 1, it is an annual and short-lived perennial plants. Amaranthus leaves are a good source of protein, dietery fibres, carotenoids vitamin A, vitamin C, and folate, thiamine, niacin, and riboflavin[1] and some dietary minerals including calcium, iron, potassium, zinc, copper, and manganese[2-3].

Amaranthus helps to enhance eyesight. *Amaranthus giganticus* L. (red) and *Amaranthus Palme* L. (green) has high vitamin C content[4]. Carotenoids exhibit a central role against cancers, Cardiovascular diseases and HIV infection and other age-related disorders. Phenolic content in *Amaranthus* species have been studied by[5-8]. Generation of Reactive Oxygen Species (ROS) or free radicals during cellular metabolism and other activities beyond the antioxidant capacity of a biological system gives rise to oxidative Stress. These free radicals may oxidize nucleic acids, proteins or lipids a variety of diseases such as cancer, cardiovascular diseases, cataract, diabetes, asthma, macular degeneration, inflammatory diseases etc[9].

Oxidation of lipids by free radicals (Lipid peroxidation) is associated with various human degenerative diseases, since it alters the fluidity of biological membranes and causes cell degradation affecting the biological defense mechanism[10]. Antioxidants are compounds that inhibit or delay the oxidation of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions. Fruits and vegetables are good source of natural antioxidants.

Reactive oxygen species (ROS) are constantly formed in the human body by normal metabolic action and these are exert oxidative damaging effects by reacting with nearly every molecule found in living cells including nucleic acids, proteins, lipids or DNA and may involve in several chronic and degenerative diseases including gastritis, reperfusion injury of many tissues, atherosclerosis, ischemic heart disease, ageing, diabetes mellitus, cancer, immunosuppression, neurodegenerative diseases and others[11-13], if excess ROS and free radicals are not eliminated by endogenous antioxidant system.

Therefore, investigations of antioxidants are focused on naturally occurring substances, especially plant phytochemicals.

MATERIALS AND METHODS

Chemicals and Reagents

Ferric reducing antioxidant property(FRAP), 2,2–Diphenyl-2-picryl hydrazyl (DPPH), Folin ciocalteau, TPTZ(2,4,6-Tri(2-pyridyl)-*s*-triazine), NADP (Nicotiamide adenine dinucleotide phosphate), ABTS (2,2′-azinobis3-ethylbenzthiazoline-sulphonic acid), aluminium chloride was obtained from Sigma–Aldrich Co., St. Louis, USA. 2, 6–dichlorophenolindophenol (DCIP), methanol Folin ciocalteau, phenol, sodium carbonate, ethlenediaminetetraacetic acid disodium salt EDTA. ascorbic acid, pyragallol, metaphosphoric acid (MPA), acetic acid, potassium persulphate, acetone, sodium hydroxide. All chemicals used were of G R grade only.

Spectrophotometric Measurements

Spectrophotometric measurements were performed by UV-VIS 116 Double Beam Spectrophotometer.

Plant Material

The experimental plant materials were procured and collected from different Research Stations as mentioned below.

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Leafy Vegetables

Samples were collected from Agricultural Research Station, Rajendernagar, Hyderabad, Telangana state.

Preparation of Extracts

The fresh whole leafy vegetables were chopped and dried in shade. The dried masses were blended into fine powder by frequent sieving and powders were extracted by soxhlet process with methanol. After extraction the contents were concentrated at maintained proper conditions and dried in desiccators to get corresponding extracts. All the extracts were stored at 0 °C in airtight containers until need for further studies. The GLV were weighed separately and ground in water at a concentration of lg/5 ml. The extracts were centrifuged at 10,000 rpm for 10 minutes and the supernatants were kept under refrigerated conditions and used for biochemical estimations.

Biochemical Analysis

Assessment of Nonenzymatic Antioxidants and Phytochemicals

Non enzymatic antioxidants and phytochemicals such as Total Phenols, Ascorbic Acid, Flavonoids, Total Carotenoids were analyzed.

Total Phenol

Total phenol contents in the extracts were determined by the modified Folin ciocalteau method[14].

An aliquot of the extract was mixed with 5ml folin-ciocalteau reagent (diluted with water 1:10 v/v) and 4ml (75 g/l) of sodium carbonate.

The tubes were vortexed for 15 sec and allowed to stand for 30min at 40°C for color development. Absorbance was then measured at 765 nm using Shimadzu 160A UV-VIS double beam spectrophotometer.

Determination of Flavonols

Total flavonols in the plant extracts were estimated using the method of[15]. To 2.0 ml of the sample, 2.0 ml of 2% AICI, ethanol and 3.0ml (50g/l) sodium acetate solutions were added.

The absorption was read at 440nm by Shimadzu 160A UV-VIS double beam Spectrophotometer was read after 2.5h at 20° C.

Flavonoids

Aluminium chloride colorimetric method was used for the determination of flavonoids[16]. Each plant extracts (0.5ml) in methanol were separately mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1ml of 1M potassium acetate and 2.8 ml of distilled water.

It remained at room temperature for 30 minutes: the absorbance of the reaction mixture was measured at 415nm with a Shimadzu 160A UV-Visible double beam spectrophotometer.

Ascorbic Acid

The 10gms of the sample (leaf) were sliced, frozen into liquid nitrogen and stored at -80°C until the analyses were carried out. Frozen pulverized samples were weighed and mixed with 2.5 ml of the extract solution (3% MPA and 8% acetic acid for MPA-acetic acid extraction and 0.1% oxalic acid for oxalic acid extraction). The mixture was homogenized in a high-speed blender at 18000 g (in ice and darkness) for 1 min and then centrifuged at 9000 rpm (refrigerated at 4°C) for 20 min[17]. Ten millilitres of the sample (leaf) was titrated against standard 2, 6-dichlorophenolindophenol dye which was already standardized against standard ascorbic acid. Results were expressed in percentage.

Determination of Total Carotenoids

10g sample (fruit and leaf) were rinsed with distilled water to remove sand, cut into pieces and lyophilized to remove the moisture content. Resulting dried samples were powdered using blender. These ground samples were extracted twice with a total volume of 100 ml of 70% aqueous methanol. The mixture was shaken on an orbital shaker for 75 min at 2500rpm and then filtered through Whatman No 1 filter paper[18]. The absorbance of the filtrate was measured at λ = 453, 505, 645 and 663 nm by Shimadzu 116 A UV-VIS Spectrophotometer.

Assessment of Enzymic Antioxidants

The leaf extracts were assayed for enzymatic antioxidants such as Catalase, Peroxidase, Polyphenol Oxidase, glutathione reductase were also analyzed Catalase.

Catalase activity was estimated as per the method of[19]. The reaction mixture consists of 1ml of enzyme, 2ml of hydrogen peroxide and 3ml of 0.05M Tris-Hcl buffer (pH7.0). The reaction was stopped by 1ml of 2.5N H_2SO_4. After 5 minutes of incubation at 20°C, the residual H_2O_2 was titrated with 0.01 $KMNO_4$. A blank was prepared by adding 1ml of 2.5N H_2SO_4 initially to the reaction mixture at zero time.

Peroxidase

Peroxidase activity was estimated as per the method of[20]. The reaction mixture consisted of 2ml of Tris–HCl buffer 0.1 M (pH 7.0), 1ml of pyrogallol (0.01M), 1ml of H_2O_2 (0.05M) and 1ml enzyme, 1ml of 0.05 M H_2O_2 and 1ml of enzyme extract. The reaction mixture was incubated at 25°C for 5 minutes. The reaction was stopped by adding 1ml of 2.5N H_2SO_4. The amount of purpurogallin formed was estimated by measuring the absorbance at 425nm in Shimadzu 160A UV-Visible double beam spectrophotometer.

Polyphenol Oxidase

Polyphenol oxidase was estimated as per the method of[21]. The reaction mixture consists of 2ml of Tris–HCl buffer 0.1M (pH7.0), 1ml of pyrogallol (0.01M) and 1ml of enzyme extract. The assay mixture was incubated for 5minutes at 25°C. The reaction was stopped by adding

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1ml of 2.5 N H_2SO_4. The absorbance at 425nm was recorded using Shimadzu 160A UV-visible double beam spectrophotometer. Enzyme Activity was expressed in absorbance units per gram.

Glutathione Reductase

Glutathione reductase activity was determined according to the method[22]. 0.2 ml of sample, 1.5ml of 0.3 M phosphate buffer, pH 6.8. 0.5ml of 25 mM EDTA, 0.2 ml of 12.5 mM oxidized glutathione and 0.1 ml of 3 mM NADPH was added.

Decrease in absorbance was measured against that of blank at 340 nm.

Assessment of Free Radical Scavenging Activity

Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP reagent was prepared from sodium acetate buffer (300 mM, and pH 3.6), 10 mM TPTZ Solution (40 mM HCl as a solvent) and 20 mM iron (Fe^{+3})[23] chloride solution in a volume ratio of 10:1:1, respectively.

The FRAP Reagent was prepared freshly and warmed to 37°C in a water bath before use. One hundred µL of the diluted sample was added to 3 ml of the FRAP reagent. The absorbance of the reaction mixture was then detected at 593nm by using Shimadzu 160 A UV-VIS double beam Spectrophotometer.

Determination ABTS Radical Scavenging Activity

The ABTS cation radical scavenging activity of the extracts was determined according to the modified method of[24].

A stock solution of ABTS was produced by mixing 7 mM aqueous solution of ABTS with potassium per sulfate (2.45 mM) in the dark at ambient temperature for 12–16 h before use, where initial reading is absorbance at 0 min and final reading is absorbance at 6 min.

Determination of DPPH Radical Scavenging Activity

The DPPH radical scavenging activity was estimated by measuring the decrease in the absorbance of methanolic solution of DPPH[25].

In brief, to 5 mL DPPH solution (3.3 mg of DPPH in 100 mL methanol), 1mL of each plant extracts were added, incubated for 30 min in the dark and the absorbance (A1) was read at 517 nm.

Table 1: Antioxidant non-enzymatic content in *Amaranthus* varieties

S. No	Leafy Vegetable varieties	Phenols mg/g dry wt	Flavonols mg/g dry wt	Flavonoids mg/g dry wt	Ascorbic acid in percentage
1	Amaranthus (RNA1)	9.5 ± 0.3	10 ± 0.34	69.6 ± 0.07	0.35 ± 0.004
2	Amaranthus (Arkasuguna)	10 ± 0.34	10.2 ± 0.05	66 ± 0.07	0.4 ± 0.004

Each value in the table is represented as mean ± SE (n=3) of triplicates, statistically significant ($p<0.05$) and are expressed in mg/g dry

Table 2: Total chlorophyll, total carotenoid, β carotene content in *Amaranthus* varieties

S. No	Leafy Vegetable varieties	Total chlorophylls mg/g Fr wt	Total carotenoids mg/g Fr wt	β carotene content in mg/g Fr wt.
1	Amaranthus (RNA 1)	5.65 ± 0.05	2 ± 0.004	3 ± 0.005
2	Amaranthus (Arka suguna)	6.2 ± 0.003	1.9 ± 0.005	3.4 ± 0.008

Each value in the table is represented as mean ± SE (n=3) of triplicates, statistically significant ($p<0.05$) and are expressed in mg/g dry

Table 3: Antioxidant enzymatic activity in Amaranthus varieties

S. No	Leafy Vegetable varieties	Catalase u/g Fr wt	Peroxidase u/g Fr wt	Polyphenol oxidase u/g Fr wt	Glutathione reductase u/g Fr wt
1	Amaranthus (RNA1)	0.91 ± 0.003	2.42 ± 0.005	0.6 ± 0.06	0.09 ± 0.003
2	Amaranthus (Arka suguna)	0.8 ± 0.002	2.11 ± 0.04	0.008 ± 0.003	0.02 ± 0.005

Each value in the table is represented as mean ± SE (n=3) of triplicates, statistically significant ($p<0.05$) and are expressed in mg/g dry

Table 4: Antioxidant activity by FRAP, ABTS and DPPH assay in *Amaranthus* varieties

S. No	Leafy Vegetable varieties	FRAP percentage	ABTS Percentage	DPPH percentage
1	Amaranthus (RNA 1)	35 ± 0.231	3 ± 0.03	5.7 ± 0.005
2	Amaranthus (Arka suguna)	30 ± 0.289	2.3 ± 0.005	4.5 ± 0.005

Each value in the table is represented as mean ±SE (n=3) of triplicates, statistically significant ($p<0.05$) and are expressed in mg/g dry. (Ferric reducing antioxidant property (FRAP), 2,2–Diphenyl-2-picryl hydrazyl (DPPH), ABTS (2,2'-azinobis3-ethylbenzthiazoline-sulphonic acid).

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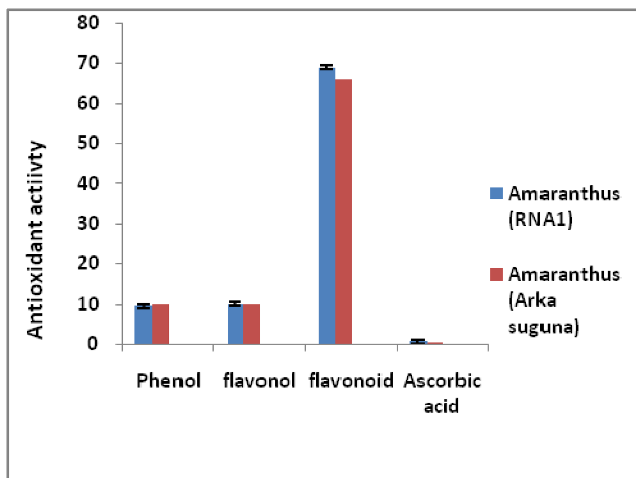

Figure 1: Showing antioxidant activity in *Amaranthus* Varieties.

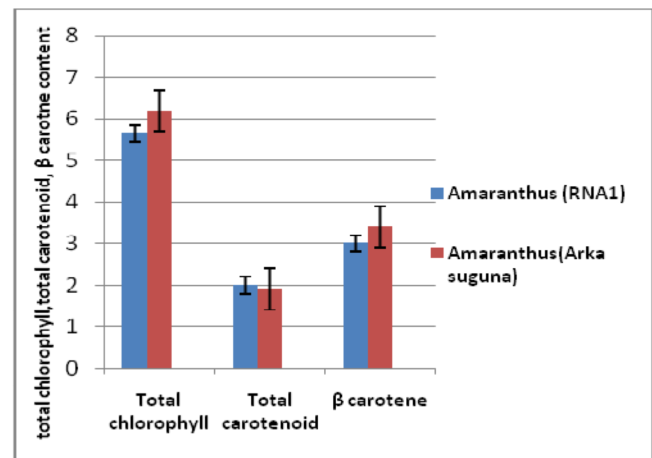

Figure 2: Showing total chlorophyll, total carotenoid, carotene content in *Amaranthus* varieties

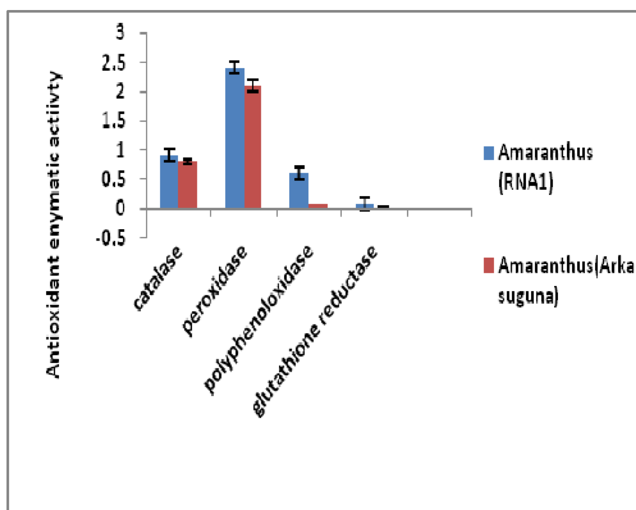

Figure 3: Showing the antioxidant enzymatic content in *Amaranthus* varieties

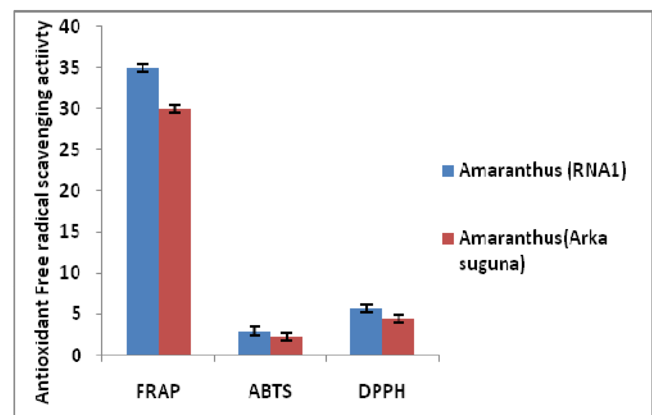

Figure 4: Showing Free radical scavenging antioxidant activity by FRAP, ABTS, DPPH assays (Ferric reducing antioxidant property (FRAP), 2,2–Diphenyl-2-picryl hydrazyl (DPPH), ABTS (2,2′-azinobis3-ethylbenzthiazoline-sulphonic acid)

RESULTS AND DISCUSSION

The non antioxidant activity of phenol content was more in arka suguna10 ± 0.34 mg/g dry wt, similar studies were done[26], the values are significant ($p<0.05$) flavonol content was more in Amaranthus arka suguna 10.2 ± 0.05, flavonoid content was more in Amaranthus RNA 1. 69.6 ± 0.07 mg/g dry wt, similar studies on flavonoids content was done[27], were ascorbic acid content was more in arka suguna 0.4 ± 0.004% *Basella alba* recorded highest maximum ascorbic content 150 mg/g studies[28]. (Table 1, Figure 1)

Total chlorophyll content was more in Arka suguna 6.2 ± 0.003 mg/g Fr wt, the chlorophyll content was 1.12 mg/g in *Amaranthus tritis*, similar results were obtained by[29-30], followed by total carotenoid content was more in RNA 1, 2 ± 0.004. Carotenoid exhibits a central role against cancers, cardiovascular diseases and HIV infection and other age-related disorders[31], followed by beta carotene content which was more in Arka suguna 3.4 ± 0.008[32], reported higher concentration of β-carotene in spinach and lettuce, which are green leafy vegetables (Table 2, Figure 2).

Catalase enzymatic activity was more in Amaranthus RNA 1 variety 0.91 ± 0.003 u/g Fr wt and the peroxidase activity was more in Amaranthus RNA 1 variety 2.42 ± 0.005 u/g Fr wt. Enzyme activity studies was done by enzymatic studies[33-34], followed by polyphenol oxidase activity was more in amaranthus RNA 1 variety 0.6 ± 0.06 u/g Fr wt, followed by glutathione reductase activity which was more in Amaranthus RNA 1 variety 0.09±0.003 u/g Fr wt [35-36]. (Table 3, Figure 3).

Free radical scavenging activity was more in RNA 1, 35 ± 0.231 followed by ABTS 3 ± 0.03 followed by 5.7 ± 0.005[37]. (Table 4, Figure 4)

Statistical Analysis

All results are expressed as mean ± Standard deviation. All results are means of three replicates. The data were correlated using Pearson correlation coefficient at $p<0.05$. Correlations among data obtained were

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calculated using Pearson's correlation coefficient (*r*) and P<0.05 was considered significantly different; SPSS 15 Version was used for the statistical analysis.

CONCLUSION

The present experiments were designed to find out the important antioxidants, which are useful as a strategy of preventing and protecting the human populations from various stress, nutrient and age related diseases and disorders. Nature has gifted us with rich varieties and diversities of fruits which had to be carefully and judiciously used in a proper way for the benefit of human which are easily available and affordable and easy for the benefit of human welfare by implementing for curing and treating diseases.

It has been suggested that it is an wonderful drug plant for radical scavenging activity and the phenols contribute maximum antioxidant activity with rich polyphenol content. Strong antioxidant capacity scavenges the free radicals from oxidative stress leading to various diseases. Thus, Amaranthus leaves are cheap and accessible source of antioxidants for medicinal and commercial purposes for the better health.

Thus Amranthus RNA-1 was showing the maximum antioxidant activity to scavenge the free radicals against the reactive oxygen species molecules and thereby can overcome many diseases such as stress, blindness, anaemia, age related diseases as a dietary antioxidant.

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