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Rich Foliar Antioxidant Based Phytonutrient Potential of a Grain Amaranth (*Amaranthus hypochondriacus* L.): RP-HPLC Based Evidences

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Abstract: The antioxidant potential of the leaf tissue of a promising accession of a seed amaranth, *Amaranthus hypochondriacus* (Accession no. IC47434) was evaluated in terms of metal chelating activity, anti-lipid peroxidation, and reducing properties, availability of flavonoids and phenolic acids. Selective RP-HPLC for some important health promoting phenolic acids and flavonoids of the lead accession demonstrated the significant availability of gallic acid, caffeic acid, syringic acid, p-coumaric acid, ferullic acid, rutin, kaempferol, elagic acid. The present study reports antioxidant-based multifunctional nutritional and pharmacological promise of leaf tissue of an established seed amaranth.

Keywords: *Amaranathus hypochondriacus*, Antioxdant Potential, Anti-lipid Peroxidation, RP-HPLC Analysis

1. Introduction

Oxidative stress or loss of ability to maintain redox homeostasis is one of the major causative factors in induction of degenerative diseases including atherosclerosis, ischemic heart disease, ageing, diabetes mellitus, cancer, immune-suppression, neurodegenerative diseases and others [1-5]. Antioxidant therapy has been gaining importance for the effective treatment of those degenerative diseases and health problems [6].

Studies conducted on health effect of polyphenols (flavonoids and phenolic acids) exhibited their potential role in combating degenerative diseases like cancer, atherosclerosis, diabetes etc [4, 5]. In fact, majority of the degenerative diseases or disorders are linked directly or indirectly to oxidative stress or loss of redox homeostasis of the cell due to over accumulation of Pro- oxidants. The oxidative damage caused by ROS ($0^{\cdot-}$, H_2O_2, OH, $ROO^{\cdot}$, $^1O_2$ etc) can trigger various chronic degenerative disease (Finkel 2002). Antioxidants can either interfere with the production of ROS and / or also by triggering oxidations detoxify oxyfree radicals. They can also chelate catalytic metals responsible for generation of ROS [3, 4]. Polyphenols, particularly flavonoids have the ability to scavange O_2 and OH and can also chelate transition metal ions necessary for the generation of ROS.

Amaranthus hypochondriacus is essentially recognized as a grain crop for their outstanding nutritional and functional attributes associated with their seeds [7]. However, no detailed data were published about antioxidant potential of leaf tissue (both quantitative and qualitative) of unexplored germplasms of *Amaranthus hypochondriacus*.

In fact, the characterization of germplasm-specific availability of antioxidant and associated properties of *Amaranthus hypochondriacus* is also the subject of interest. Therefore, in this investigation an effort have been made to access the antioxidant potential of the leaf extracts of a promising accessions of *A hypochondriacus* (Accession No IC47434) supplied from NBPGR, New Delhi, through analysis of antioxidant based markers (in term ofmetal chelating activity, anti-lipid peroxidation, superoxide radical scavenging and reducing properties). The RP-HPLC study was also conducted to identify and quantify the presence of eighteen pharmacognosically important flavonoids and phenolic acids experimental accessions of *A*

hypochondriacus.

2. Materials and Methods

Seeds of the experimental accession of *Amaranthus hypochondriacus* L. (IC47434) were collected from National Bureau of Plant Genome Research (NBPGR), New Delhi, India, and were cultivated in Crop Research and seed Multiplication Farm, University of Burdwan, West Bengal, India, based on Standard Procedure.

2.1. RP- HPLC Study

2.1.1. Sample Preparation for HPLC Study

For HPLC seperation, the mature leaf tissue (six weeks old) of two experimental accessions of *A. Hypochondriacus,* grown at Crop Research and Seed Multiplication Farm, The University of Burdwan, Burdwan, West Bengal, India were collected and washed thoroughly with normal tap water followed by sterile distilled water. Then leaves were dried at 45°C for 48 hours in hot air oven. Leaves were crushed to powder using mixer grinder. Powder was stored in tight air container bottle. The dried leaf tissue was extracted with methanol. The supernatant were stored in refrigerator for their future use for the following phytochemical analysis. The filtrate collected was concentrated by in a rotary vacuum evaporator. 20 µl of solution was taken for HPLC study.

2.1.2. RP -HPLC Analysis of Phenolic Acids and Flavonoids

HPLC analyses were performed using Dionex Ultimate 3000 liquid chromatograph including a diode array detector (DAD) with 5 cm flow cell and with Chromeleon system manager as data processor. Separation was achieved by a reversed-phase Acclaim C18 column (5 micron particle size, 250 x 4.6 mm). All extracted solutions were filtered through HPLC filter 0.45 mm membrane filter (Milipore).20 μL of sample was introduced into the HPLC column.

The mobile phase contains methanol (Solvent A) and 0.5% aq. acetic acid solution (Solvent B) and the column was thermostatically controlled at 25°C and the injection volume was kept at 20 µl. A gradient elution was performed by varying the proportion of solvent A to solvent B. The mobile phase composition back to initial condition (solvent A: solvent B: 10: 90) in 101 min and allowed to run for another 4 min, before the injection of another sample. Total analysis time per sample was 105 min. HPLC chromatograms were detected using a photo diode array UV detector at three different wavelengths (272, 280 and 310 nm) according to absorption maxima of analysed compounds. For the preparation of standard stock solutions of twenty one phenolic acids and flavonoids like Gallic acid Protocatechuic acid, Gentisic acid, p-Hydroxy benzoic acid, Catechin, Chlorogenic acid, Vanillic acid, Caffeic acid, Syringic acid, p-Coumaric acid, Ferullic acid, Sinapic acid, Salicylic acid, Naringin, Rutin, Ellagic acid, Myricetin, Quercetin, Naringenin, Apigenin and Kaempferol were prepared in methanol at 10 µg ml^{-1}. All standard solutions were filtered through HPLC filter 0.45 mm membrane filter (Milipore).

2.2. Metal Chelating Property & Reducing Property

Metal chelating property: For the estimation of metal chelating property of experimental plant tissue, the process of Lin et al. (2009) (8) was followed with slight modifications. Shortly, 1 ml water extract (extraction procedure described earlier) was added to a solution of .02 ml 2 mM ferrous chloride and .04 ml 5 Mm ferrozine. The mixture was vigorously shaken and incubated for 10 mins. Absorbance was taken at 562 nm. Metal chelating activity was expressed as:

Activity (%): [Ac- As / Ac] x 100 where Ac= Absorbance of control, As= Absorbance of sample

Reducing power: For the estimation of reducing power of experimental plant tissue the process of Lin et al (2009) (8) was followed with slight modifications shortly, 1 g of dry powder was extracted with 50 ml of distilled water at 70°C under reflux for 4 hours and then centrifuged for 3000 rpm for 10 mins. 25 ml of supernatant was taken and added with 200 Mm sodium- phosphate buffer (pH 6.6) and .1% potassium ferricyanide. The mixture was incubated for 20 mins at 50°C and then added with .25ml 10% TCA. Subsequently the mixture was centrifuged at 3000 rpm for 10 mins. Supernatant was collected and mixed with deionised water and 1% ferric chloride solution. The mixture was kept for 10 mins and absorbance was taken at 700 nm. Reducing power was expressed as activity (%).

Activity (%): [Ac- As/ Ac] x 100

Where, Ac= Absorbance of control, As= Absorbance of sample respectively.

2.3. Anti-lipid Peroxidation Assay in Linoleic Acid System

For the estimation of anti-lipid peroxidation assay experimental plant tissue the process of Amabye (2015) [9] was followed with slight modifications. The antilipid peroxidation activity of the different accessions of Amaranthus hypochondriacus extracts were determined by measuring the oxidation of linoleic acid. 5 mg of Amaranthus spinosus extracts were added separately to a solution of linoleic acid (0.13 mL), 99.8% ethanol (10 mL) and 10 mL of 0.2 M Sodium Phosphate buffer (pH=7). The mixture was made up to 25 mL with distilled water and incubated at 40°C up to 360 hours. Extent of oxidation was measured by peroxide value applying thiocyanate method. Briefly, 10 mL of ethanol (75% v/v), 0.2 mL of an aqueous solution of Ammonium thiocyanate (30% w/v), 0.2 mL of sample solution and 0.2 mL of ferrous chloride ($FeCl_2$) solution (20 mM in 3.5% HC1; v/v) added sequentially. After 3 min of stirring, the absorption was measured at 500 nm using a spectrophotometer (U-2001, Hitachi Instruments Inc. and Tokyo, Japan). A negative control contained all reagents with exception of extracts. Synthetic antioxidants Butylated Hydroxytoluene (BHT) (also we can use ascorbic acid) was

used as positive control. The maximum per oxidation level was observed at 360 h (15 days) in the sample that possesses no antioxidant component percent inhibition of linoleic acid oxidation was calculated with the following equation: % inhibition of Linoleic acid peroxidation: [1- Change in absorbance of treated sample / Change in absorbance of Control sample] x 100.

2.4. Flavonoids

Total flavonoid content was determined as per the method described by Chang et al. (2002) [10]. Different solvent extracts of leaf were separately mixed with 0.1 ml of 10% aluminium chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. The mixture was kept at room temperature for 30 minutes and the absorbance was recorded at 415 nm with the help of UV Visible spectrophotometer. The calibration curve was prepared by preparing quercetin solutions in methanol.

2.5. Total Phenols

Total phenols were determined by Folin Ciocalteu reagent following the procedure of (McDonald et al. 2001) [11]. Different solvent extracts (diluted) of each plant or gallic acid (standard phenolic compound) was mixed with Folin Ciocalteu reagent. The reaction mixtures were allowed to stand for 15 min and the absorbance was taken by UV-VIS spectrophotometer at 765 nm. The standard curve was prepared using 0, 50, 100, 150, 200, 250 mg L^{-1} solutions of gallic acid in methanol: water (50:50, v/v). Total phenol values are expressed in terms of gallic acid equivalent (mg g^{-1} of dry mass), which is a common reference compound.

3. Result & Discussion

The RP-HPLC procedure exploited in the present work provided excellent identification and qualification of 19 phenolic acids and flavonoids in leaves of the experimental accession of the seed amaranth, *Amaranthushypochondriacus*. The study confirm the presence of rich availability of phenolic acids like Gallic acid, Protocatechuic acid, Gentisic acid, p-Hydroxy benzoic acid, Chlorogenicacid, Caffeic acid, Syringic acid, p-Coumaric acid, Ferullic acid, Sinapicacid, Ellagic acid and flavonoids like Catechin, Naringin, Rutin, Myricetin, Quercetin, Naringenin, Apigenin, Kaempferol (Figure 1). The present study therefore has a potential application widely to identify flavonoids and phenolic acids in experimental accessions of *Amaranthus hypochondriacus* [12] [13].

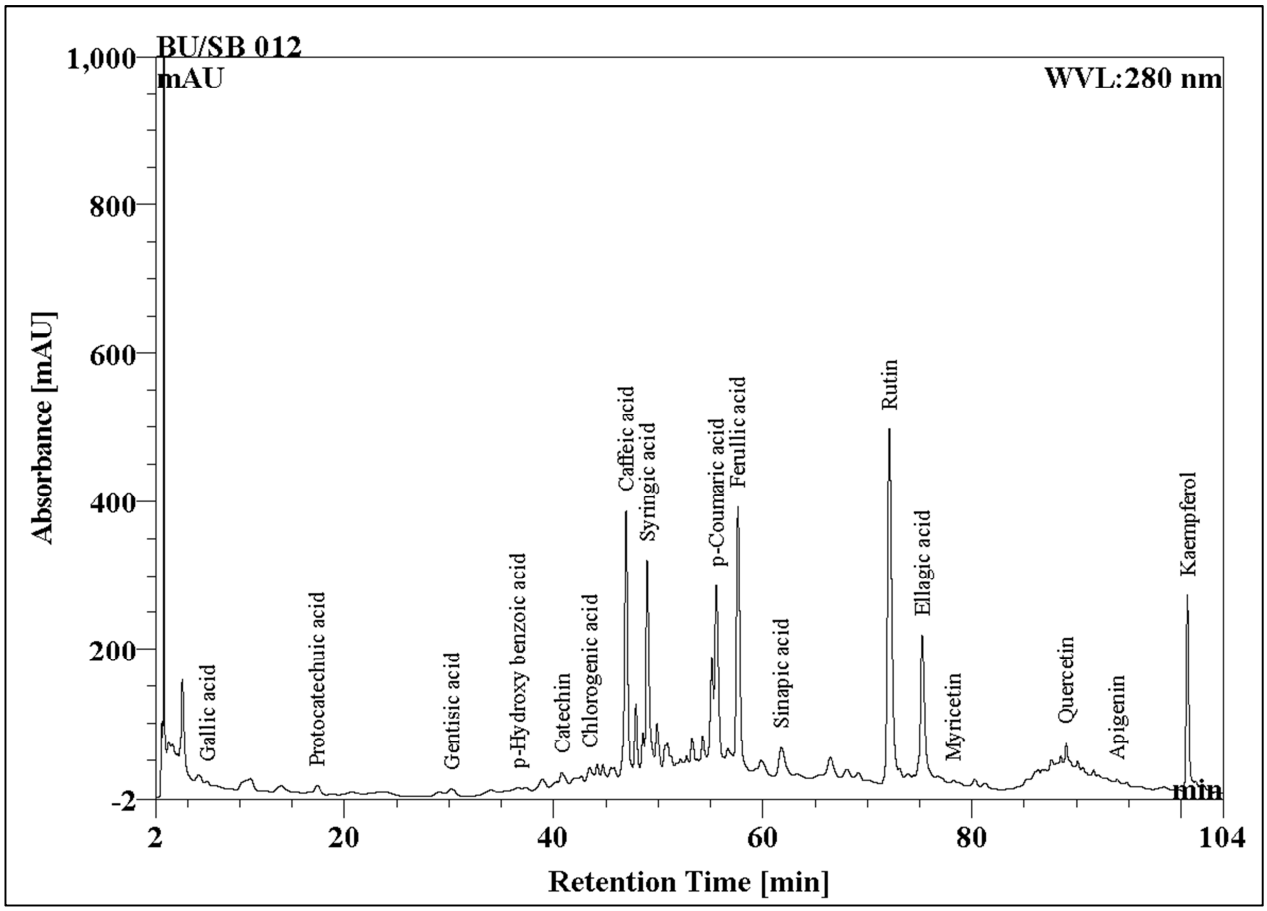

Figure 1. *RP-HPLC Chromatogram of separated phenolic acids and flavonoids of hydromethanolic leaf extracts of Amarathushypochondriacus L, Accession no. IC47434.*

Table 1. *Validation data of separated phenolic acids and flavonoids of hydromethanolic leaf extracts of Amarathushypochondriacus L, Accession no. IC47434.*

No.	Ret. Time min	Peak Name	Height mAU	Area mAU*min	Rel. Area %	Amount µg/ml	Type
1	6.81	Gallic acid	3.394	1.095	0.15	1.579	bMB
2	17.10	Protocatechuic acid	11.203	8.375	1.12	17.518	BMB
3	29.88	Gentisic acid	5.342	5.009	0.67	86.108	MB
4	36.12	p-Hydroxy benzoic acid	8.006	11.272	1.50	17.923	MB
5	40.39	Catechin	6.385	4.122	0.55	17.823	bMB
6	44.02	Chlorogenic acid	20.009	6.984	0.93	18.916	M
7	46.76	Caffeic acid	157.860	55.531	7.41	75.567	BMb
8	48.92	Syringic acid	162.663	51.613	6.88	60.822	M
9	55.58	p-Coumaric acid	188.277	67.488	9.00	39.410	M
10	57.60	Ferullic acid	234.720	91.181	12.16	71.842	MB
11	61.90	Sinapic acid	4.486	4.710	0.63	1.036	BMb
12	70.87	Naringin	3.785	2.623	0.35	4.737	BMB
13	72.22	Rutin	180.387	78.776	10.51	191.265	BM
14	75.86	Ellagic acid	6.087	2.968	0.40	5.701	bM
15	76.79	Myricetin	5.790	4.224	0.56	6.740	MB
16	88.90	Quercetin	13.253	3.122	0.42	5.525	MB
17	93.77	Apigenin	3.723	0.988	0.13	1.588	BMB
18	100.51	Kaempferol	237.581	69.466	9.27	89.735	BM

The methanol leaf extract of both the experimental accession of *Amaranthus hypochondriacus* and its solvent extract exhibited significant levels of antioxidant accumulation like total phenol and flavonoids and exhibit important marker antioxidant activities as well (Table 1). In fact, the methanol fraction showed significantly high amount of phenolics and flavonoids which might be subsequently responsible for better metal chelating and anti-lipid peroxidation properties. This is in agreement with similar reports [14, 15].

Table 2. *Antioxidant potential (assessed in terms of Anti-lipid peroxidation property, Reducing power, Metal chelating activity, total phenol and flavonoid content) of foliar hydro-ethanolic extracts of an experimental accession of Amaranthus hypochondriacus L. (Accession no. IC47434). Results are mean of three replicates ± standard error.*

Accession No. of Amaranthus hypochondriacus L.	Anti-lipid peroxidation (% inhibition g^{-1}dm)	Reducing Power (% g^{-1}dm)	Metal chelating activity (% g^{-1}dm)	Flavonoids (mg g^{-1} d.m.)	Total Phenol (mg g^{-1} d.m.)
IC47434	482.20±0.32	27.04±0.06	286.81±0.60	10.20± 0.16	18.19 ± 0.19

Phenolic compounds generally act as antioxidants through radical scavenging in which they disrupt the free radical chain reaction (by H atom donation), resulting phenoxy radical, which subsequently got reduced enzymatically or non-enzymatically into parent compounds [16]. Phenolic compounds may also cause metal chelation and reduce the availability of metals required for generation of toxic ROS like $OH^{\cdot}$ [17], thereby can provide necessary components for radical scavenging properties and well corroborate with the present data of accession specific variations of antioxidant availability [18-20]. So, it can be assumed that discrepancies of the phenolic contents (total polyphenol, flavonoids) together with antlilipid peroxidation property, reducing property and metal chelating property may contribute important role in better total antioxidant capacity of experimental accession of *Amaranthus hypochondriacus*. It can also be possible that better radical scavenging property of leaf extract of the experimental accession may be be due to –OH group present in phenolic compounds [20-22].

4. Conclusion

The foliar extracts with property of bioavailabilty of phenolic acids and flavonoids of *Amaranthus hypochondriacus* L. (Accession no. IC47434) can be recommended for their use as natural source of antioxidants and as an alternative anti-infective agent in the treatment of degenerative diseases. Moreover, due to entirely multifaceted nature of phytochemicals, it is not judicious to use a solitary method for assessing antioxidant potential of plant extract, we have used different biomarkers like, metal chelating activity, and anti-lipid peroxidation property and reducing property to validate the nature of leaf extracts from the experimental accessions of *A. hypochondriacus* in terms of desired antioxidant potential. Based on that, our result unequivocally identified a red accession IC47434 as a promising crop with neutraceutical value.

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