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Original Article

Bioactive Polyphenolic Compounds and *In Vitro* Anti-degenerative Property-based Pharmacological Propensities of Some Promising Germplasms of *Amaranthus hypochondriacus* L.

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Abstract

Background and objectives: Amaranth is conventionally consumed as a significant source of nutrients and bioactive compounds and is a potential alternate crop. The present study aimed to validate the folklore and ethnomedicinal claims regarding the utilization of foliar tissues of the pseudocereal *Amaranthus hypochondriacus* L. for their pharmacological propensities, primarily focusing on bioactive polyphenolic compounds and associated anti-degenerative properties, in view of the scarce evidence available on the same.

Methods: Reverse-phase high-performance liquid chromatography coupled with a photodiode array assay of nineteen significant bioactive polyphenolic compounds, along with their *in vitro* antioxidant-based pharmacological properties (superoxide and hydroxyl radical scavenging properties, metal-chelating and reducing properties, radical scavenging properties, anti-lipid peroxidation and protein coagulation properties, and α-glucosidase and α-amylase inhibitory activities), were assessed and compared for foliar extracts of ten promising experimental accessions of *Amaranthus hypochondriacus*, grown in two different seasons (summer and winter).

Results: The results exhibited germplasm-specific variations in the pharmacological potential of foliar tissues of the experimental amaranths, which can be substantiated by data showing a close correlation between the abundance of bioactive polyphenolic compounds (naringin, myricetin, naringenin, apigenin, rutin, catechin, quercetin) and *in vitro* antioxidant (2,2-diphenyl-1-picrylhydrazyl, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) assay, hydroxyl radical scavenging, reducing, and metal-chelating) properties, as well as anti-diabetic (inhibition of α-glucosidase and α-amylase activities) and anti-inflammatory (anti-lipid peroxidation) attributes. Accessions IC107144 and IC47434 stood out as the most promising medicinal crops based on overall *in vitro* anti-degenerative properties and the bioavailability of polyphenolic compounds.

Conclusions: Overall, the results validated the traditional ethnomedicinal claim regarding the utilization of foliar tissues of the underutilized pseudocereal *Amaranthus hypochondriacus* L., and identified lead germplasms (IC107144 and IC47434) as low-cost natural sources of bioactive compounds, potentially promoting their pharmacological utilization.

Keywords: *Amaranthus hypochondriacus*; Bioactive polyphenolic compounds; Nutraceuticals; Reverse-phase high-performance liquid chromatography; Therapeutic propensity.

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Introduction

Vegetable-derived polyphenolic compounds have recently drawn attention in the field of natural product chemistry due to their involvement in the prevention of degenerative diseases and several

health-related issues.[1–3] Chemically, polyphenolic compounds are classified according to their structures associated with aglycones. The major categories include phenolic acids (hydroxycinnamic acids, hydroxybenzoic acids), flavonoids (flavanols, flavonols, flavanones, flavones), polyphenolic amides (avenanthramides, capsaicinoids), and other polyphenols (tannins, coumarins).[4,5] In general, plants make these polyphenolic compounds using phenylalanine and tyrosine during secondary metabolism. Flavonoids are mostly made from the chalcone synthase-dependent phenylpropanoid pathway, whereas the cinnamate-dependent pathway contributes major phenolic acids.[5,6] The amino acid tyrosine can also be converted into p-coumaric acid using tyrosine ammonia lyase.

Dietary polyphenolic compounds with low digestibility have been found to exhibit a strong impact on gut health through their interaction with gut microbiota. Gut microbiota facilitates the metabolism of non-digestible polyphenols and converts them into bioactive, bioavailable metabolites, thereby revealing great phytonutrient potential.[7–9] Apart from this, dietary polyphenolic compounds can also have a profound impact on the gut microbial population, particularly in promoting the growth of beneficial bacteria.[10,11] Moreover, several works vouch for the bioactive functional food properties of polyphenolic compounds, particularly in relation to antioxidant, anti-inflammatory, antidiabetic, and anticancer properties.[12,13]

Amaranth research over the last few decades has established *Amaranthus hypochondriacus* as a pseudocereal with its seeds having extremely high phytonutrient promise due to their polyphenolic compound content.[14,15] In the tropical and subtropical highlands of South America and Asia, *Amaranthus hypochondriacus* is one of the most important auxiliary food crops used as a pseudocereal, forage crop, and green leafy vegetable. In fact, the use of amaranths as a possible alternative crop has gained a lot of interest in the last 20 years due to their exceptionally high nutrient content and the availability of bioactive molecules, primarily polyphenolic compounds.[14,15] According to certain research, amaranth seeds contain a variety of polyphenolic component groups.[14] Research has also shown that several other amaranth plant parts are significant sources of these bioactive polyphenolic compounds.[16,17] Domesticated more than 4,000 years ago, amaranth is highly valued for its many uses as a source of proteins, minerals, vitamins, phenolics, carotenoids, betalains, fibers, nitrogen-containing pigments, vital amino acids, and more.[18,19] According to review of the literature, amaranth extracts (seed and leaf), especially those from *Amaranthus hypochondriacus*, have a wide range of applications in the treatment of degenerative disorders and chronic illnesses. Additionally, amaranth's traditional uses in treating various chronic illnesses are supported by folklore-based phytochemical claims.[20] Numerous studies have been conducted to support the chemistry behind the beneficial effects of polyphenols derived from *Amaranthus* species, focusing on their anti-inflammatory and antioxidant properties.[14] Other studies also report anticancer,[21,22] antinociceptive,[23] antimicrobial,[24,25] antilipidemic,[26] hepatoprotective,[27,28] and antidiarrheal properties.[29,30]

Furthermore, investigating the antioxidant properties of bioactive natural substances, particularly polyphenolic compounds, is becoming increasingly important in the treatment of such diseases through anti-degenerative therapy.[14] Indeed, it has been demonstrated that antioxidants are highly beneficial in treating numerous infectious diseases, degenerative disorders, and other health problems. Furthermore, the recent research trend toward identifying plant-based natural antioxidants without side effects has intensified due to the banning of several synthetic antioxidants—such as butylated hydroxyanisole, butylated hydroxytoluene, and tert-butylhydroquinone—because of their adverse effects.

Several flavonoids, phenolic acids, glycosides, and other compounds have been identified in *Amaranthus* species, with some researchers reporting high antioxidant and anti-carcinogenic activity. In addition to these bioactive compounds, grain amaranths also offer high-quality proteins, fatty acids, fibers, and vitamins. This places amaranth among the few multipurpose crops that provide both grains and leafy vegetables of excellent nutritional value, making it a widely recognized subsidiary food crop.[18,19] To our knowledge, scant data are available on the profiling of polyphenolic compounds of *Amaranthus hypochondriacus* leaf as compared to seed, though the information on polyphenolic compound profiles is essential to assess the relevance of *Amaranthus hypochondriacus* as a vegetable crop and a potential source of dietary bioactive compounds.

Although rarely observed, the variations in bioactive polyphenol profiles and their content based on plant accessions, varieties or species, plant parts, and growing seasons warrant further study for their prudent use as inexpensive dietary supplements. Though many studies on the genetic variability of crops have exhibited interrelationships with bioactive phytonutrients, similar studies on the foliar tissues of *Amaranthus hypochondriacus* are very rare, despite wide genotype diversity.[31,32] Therefore, in the context of understanding the significance of efficient utilization of plant genetic resources for nutritional and pharmacological purposes, and given the inadequate information on the systematic utilization of underutilized plants, along with the need to understand genotype influence on the accumulation of bioactive polyphenolic compounds, the current work was undertaken to assess and compare the bioactive polyphenolic compounds and *in vitro* anti-degenerative property-based pharmacological propensities from foliar tissues of some promising germplasms of the pseudocereal *Amaranthus hypochondriacus* L. Furthermore, *in vitro* antioxidant, anti-inflammatory, and antidiabetic properties were also assessed and compared to authenticate the claim of utilizing this established pseudocereal as a medicinal crop and a cheap natural source of bioactive compounds.

Materials and methods

Seeds of all ten accessions of experimental *Amaranthus hypochondriacus L.* (accession numbers EC42352, IC47434, IC94661, IC95251, IC95316, IC95322, IC95326, IC107144, EC146543, and IC42397) were collected from the National Bureau of Plant Genetic Resources, New Delhi, India. These seeds were sown in the Crop Research and Seed Multiplication Farm, University of Burdwan, India, in two different seasons (summer and winter). The crops were cultivated with a unit plot size area of 1 m^2, with spacing between plants and rows maintained at 5 cm and 20 cm, respectively. Appropriate cultural practices with randomized organic fertilizers and compost doses were maintained throughout the cultivation. Thinning, weeding, and hoeing were performed at the appropriate stages of cultivation. Irrigation was provided at six- to eight-day intervals. Leaves of the mature plants (eight weeks old) were harvested for two consecutive years of cultivation during winter and summer. Leaves were dried at 45°C for 48 h in a hot air oven and crushed to powder for storage in an airtight container for further chemical investigations.

Foliar extraction of polyphenolic compounds

Fifteen grams of dried and pulverized mature leaf tissue of each

accession were extracted with 70% ethanol in a Soxhlet apparatus, maintaining a temperature of 60°C ± 2°C, and repeated for five cycles. The extract was collected and concentrated in a rotary vacuum evaporator (Eyela). All extracted solutions were filtered through a 0.45 µm high-performance liquid chromatography (HPLC) membrane filter (Millipore). A 20 µL sample was injected into the HPLC column.

Reverse-phase high-performance liquid chromatography (RP-HPLC) analysis

HPLC analyses were performed using Dionex Ultimate 3000 liquid chromatography, including a diode array detector with a 5 cm flow cell and Chromeleon system manager as the data processor. Separation was completed using a reversed-phase Acclaim C-18 column (5 µm particle size, 250 × 4.6 mm) following the procedure of Kar and Bhattacharjee.[33] Standard stock solutions of twenty-one phenolic acids and flavonoids—gallic acid, chlorogenic acid, vanillic acid, caffeic acid, protocatechuic acid, gentisic acid, p-hydroxybenzoic acid, catechin, sinapic acid, salicylic acid, syringic acid, p-coumaric acid, ferulic acid, ellagic acid, myricetin, naringin, rutin, quercetin, naringenin, apigenin, and kaempferol—were prepared in methanol at 10 µg/mL. All standard solutions were filtered through a 0.45 µm HPLC membrane filter (Millipore).[34]

Estimation of total phenols, monophenols, diphenols, and flavonoids

Two milliliters of Folin–Ciocalteu reagent and 2 mL of saturated $NaHCO_3$ were added to the extract solutions for the estimation of total phenols. After incubating the reaction mixture at room temperature, spectrophotometric absorbance (Shimadzu ultraviolet-visible (UV-VIS) spectrophotometer, model 1900i) was measured at 765 nm. The total phenolic content in different plant parts was evaluated using the standard curve of gallic acid and expressed as mg gallic acid equivalents/g dm (Djeridane *et al*., 2006).[35]

To prepare the assay mixture for monophenols, 0.1 mL of plant extract, 0.1 mL of 4-aminoantipyrine, 1 mL of saturated $NaHCO_3$, and 1 mL of potassium ferrocyanide were mixed. After 45 m of incubation at 37°C, spectrophotometric absorbance (Shimadzu UV-VIS spectrophotometer, model 1900i) was measured at 520 nm. The concentration was calculated based on the standard curve of phenol and expressed as mg PE/g dm (Keshavkant *et al*., 2008).[36]

For the estimation of diphenols, 1 mL of 1 N NaOH solution, 1.5 mL of Arnow's reagent, and 0.1 mL of plant extract were mixed. The assay mixture was then incubated at room temperature for 45 m. The absorbance was measured at 525 nm using a Shimadzu UV-VIS spectrophotometer (model 1900i). Catechol was used for the preparation of the standard curve, and the content was expressed as mg catechin equivalents/g dm.[36]

For the estimation of flavonoids, 0.1 mL of experimental sample was mixed with 0.1 mL of $AlCl_3$ solution and 0.1 mL of CH_3COOK solution, and the volume was made up to 3 mL with distilled water. The spectrophotometric absorbance (Shimadzu UV-VIS spectrophotometer, model 1900i) was measured at 415 nm. The total flavonoid content in different plant parts was evaluated using the standard curve of quercetin and expressed as mg QE/g dm (Chang *et al*., 2002).[37]

Extraction and estimation of antioxidative pigments

The process of Ali *et al*.[38] was followed for the extraction and estimation of β-cyanin. For β-cyanin extraction, 200 mg of leaf disc (without veins) was weighed and homogenized in 5 mL of 80% aqueous methanol containing 50 mM ascorbic acid. The sample was then centrifuged at 14,000 g for 10 m at 4°C. The absorbance of the supernatant was measured at 540 nm using a Shimadzu UV-VIS spectrophotometer (model 1900i). The pigment content was estimated using a molar extinction coefficient value of 62×10^6 mol^{-1} cm^{-1}.

For the extraction and estimation of carotenoids, the absorbance of the supernatant from the ethanolic extract (96% aqueous ethanol) was measured at wavelengths of 470, 537, 647, and 663 nm, respectively, using a Shimadzu UV-VIS spectrophotometer (model 1900i). The concentrations (µmol g^{-1} dry mass) of the pigments were calculated using the equations of Lichtenthaler and Wellburn.[39]

Assessment of total antioxidant capacity (TAC)

The DPPH (2,2-diphenyl-1-picryl-hydrazyl) assay was conducted using the process of Shyu *et al*.[40] To estimate the radical scavenging activity, 1 mL hydro-methanolic extract of seedling was mixed with 3 mL DPPH (0.04 mg mL^{-1} ethanol) and incubated for 30 m at room temperature, after which absorbance was measured at 517 nm. The scavenging activity of radicals by DPPH was assessed using the formula:

$$\% \text{ inhibition} = \frac{A_b - A_s}{A_b} \times 100$$

where A_b = absorbance of the blank sample; A_s = absorbance of the sample containing plant part extract. Finally, the DPPH radical scavenging property was expressed as % inhibition per dry mass.

The process of Re *et al*.[41] was followed for the determination of TAC using 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). The addition of potassium persulfate to the ABTS stock solution resulted in the formation of $ABTS^+$ radicals, which were maintained in the dark at room temperature for 12–16 h. The stock solution was diluted with ethanol to achieve an absorbance of 0.7 ± 0.02 at 734 nm. After that, 0.3 mL of plant extract was mixed with the ABTS solution, stirred, and the spectrophotometric value was measured after 30 s. The Trolox equivalent antioxidant capacity per gram dry mass was determined using the standard curve.

For the estimation of the metal-chelating property of experimental leaf tissue to assay the metal chelation activity, 1 mL of plant extract was mixed with 0.02 mL of ferrous chloride (20 mM) and 0.04 mL of ferrozine (5 mM). After shaking the solution for 10 m at 25°C, the absorbance at 562 nm was measured. The result was estimated using the following formula: The activity (%) is calculated by dividing the absorbance of the blank sample by the absorbance of the sample containing plant part extract, multiplied by 100.

For the estimation of the reducing power of experimental plant tissue, the process of Lin *et al*.[42] was followed. To estimate reducing power, the experimental plant extract (1 mL) was combined with 2.5 mL potassium ferrocyanide and 2.5 mL sodium phosphate buffer and incubated. Then, 2.5 mL of 10% TCA was added and centrifuged. To measure absorbance at 700 nm, 0.5 mL of ferric chloride solution was added to the collected supernatant. The result was calculated using the formula: The activity (%) is calculated by dividing the absorbance of the blank sample by the absorbance of the sample containing plant part extract, multiplied by 100.

The method of Jan *et al*.[43] was followed for the estimation of hydroxyl radical scavenging capacity. The methanolic extract was diluted with potassium phosphate buffer (10 mM, pH 7.2); ascorbic acid, hydrogen peroxide, 2-deoxy-ribose, and $FeCl_3$ were added after 1 h of incubation at 37°C. After boiling the mixture for 15 m, the spectrophotometric value was taken at 532 nm, and the

inhibition percentage was calculated.

Anti-lipid peroxidation property (ALPP) in the linoleic acid system

For the estimation of ALPP of the experimental leaf tissue, the process of Amabye was followed with minor modifications.[44] The ALPP of different accessions of *Amaranthus hypochondriacus* L. leaf extracts was determined by measuring the oxidation of linoleic acid. Five milligrams of different solvent 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 for up to 360 h. The extent of oxidation was measured by peroxide value using the thiocyanate method. Synthetic antioxidants (butylated hydroxytoluene or ascorbic acid) were used as positive controls. The maximum peroxidation level was observed at 360 h (15 days) in the sample that possessed no antioxidant component. The percentage 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)] × 100.

Assessment of anti-diabetic property (in terms of inhibition of α-amylase and α-glucosidase activity)

The process of Wong *et al*.[45] was followed with minor modifications for the extraction and estimation of α-amylase inhibition activity of the experimental plant tissue. The extract of the plant sample was mixed with 0.3 mL of 0.02 M sodium phosphate buffer (pH 6.9) and 100 µL of α-amylase solution (4.5 units/mL/m). The mixture was incubated for 10 m at 25°C. Then 1% starch was added and incubated for 30 m at 25°C. The reaction was stopped by the addition of 1 mL of dinitrosalicylic acid reagent. After that, the reaction mixture was diluted tenfold with distilled water, and the absorbance was measured at 540 nm using a Shimadzu UV-VIS spectrophotometer (model 1900i). The reading was compared with the control (extract replaced by buffer), and α-amylase inhibition activity (%) was calculated.

For the extraction and estimation of α-glucosidase inhibition activity of the experimental plant tissue, the process of Wong *et al*.[45] was followed. Ninety-six–well plates (Merck India) were incubated at room temperature for 10 m with 50 µL of 0.1 M potassium phosphate buffer and α-glucosidase solution, diluted with 50 µL of experimental sample extract. After incubation, p-nitrophenyl-α-D-glucopyranoside solution was added to each well at scheduled intervals. The reaction mixtures were incubated at room temperature for 5 m, and absorbance was measured at 405 nm. The inhibitory activity of experimental samples was estimated using the same formula as the α-amylase inhibition assay.

Statistical analysis

Each experiment consisted of three replicates and was carried out twice at different times. Results are the mean of three replicates ± standard error. Analysis of variance was performed using MS Excel software (analysis of variance single-factor analysis) for the statistical analysis of the data, and the means of the significant differences were separated using Fisher's least significant difference test at the 0.05 level of probability.

Hierarchical cluster analyses were performed to identify close as well as distant relationships based on functional food properties and the bioavailability of flavonoids and phenolic acids assessed among the 10 experimental accessions of *Amaranthus hypochondriacus*, using IBM SPSS Statistics 20.

Result

RP-HPLC coupled photodiode assay of foliar extracts of experimental amaranths revealed germplasm and seasonal impact on the presence of important bioactive polyphenolic compounds

RP-HPLC-based analyses of nineteen nutritionally important bioactive polyphenolic compounds from foliar extracts of summer-grown experimental germplasms of *Amaranthus hypochondriacus* were performed to explore genotype impact on secondary metabolite-based functional food properties of one of the underutilized potential alternate crops. The results, in general, showed accession-specific variations of tested phenolic acids and flavonoids of *Amaranthus hypochondriacus* L. in summer-grown crops. RP-HPLC of hydroethanolic leaf extracts showed the presence of 18,19,17,17,18,18,18,19,18 and polyphenolic compounds, respectively (Figs. 1 and 2).

A comparative account of the bioavailability of 11 pharmaceutically important phenolic acids (gallic acid, protocatechuic acid, gentisic acid, p-hydroxybenzoic acid, chlorogenic acid, caffeic acid, syringic acid, p-coumaric acid, ferulic acid, sinapic acid, and ellagic acid) in ten experimental accessions of *Amaranthus hypochondriacus* L. is depicted in Table 1. The results, in general, showed accession-specific variations. Pharmaceutically important bioactive phenolic acids such as chlorogenic acid, syringic acid, p-coumaric acid, ferulic acid, and caffeic acid were found to be significantly higher in the green accession IC95326 (Table 1). Protocatechuic acid was found to be maximally accumulated in the red accession IC107144. The accession IC42397, a red accession, showed maximum accumulation of gentisic acid and gallic acid. The pharmaceutically important phenolics ellagic acid and sinapic acid were present in excessively higher amounts in another green accession, IC94661 (Table 1). The other phenolic acid tested, p-hydroxybenzoic acid, showed maximum accumulation in the green accession IC95251 (Table 1).

RP-HPLC-based comparative evaluation of quantitative accumulation of eight pharmaceutically important flavonoids in hydroethanolic leaf extracts of ten experimental accessions of *Amaranthus hypochondriacus* L. is shown in Table 2. The flavonoid naringenin was found to be present exclusively in the accessions IC107144 (red accession) and IC47434 (green accession). The same red accession, IC107144, possessed a significantly higher amount of myricetin and apigenin (Table 2).

The quantitative accumulation of rutin, quercetin, and catechin was found to be significantly higher in accession no. IC95326 (Table 3). The accessions IC94661 and IC42397 showed greater accumulation of the flavonoids kaempferol and naringenin (Table 2). The RP-HPLC data identified the green accession IC95326 as one of the lead germplasms, exhibiting significantly greater accumulation of as many as eight pharmaceutically significant polyphenolic compounds compared to other accessions and hence may be targeted for pharmaceutical purposes. Similarly, the red accession IC107144 also exhibited greater bioaccumulation of protocatechuic acid, myricetin, and apigenin (Tables 1 and 2). Moreover, the flavonoid naringenin was exclusively present in accession IC107144 along with another green accession, IC47434.

Analysis of in vitro antioxidant, anti-inflammatory, and anti-diabetic properties of experimental amaranths confirmed their functional food and phytomedicinal properties and their close relationship with the availability of bioactive polyphenolic compounds

Figure 3a illustrates the DPPH radical scavenging property of methanolic leaf extracts of winter- and summer-grown experi-

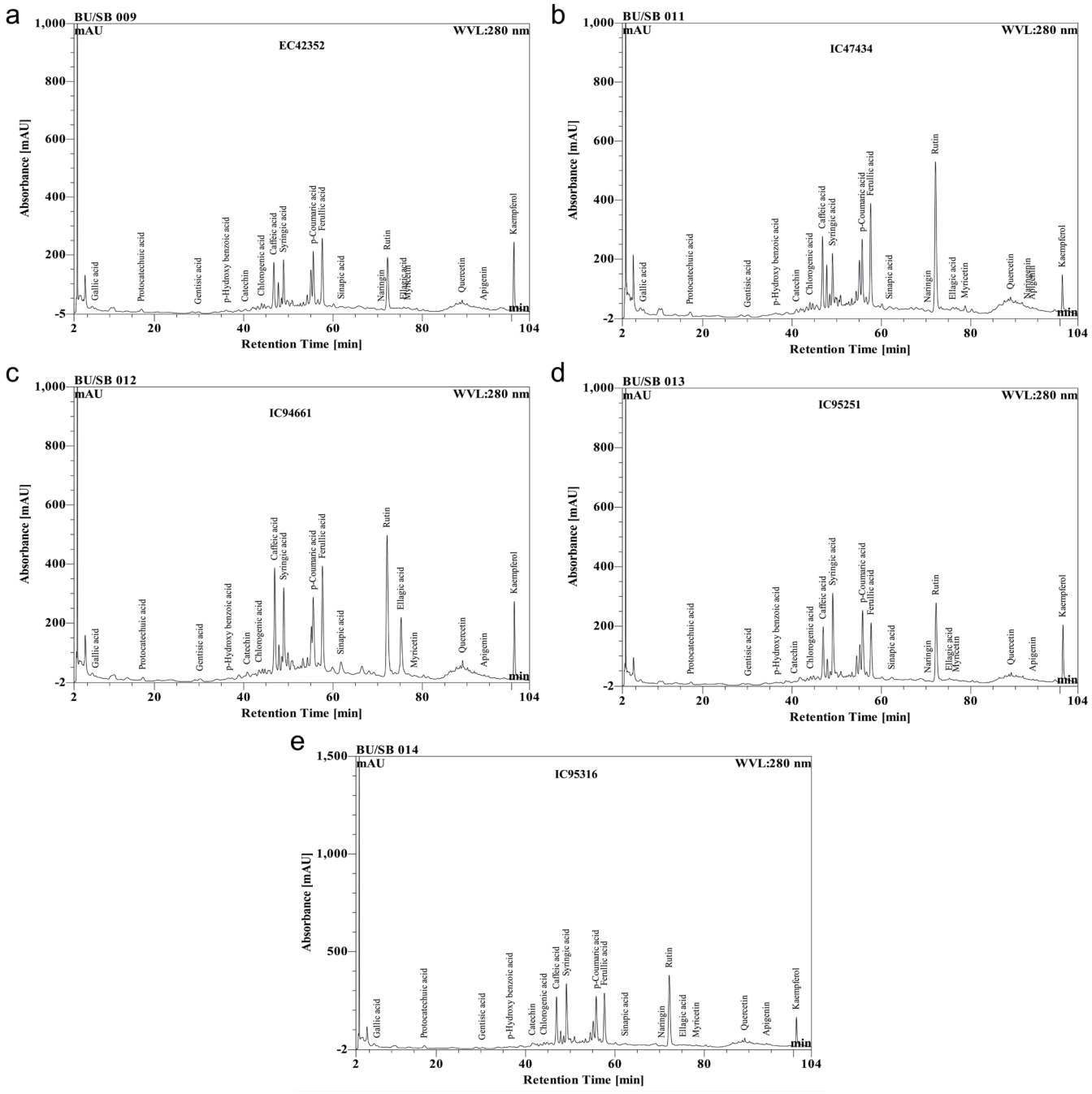

Fig. 1. RP-HPLC derived chromatogram of pharmacologically important phenolic acids and flavonoids of hydroethanolic leaf extracts of accessions EC42352, IC47434, IC94661, IC95251, and IC95316 of *Amaranthus hypochondriacus* L. HPLC analyses were performed using Dionex Ultimate 3000 liquid chromatography, including a diode array detector (DAD) with Chromeleon system manager as the data processor. Separation was completed using a reversed-phase Acclaim C-18 column (5-micron particle size, 250 × 4.6 mm). BUSB, sample nomenclature; HPLC, high performance liquid chromatography; mAU, milli-absorbance units of reactive oxygen species; RP, reverse phase; WVL, wave length.

mental accessions of *Amaranthus hypochondriacus* L. The results, in general, showed significantly higher DPPH radical scavenging property in summer-grown methanolic leaf extracts compared to their corresponding winter-grown leaf extracts (Fig. 3a), corroborating well with the data on flavonoids and other phenolic contents. When compared between the accessions, IC107144 and IC47434 exhibited the maximum antioxidant potential (DPPH radical scavenging property) among all experimental germplasms (Fig. 3a). The accessions that exhibited the least potential for the accumulation of flavonoids, monophenols, diphenols, and total phenols were also found to be comparatively inefficient in reducing the DPPH radical. Thus, the results of the DPPH assay strongly correlated with the

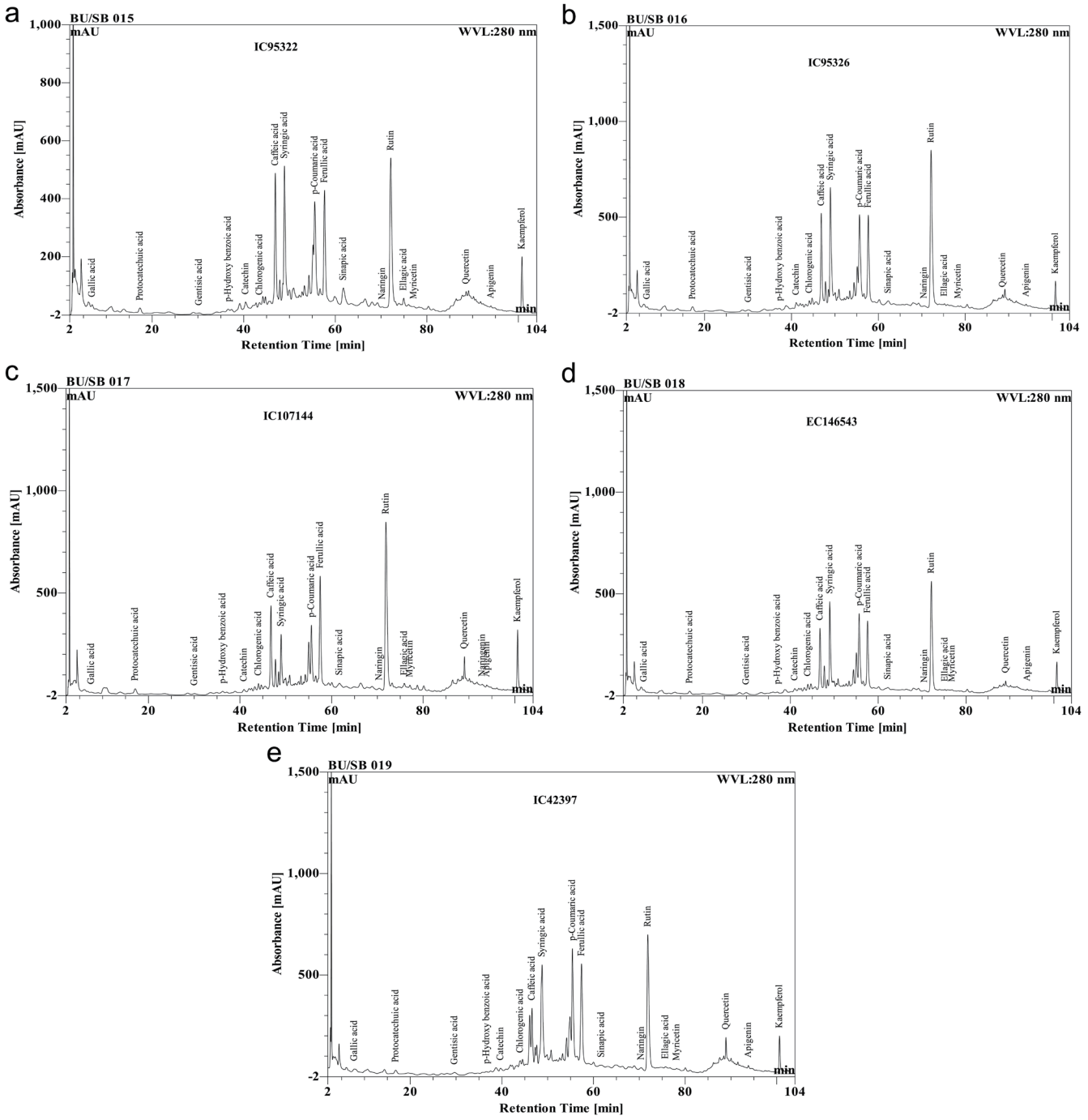

Fig. 2. RP-HPLC derived chromatogram of pharmacologically important phenolic acids and flavonoids of hydroethanolic leaf extracts of accessions IC95322, IC95326, IC107144, EC146543, and IC42397 of *Amaranthus hypochondriacus* L. HPLC analyses were performed using Dionex Ultimate 3000 liquid chromatography, including a diode array detector (DAD) with Chromeleon system manager as the data processor. Separation was completed using a reversed-phase Acclaim C-18 column (5-micron particle size, 250 × 4.6 mm). BUSB, sample nomenclature; HPLC, high performance liquid chromatography; mAU, milli-absorbance units of reactive oxygen species; RP, reverse phase; WVL, wave length.

quantitative accumulation of flavonoids and phenolics (Table 3). The accessions capable of greater accumulation of flavonoids and other phenolics were superior in their antioxidant potential, assessed in terms of DPPH radical scavenging property. The results of the radical scavenging properties of foliar extracts of the experimental amaranths, assessed using the ABTS decolorization assay (Fig. 3b), also exhibited the same trend, with summer-grown crops of accessions IC107144 and IC47434 showing significantly higher activities, thereby confirming the RP-HPLC data as well.

The maximum -OH radical scavenging property was observed in

Table 1. RP-HPLC coupled photodiode assay-based quantification of pharmacognosically important bioactive phenolic acids from foliar tissue of ten different accessions of *Amaranthus hypochondriacus* L

Accessions of *Amaranthus hypochondriacus* L.	Phenolic acids (µg g-100 d.m.)										
	Gallic acid	Protocatechuic acid	Gentisic acid	p-Hydroxy benzoic acid	Chlorogenic acid	Caffeic acid	Syringic acid	p-Coumaric acid	Ferullic acid	Sinapic acid	Ellagic acid
EC42352	1.316	14.598	71.756	14.935	15.763	62.972	50.684	32.841	59.867	0.863	4.750
IC47434	2.594	15.397	82.845	12.971	18.599	75.087	43.779	29.111	65.517	1.407	7.926
IC94661	2.105	22.130	204.207	6.880	34.093	227.986	180.617	79.423	144.834	90.612	221.983
IC95251	–	23.439	121.086	18.370	23.117	139.550	171.294	83.607	92.261	2.152	6.385
IC95316	3.635	34.512	207.439	13.203	29.718	250.880	266.032	120.289	174.149	4.862	2.589
IC95322	5.750	28.159	101.581	15.792	22.293	251.252	254.006	100.508	136.845	9.825	20.419
IC95326	3.282	36.197	252.914	11.207	64.345	294.758	414.057	172.854	200.092	5.995	4.065
IC107144	1.464	36.501	100.952	11.782	24.103	152.964	77.479	54.390	131.866	29.737	23.974
EC146543	2.081	21.400	203.564	6.729	61.020	187.594	273.185	121.010	146.682	12.978	2.071
IC42397	19.081	24.482	293.684	5.205	35.489	136.219	333.359	125.720	186.965	1.871	5.792

HPLC analyses were performed using Dionex Ultimate 3000 liquid chromatography, including a diode array detector (DAD) with Chromeleon system manager as the data processor (Separation was completed using a reversed-phase Acclaim C-18 column (5-micron particle size, 250 × 4.6 mm). HPLC, high performace liquid chromatography; RP, reverse phase.

the methanolic leaf extracts of accessions IC107144 and IC47434, followed by IC95322, IC95326, and IC42397 (Fig. 3c). When the -OH radical scavenging property of methanolic leaf extracts of each accession grown under summer and winter conditions was compared, the results showed a mixed pattern, where accessions IC47434, IC95322, IC95316, and EC42352 exhibited significantly better -OH radical scavenging property in summer-grown crops, whereas accessions IC107144 and IC42397 showed marginally better -OH radical scavenging property in winter-grown crops. The trend of accession-specific variations in -OH radical scavenging property matched well with the availability of phenolics, flavonoids, and antioxidant pigments. Accessions IC107144 and IC47434 exhibited significantly higher OH radical scavenging properties, corroborating well with the data on greater availability of flavonoids and phenolics.

The antioxidant property of plant extracts usually shows a reciprocal correlation with their reducing power. This property serves as a significant indicator of the antioxidant potential of plant tissue extracts. Here, the extracts of plants acted as electron donors and could react with reactive oxygen species to convert them into stable products and terminate chain reactions; hence, plant extracts with rich antioxidant activities showed a reciprocal correlation with reducing power. The accession-specific and seasonal variation in the reducing power of leaf extracts of ten experimental accessions of *Amaranthus hypochondriacus* L. is shown in Figure 3d.

Figure 3e shows the ALPP of methanolic leaf extracts of ten experimental accessions of *Amaranthus hypochondriacus* L. grown in two different seasons (summer and winter). The results, in general, showed significant accession-specific variation, with accessions IC107144 and IC47434 exhibiting significantly higher ALPP, fol-

Table 2. RP-HPLC coupled photodiode assay-based quantification of pharmacognosically important bioactive flavonoids from foliar tissue of ten different accessions of *Amaranthus hypochondriacus* L

Accessions of *Amaranthus hypochondriacus* L.	Flavonoids (µg g-100 d.m.)							
	Catechin	Naringin	Rutin	Myricetin	Quercetin	Naringenin	Apigenin	Kaempferol
EC42352	14.852	3.949	159.387	5.616	4.604	–	1.323	74.779
IC47434	20.717	4.648	367.58	7.408	3.606	2.371	1.270	33.049
IC94661	65.909	–	740.554	71.193	21.562	–	2.452	134.117
IC95251	8.801	4.889	491.799	3.125	8.331	–	2.179	122.143
IC95316	85.265	10.551	874.024	6.318	25.531	–	5.908	125.236
IC95322	85.315	1.561	703.295	2.181	6.006	–	2.573	85.005
IC95326	127.498	7.913	1,177.41	4.264	100.222	–	4.069	65.355
IC107144	22.772	7.388	849.248	93.557	44.980	6.923	50.395	94.758
EC146543	97.839	13.511	696.831	2.788	14.785	–	2.988	63.696
IC42397	31.753	16.197	898.333	10.672	142.184	–	6.435	73.980

HPLC analyses were performed using Dionex Ultimate 3000 liquid chromatography, including a diode array detector (DAD) with Chromeleon system manager as the data processor [Separation was completed using a reversed-phase Acclaim C-18 column (5-micron particle size, 250 × 4.6 mm)]. HPLC, high performace liquid chromatography; RP, reverse phase.

Table 3. Correlation coefficients showing the relationship between total antioxidant capacity (ABTS, FRAP, and DPPH radical scavenging properties) and individual antioxidant contents [total polyphenol (TPP), diphenol (DP), monophenol (MP), flavonoids (FLAV), betacyanin (BCY), anthocyanin (ANTHCY), and carotenoids (CARO)] of foliar tissue of accessions IC107144 & IC94661 of Amaranthus *hypochondriacus* L., using SigmaPlot software

	FRAP	DPPH	TPP	DP	MP	FLAV	BCY	ANTHCY	CARO
Accession IC107144									
ABTS	0.945	0.870	0.488	0.154	0.935	0.019	0.134	0.829	0.982
FRAP	–	0.983	0.747	0.178	0.767	0.309	0.198	0.966	0.990
DPPH	–	–	0.855	0.353	0.638	0.477	0.0372	0.997	0.948
TPP	–	–	–	0.788	0.146	0.864	0.800	0.893	0.645
DP	–	–	–	–	0.494	0.991	1.000	0.425	0.037
MP	–	–	–	–	–	0.372	0.477	0.576	0.850
FLAV	–	–	–	–	–	–	0.993	0.544	0.172
BCY	–	–	–	–	–	–	–	0.443	0.057
ANTHO	–	–	–	–	–	–	–	–	0.920
Accession IC94661									
ABTS	0.97	0.896	0.677	0.999	0.442	0.714	0.475	0.590	0.846
FRAP	–	0.126	0.666	0.073	0.936	0.628	0.922	0.861	0.613
DPPH	–	–	0.934	0.906	0.003	0.904	0.034	0.170	0.521
TPP	–	–	–	0.695	0.361	0.999	0.326	0.194	0.181
DP	–	–	–	–	0.420	0.731	0.453	0.570	0.883
MP	–	–	–	–	–	0.313	0.999	0.985	0.852
FLAV	–	–	–	–	–	–	0.277	0.144	0.231
BCY	–	–	–	–	–	–	–	0.991	0.871
ANTHCY	–	–	–	–	–	–	–	–	0.930

ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); DPPH, 2,2-diphenyl-1-picrylhydrazyl; FRAP, ferric reducing antioxidant power.

lowed by IC94661, IC95251, EC146543, IC42397, IC95316, and EC42352. The accessions IC95322 and IC95326, on the other hand, exhibited significantly lower ALPP. When compared, the ALPP data showed a strong correlation with the accumulation of phenolics, flavonoids, antioxidative pigments, metal-chelating property, and reducing power. The summer-grown crops, in general, exhibited better ALPP, except for accessions EC42352, IC95322, and IC95316, which showed only marginal and insignificant differences.

Figure 3f shows the chelating effect of methanolic leaf extracts of ten experimental accessions on ferrous ions. The results, in general, showed both seasonal and accession-specific variations in the metal-chelating property of experimental leaf extracts. The lead accessions IC107144 and IC47434 exhibited better metal-chelating properties for both summer- and winter-grown crops compared to other accessions. This result significantly validated the greater abundance of monophenols, diphenols, flavonoids, and total phenol contents in the corresponding tissue samples. All accessions except IC94661, EC146543, and EC42352 exhibited no significant difference in metal-chelating property between winter- and summer-grown crops (Fig. 3f).

Comparative estimation of total phenol, monophenol, diphenol, and flavonoid contents of foliar extracts of winter- and summer-grown experimental accessions of *Amaranthus hypochondriacus* L. revealed accession-specific and seasonal variations and strongly supported the *in vitro* antioxidative properties (Fig. 4a–d). When comparing the winter- and summer-grown crops, as with other attributes of *in vitro* antioxidative properties, a general trend of augmented accumulation in summer-grown crops was noticed. The green and red accessions IC47434 and IC107144, respectively, proved to be the lead germplasms, exhibiting significantly greater accumulation of total phenol, monophenol, diphenol, and flavonoid contents.

Figure 5a–c represent accumulations of important antioxidant pigments (anthocyanin, betacyanin, and carotenoids) in summer- and winter-grown experimental amaranths. The data revealed a different trend from that of the phenolic pool. Although the red accession IC107144 exhibited significantly better potential for accumulation of phenolics, another red accession, IC95316, showed significantly better antioxidant promise in terms of anthocyanin, betacyanin, and carotenoid accumulation. Overall, the red accessions IC95316 and IC107144 proved to be the better germplasms with potential for the accumulation of all three antioxidant pigments compared to others.

α-amylase and α-glucosidase are important enzymes of carbohydrate metabolism. Inhibition of these enzymes results in deferred digestion and glucose absorption, subsequently causing attenuation of postprandial hyperglycemic effects. Thus, these two enzymes are the prime targets for diabetes therapeutics. Methanolic leaf extracts of ten experimental accessions, in general, exhibited significant inhibition of α-amylase and α-glucosidase enzymes. As evident from the different attributes of antioxidant properties, the anti-diabetic properties of methanolic leaf extracts also exhibited both seasonal and accession-specific variations (Fig. 5d and e). The summer-grown crops, in most cases, exhib-

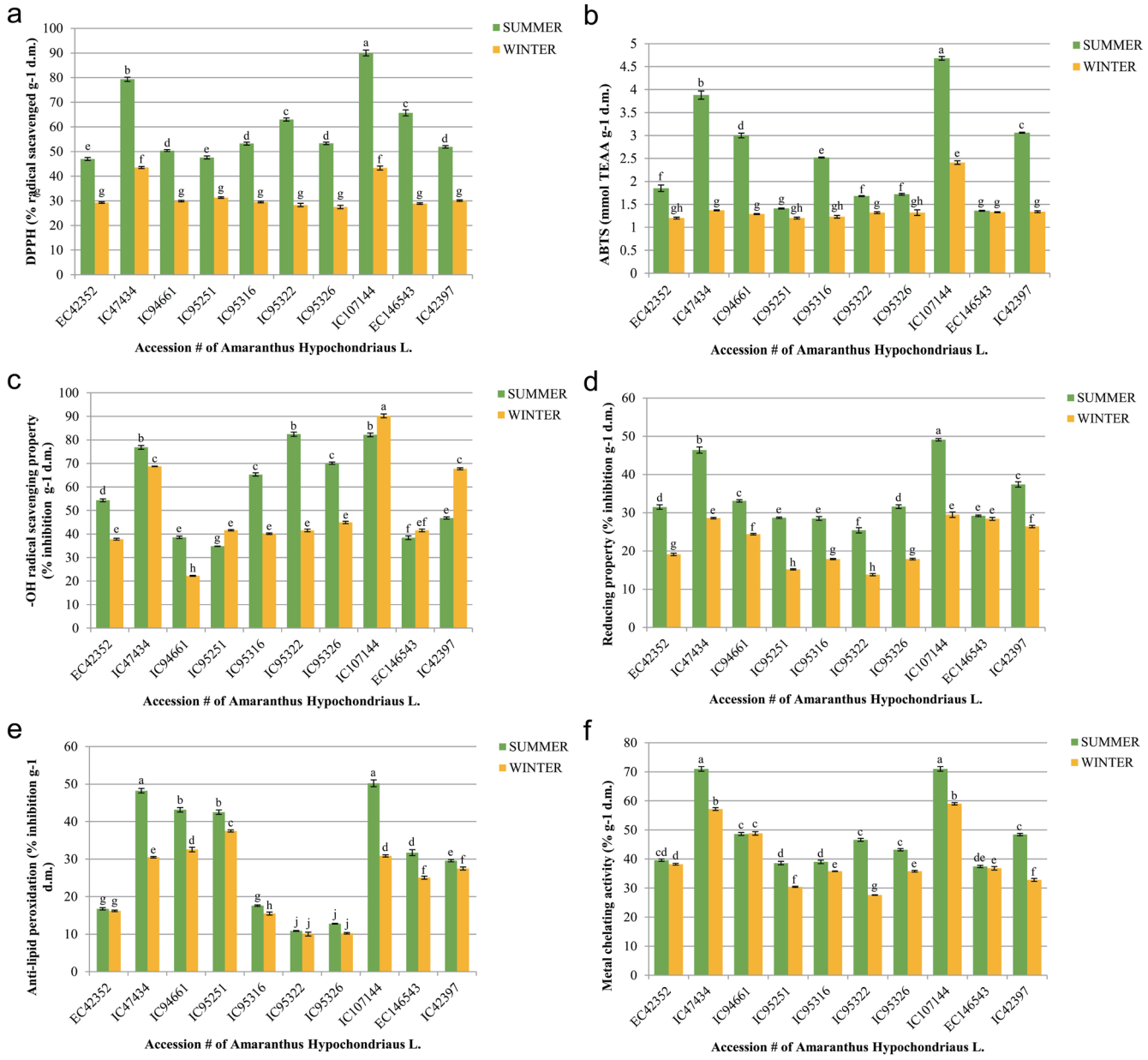

Fig. 3. *In vitro* antioxidant (assessed in terms of DPPH, ABTS radical scavenging properties, hydroxyl radical scavenging, reducing, and metal chelating properties) and anti-inflammatory properties (anti-lipid peroxidation) of foliar extracts of ten experimental accessions of *Amaranthus hypochondriacus* L. grown in two different seasons. Values are the mean of three replicates ± SE. Different letters indicate significant differences, compared by Fisher's least significant difference test ($p < 0.05$). ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); DPPH, 2,2-diphenyl-1-picrylhydrazyl ; SE, standard error.

ited better anti-diabetic properties (% inhibition of α-amylase and α-glucosidase) compared to the winter-grown crops. The lead accessions, adjudged or screened based on antioxidant potential—IC47434 and IC107144—exhibited maximum inhibition of α-amylase and α-glucosidase activity (Fig. 5d and e). Accessions IC95251, IC95316, IC94661, and IC95326 also exhibited significant anti-diabetic properties in terms of inhibition of α-amylase and α-glucosidase activity. Accessions IC95322 and EC146543 showed minimal anti-diabetic properties. When the inhibitory properties of α-amylase and α-glucosidase were compared, it was noticed that the enzyme α-amylase was more susceptible than α-glucosidase under the influence of the same quantity of leaf extract. The results also showed that accessions capable of producing higher quantities of flavonoids, other phenolics, and antioxidant pigments possessed better anti-diabetic properties.

Cluster analysis, based on phytomedicinal properties and the presence of bioactive compounds in experimental amaranths, classified experimental germplasms and identified lead accessions with superior phytomedicinal properties

On the basis of functional food propensities (*in vitro* antioxidant, anti-inflammatory, and anti-diabetic properties) and the abundance

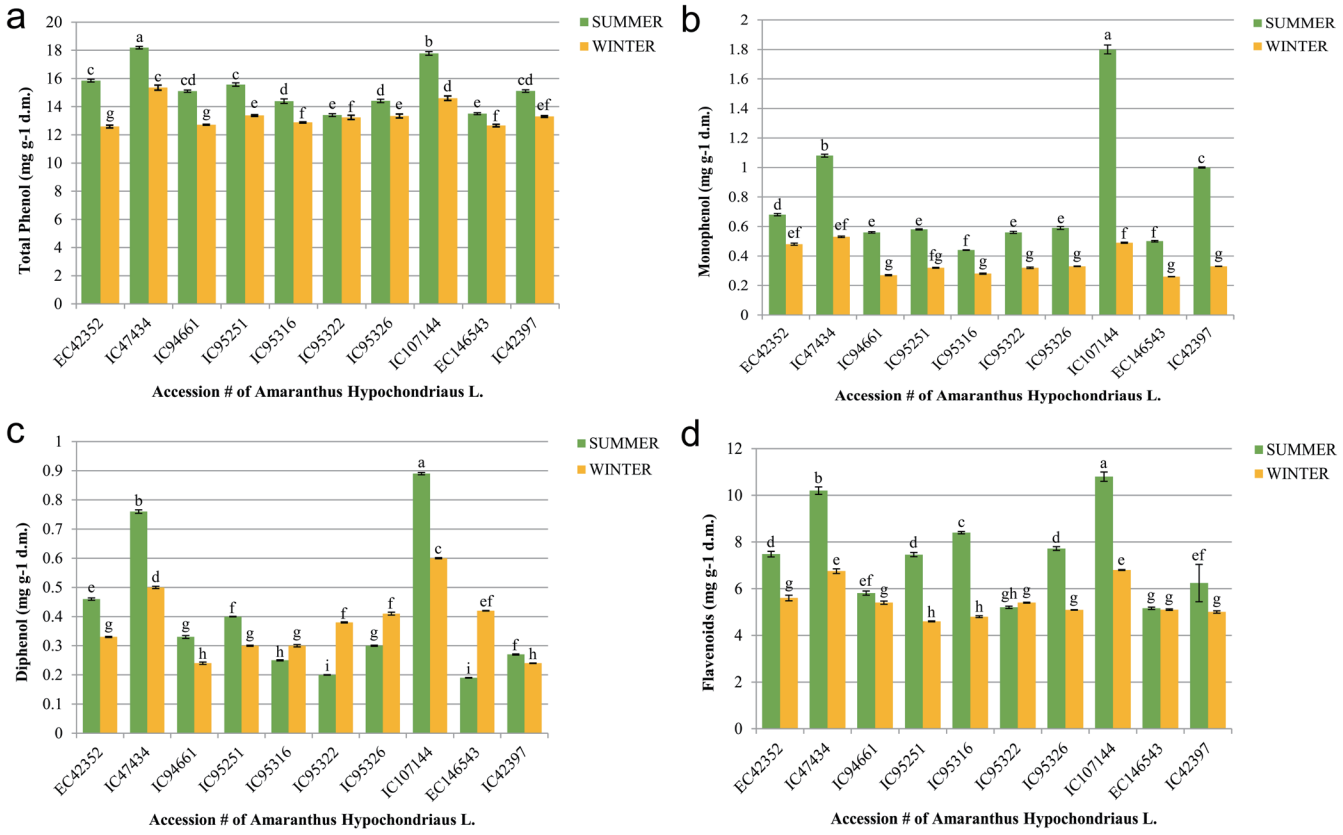

Fig. 4. Total phenol, monophenol, diphenol, and flavonoid content of foliar extracts and their seasonal variations of ten experimental accessions of *Amaranthus hypochondriacus* L. Values are the mean of three replicates ± SE. Different letters indicate significant differences, compared by Fisher's least significant difference test ($p < 0.05$). SE, standard error.

of bioactive phenolic acids and flavonoids tested, cluster analysis of ten accessions of *Amaranthus hypochondriacus* was conducted, and a dendrogram was constructed based on the square Euclidean distance using the "between-group linkage" method as the agglomerative clustering technique. The results obtained are presented in Figure 6a–c. The positions of the ten accessions in the dendrogram based on functional food propensity were distributed into three groups, with IC107144 and IC47434 forming the first group and classified as accessions with superior food propensity. The accessions IC94661, IC95251, IC42397, and EC146543 formed the second group, i.e., accessions with moderate food propensity, and IC95361, IC95326, EC42352, and IC95322 formed the third group, which can be classified as accessions with inferior food propensity (Fig. 6a). However, slightly different positions of the experimental accessions were observed in the dendrogram based on the bioavailability of bioactive phenolic acids and flavonoids tested (Fig. 6b). Although both accessions IC107144 and IC47434, classified as accessions with superior food propensity, held the same position when dendrograms were prepared based on the bioavailability of phenolic acids, accession IC47434 secured a different position when dendrograms were prepared based on the bioavailability of flavonoids. Moreover, the positions of some other accessions did not match exactly when the dendrograms were compared, suggesting contributions of polyphenolic compounds in exhibiting cumulative in vitro functional food propensities. Based on the coefficient value of the agglomeration schedule, the highest similarity was observed between accessions IC107144 and IC47434. Accessions IC95322, IC95326, and IC95361 were also very close to each other. Intense similarity was also observed between IC107144 and IC47434.

Discussion

Our study focused on a significant category of foliar polyphenolic compounds found in promising germplasms of pseudocereal *Amaranthus hypochondriacus* (including bioactive flavonoids, flavones, flavanones, flavonols, and phenolic acids), which have antioxidant properties of pharmacological significance. The RP-HPLC-based separation, depending on the retention time, was done for phenolic acids like gallic acid, protocatechuic acid, gentisic acid, p-hydroxybenzoic acid, chlorogenic acid, vanillic acid, caffeic acid, syringic acid, p-coumaric acid, ferulic acid, sinapic acid, salicylic acid, and ellagic acid. The pharmaceutically important flavonoids, flavones, flavanones, and flavonols such as naringin, rutin, catechin, myricetin, quercetin, naringenin, apigenin, and kaempferol were also tested and compared for the experimental accessions of *Amaranthus hypochondriacus* L. This RP-HPLC-based result, depending on computation against the retention time of standards, revealed accession-specific variations of individual polyphenolic compounds tested.[18,46] The lead accessions identified based on quantitative antioxidant profiling, i.e., IC107144 and IC47434, exhibited greater accumulation or exhibited the presence of a pharmaceutically important flavonoid, naringenin, which was altogether absent in other experimental accessions. The red acces-

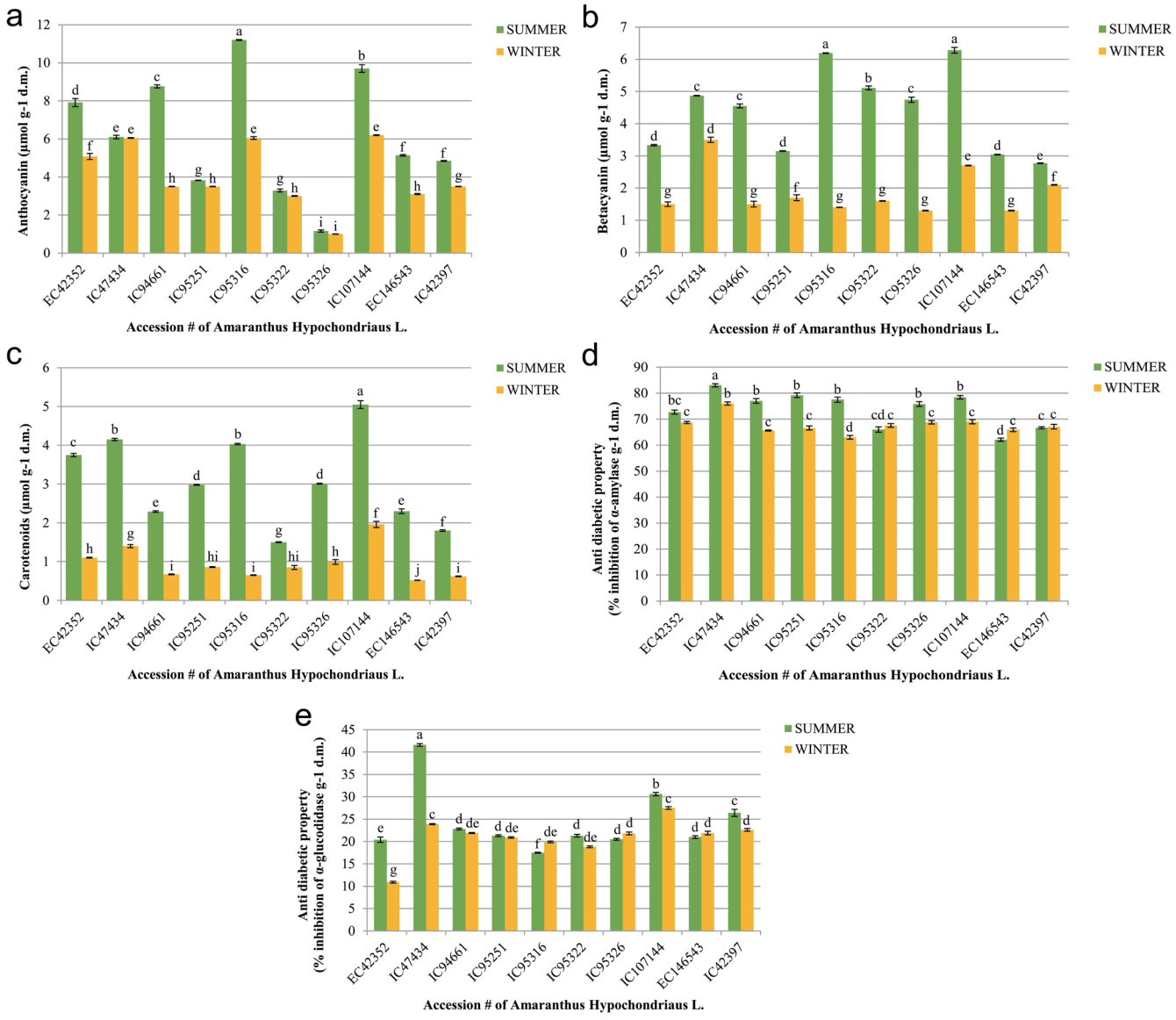

Fig. 5. Bioactive antioxidant pigment (anthocyanin, betacyanin, carotenoid) content and antidiabetic properties (assessed in terms of inhibition of α-glucosidase and α-amylase activities) of foliar extracts and their seasonal variations of ten experimental accessions of *Amaranthus hypochondriacus* L. Values are the mean of three replicates ± SE. Different letters indicate significant differences, compared by Fisher's least significant difference test ($p < 0.05$). SE, standard error.

sion IC107144 also showed greater accumulation of the flavonoids myricetin and apigenin and the phenolic acid protocatechuic acid, which were found to be relatively low in other experimental accessions, including another lead accession, i.e., IC47434. The present study also confirmed significantly greater accumulation of flavonoids like naringin, myricetin, naringenin, and apigenin in the red accessions (IC107144 and IC42397). However, when comparing the overall accumulation of all 21 phenolic acids and flavonoids, the green accession IC95326 proved to be the most important one for its ability to accumulate as many as five phenolic acids (chlorogenic acid, caffeic acid, syringic acid, p-coumaric acid, and ferulic acid) and three flavonoids (rutin, catechin, and quercetin). Another green accession, IC94661, showed significantly greater accumulation of the three pharmaceutically important phenolic acids and flavonoids: sinapic acid, ellagic acid, and kaempferol. Thus, the result of qualitative and quantitative antioxidant profiling based on RP-HPLC did not match exactly with the result of spectrophotometric quantitative antioxidant profiling. The lead accessions (in terms of quantitative antioxidant profiling and antioxidant markers), i.e., IC107144 and IC47434, though containing one unique flavonoid (naringenin) and exhibiting accumulation of all the targeted phenolics, in terms of the quantitative accumulation of targeted antioxidants (RP-HPLC data), the green accession IC95326 seemed to be the more promising one for its ability to accumulate eight phenolics in significantly greater amounts compared to other accessions. These results strongly corroborate the genotype impact on the accumulation of phenolic acids and flavonoids.[18,31,34,46]

The unique flavonoid naringenin, which was absolutely con-

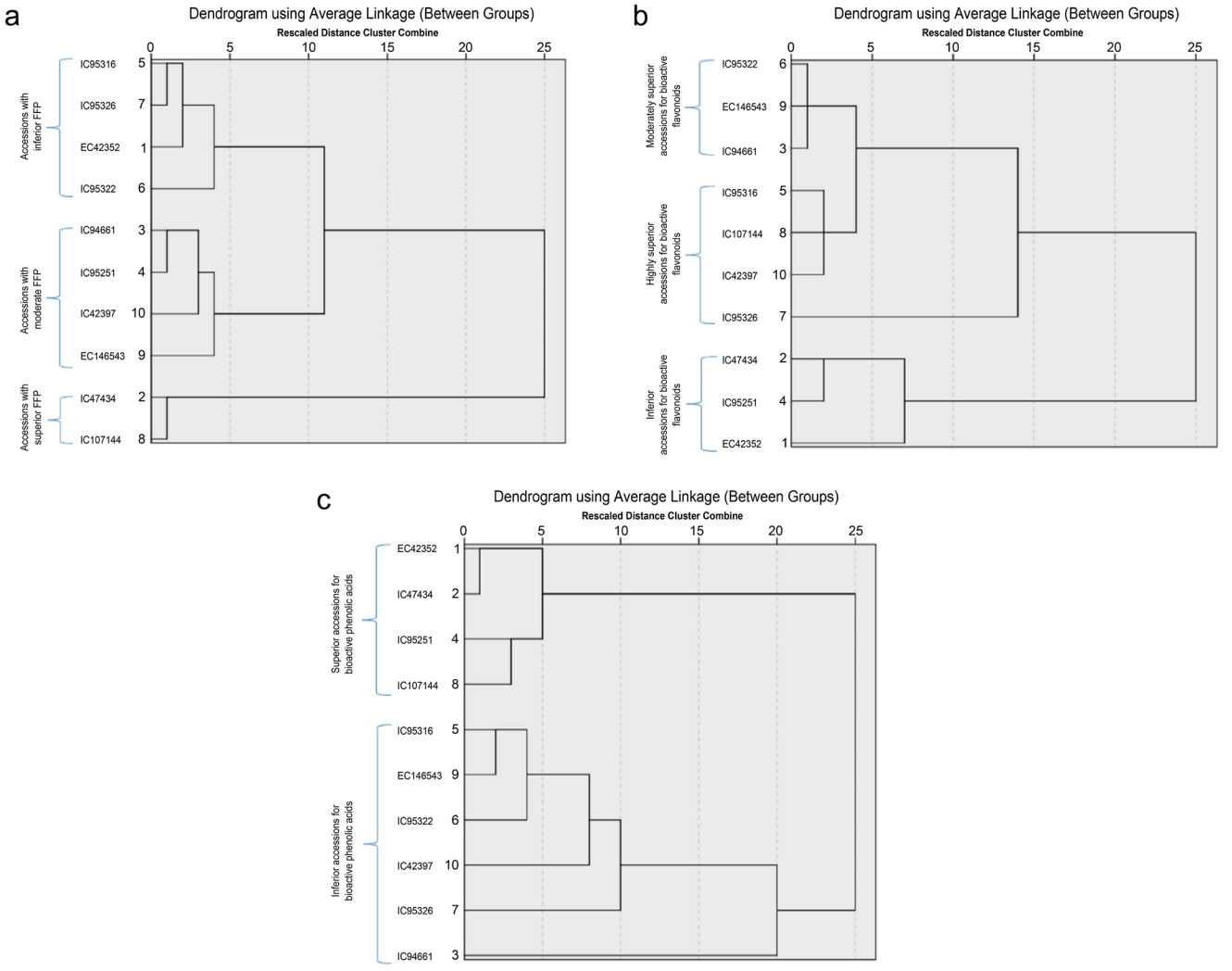

Fig. 6. Dendrogram of hierarchical cluster analysis showing clusters of experimental accessions of *Amaranthus hypochondriacus* L., separated on the basis of overall therapeutic food propensity (a), bioavailability of bioactive phenolic acids (b), and bioactive flavonoids (c). FFP, functional food propensity.

fined to the lead accessions (IC107144 and IC47434), identified based on RP-HPLC quantitative antioxidant profiling, has intense antibacterial activity, and hence these two accessions may be recommended for the prevention of infectious diseases. Quercetin is also known for its iron-chelating and iron-stabilizing properties, which have direct relevance in reducing reactive oxygen species formation and oxidative stress.[47,48] Quercetin, which was present in significantly high amounts in the accession IC95326, is known to evoke cell cycle arrest in proliferating lymphoid cells in addition to its growth-inhibitory effect on several malignant tumor cell lines.[49] Rutin, a flavonoid found in significantly higher amounts in the same accession IC95326, has several beneficial effects on health,[50] including antiviral properties.[51] The flavonoids apigenin and naringin, in general, have significant antiviral properties, particularly against human immunodeficiency virus, herpes virus, and respiratory syncytial virus.[52] Myricetin, another flavonoid maximally present in the red accession IC107144, serves as a functional food ingredient with partial anticancer and antihyperglycemic activity. Catechin, another flavonoid present in greater abundance in IC95326, has nutraceutical value due to its inhibitory effect on the development of malignancy.[53] The flavonoid kaempferol, in general, exhibits downregulation of mutant p53 protein expression. An experiment of rat protein tyrosine kinase, which is associated with the promotion of carcinogenicity, also proved the role of kaempferol in their downregulation.

Out of 11 phenolic acids, five phenolic acids—chlorogenic acid, caffeic acid, syringic acid, p-coumaric acid, and ferulic acid—were found to be significantly more abundant in the accession IC95326, making it a pharmaceutically promising one. Previous work indicated that chlorogenic acid may act as a defensive compound.[54]

The maximum extent of accession-specific genotypic variation of individual flavonoids ranged from 65.75% (naringenin) to 97.66% (myricetin). Similarly, when the extent of accession-specific genotypic variation of phenolic acids was estimated, it ranged from 60% to 99.06%. Kalinova and Dadakova reported species-specific genotypic variation of flavonoids such as rutin and speculated that this variation may be due to species-specific metabolism.[55] Moreover,[56] they observed qualitative and quantitative profiles of flavonoid glucosidase and hydroxycinnamic acids and found a species-specific composition in amaranth. The corre-

sponding profile not only indicated genotype-specific composition but also hinted at the identification of proper amaranth genotypes as food crops due to their strong diversity of plant secondary metabolites.[57] Previous works on polyphenols in various amaranth species identified some important phenolic acids and flavonoids and their glucosides. Free phenolic acids were found in the leaves of *Amaranthus caudatus* and *Amaranthus paniculatus*. Rutin was found to be best present in the leaves of *Amaranthus hybridus and Amaranthus cruentus*.[55,58] Similarly, quercetin and rutin were found in some mature leaves of amaranthus.[55] Genotype-specific variation of phenolic acids and flavonoids was also noticed.[59,60] Among flavonoids, rutin showed great variation among some genotypes,[59] though their experimentation showed that, among species, environmental factors had a lesser effect on the accumulation of polyphenolic compounds. This group held the view that variation of polyphenolic compounds due to environmental factors in amaranths does exist; however, the traits did not reveal any consistent difference between species and genotypes. Therefore, it is most likely that more comprehensive cultivation traits would be needed to evaluate possible effects of plant genotype on the accumulation of polyphenolics in amaranths.[18,31,59]

Significantly, understanding germplasm-specific anti-degenerative and phytomedicinal properties (assessed in terms of bioavailability of important bioactive polyphenolic compounds, radical scavenging property, ALPP, and anti-diabetic property) validates the chemical basis of diverse ethnomedicinal utilization of foliar tissue of seed amaranth *Amaranthus hypochondriacus*. Moreover, the study also finds its future prospects as it paves the way for further pharmacological research for elucidation of the chemistry and the functional role of those compounds in mitigating degenerative diseases.

Though our experiment explored the phytomedicinal propensity of *Amaranthus hypochondriacus* with ten promising germplasms, the study has its own limitation in establishing this crop as an industrial medicinal crop with potential sources of bioactive compounds, as it requires more complete investigation because of the abundance of bioactive chemicals, such as flavonoids, betalains, and peptides, which provide a variety of therapeutic benefits, including anti-inflammatory, anti-diabetic, and antibacterial properties. Another limitation of the present investigation was the targeted RP-HPLC-based profiling of some bioactive compounds, which necessitates more research to isolate, purify, and standardize these chemicals, to build dependable extraction and purification processes, to conduct clinical trials to prove health benefits, and to develop innovative technology for their integration into functional foods and pharmaceutical products.

Conclusions

The present work finds its novelty in confirming that foliar tissues of experimental accessions of pseudocereal *Amaranthus hypochondriacus* L., otherwise known for their seed nutraceutical potential, are also an excellent source of bioactive polyphenolic compounds with promising *in vitro* anti-degenerative properties, which may be of benefit to human health and may be recommended as a low-cost subsidiary medicinal vegetable crop to prevent degenerative diseases and also as a promising industrial crop.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

Study concept and design (SB), acquisition of data (MA, SG), analysis and interpretation of data (SB, MA), drafting of the manuscript (SB, MA), critical revision of the manuscript for important intellectual content (SB), administrative, technical, or material support (SB, SG), study supervision (SB), and funding acquisition (SB). All authors have made significant contributions to this study and have approved the final manuscript.

Ethical statement

Not applicable.

Data sharing statement

Data were generated from the experimental outcomes performed in the communication laboratory. Datasets associated with this article are included within the article and its supplementary file. Material was collected from the National Bureau of Plant Genome Research (NBPGR), New Delhi, India.

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