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Betalains from *Amaranthus tricolor* L.

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Betalains in *Amaranthus tricolor* leaf were identified by means of reversed phase high-performance liquid chromatography (HPLC) and Liquid chromatography-Mass spectrometry (LC-MS). In addition to the known compound red-violet amaranthin, two yellow pigments were detected in *Amaranthus tricolor*. A novel betaxanthin, methyl derivative of arginine betaxanthin was identified on the basis of UV-Vis spectra and mass spectrometric characteristics, as well as by comparison with literature data, which to the best of our knowledge has not been reported in *Amaranthus tricolor*. Amaranthin was the major betacyanin pigment present in *Amaranthus tricolor* although methylated arginine betaxanthin, a novel betaxanthin hitherto not observed naturally and betalamic acid were also present. The identified compounds were then evaluated for alpha-amylase inhibitory potential using Bernfeld method. Amaranthin and betaxanthin did not show alpha-amylase inhibitory activity. Betalamic acid displayed significant alpha-amylase inhibitory activity compared to that of a reference standard, acarbose

Keyword: *Amaranthus tricolor*; Betalains; Amaranthin; Methylated Arginine Betaxanthin; Betalamic Acid; Liquid Chromatography-Mass Spectrometry

1. Introduction

Preparations of plants or parts of them were widely used in popular medicine since ancient times and till today the use of phytomedicines is widespread in most of the world's population[1].

Betalain pigments are water-soluble red-violet (betacyanins) and yellow (betaxanthins) pigments. These pigments replace anthocyanins in most plant families of the order Caryophyllales[1]. Betalains occur only in the plants of the order Caryophyllales (Old name: Centrospermae), such as the family Amaranthaceae which includes several important genera, i.e. *Amaranthus*, *Celosia*, *Gomphrena* and *Iresine*[2,3]. Several betalains (16 red-violet betacyanins and 3 yellow betaxanthins) were reported to be isolated and identified in different portions (viz. stems, leaves, and inflorescences) from plants of the Amaranthaceae family[4-6]. Amaranth species are cultivated and consumed as a leafy vegetable in India and Bangladesh. There are four species of *Amaranthus* documented as cultivated vegetables in eastern Asia: *Amaranthus cruentus*, *Amaranthus blitum, Amaranthus dubius* and *Amaranthus tricolor*[3]. Betalains are conjugates of the chromophore betalamic acid that is derived from dihydroxyphenylalanine by an oxidative 4, 5-extradiol ring opening mechanism[7].

Betalain pigments are of particular interest because of their limited biological distribution, occurring only in those plant species confined to the order Caryophyllales, notably the red beet (Chenopodiaceae) and certain fungi such as the fly-agaric mushroom (*Amanita muscaria*)[8].

Betalains are attracting increasing attention because of their use for food coloring and their antioxidant and radical scavenging properties against certain oxidative stress-related disorders, anticancer, antiviral and antiparasitosis properties[1,9-21]. The present investigation deals with the isolation and characterization of betalains from *Amaranthus tricolor*. Additionally, all the compounds were tested for their alpha-amylase inhibitory activity.

2. Materials and Methods

2.1 General

An 1100 series HPLC System from Agilent was used, consisting of binary pump and a diode array detector (DAD) and operated at room temperature throughout the analysis. Data processing was performed using Agilent HPLC 2D ChemStation software. Fourier transform infrared (FTIR) spectra was recorded at 4000–400 cm^{-1} using a Perkin Elmer FTIR spectrophotometer (Bio-Rad Co., Hercules, CA). UV-Vis spectra of the HPLC purified pigment liquid samples were measured on a double beam spectrophotometer (Chemito UV-Vis spectrophotometer, Model Spectrascan UV 2600 PC). HPLC purified pigment samples were reconstituted in water and analyzed by LC-MS using a QuattroMicro^{TM} API (WATERS).

2.2 Plant Material

The leafy vegetable, amaranth leaves (*Amaranthus tricolor* L.), locally called lalsaag, was bought from the local market at Kharagpur (West Bengal, India).

2.3 Preparation of crude extracts

A sample (200 g) of fresh plant material (leaves) was taken at the 90 days growth stage. Frozen samples were homogenized in a mortar with liquid nitrogen and extracted with 80% methanol. The homogenates were kept at room temperature (25 ± 2 °C) for 40 min with continuous stirring and were filtered through multiple layers of gauze. The filtrates were centrifuged at 10,000 rpm for 30 min. The extracts were concentrated under vacuum at room temperature (25 ± 2 °C).

2.4 Purification of *Amaranthus tricolor* pigments

Amaranthus tricolor pigments were purified following the method of Cai et al.[5] with slight modifications. The concentrated crude pigment samples were applied to a Sephadex LH-20 column (20 × 1.0 cm i.d.) and eluted with Milli-Q water. Three pigment bands were collected and freeze-dried. The freeze-dried samples were dissolved in water and filtered through a 0.22 µ Millipore filter. The final purification of samples was done by TLC, HPTLC and HPLC.

2.5 HPLC analysis

Conditions for HPLC were as follows: Zorbax SB-C18 column (5 µm, 250 × 4.6 mm) was used. The mobile phase consisted of solvent A, 0.005 mol/L potassium phosphate (KH_2PO_4), and solvent B, 5% acetonitrile (CH_3CN)/0.005 mol/L KH_2PO_4. The following elution profiles were used: 0–5 min, 100% A; 5–10 min, 50% B; 10–20 min, 100% B; 20–30 min, 100% A. The flow rate was 1.0 mL/min. The samples were injected with a 100 μL loop. Peaks were determined at the wavelength of 536, 475 and 430 nm.

FTIR Spectroscopy

HPLC purified pigment sample was ground with IR grade KBr (1:10) and pressed into disks under high pressure using a pellet maker[22].

LC-MS of Betalains

Analyses were performed by using a Waters XTerra MS C_{18} column (100 × 2.1 mm i.d.; particle size 5 µm) and as mobile phase a gradient of 0.1% formic acid as eluent A and acetonitrile (LC MS grade, J. T Becker) as eluent B. The flow rate was 0.250 mL/min and the amount injected was 10 µL. The temperature was set to 25 °C and the column back pressure was 1490 bar. An electrospray ionization source (source block temperature 130 °C, desolvation temperature 300 °C, capillary voltage 3 kV, cone voltage 35 V) was used whereas the desolvation and cone gas flow rate were 650 and 50 L/h respectively. The flow generated by chromatographic separation was directly injected into the electrospray ion source for mass detection.

Positive ion as well as negative ion mode was used for mass detection.

2.6 Quantitative Determination of *Amaranthus* Pigments

Total betalain contents (milligrams per 100 gram of fresh tissue) were determined on a spectrophotometer (Chemito UV-Vis spectrophotometer). The betalain content (AC_a) was calculated using the formula reported by Cai et al.[4] (Eq. 1), where A is the absorption value at the absorption maximum of 536, 480 and 430 nm for betacyanins, betaxanthins and betalamic acid, respectively, DF is the dilution factor, Va is the total extract volume (mL), Wa is the fresh weight of extracting material (g), and L is the path-length (1 cm) of the cuvette. The molecular weight (MW) and molar extinction coefficient (ε) of amaranthin (MW=726.6; $\varepsilon = 5.66 \times 10^4$ $M^{-1}cm^{-1}$) were applied in order to quantify the betacyanins. Quantitative equivalents of the major betaxanthins (Bx) were determined by applying the molecular weight (MW=309) and the mean molar extinction coefficient (ε= 48 000 $M^{-1}cm^{-1}$). For quantitative determination of betalamic acid, molecular weight (MW=212) and molar extinction coefficient (ε= 24 000 $M^{-1}cm^{-1}$) were applied.

$$AC_a = \frac{A(MW)V_a(DF)x10^2}{\varepsilon L W_a} \text{ (mg/100g of fresh tissue) (1)}$$

2.7 Alpha-amylase inhibition assay

The amylase inhibition assay was performed using the chromogenic method adopted from Bernfeld[23].

3. Results and Discussion

3.1 Isolation and Characterization of Betalains from *A. tricolor*

Three pigment fractions were isolated by gel filtration chromatography on Sephadex LH-20 column for leaf extract (Fig. 1). These fractions were analyzed for absorption maxima using a UV-Vis spectrophotometer over a range of 350–700 nm. The pigments absorb light as strongly as betacyanins from red beet[24] and garambullo[25], with only one maximum absorption peak (536 nm) at the range of visible light for the first pigment fraction, other two pigment fractions absorb at 478 and 430 nm. Each column elutes were then subjected to HPLC for further purification.

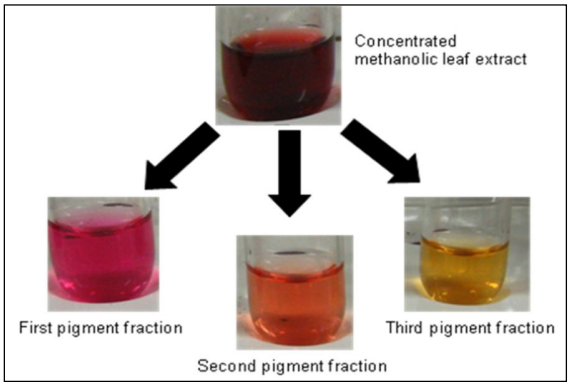

Fig. 1: Pigment fractions collected from Sephadex LH-20 column of the leaf extract

The typical HPLC chromatograms of the Sephadex column purified pigment fractions of the methanolic extracts of *A. tricolor* leaves monitored at 430, 450, 478 and 536 nm are shown in Fig. 2. The wavelengths of absorption bands of the FTIR spectrum (Fig. 3) and some selected assignments to the type vibration (Table 1) of *A. tricolor* pigment (HPLC purified pigment fraction of the Sephadex LH- 20 column fraction 1) from *A. tricolor* leaves was determined. The IR spectrum behavior showed the pigment to be betacyanin, similar to those of red beet[4,26]. The FTIR spectral data in Table 1 further demonstrated that *A. tricolor* pigments had typical functional groups (1653–918 cm^{-1}) of betacyanins. However, the vibration in the range from wave number 3400–3100 cm^{-1} and from 600–474 cm^{-1} might be caused by $-NH_2$, $-OH$, $-H_2O$ and C−S functional groups from the protein or amino acid components and trace water in the pigments (Fig. 3). Thus the presence of betacyanin was confirmed in the first pigment fraction of the leaf extract obtained from Sephadex column after HPLC.

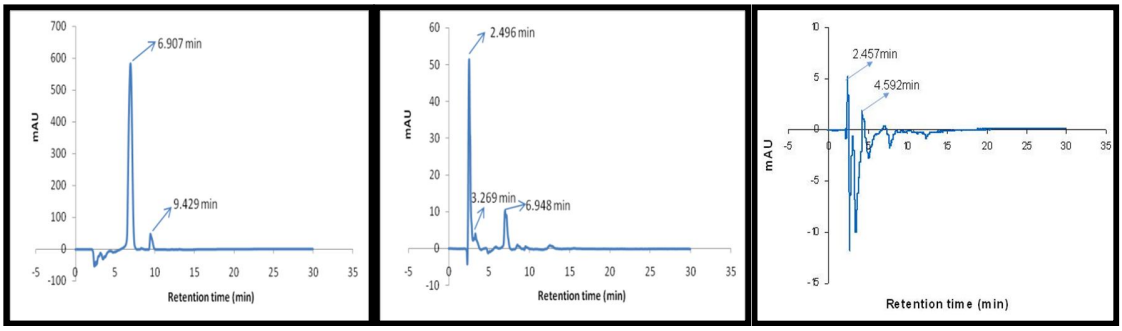

Fig. 2: HPLC profiles of pigment fractions of *A. tricolor* leaves collected from Sephadex LH-20 column

Table 1: FTIR spectral data for *Amaranthus* betacyanins (HPLC purified fraction of the Sephadex LH-20 column fraction1 of leaf extract)

Peaks	Wavenumber (cm^{-1})	Assignment
A	2926.14	O-H str[a]
B	1653.45	C=N str and C=O str[a] (represented by shoulder)
C	1297.61	C-O str[a]
D	1100.15	C-O str[a]
E	918.38	C-H def[b] (benzene ring)

[a]str: stretch; [b]def: deformation

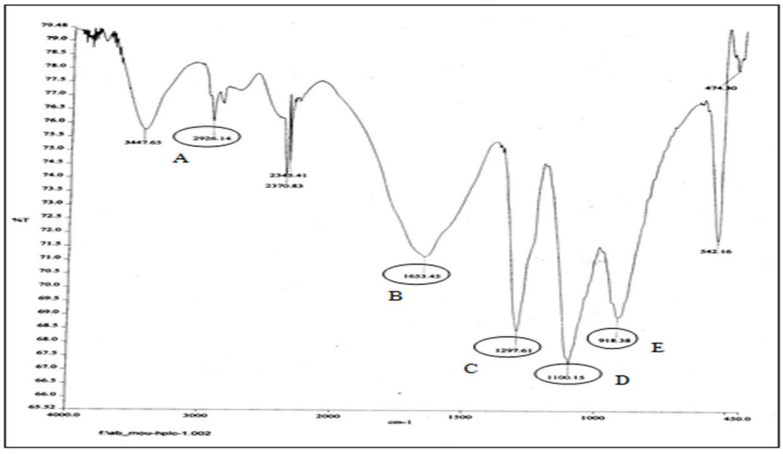

Fig. 3: FTIR spectra of HPLC purified pigment fraction of the Sephadex LH-20 column collected fraction 1 of leaf extract

Electrospray mass spectrometric technique has been successfully used to confirm the molecular mass of betalains and thus provides important structural information[27-29]. The mass spectra of the HPLC purified fractions of the leaf extract are shown (Fig. 4, Fig. 5 and Fig. 6). From the mass spectral analysis, presence of amaranthin as betacyanin, methyl derivative of arginine-betaxanthin as betaxanthin and betalamic acid in the HPLC purified fractions of the pigment fractions 1, 2 and 3 collected from Sephadex LH-20 column of *Amaranthus tricolor* leaf extract was detected respectively (Fig. 4, Fig. 5 and Fig. 6).

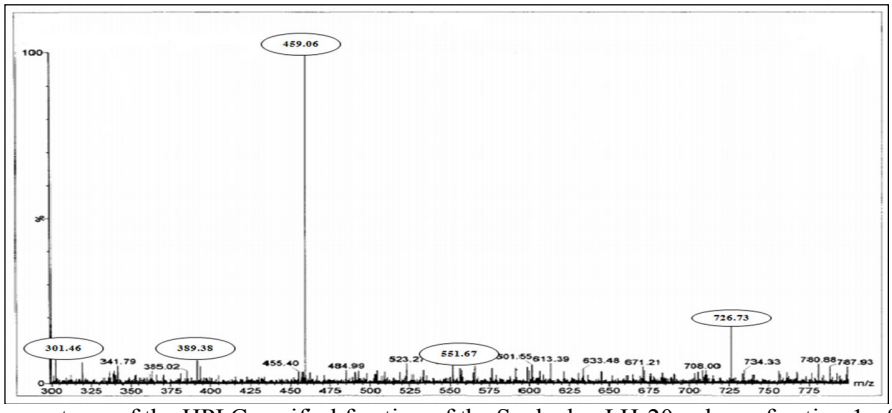

Fig. 4: Mass spectrum of the HPLC purified fraction of the Sephadex LH-20 column fraction 1 of leaf extract

Fragmentation pattern of the HPLC purified fraction of the Sephadex LH-20 column fraction 1 of leaf extract:

a. Amaranthin--------727
b. Amaranthin-Glucuronosyl-----551
c. Neobetanidin 5-O-β-glucoside-2H-2CO_2-----459
d. Betanidin----389
e. Amaranthin-Glucuronosyl-Glucose-2CO_2----301

Thus the presence of amaranthin-type betacyanin in pigment fraction 1 of leaf extract was confirmed through the above fragmentation pattern of the molecule in the mass spectrum.

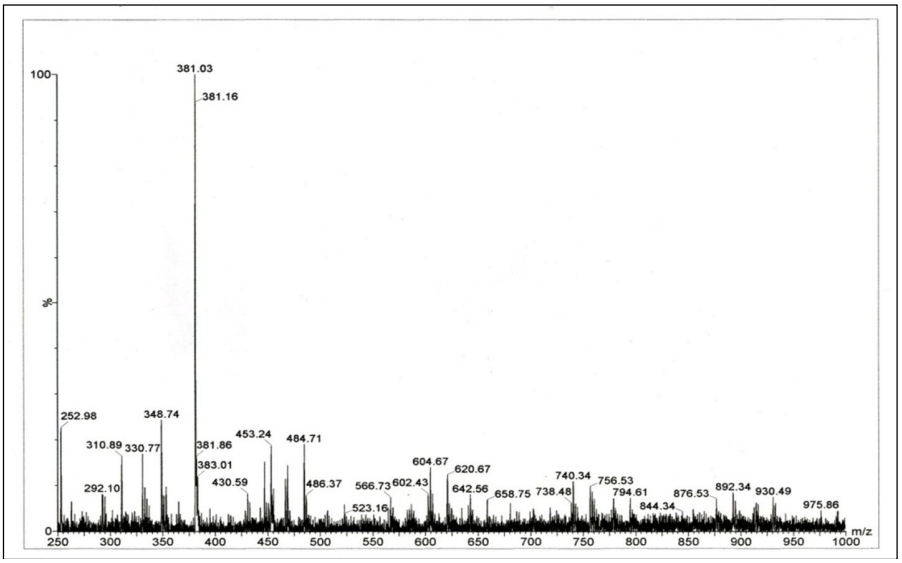

Fig. 5: Mass spectrum of the HPLC purified fraction of the Sephadex LH-20 column fraction 2 of leaf extract

To the best of our knowledge, presence of betaxanthin in *Amaranthus tricolor* leaves has not been reported so far. Arginine-betaxanthin was detected as a novel betaxanthin in *Gomphrena globosa* inflorescences and yellow Ulluco, which has not been reported as a pigment that occurs naturally so far[30,31]. In the present study, their characterization was performed based on UV-Vis and mass spectrometric characteristics, as well as by comparison with literature data[32,33]. Pigment isolated from *A. tricolor* leaf showed in LC-MS a protonated molecular ion at m/z 383 indicative for a methyl derivative of arginine-betaxanthin (m/z 368). Methyl derivative of arginine-betaxanthin was found to be a novel betaxanthin in *A. tricolor*. The first methylated betaxanthin (3-methoxytyramine-betaxanthin) was reported in *Celosia argentea*[6].

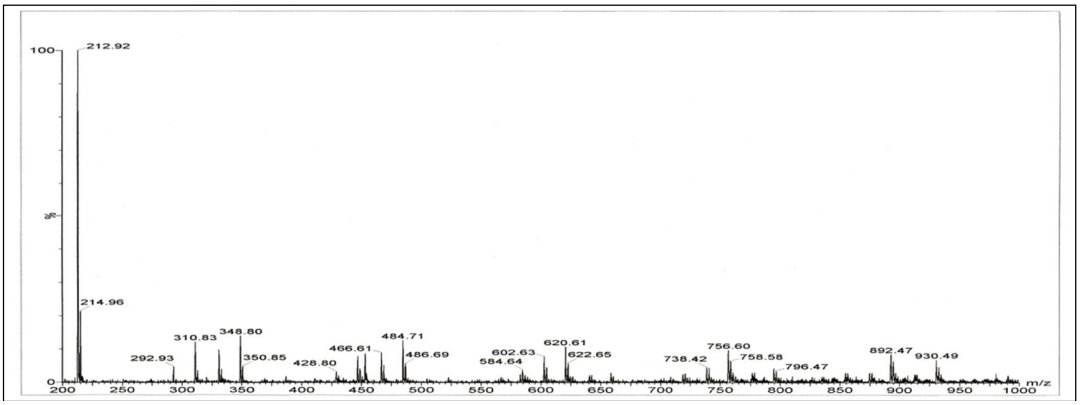

Fig. 6: Mass spectrum of the HPLC purified fraction of the Sephadex LH-20 column fraction 3 of leaf extract

The presence of amaranthin (peak 1 in Fraction 1 HPLC chromatogram; Fig. 2) and isoamaranthin (peak 2) were confirmed first by their identical spectral properties (maximum absorbance at 536 nm), by the presence of their protonated molecular ions $[M + H]^+$ with m/z 727 and by the prominent secondary ion at m/z 389 due to the presence of the protonated aglycones $[betanidin + H]^+$ or $[isobetanidin + H]^+$ (mass spectrum of isoamaranthin is not shown). Both molecules differ only in the absolute configuration of their C-15 chiral center[34]. The maximum absorbance (536 nm) and molecular ion ($[M + H]^+$ at m/z 727) of peak 1 (Fig. 2) suggested that this peak should correspond to a betacyanin structure very close to amaranthin and isoamaranthin. All betacyanins produced a daughter ion at m/z 389, corresponding to $[betanidin + H]^+$ (Fig. 4). The retention of betacyanins decreased with the increase of glycosyl substitution; thus, amaranthine-type betacyanins with two sugar units attached had a shorter retention time than the betacyanins with only one sugar unit attached (betanin or gomphrenin-type); and because the configuration of C-15 isomeric forms for betacyanins allowed greater interaction with the stationary phase, isobetacyanins were retained slightly longer than their parents[5]. Not only did the results agree well with those predicted by reversed-phase chromatography principles, they were also similar to earlier results reported by Heuer et al.[27], Jackman and Smith[35] and Piattelli and Minale[36].

Amaranthin is the major pigment (Table 2) present in *Amaranthus tricolor* leaves along with betaxanthin (methylated arginine-betaxanthin) and betalamic acid. Previous analysis [4, 5, 27, 36] of the betacyanin patterns of *Amaranthus tricolor* and other species, *Iresine herbstii*, *Celosia crisata* and inflorescences from *Gomphrena globosa* revealed the presence of amaranthin and isoamaranthin. Besides the phytochemical findings, the present study is

useful for the evaluation of betalainic Amaranth as a potential coloring foodstuff with high antioxidative properties. Therefore, consumption of red amaranth may provide nutritional and health benefits. The red-violet betacyanins and betaxanthins detected in *Amaranthus tricolor* leaves are summarized in Table 3.

Table 2: Quantification of betalain pigments in *Amaranthus tricolor* leaves

Material used	Individual Betalain composition (mg/100 g fresh tissue)			Total betalain content (mg/100 g fresh tissue)
Amaranthus tricolor leaves	Amaranthin 4.85	Methylated arginine-betaxanthin 1.45	Betalamic acid 1.43	7.73

Table 3: Betacyanins and betaxanthins detected in *Amaranthus tricolor* leaves

Pigment fraction	HPLC Peak	Betacyanin/Betaxanthin	t_R[a] (min)	λ_{max} (nm)	m/z $[M+H]^+$
1st (leaf extract)	1	Amaranthin	6.9	536	727
	2	Isoamaranthin	9.4	536	727
2nd (leaf extract)	2	Methyl derivative of arginine-betaxanthin	3.3	478	383
	3	Amaranthin	6.9	536	727
3rd (leaf extract)	3	Betalamic acid	4.6	430	212

[a] t_R: Retention time

3.2 Alpha-amylase inhibitory activity:

Betalamic acid at 250 µg/mL concentration inhibited the porcine pancreatic α-amylase activity by 22% whereas amaranthin and betaxanthin didn't show any inhibition. However, acarbose used as a reference standard at 250 µg/mL concentration inhibited the porcine pancreatic α-amylase activity by 40.9%. These data are a preliminary result. Further study is needed for developing betalamic acid as an anti-diabetic drug. Drugs that lessen post-prandial hyperglycemia by suppressing hydrolysis of starch such as PPA (porcine pancreatic amylase) inhibitors have been found beneficial in the control of diabetes mellitus[37]. Many plant extracts have been reported for their anti-diabetic activities and are currently being used for the treatment of diabetes[38-43]. The search for natural compounds as potential pancreatic α-amylase inhibitors has gained an increased importance in the recent years. Alpha-amylase inhibitory activities of *Amaranthus spinosus* leaves (methanolic extract) and *Amaranthus caudatus* seeds and methanolic extract of the whole plant have been reported[44,45].

Conclusion:

Amaranthin was the major pigment found to be present in *A. tricolor* leaves, confirmed by the spectral properties, λmax at 536 nm, FTIR spectra demonstrated by the presence of functional groups (1653–918 cm^{-1}) of betacyanin and presence of the protonated molecular ion with m/z 727. A betaxanthin was identified for the first time in *Amaranthus tricolor* species. Methylated arginine-betaxanthin was isolated both from *Amaranthus tricolor* leaves and characterized based on spectral properties such as maximum absorption peak at 478 nm and presence of a protonated molecular ion at m/z 383 in the mass spectrum. Betalamic acid was also detected in *A. tricolor*. The present study demonstrates that

betalamic acid has alpha-amylase inhibitory potential whereas amaranthin and betaxanthin do not.

5. Acknowledgements

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6. Conflict of Interest Statement:

There is no conflict of interest.

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