Blood fruit (*Haematocarpus validus* (Miers.) Bakh. f. ex Forman): a novel source of natural food colourant

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Abstract Plant based pigments have widely been used by human beings since ancient times. These pigments, being natural in origin, have better acceptability amongst the consumers and offer diversified applications worldwide. For identification of novel pigment sources, lesser known species need to be studied systematically. In the present report, a tropical fruit species viz. blood fruit was studied for its physicochemical parameters and anthocyanin content. Fruit pulp had total soluble solids of 17 °B with acidic pH (3.01) and total phenolic content of 13.44 mg GAE/100 g of fresh pulp. Pulp of fully ripe fruits contained 8.76 mg/g of total anthocyanins. Pelargonidin was the dominant anthocyanin, followed by Cyanidin, Peonidin and Petunidin. Considering these qualities, the species could be a potential candidate for food and dye industries.

Keywords Anthocyanins · Food colourant · India · Liana · Menispermaceae

Introduction

Natural colourants have been regarded as safer alternatives to potentially harmful synthetic dyes, especially in food industries. In recent past, demand for natural colorants

including anthocyanins has increased due to their health promoting properties and overall increase in consumer demand for natural foods (Rymbai et al. 2011). Anthocyanins are known to possess industrially desirable attributes as they are attractive in colour and hence, have high consumer preference; soluble in water so could be easily used for colouring processed products such as beverages; exhibit moderate stability during processing and storage and provide health/nutritional benefits (Bagchi et al. 2004; Bohra et al. 2012; Einbond et al. 2004; Ozela et al. 2007; Torslangerpoll and Andersen 2005). A number of plant species have been identified and are being exploited as sources of anthocyanins (Bohra et al. 2012; Fan et al. 2007; Einbond et al. 2004; Pliszka et al. 2008). However, many species are still being used on regional level and their industrial potential is yet to be explored. Identification of such potential species is thus a continuous process as these could probably reveal better sources of dyes for various food processing applications.

Haematocarpus validus (Miers.) Bakh. f. ex Forman is a species of Menispermaceae family found distributed in India, Bangladesh, Indonesia, Singapore, Pakistan and Thailand (Bohra et al. 2018; Singh and Bedi 2016). In India, it is naturally distributed in Tripura, Meghalaya, Assam, Arunachal Pradesh, Sikkim, West Bengal and Andaman and Nicobar Islands. In most of the regions where it is naturally grown, fruits of this species are being relished by the local masses for sweet–sour blood red pulp, and hence the local names *khoon phal* or *rakta phal*, literally meaning blood fruit (Bohra et al. 2018).

Blood fruit is known to be a good source of iron and β -carotene, while the anti-nutritional factors are meager in it (Singh et al. 2014). In the Northeastern Indian states, the species is valued ethno-botanically for preparation of beverages, dyeing local handicrafts and also in medicines

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(Singh and Bedi 2016; Bohra et al. 2018). Considering potential of this species, efforts have been made for its domestication in the Andaman and Nicobar Islands of India (Bohra et al. 2018). Though the species is traditionally used for its dye value, no reports are so far available that describe its colouring compounds. Present is apparently the first report on the anthocyanin profiling in this novel species.

Materials and methods

Collection of sample

Freshly harvested fruits (Fig. 1) were collected from a farmer's field in Kalsi village of Middle Andaman island, India and brought to ICAR-Central Island Agricultural Research Institute, Port Blair for further analysis. Fruits were separated from bunches, kept at room temperature ($28 \pm 2^\circ\text{C}$) and allowed to ripen naturally. One set of fruits was sent to ICAR-Indian Institute of Horticultural Research, Bengaluru for anthocyanin estimation.

Fig. 1 Harvested fresh fruit bunches of *Haematocarpus validus* used in the present study

Physico-chemical analysis

Uniformly ripe twenty fruits were used for recording data on physicochemical parameters. Morphological parameters such as fruit polar/equatorial length (cm), fruit polar/equatorial circumference (cm), fruit weight (g), pulp weight (g), pulp content (%), peel weight (g), peel thickness (cm), seed weight (g), seed length (cm) and seed width (cm) were recorded following standard practices. Total soluble solids (TSS) and pH were estimated using hand held refractometer (Optics Technologies, India) and digital pH meter (Hanna, Romania), respectively. For determination of total phenolic content, Folin–Ciocalteu method was used. Total phenolic content was calculated using the standard gallic acid curve with equation: $Y = 0.176X$ ($R^2 = 0.9916$) and expressed as mg gallic acid equivalent (GAE) per 100 g fresh sample.

Determination of anthocyanins

Extraction of anthocyanins

Anthocyanins were extracted from fresh fruits using method described by Shivashankara et al. (2010). For identification and quantification, single-ion reaction (SIR) and multiple reaction monitoring (MRM) methods were followed as described earlier (Karanjalkar et al. 2018). Anthocyanin standard was procured from Sigma-Aldrich, and Cyanidin was used as internal standard and co-chromatographed with extracted sample. For this, 4 g of pulp sample was extracted in pestle and mortar using acidified methanol (1%) under dark condition, centrifuged at $14,000 \times g$ for 10 min and volume of supernatant was made up to 25 ml using acidified methanol. After eluting 5 ml extract through C-18 SEP pack, the elutant was evaporated in vacuum evaporator, dissolved with 2 ml mobile phase and filtered through $0.2 \mu\text{m}$ nylon filter prior to injection in LC–MS. Alternatively, total anthocyanin content (mg/g) was determined using spectrophotometric method at 540 nm and presented as Cyanidin equivalent.

Table 1 MRM Parameters of anthocyanins standards

Compound	Formula/mass	Parent (m/z)	Cone voltage	Daughters (m/z)	Collision energy	Ion mode
Cyanidin	286.0	287.10	56	137.07	34	ES+
Delphinidin	301.98	303.08	66	229.06	32	ES+
Malvidin	330.0	331.16	40	242.08	34	ES+
Pelargonidin	270.0	271.10	56	121.05	34	ES+

Fig. 2 Chromatographs of **a** MS, **b** MS–MS and **c** calibration curve of Quantifier Daughter for Cyanidin

Fig. 3 Chromatographs of
a MS, **b** MS–MS and
c calibration curve of Quantifier
 Daughter for Delphinidin

Fig. 4 Chromatographs of **a** MS, **b** MS–MS and **c** calibration curve of Quantifier Daughter for Malvidin

Fig. 5 Chromatographs of
a MS, **b** MS–MS and
c calibration curve of Quantifier
 Daughter for Pelargonidin

LC–MS/MS

An Acquity UPLC-H class coupled with TQD-MS/MS (Waters, USA) equipped with a degasser, quaternary pump, automatic injection system (0–10 µL), MSD with ESI(±) probe and a temperature control compartment for the analytical column was used for anthocyanin profiling. The overall system was controlled by the Mass lynx software, which also administered the data collection and processing.

The mobile phase consisted of an aqueous phase of 0.25% formic acid in water (A) and organic phase of 0.25% formic acid in acetonitrile (B). With the flow rate of 0.25 mL/min, gradient flow was initially set at 100% aqueous phase (A), for 0.5 min, then linearly the mobile phase composition changed to 30% A and 70% B at 10 min held for 0.5 min and further a linear gradient followed, arriving at 100% A in 18 min. The analytical column 2.1 × 50 mm UPLC BEH-C18 column (Waters) with 1.7 µm particles, protected by a Vanguard BEH C-18 with 1.7 µm guard column (Waters) was used at 25 °C. The sample injection volume was 5 µL.

Anthocyanins eluted from UPLC column effluent was pumped directly without split into TQD-MS/MS (Waters, USA) system, optimized for anthocyanins analysis (Table 1). The samples were analyzed using TQD Mass spectra (Waters) through ES + (positive ionization) mode with following MS conditions: cone voltage (32 V); capillary voltage (3.5 kV); source temperature (135 °C); desolvation temperature (350 °C); cone gas flow (50 L/h); desolvation gas (N₂); flow rate (600 L/h) with MRM and in SIR mode for some of the selected compounds as Peonidin and Petunidin that showed most abundant forms of protonated [M + H]⁺ molecules of anthocyanidin compounds. The relative quantity was calculated from peak area of sample as compared with standard value equivalent to anthocyanidin standards viz. Cyanidin (catalog no. 79457) purchased from Sigma Chemical Co., USA used as internal standard recorded and the relative amount of the compound was calculated by the standard value equivalent to Cyanidin.

Linearity, detection and quantification limits and repeatability were used as parameters to validate the methodology for determination of available anthocyanins. Series of standards of targeted anthocyanidins were prepared by serial dilution in HPLC grade methanol for creating a calibration curve and linearity was determined by constructing calibration curves, and three injections were made at each level (Figs. 2, 3, 4, and 5). The values for the correlation coefficients (r) were 0.95 ± 0.05. The multiple reaction monitoring (MRM), the most sensitive detection mode, was employed for analysis and quantification except for Petunidin and Peonidin, wherein SIR method was used and results were expressed as equivalent to Cyanidin.

Results and discussion

Physico-chemical analysis

Fruits of blood fruit are borne as a cluster directly on stem during the fruiting season (April–June) and whole vine is laden with fruit bunches. As the fruits mature and start ripening, the peel colour changes from green to dark red or purple. At colour break stage, fruits are harvested and sold in the local markets for fresh consumption. Various physicochemical parameters of fruits have been presented in Table 2. Fruits were 3.6 cm long and 2.7 cm wide with polar circumference of 10.6 cm and equatorial

Table 2 Physicochemical parameters of blood fruit

Parameters	Value
<i>Fruit parameters</i>	
Fruit polar length (cm)	3.6 ± 0.05
Fruit polar circumference (cm)	10.6 ± 0.17
Fruit equatorial length (cm)	2.7 ± 0.03
Fruit equatorial circumference (cm)	9.2 ± 0.08
Fruit weight (g)	12.7 ± 0.34
Peel weight (g)	7.2 ± 0.19
Peel thickness (mm)	3.4 ± 0.13
Pulp weight (g)	2.9 ± 0.2
Pulp (%)	22.7 ± 1.17
<i>Seed parameters</i>	
Seed length (cm)	2.3 ± 0.04
Seed width (cm)	1.3 ± 0.04
Seed thickness (cm)	1.0 ± 0.02
Seed weight (g)	2.6 ± 0.09
<i>Biochemical parameters</i>	
Total soluble solids (°B)	17.0 ± 0.00
pH	3.01 ± 0.00
Total phenolic content (mg GAE/100 g)	13.44 ± 0.077

Table 3 Major anthocyanins identified in the pulp of blood fruit

Compound	Content (mg/g) as Cyanidin equiv
Pelargonidin	4.997 ± 1.673
Cyanidin	2.939 ± 0.585
Peonidin	0.729 ± 0.115
Petunidin	0.084 ± 0.005
Delphinidin	0.009 ± 0.000
Malvidin	0.001 ± 0.000
Total anthocyanins	8.760
Total anthocyanins (as Cyanidin equivalent) spectrophotometrically at 540 nm	10.850

circumference of 9.2 cm. Individual fruits weighed to 12.7 g in which 22.7% i.e. 2.9 g of edible pulp was present. The peel is inedible but quite thick (3.4 mm), which could help in facilitating long distance transportation of the produce. Each fruit has a seed of about 2.3 cm length, 1.3 cm width and 1.0 cm thickness. Total soluble solids content in pulp of fruits was found to be 17.0 °B with acidic pH of 3.01. This blend of sugar and acidity makes the fruits sweet–sour, which are relished by the native populations of the Andaman and Nicobar Islands. Total phenolic content of 13.44 mg GAE/100 g fresh sample was observed in the pulp of blood fruit.

Pulp is the economic portion of the fruit; however, pulp recovery of mere 18.38% and 22.00% have been reported in fruits collected from Andaman islands and Bangladesh (Bohra et al. 2018; Khatun et al. 2014). Phenolic compounds are known to possess antioxidant activities and hence, use of natural colourants such as blood fruit in processing would improve the nutritional properties of the processed products. Considering the industrial potential of the species, identification of genotypes with higher pulp content could be of great significance (Bohra et al. 2018). Also, types in which pulp doesn't adhere to seeds (free seeds type) will be a boon for getting more efficient and easier recovery of pulp for value addition. As the natural populations are of seedling origin, they need to be tapped for such traits.

Anthocyanins content in blood fruit

Literature survey suggested that detailed chromatographic studies pertaining to species of the family Menispermaceae have not been attempted so far. However, presence of anthocyanins has been reported in the fruits of *Cocculus hirsutus* and *Tinospora cordifolia* (Sharma et al. 2014; Rakkimuthu et al. 2016). Total anthocyanins to a tune of 8.76 mg/g were present in blood fruit pulp during present study (Table 3), which was comparatively higher than the values reported from the fruits of *Tinospora cordifolia* i.e. 19.061 mg/100 g total anthocyanins (Sharma et al. 2014).

Various anthocyanidins found in the pulp of ripe blood fruit are presented in Table 2. Pelargonidin was found to be the most dominant anthocyanin (4.997 ± 1.673 mg/g) in the pulp, which was followed by Cyanidin (2.939 ± 0.585 mg/g), Peonidin (0.729 ± 0.115 mg/g) and Petunidin (0.084 ± 0.005 mg/g). Malvidin and Delphinidin were found in trace amounts. Earlier researchers have identified various natural sources of anthocyanins viz. Mulberry (*Morus indica*), grapes (*Vitis* spp.), cranberries (*Vaccinium* spp.), red cabbage (*Brassica oleracea* var. *capitata* f. *rubra*), black chokeberry (*Aronia melanocarpa*), strawberry (*Fragaria ananassa*), bilberry (*Vaccinium myrtillus*), Surinam cherry (*Eugenia uniflora*), jaboticaba

(*Myrciaria cauliflora*), star apple (*Chrysophyllum cainito*), kokum (*Garcinia indica*), basella (*Basella rubra*), banana (*Musa* spp.) bracts etc. (Einbond et al. 2004; Fan et al. 2007; Pliszka et al. 2008; Shivashankara et al. 2010; Bohra et al. 2012).

The composition of component pigments and their relative proportion varies greatly with species. As proposed by Fang (2015), anthocyanin rich fruits could be broadly classified into three major categories viz. Pelargonidin group, Cyanidin/Peonidin group and multiple anthocyanins group. Fruits of *Myrciaria cauliflora* and *Chrysophyllum cainito* had Cyanidin-3-glucoside as dominant constituent, while fruits of *Eugenia uniflora* had delphinidin-3-glucoside as additional compound to Cyanidin-3-glucoside (Einbond et al. 2004). Identification of novel sources is of prime importance to diversify the income avenues to the cultivators and also to offer new products in the markets. For example, banana inflorescence bracts were found to yield 14–32 mg anthocyanin/100 g bracts in which Cyanidin-3-rutinoside was the dominant compound (Padam et al. 2014). Hence, high anthocyanin content of blood fruit in the present report could make it a potential source of anthocyanins for food and dye based industries. Interestingly, Fang (2015) in his review observed a typical trend suggesting reproducible potency for anti-inflammatory property in fruits containing Cyanidin, Pelargonidin or Peonidin, all of which were also dominant in blood fruit pulp as observed in the present study. This further opens up opportunities for exploring the medicinal potential of this novel anthocyanin source.

Conclusion

Present study revealed the potential of blood fruit, an underutilized fruit species, as a novel source of anthocyanins with Pelargonidin and Cyanidin as dominant compounds. Hence, the species could be utilized as a natural colourant in various food industries.

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