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Elucidation of the betalainic chromoalkaloid profile of *Pilosocereus catingicola* (Gürke) Byles & Rowley subsp. *salvadorensis* (Werderm.) Zappi (Cactaceae) from Paraíba, Brazil

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The cacti are important plant genetic resources of the Brazilian semiarid region, with potential for the extraction of bioactive compounds such as flavonoids and alkaloids. The objective of this research was to characterize and quantify the chemical constituents of *Pilosocereus catingicola* (Gürke) Byles & Rowley subsp. *salvadorensis* (Werderm.) Zappi occurring in *caatinga* in the Westland of Paraíba, Brazil. We collected roots, stems and fruit of plants growing in populations at Arara, Areial and Boa Vista. Chemical characterization of the different plant tissues showed that roots and stems of *P. catingicola* contained steroids, flavonoids and saponins whereas fruit was dominated by high levels of betalainic chromoalkaloids (betalains). Tests performed to optimize extraction of betalains from *P. catingicola* fruit showed highest yields were from freeze-dried nuts extracted for 95 min, with the crude extract stored at -20°C for a maximum of 48 h. The betalains of all fruit samples were dominated by betacyanins with much lower amounts of betaxanthins observed in each population. Numerous betacyanin constituents were detected in fruit extracts, with the key constituents identified as betanin and phyllocactin. The Arara population yielded relatively more betaxanthins compared to plants from the Areial and Boa Vista regions.

Key words: Betacyanin, betanin, betaxanthin, caatinga, cactus, facheiro, phyllocactin.

INTRODUCTION

Plants in the Family Cactaceae (Order Caryophyllales) are indigenous to the Americas, possibly arising there some 30 million years ago during the mid-Tertiary period (Hershkovitz and Zimmer, 1997). The family is morphologically diverse, ranging from tree-sized specimens (e.g. *Pereskia sacharosa*) to creeping forms (e.g. *Ariocarpus retusus* subsp. *retusus*). Species occurrence is widespread throughout the tropical region

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of America, and in particular, Cactaceae members occupy vast areas in the semi-arid region of northeastern Brazil (Anderson, 2001).

An increasing variety of metabolites expressed by plants are targets of pharmacology studies due to their beneficial effects on living organisms such as antioxidant activity, whereby they prevent the oxidative stress caused by cellular metabolism, particularly by peroxidases and reactive oxygen species. Moreover, two classes of compounds extracted from within the Order Caryophyllales have received a great deal of attention due to their potential pharmaceutical applications - anthocyanins and betalainic chromoalkaloids (betalains), the latter restricted to a small group of only ten species (Chauhan et al., 2013).

Betalains are a group of nitrogen-based plant-products belonging to the alkaloid class and are biosynthesised via the shikimic acid pathway. They have known roles in preserving the integrity of cells by preventing oxidative processes caused by the action of free radicals that lead to apoptosis. Most reported betalains occur in flowers and fruits, but they can also be found in roots and leaves of plants belonging to the Order Caryophyllales where they effectively replace flavonoids such as anthocyanins in roles related to plant coloration (Gandía-Herrero and García-Carmona, 2013).

Betalains are characterized by vivid colors ranging from purple to violet or yellow and orange depending on the chains linked to the betalamic acid precursor of this metabolite. They are divided into two subgroups: the betaxanthins characterized by yellow, orange and red colours, which include the compounds vulgaxanthin, miraxanthin, portulaxanthin and indicaxanthin; and the purple-colored betacyanins found in certain vegetables as the compounds betanin, isobetanin, neobetanin, protobetanin and phyllocactin (Strack et al., 2003).

Several studies with cultured cacti have shown the nopal fruit (*Opuntia ficus-indica*) to be a promising commercial source of antioxidant compounds, particularly betalains (Stintzing et al., 2002; Wybraniec and Nowak-Wydra 2007; Castellanos-Santiago and Yahia, 2008; Jerz et al., 2008; Wybranec et al., 2009; Gandía-Herrero and García-Carmona, 2013). In addition, Anderson (2001) reported the presence of nitrogenous natural pigments, which include the betacyanins (violet color) and betaxanthins (orange), in *Lophophora williamsii* together with over 50 different types of other alkaloids.

The aforementioned studies were undertaken to characterise the diversity of alkaloids in plants within the genus *Opuntia*. Despite this work, there have been no such chemical studies of native cacti in Brazil. Surveys that have been done have related exclusively to the occurrence and distribution of plants in the genera *Cereus*, *Pilosocereus*, *Harrisia*, *Melocactus* and *Tacinga* in the arid zone of Paraíba. These plants have wide distribution in local ecosystems, but there has been a lack of investigations on their chemical constituents or potential uses and applications. It is likely that species in these genera may be important sources of functional compounds such as betalains.

The objective of this research was to characterize and quantify the betalainic constituents extracted from different tissues of *Pilosocereus catingicola* (Gurke) Byles & Rowley subsp. *salvadorensis* (Werderm.) Zappi occurring in populations within the *caatinga* areas of the state of Paraíba, Brazil.

MATERIALS AND METHODS

Sample collection

Plant samples were collected from three forest fragments of *caatinga* in the regions of Arara, Areial and Boa Vista, belonging to the middle Westland of Paraíba, Brazil (Figure 1). The three areas were chosen based on the criterion of having large number of specimens of *Pilosocereus* spp. reported by Barbosa et al. (2015).

Material collection and preparation of samples

Four samples were collected from each of the three populations (N = 12). The collection proceeded randomly at 8 am observing the cardinal points: North, South, East and West, where North was the reference point, and samples were harvested using a cleaned and sanitized knife hoe. The material was placed in a thermal box and taken to the Laboratory of Phytochemistry - Pharmaceutical Technology Laboratory (Prof. Raimundo Braz Filho) of the Universidade Federal da Paraíba, João Pessoa, Brazil and dried at 40°C for 120 h. After drying, samples were ground to a fine powder using a grinding mill (CQA m120) and then 50 g of each sample was packed into 250 mL plastic containers.

Extraction and determination of betalains in fruit

Dried samples were transported to the Plant Physiology Laboratory (Prof. Ian Woodrow), School of BioSciences, University of Melbourne, Australia. The protocol for extraction was adapted from Stintzing et al. (2002), with the modifications that 2 g of the dried pericarp was mixed with 5 mL of 50% MeOH and 50 mmol L^{-1} sodium ascorbate. Initially, the dried product was ground and then packed into 50 mL tubes. Each extraction was repeated five times. After solubilization with MeOH, the material was stirred by vortexing for 5 min at 25 °C, then centrifuged at 2000 rpm (25 °C). The supernatant was collected and concentrated under full vacuum centrifuge (SpeedVac) and then dissolved in 1 mL 100% MeOH and stored at -20°C.

For the hydrocolloid precipitation, 2 mL of 96% ethanol was added to 1 mL of the ground sample; the material was then left for 20 min for precipitation of proteins and mucilage. For separation, a 0.45 µM membrane was used (Phenomenex, Torrance, CA, USA) and samples were washed with 2 mL of ethanol/water (2:1, v/v). The ethanol was removed under reduced temperature using a SpeediVac at a temperature of 30°C and then the residue was redissolved in acidified water (pH 3).

The absorbance of extracts was measured using a spectrophotometer Mertek® SP-870 (600 nm; Sanford, North Carolina, USA) and the betalain content mg 100 g^{-1} = (A*F*MW*100/ε*l) where A = Absorbance; F = Dilution factor; MW = Molecular weight (indicaxanthin = 308 g mol^{-1} and betanin = 550 g mol^{-1}), ε is the molar extinction coefficient (indicaxanthin = 48,000 L

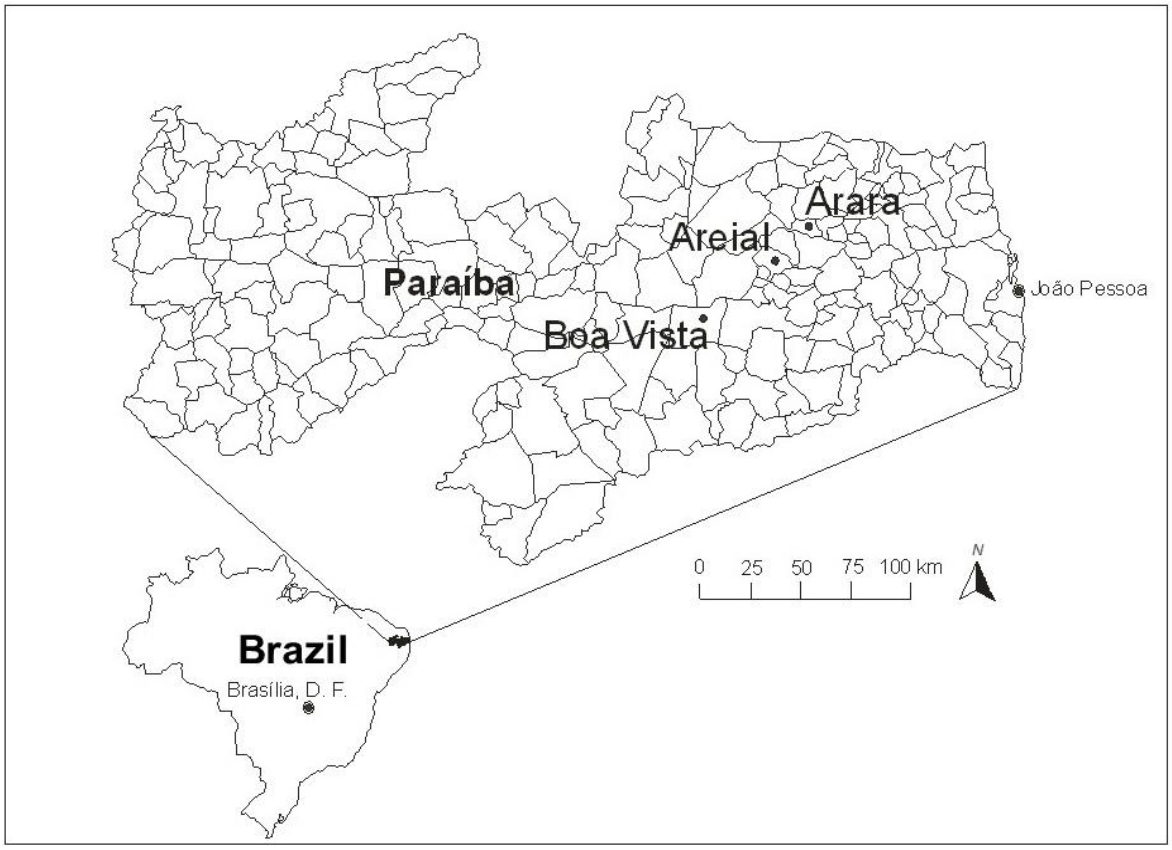

Figure 1. Location of sampled populations of *Pilosocereus catingicola* (Gurke) Byles & Rowley subsp. *salvadorensis* (Werderm.) Zappi within Brazil. (a) Map of Brazil showing the location of the region of Paraíba (box); scale bar represents 500 km. (b) Magnified map of the region of Paraíba showing the location of the three sampled populations; scale bar represents 50 km.

mol^{-1} cm^{-1} and betanin=60,000 L mol^{-1} cm^{-1}), and 1 = path length (1 cm) of the cuvette.

The separation of betalains was performed using reversed phase high performance liquid chromatography (RP-HPLC) and identification based on liquid chromatography mass spectrometry (LC-MS). We used an Agilent HPLC system (Santa Clara, CA, USA) 1200 with photodiode array detector and a LUNA C18 column (Phenomenex). Eluent A consisted of 0.2% trifluoroacetic acid (TFA) and 10% formic acid [HCOOH (65:35, v v^{-1})] and eluent B prepared with a mixture of 100% acetonitrile and 10% HCOOH (80:20, v:v). The system was coupled to an Agilent 6520 Quadrupole with time of flight mass spectrometer (QTOF MS) with electro-spray ionisation (ESI). The ESI-QTOF MS was operated in positive mode using the following conditions: pressure of nebulizer 35 psi, the gas flow 11 L min^{-1}, the gas temperature 325°C, capillary voltage 4000, Fragmenter 150 and skimmer 65 V. Data were collected based on three replicate runs and compounds identified based on UV absorbance, retention times and comparison of parent and MS2 fragment masses with literature values.

RESULTS AND DISCUSSION

Quantification and profile of betalains

The betacyanin concentration (mg 100 g^{-1} DM) of plants did not differ significantly between populations (Figure 2). The mean betacyanin contents were 17.4; 18.3 and 14.0 mg 100 g^{-1} for Arara, Areial and Boa Vista, respectively. Nevertheless, the betaxanthin content was significantly different between populations, with the highest content found in Arara (11.2 mg 100 g^{-1}) compared to Areial (8.1 mg 100 g^{-1}) and Boa Vista (6.3 mg 100 g^{-1}; Figure 2). The mean total content of betalains (betacyanins + betaxanthins) was 28.7; 26.4 and 20.4 mg 100 g^{-1} for populations of Arara, Areial and Boa Vista, respectively (Figure 2).

The betalain concentration found here for *P. catingicola* is quite similar to that found in other Cactaceae species such as *Opuntia stricta* (80.1 mg 100 g^{-1}), *O. ondulata* (19.6 mg 100 g^{-1}) and *O. ficus-indica* (15.2 mg 100 g^{-1}; Castellar et al., 2003), and the report by Castellanos-Santiago and Yahia (2008) of 81.0 mg 100 g^{-1} in *O. ficus-indica* varieties. However, the betalain concentrations in *P. catingicola* are lower than those of other groups such as *Beta vulgaris* plant varieties with 40-60 mg 100 g^{-1} (Von Elbe et al., 1981), and some Amaranthaceae reported by Cai et al. (2005) where values between 15.4 and 46.9 mg 100 g^{-1} were found in the dry tissue.

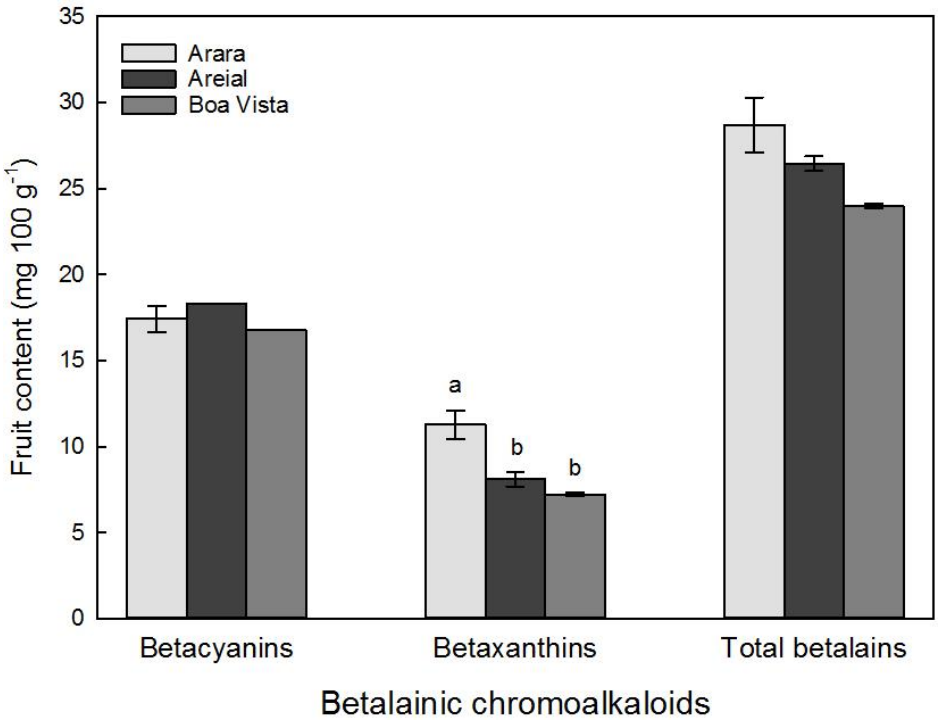

Figure 2. Total betalains (betacyanins and betaxanthins) quantified in extracts of *P. catingicola* subsp. *salvadorensis* fruit collected from three populations in Paraíba, Brazil. Data were analysed with one-way ANOVA with Tukey's post-hoc tests. The only significantly different result was observed for betaxanthins extracted from the Arara population (ARA, Arara; AR, Areial; BV, Boa Vista).

The betacyanin HPLC profile was qualitatively similar for all specimens studied (Figure 3); however, the relative amounts of some constituents differed between sampling sites, although no clear trend could be detected.

The first peak in the chromatogram was consistently the most abundant compound and was identified as the betacyanin betanin (Figure 3) at a retention time (RT) of 10.53 min. The compound had a parent mass of *m/z* 551.15 corresponding to $[M+H]^+$ $C_{24}H_{27}N_2O_{13}$ with key MS2 fragments of *m/z* 389, 309, and 507. This is identical to the fragmentation pattern found for betanin in numerous studies (e.g. Wybraniec and Nowak-Wydra (2007) in cactus fruit of *Mammilaria gender*; Strack et al. (2003) in Amaranthaceae plants; Stintzing et al. (2002) in fruits of *O. ficus-indica*; Jerz et al. (2008) in fruits of *Phytolacca americana*; and Wybraniec et al. (2009) in fruits of *Hylocereus polyrhizus* (Cactaceae).

The second peak was present in only trace amounts in all samples and was characterized as a 2'-O-apiosyl-isobetanin (RT 12.73 min; *m/z* 683.19 corresponding to $[M+H]^+$ $C_{29}H_{34}N_2O_{17}$ with fragment ions at *m/z* 551, 389 and 345; Figure 3). This betacyanin was previously unknown in Cactaceae, but recently it was elucidated by means of HPLC-ion interaction chromatography and mass spectrometry by Wybraniec et al. (2009) as an isomeric form of betanin.

The third peak was characterized as isobetanin (RT 12.96 min; *m/z* 551.15 corresponding to $[M+H]^+$ $C_{24}H_{26}N_2O_{13}$ with fragment ions 389, 507 and 344; Figure 3), and this matched the mass fragmentation pattern observed by Wybraniec and Nowak-Wydra (2007), Stintzing et al. (2002) and Jerz et al. (2008) in *Mammilaria*, *O. ficus-indica*, and *Phytolacca americana,* respectively.

The fourth peak was observed at low levels and was characterized as 17-decarboxybetanin (RT 13.51 min; *m/z* 507.16 corresponding to $[M+H]^+$ $C_{23}H_{26}N_2O_{11}$ with fragment ions of 345, 399 and 307 (Figure 3). This compound has also been characterized by Wybraniec-Wydra and Nowak (2007) in *Mammilaria*. Similarly, tests conducted by Jerz et al. (2008) using Ion-Pair High-Speed Counter-Current Chromatography (IP-HSCCC) analysis confirmed the presence of decarboxylated (2-decarboxy and 17-decarboxy) betanin/isobetanin and neobetanin, betanin derivatives in *P. americana* extracts. Such decarboxylated betacyanins can be produced as degradation products when alcoholic extracts are heated (Wybraniec et al., 2009). Therefore it is possible that the presence of decarboxy-betacyanins in the samples presented here may be indicative of decarboxylation occurring during the extraction process, but given extractions were performed under ambient temperature,

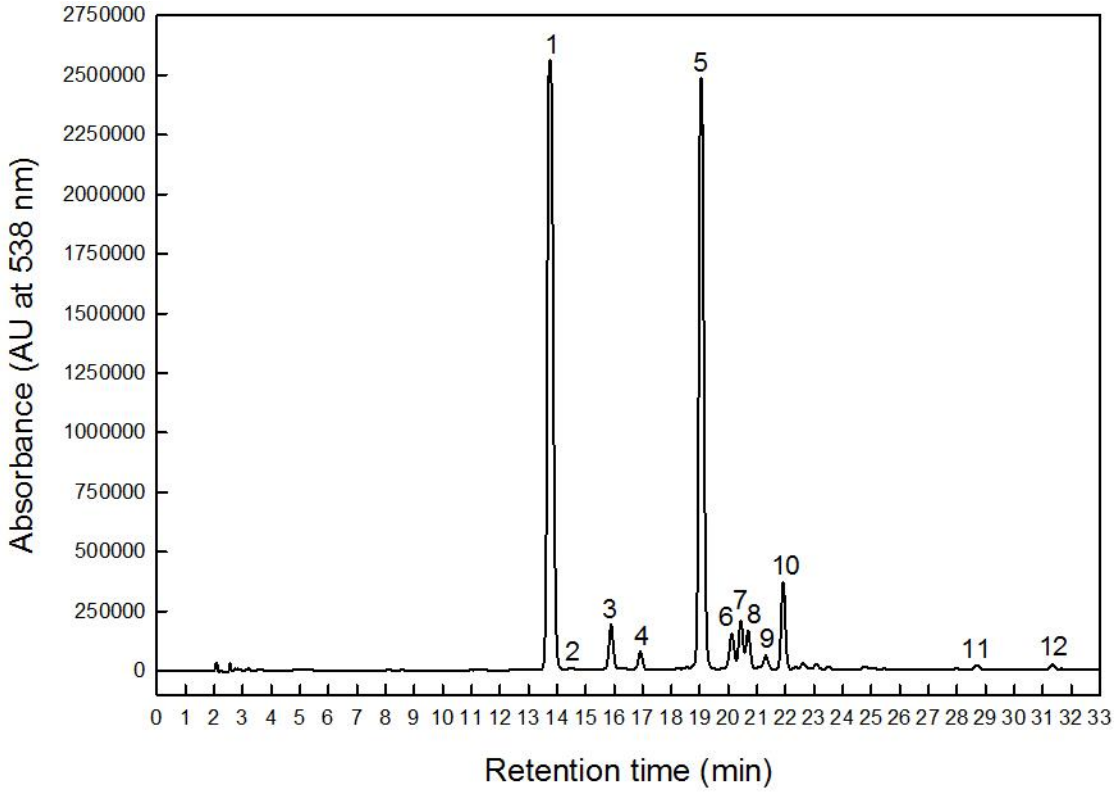

Figure 3. Representative HPLC chromatogram (λ 538 nm) of methanol extracted betalains from fruit of *P. catingicola* subsp. *Salvadorensis* collected from Paraíba, Brazil. Twelve betalainic chromoalkaloids were present in all samples collected from populations at Arara, Areial and Boa Vista. Compounds were identified by mass spectometry as: 1) betanin, 2) 2'-O-apiosyl-isobetanin, 3) isobetanin, 4) 17-decarboxybetanin, 5) phyllocactin, 6) 15-decarboxy-betanin, 7) isophyllocactin, 8) 6'-O-malonyl-2-decarboxybetanin, 9) unknown phyllocactin derivative, 10) 2'-*O*-apiosyl-phyllocactin, 11) 2'-(5''-O-E-Feruloylapiosyl)betanin, and 12) lampranthin II.

this is perhaps unlikely. Nonetheless, it has also been shown that decarboxylation of pigments can occur if the time between harvesting and freezing of the material is prolonged (Wybraniec and Nowak-Wydra, 2007). Although the time was minimised in this study, it remains possible that the observed decarboxy-betacyanins are artefacts of extraction.

The second most abundant compound in all samples was the fifth peak, identified as phyllocactin (RT 15.2 min, *m/z* 637.15 corresponding to $[M+H]^+$ $C_{27}H_{28}N_2O_{16}$ with MS2 ions 593, 389 and 551; Figure 3). Strack et al. (2003) characterised a compound with matching mass as a betacyanin from Cactaceae fruit. In that study, the connection of the malonyl residue of phyllocactin to glucose was deduced by analysis of permethylation and was confirmed by nuclear magnetic resonance (NMR) of phyllocactin isolated from *Schlumbergera buckleyi* flowers (Cactaceae; Minale et al., 1966; Kobayashi et al., 2000).

The sixth peak was characterized as 15-decarboxy-betanin (RT 16.06 min; *m/z* 507 corresponding to $[M+H]^+$ $C_{23}H_{25}N_2O_{11}$ with MS_2 ions 389 and 375; Figure 3). This compound has previously been described as being exclusive to *B. vulgaris* and its decarboxylated form as an artifact of extraction under elevated temperature (Herbach et al., 2004).

The seventh peak was characterized with an isophyllocactin (RT 16.06 min; *m/z* 637.16 corresponding to $[M+H]^+$ $C_{27}H_{28}N_2O_{16}$ with *m/z* 389 and 375 daughter ions; Figure 3) consistent with the molecular fragmentation found by Wybraniec and Nowak-Wydra (2007) and Wybraniec et al. (2009) in extracts from *H. polirhizus*.

The eighth peak was identified as 6'-O-malonyl-2-decarboxybetanin (RT 16.25 min; *m/z* 593.16 corresponding to $[M+H]^+$ $C_{26}H_{28}N_2O_{14}$ with *m/z* 345 and 389 daughter ions; Figure 3) with identical molecular fragmentation found by Wybraniec and Nowak-Wydra (2007) in *H. polirhizus* extracts.

A low abundance compound was observed as the ninth peak and remains unidentified. It is likely an unknown betacyanin (RT 16.89 min; *m/z* 785.24 corresponding to $[M+H]^+$ $C_{32}H_{36}N_2O_{21}$ with *m/z* 311 452 and 637 daughter ions; Figure 4). A search of the Scifinder database found

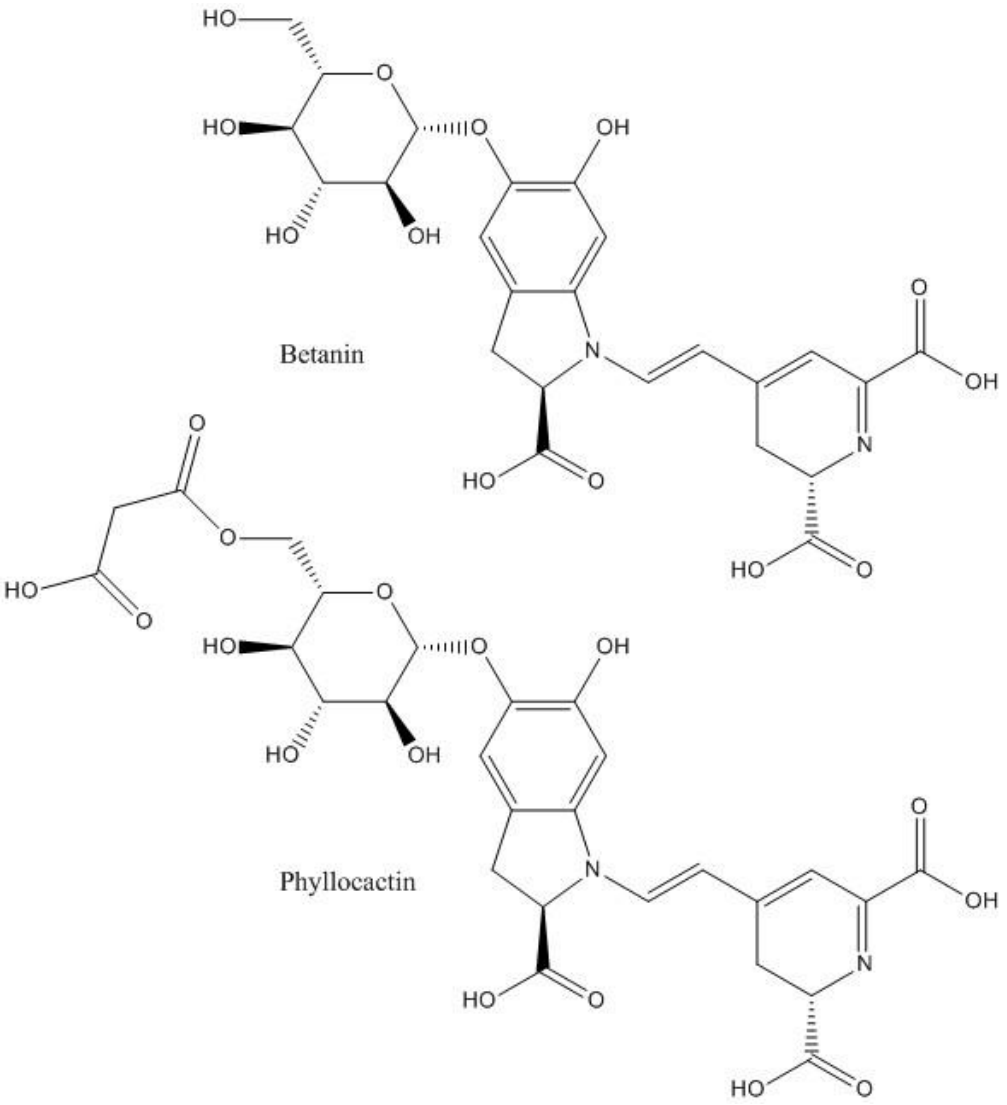

Figure 4. Chemical structures of the betacyanina betanin and phyllocactin, the two most abundant betalains found in fruit of *P. catingicola* subsp. *salvadorensis* collected from Paraíba, Brazil.

no known betacyanins matching that molecular formula. We suggest this compound is a novel betacyanin with a similar base structure to phyllocactin due to the 637 daughter ion, but with additional ornamentation. Further experiments using NMR spectroscopy are required to elucidate its structure.

The third most abundant compound in all samples was the tenth peak which was identified as 2'-*O*-apiosyl-phyllocactin (RT 17.5 min; *m/z* 769.19 corresponding to $[M+H]^+$ $C_{32}H_{36}N_2O_{20}$ with *m*/*z* 549, 637 and 389 daughter ions; Figure 3). This compound had previously been identified from Cacti by Wybraniec et al. (2010).

The eleventh peak was present in very low amounts, but could be identified as 2'-(5''-O-E-Feruloylapiosyl)betanin (RT 22.28 min; *m/z* 859.24 corresponding to $[M+H]^+$ $C_{39}H_{42}N_2O_{20}$ with *m*/*z* 333, 377 and 571 daughter ions). This compound has been reported by Strack et al. (2003) in experiments with plants of the Amaranthaceae family and by Kobayashi et al. (2000) examining betalains from Christmas cactus.

Despite also occurring in very low amounts, the twelfth peak was identified as lampranthin II (RT 30.3 min, *m/z* 727.2 corresponding to $[M+H]^+$ $C_{34}H_{34}N_2O_{16}$ with *m*/*z* 303, 585 and 389 daughter ions; Figure 3). Strack et al. (2003) analyzing the structure of lamprantin II (6'-OE-feruloyl-betanin) confirmed the presence of a pentose using NMR analysis. In addition, Vogt et al. (1999) observed accumulation of this compound in response to high light stress in *Mesembryanthemum crystallinum* epidermal layers (Aizoaceae).

Liquid chromatography coupled to mass spectrometry and photodiode array detector enabled us to successfully characterise the betalainic constituents of *P. catingicola* subsp. *salvadorensis* occurring in the *caatinga* areas in the state of Paraíba, Brazil. Similar approaches have achieved betalain separation and identification in fruits of *Opuntia* spp. (Castellano-Santiago and Yahia 2008) and fruits from *B. vulgaris* and *O. ficus-indica* (Stintizing et al. 2002). Another, potentially superior technique for identifying phytochemicals such as betalainic chromoalkaloids in extracts is LC-NMR, which can provide unambiguous structure of the molecule (Stintizing et al., 2003). For example, studies undertaken by Stintizing et al. (2005) analyzed betacyanin pigments by LC-NMR and 2D NMR spectroscopy in *Hylocereus polyrhizus* at neutral pH. Similarly, Wybraniec et al. (2006) and Wybraniec-Wydra and Nowak (2007) characterized phyllocactin, hilocerenin and betanin in *H. polyrhizus* and *H. mamilarinina* using 1H and 13C NMR spectroscopy.

In recent years there has been increasing interest in the development of natural dyes, mainly due to the apparent toxicity of synthetic dyes and their roles as environmental pollutants - this demand is being driven by strong consumer pressure. Consumer awareness is increasing day by day and is culminating in the interests of consumers to acquire natural food with integrity. Therefore, identification of natural alternatives to synthetic pigments is an important avenue of research for the food industry (Wang et al., 2006).

The cacti are prime candidates for providing natural dyes to replace synthetics. Among these plants, Park et al. (1998) and Stintizing et al. (2005) reported *O. ficus-indica* as a promising source of betalain compounds, not only as coloring agents but also an agents with the purpose of promoting cellular integrity through removal of free radicals and additional chemical reducing properties. Extracts of *O. ficus-indica* fruits exhibited antioxidant activity in various *in vitro* assays, including the oxidation of lipids in red blood cells of the blood and the oxidation of human LDL induced by copper and 2,2-azobis (2-amidinopropane) dihydrochloride (Tesoriere et al., 2004), and the ethanolic extract from cladodes show anti-inflammatory and analgesic effect (Park et al., 1998).

Natural dyes based on betacyanins and betaxanthins, such as those demonstrated in this work, have excellent nutritional characteristics, and also act as antioxidants and scavengers of free radicals (Strack et al., 2003), and their presence in the diet can reduce the risk of cardiovascular disease, cancer and age-related diseases (Delgado- Vargas et al., 2000). In addition, assays using betalains from *Rivina humilis* L. (Phytolaccaceae) have demonstrated effective action against lipid peroxidation and *in vitro* cytotoxicity towards cancer cells (Khan et al., 2012).

Conclusion

The fruits of *P. catingicola* subsp. *salvadorensis* growing in the *caatinga* areas of the state of Paraíba, Brazil contain high levels of betacyanins, particularly betanin and phyllocactin, together with lower levels of betaxanthin chromoalkaloids. Samples harvested from three different populations all showed similarly high abundances of betacyanins. Consequently *P. catingicola* has the potential for agricultural development and commercial harvesting as a source of antioxidants and natural dye compounds for use in in the pharmaceutical, cosmetic and food industries.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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