

Comparative estimations of betalains and sugars in fruits of five species of *Selenicereus* (Cactaceae)

Comparación del contenido de betalaínas y azúcares en frutos de cinco especies de *Selenicereus* (Cactaceae)

Arith F. Pérez-Orozco^{1,2}, Victoria Sosa

Abstract:

Background and Aims: Betalains are water pigments reported for most species in the order Caryophyllales, to which the family Cactaceae belongs, with benefits as antioxidants in nutrition and human health. The objective of this paper is to determine the quantity of the betalains (betaxanthins and betacyanins) and sugars present in five taxa of Selenicereus, from the Hylocereus clade, commonly utilized for their edible fruit, including S. ocamponis which is an underutilized species, and scarcely cultivated in Mexico.

Methods: Fruits from wild and cultivated plants were collected in Puebla, Veracruz and Yucatán (Mexico) and Valle del Cauca (Colombia) between April and June 2019, and their betalain and sugar content were estimated from frozen fruit. The method proposed by the Association of Official Analytical Chemists was followed to estimate the sugar content. A spectrophotometric method was utilized for estimation of betalains.

Key results: The highest total betalain content was found in species with fruits of purple pulp and pericarp such as S. ocamponis (5.30 and 4.03 mg/100 g of fresh weight, respectively). Glucose and fructose content was considerable in some samples: S. undatus with 5.99 and 2.87 mg/100 g and S. ocamponis with 5.30 and 4.03 mg/100 g respectively.

Conclusions: This study identified large variation in total betalain content in the pericarps of wild and cultivated fruits in S. undatus (2.47-37.11 mg/100 g of fresh weight), which might be related to their management and selection. Estimated total betalain content of wild plants of this species was higher than in some cultivated plants. These results suggest that wild S. undatus plants might represent a good source for breeding programs. The cultivation of species with purple pulp and pericarp should be promoted, so they can be utilized as colorants in food industry.

Key words: antioxidant, betacyanin, betaxanthin, fructose, Hylocereus.

Resumen:

Antecedentes y Objetivos: Las betalaínas son pigmentos solubles en agua reportados para la mayoría de las especies en Caryophyllales, orden al que pertenece la familia Cactaceae. Estos pigmentos poseen beneficios en la nutrición y la salud humana como antioxidantes. El objetivo de este artículo es el de determinar la cantidad de betalaínas (betaxantinas y betacianinas) y azúcares presentes en cinco taxones de Selenicereus, del grupo Hylocereus, los cuales son comúnmente utilizados por sus frutos comestibles, incluyendo S. ocamponis, una especie subutilizada y escasamente cultivada

Métodos: Se recolectaron frutos de plantas silvestres y cultivadas en Puebla, Veracruz y Yucatán (México) y el Valle del Cauca en Colombia entre los meses de abril a junio de 2019, y se estimó el contenido de betalaínas y azúcares de sus frutos una vez congelados. Se siguió el método propuesto por la "Association of Official Analytical Chemists" para estimar el contenido de azúcares. Para la determinación de betalaínas se utilizaron métodos

Resultados clave: El mayor contenido de betalaínas se encontró en especies de frutos con pulpa y exocarpio púrpura como S. ocamponis (5.30 y 4.03 mg/100 g de peso fresco, respectivamente). El contenido de glucosa y fructosa en algunas muestras fue considerable: S. undatus con 5.99 y 2.87 mg/100 g y S. ocamponis con 5.30 y 4.03 mg/100 g, respectivamente.

Conclusiones: Este estudio identificó una gran variación en el contenido total de betalaínas en el exocarpio de frutos silvestres y cultivados en S. undatus (2.47-37.11 mg/100 g de peso fresco), lo que podría estar relacionado con su manejo y selección. El contenido total estimado de betalaínas de plantas silvestres de esta especie fue más alto que en algunas plantas cultivadas. Estos resultados sugieren que las plantas silvestres de S. undatus representan un buen recurso para programas de reproducción. Se debe promover el cultivo de especies con frutos de pulpa y exocarpio de color púrpura para que puedan ser utilizados en la industria alimentaria como colorantes.

Palabras clave: antioxidante, betacianinas, betaxantinas, fructosa, Hylocereus.

¹Instituto de Ecología, A.C., Biología Evolutiva, Carretera antigua a Coatepec 351, Col. El Haya, 91073 Xalapa. Veracruz. Mexico.

²Author for correspondence: fabiana.perez@inecol.mx

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Introduction

Many species of the family Cactaceae bear edible fruits that vary in color from yellow, reddish to purple, resulting from the presence of betalains, and are considered promising new crops suitable for cultivation in semi-arid and arid regions (Stinzing et al., 2003; Mizrahi, 2014). Among them are several hemiepiphytic species in the *Hylocereus* clade of *Selenicereus* (A. Berger) Britton & Rose (Korotkova et al., 2017). They are cultivated around the world and known as dragon fruit, pitahaya or pitaya, although the latter is also used for fruits of *Stenocereus* (A. Berger) Riccob. in some regions of Mexico (Pimienta-Barrios and Nobel, 1994). Species in this clade are native from Mexico to South America and have been introduced and cultivated, for instance in China, Malaysia, Vietnam and Israel (Vaillant et al., 2005).

Five or six species are the most frequently sold in fruit markets, such as the yellow-fruited *Selenicereus megalanthus* (K. Schum. ex Vaupel) Moran, native to South America (Fig. 1A, D) (Bauer, 2003; Tel-Zur et al., 2004). It is currently planted in many parts of the Andes in Colombia and Ecuador (Galán Saúco et al., 2014; Buriticá and Cartagena Valenzuela, 2015). *Selenicereus undatus* (Haworth) D.R. Hunt is the most widely cultivated (Fig. 1C), with its wild distribution ranging from Mexico to Guatemala and the Lesser Antilles, and it has been introduced and is grown in many countries in the Americas and in the rest of the world (Bauer, 2003; Arias and Véliz-Pérez, 2006; Bravo-Hollis and Arias, 2011). This species is characterized by fruits with a whitish pulp and a purplish or dark-pink pericarp (see García de Almeida et al., 2018); a subspecies was described as having a yellow

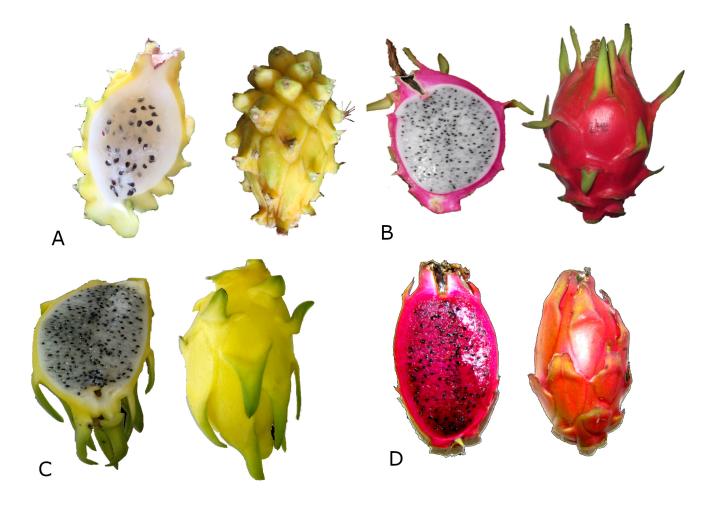


Figure 1: Fruits of pitahayas, longitudinal section showing pulp and seeds left, fruit showing bracts and spines right: A. Selenicereus megalanthus (K. Schum. ex Vaupel) Moran (Valle del Cauca, Colombia); B. Selenicereus ocamponis (Salm-Dyck) D.R. Hunt (Puebla, Mexico); C. Selenicereus undatus (Haw.) D.R. Hunt (Puebla, Mexico); D. Selenicereus undatus subsp. luteocarpus (Cálix de Dios) M.H.J. van der Meer (Quintana Roo).

pericarp: S. undatus subsp. luteocarpus Cálix de Dios (Cálix De Dios, 2005; Fig. 1B). Selenicereus ocamponis (Salm-Dyck) D.R. Hunt is native to the western Pacific coast in Mexico and Guatemala with purplish pericarp and pulp, and cultivated in home gardens on the Pacific slopes in Mexico (Fig. 1D) (Sosa et al., 2020). Other important cultivated species are S. polyrhizus (F.A.C. Webber) Britton & Rose and S. costaricensis (F.A.C. Webber) S. Arias & N. Korotkova, both with red pulp and red to pink pericarp. Their betalain biochemistry and biosynthesis have been widely studied (Qingzhu et al., 2016; Xi et al., 2019). These two latter species have been documented as rich sources of betalains, such as betanidin, betanin, isobetanin, neobetanidin, hylocerein, isohylocerein, phyllocactin, isophyllocactin, betaxanthin and miraxanthin, and variation in pigment concentrations is responsible for the different hues in the pulp and pericarp of their fruits (Wybranec and Mizrahi, 2002; Ibrahim et al., 2018).

Betalains are water soluble pigments containing nitrogen and have been reported for most species in the order Caryophyllales to which Cactaceae belongs (Timoneda et al., 2019). These compounds are present in the leaves, stems, bracts and fruits in the vacuoles of the cells, mainly in epidermal and sub-epidermal tissues (Timoneda et al., 2019). Betalains are utilized as colorants in the food and cosmetic industry; they can substitute chemical dyes with benefits to human health because they have antioxidant properties (Stintzing and Carle, 2004; 2007). Thus, there is considerable interest in documenting the presence of these pigments among plants (Stintzing and Carle, 2004; 2007). Betalains have many applications as colorants in foods, such as desserts, confections, dry mixes, dairy, and meat products (Jayasinghe et al., 2015; Song et al., 2016; Naseer et al., 2019; Rahimi et al., 2019). Among the main advantages to human health is their bioactive potential as antioxidants (Gandía-Herrero et al., 2016; Song et al., 2016; Guerrero-Rubio et al., 2020). Furthermore, estimations of these pigments have been gathered from plants under cultivation, mostly in S. polyrhizus (Wu et al., 2006; De Mello et al., 2014). Nevertheless, evaluation of the additional four species in Selenicereus cultivated for their edible fruits has not yet been carried out and they are considered in this study. These estimations may identify good sources of betalains utilized in the food and cosmetic industry.

The objective of this paper is to determine the quantity of the betalains (betaxanthins and betacyanins) and sugars present in five taxa of *Selenicereus* from the *Hylocereus* clade, commonly utilized for their edible fruit, including *S. ocamponis*, which is an underutilized species. Fruits were collected directly in the field from wild plants, or from plants cultivated in home gardens or growing on small plantations in Mexico and Colombia. Previously obtained results for these species are compared with our results, with the objective of providing data that will encourage the cultivation of these species that offer many benefits to human health.

Material and Methods

Plant material

Chemical analyses included *Selenicereus* species with fruits that have white and pinkish-purple pulp, harvested from April to June, 2019 in several localities in Puebla, Veracruz and Yucatán, in Mexico, as well as in Valle del Cauca, Colombia. Sampling sites were selected to include wild and cultivated plants in home gardens or from plantations. Vouchers and detailed localities are listed in Table 1. The pericarp was separated from the pulp of mature fruits using a steel knife. The fruit was vacuum packed, and subsequently stored at -40 °C for further analysis. No attempt was made to eliminate endogenous microorganisms, because fruits were immediately frozen until minutes before extraction, thus potential interference from microorganisms should be very low and equivalent in all samples (Pomeranz and Meolan, 1994).

Sugar determination

The Association of Official Analytical Chemists methodology was used for sugar content estimation (AOAC, 2012). Sugars were extracted with 80% (v/v) ethanol. One g of pulp from each fruit was combined with 30 ml 80% (v/v) ethanol. After stirring for 1 h, a supernatant was obtained by centrifugation (4500 g-force for 30 min) (Centrifuge 5804-R, Eppendorf Inc., Enfield, USA). After repeating this procedure, the two supernatants were combined and brought up to a final volume of 100 ml with 80% (v/v) ethanol and filtered

Table 1: Estimated sugar content (g/100 g) for the pulp of the fruits of five taxa of *Selenicereus* (A. Berger) Britton & Rose. Values are given in grams per 100 g of fresh weight in pulp. Georeferences of the vouchers for this study are provided. CUVC=Herbarium Universidad del Valle, Colombia. XAL=Herbarium Instituto de Ecología, A.C. UADY=Herbarium Universidad Autónoma de Yucatán.

Species	Locality	Voucher	Glucose	Fructose	Sucrose	
species	Georeferences	(Herbarium)	g/100g	g/100g	g/100g	
Selenicereus megalanthus (K. Schum. ex Vaupel) Moran	Valle del Cauca, Colombia (76°38'24''W, 3°48'36''N)	C. Ruiz et al. 556 (CUVC)	0.92	2.13	0.68	
Selenicereus ocamponis (Salm-Dyck) D.R. Hunt	Puebla, Mexico (97°24'00''W, 18°10'12''N)	C. Ruiz et al. 672 (XAL)	3.34	1.94	undetected	
Selenicereus ocamponis (Salm-Dyck) D.R. Hunt	Puebla, Mexico (97°21'00''W, 18°18'48''N)	C. Ruiz et al. s.n. (XAL)	5.30	4.03	undetected	
Selenicereus ocamponis (Salm-Dyck) D.R. Hunt	Puebla, Mexico (97°15'36''W, 18°22'48''N)	C. Ruiz et al. 676 (XAL)	3.22	2.50	undetected	
Selenicereus sp.	Puebla, Mexico (97°21'00''W, 18°19'48''N)	C. Ruiz et al. s.n. (XAL)	3.51	1.89	undetected	
Selenicereus sp.	Puebla, Mexico (96°25'34.8''W, 19°32'24''N)	C. Ruiz 694 (XAL)	5.67	2.48	undetected	
Selenicereus undatus (Haw.) D.R. Hunt	Puebla, Mexico (97°22'12''W, 18°10'12''N)	C. Ruiz et al. 670 (XAL)	5.99	2.87	undetected	
Selenicereus undatus (Haw.) D.R. Hunt	Veracruz, Mexico (97°10'48"W, 20°22'48"N)	C. Ruiz et al. s.n. (XAL)	1.64	0.65	undetected	
Selenicereus undatus (Haw.) D.R. Hunt	Yucatán, Mexico (89º59'24''W, 20º36'00''N)	C. Ruiz & D. Angulo 641 (UADY)	2.12	0.92	undetected	
Selenicereus undatus subsp. luteocarpus (Cálix de Dios) M.H.J. van der Meer	Veracruz, Mexico (98°10'12''W, 20°58'12''N)	C. Ruiz et al. 693 (XAL)	4.48	2.65	undetected	

through a $0.45~\mu m$ filter. Glucose, fructose and sucrose concentrations were determined by high performance liquid chromatography equipped with an isocratic solvent delivery system, manual injector, refractive index detector, and recording/computing integrator (HPLC System, Shimadzu Co. Ltd., Kyoto, Japan) with a COSMOSIL 5NH2-MS column (Nakarai Tesque Co. Ltd., Kyoto, Japan). Elution was carried out with 80% acetonitrile at a flow rate of 1 ml min⁻¹. Next, $10~\mu l$ of extract was injected directly into the column. The standard was injected in triplicate for the construction of calibration curves. Qualitative analysis of sugar was determined by comparison to the retention time of standards. All determinations were performed in triplicate.

Betalain content

Extraction and betalain determination

The method of Wu et al. (2006) was used to obtain a methanolic extract from the fruit. Two grams of pulp and one gram of pericarp were macerated with 20 ml of methanol 80% (v/v). Sonication was applied for 10 min with a Fisher ultrasonic bath (Model FS110, Waltham, USA). Mixtures were then shaken in the dark for 20 min at room temperature and centrifuged at 2200 g-force in a Hermle centrifuge (Model Z236, Gosheim, Germany). The supernatant was separated, and the residue was subjected to a similar second extraction. The supernatants were pooled, filtered with No. 1 Whatman paper, and concentrated to dryness at 40 °C in a rotary evaporator (Yamato RE201, Santa Clara, USA). Finally, residues were re-suspended in 10 ml of a solution of methanol 80% and stored in amber containers at -20 °C. Betacyanin and betaxanthin concentrations were determined using the spectrophotometric method of Castellanos-Santiago and Yahia (2008) and the calculation: $B=(A \times DF \times W \times V) / (\varepsilon \times P \times L)$, where B is the content of betacyanins or betaxanthins (mg g-1), A is the absorbance (at 538 nm for betacyanins and 483 nm for betaxanthins), DF is the dilution factor, W is the molecular weight (550 g mol⁻¹ for betanin and 308 g mol⁻¹ for indicaxanthin), ϵ is the molar extinction coefficient (60,000 L mol-1 cm-1 for betanin and 48,000 L mol⁻¹ cm⁻¹ for indicaxanthin), P is the mass of the sample (g), and L is the length (1 cm) of the cuvette used during the determination. Results were expressed as total content of betalains per 100 g of fresh weight, through the sum of the contents of betacyanins and betaxanthins. All determinations were performed in triplicate and summarized by their mean value and standard deviation.

Data analysis

For total betalains, betaxathines and betacyanins contents, we used the t-test to compare among pairs of species when appropriate (Zar, 2010). The analyses were performed in R (R Core Team, 2021).

Results

Regarding sugar content, Table 1 presents the estimates for glucose, fructose and sucrose. Glucose and fructose content in some samples was considerable. *Selenicereus undatus* with 5.99 and 2.87 mg/100 g and *S. ocamponis* with 5.30 and 4.03 mg/100 g respectively. Table 2 presents

the results of the estimated betacyanins, betaxanthins and the total betalain content for the five Selenicereus species we collected in the wild, in home gardens and small plantations. Total betalain (Table 2) content was highest (32.46 g/100 g fresh tissue) in the pericarp of the unidentified purplish red Selenicereus (t=5.7, d.f.=3, P=0.01), while S. ocamponis (16.6 g/100 g fresh tissue) and S. undatus (17.57 g/100 g fresh tissue) did not differ significantly (t=0.3, d.f.=4, P=0.72). A similar pattern of differences was observed in betacyanins in the pericarp. The unidentified Selenicereus (23.76 g/100 g fresh tissue) had the highest content (t=9.2, d.f.=3, P=0.002), but S. undatus (11.57 g/100 g fresh tissue) had on average 18% more betacyanins than S. ocamponi (13.73 g/1000 g fresh tissue) and differed significantly (t=2.8, d.f.=4, P=0.47). Moreover, the unidentified species (8.7 g/100 g fresh tissue) had the highest content of betaxanthins in the pericarp (t=6.1, d.f.=3, P=0.008), while there were no significant differences (t=1.9, d.f.=4, P=0.13) between S. ocamponis (5.03 g/100 g fresh weight) and S. undatus (3.84 g/100 g of fresh tissue). In contrast, in the pulp of fruits there were no significant differences in

Table 2: Betacyanins, betaxanthins and total betalain content estimated for five studied taxa of *Selenicereus* (A. Berger) Britton & Rose from different localities. Values are given in milligrams per 100 g of fresh weight. Vouchers and herbaria codes are the same as in Table 1. Values are means ± standard deviations of triplicate analysis (n=3).

Consider	Habitat	Country	Betacyanins		Betaxanthins		Total betalains	
Species			Pulp	Pericarp	Pulp	Pericarp	Pulp	Pericarp
Selenicereus megalanthus (K. Schum. ex Vaupel) Moran	Plantation	Colombia	undetected	0.67±0.07	undetected	2.31±0.32	undetected	2.98±0.38
Selenicereus ocamponis (Salm-Dyck) D.R. Hunt	Home garden	Mexico	3.73±0.09	7.87±0.43	1.87±0.16	3.51±0.19	5.60±0.25	11.38±0.23
Selenicereus ocamponis (Salm-Dyck) D.R. Hunt	Home garden	Mexico	4.29±0.04	8.83±0.41	1.86±0.04	4.23±0.53	6.18±0.04	13.06±0.81
Selenicereus ocamponis (Salm-Dyck) D.R. Hunt	Home garden	Mexico	3.33±0.04	18.01±0.43	1.42±0.04	7.35±0.60	4.73±0.08	25.36±0.88
Selenicereus undatus (Haw.) D.R. Hunt	Home garden	Mexico	undetected	29.85±1.04	undetected	7.27±0.56	undetected	37.11±4.0
Selenicereus undatus (Haw.) D.R. Hunt	Home garden	Mexico	undetected	1.75±0.08	undetected	0.71±0.12	undetected	2.47±0.19
Selenicereus undatus (Haw.) D.R. Hunt	Wild	Mexico	undetected	9.59±0.32	undetected	3.54±0.39	undetected	13.13±0.71
Selenicereus undatus subsp. Iuteocarpus Calix de Dios	Home garden	Mexico	undetected	0.27±0.02	undetected	0.65±0.12	undetected	0.92±0.14
Selenicereus sp. (purplish-red)	Cultivated	Mexico	4.23±0.36	16.23±0.44	2.06±0.15	6.53±0.40	6.39±0.30	22.76±0.70
Selenicereus sp. (purplish-red)	Cultivated	Mexico	5.39±0.18	31.29±1.18	1.49±0.06	10.86±0.32	6.86±0.14	42.16±1.50

betalains, betacyanins and betaxanthins among the species (t<0.77, d.f.=4, P>0.49).

Discussion

Our estimations are the first for *Selenicereus megalanthus* in which low total betalain content was determined in the pericarps of its fruits (Table 2). Fruits of this species are yellowish and although they did not contain elevated betaxanthins, the combination of betalains and betaxanthines resulted in a pale-yellow color of the studied fruits. Remarkably, elevated variation in betacyanins and betaxanthins in *S. undatus* was identified. This might be related to the management and selection that plants have been subjected to in the communities where they are cultivated, both in home gardens and on plantations. Furthermore, the hue in pericarps of fruits in Cactaceae depends on the combination of betaxanthins and betacyanins (Gandía-Herrero et al., 2016).

Previously, betalains were reported for *Selenicereus polyrhizus* cultivated in Malaysia (Gengatharan et al., 2015), Taiwan (Wu et al., 2006) and Indonesia (Priatni and Pradita, 2015) (Table 3). This species has a purplish pulp and pericarp. Higher quantities of betalains were recorded in *S. polyrhizus* compared with our estimates for *S. ocamponis*, with the same pericarp and pulp colors (Table 3). However, the methods were different from those used in our study (Stinzing et al., 2003; Stinzing and Carle, 2007; Naderi et al., 2012). Furthermore, in *S. polyrhizus* only betacyanins have been detected, as betaxanthins are absent, giving the pulp of its fruits a glowing red-purple color (Stinzing et al., 2002). In addition to previous records, the dry fruits of two undetermined *Selenicereus* sp. from Nicaragua (Montoya-Arro-

yo et al., 2014) and Costa Rica (Esquivel et al., 2007) were analyzed following different protocols but revealed a similar sugar content to that which we report for *S. ocamponis*.

Earlier estimates of betalains of the pericarps of *Selenicereus undatus* fruits from Brazil utilizing a different method detected an elevated content (101 g/100 g) (Table 3) (De Mello et al., 2014). Plants were collected in a plantation from Embrapa Cerrado in Brazil, which possibly included plants selected for having intensely colored pericarps to be utilized in the food industry (De Mello et al., 2014).

We included two different species that we could not assign to the known species of Selenicereus, with deep purplish pericarps and pulp and with a high total betalain, betacyanins and betaxanthins content. Estimates of betalain content from S. ocamponis and the two undetermined Selenicereus were elevated, indicating that they are promising crops as a source of these compounds, which as mentioned above, have many applications in the food industry. Furthermore, in the estimates obtained in this study, wild S. undatus plants with purplish fruit had a higher betalain content than some of the cultivated plants. Even though the whitish pulp of S. undatus does not contain betalains, it is worth cultivating this pitahaya for its nutritional potential. This pitahaya is also remarkable because it has very large fruits, with purplish pericarps that provide visual quality with extraordinary sensory characteristics, which adds value to these fruits on the international fruit market (Mercado-Silva, 2018).

The results of this study corroborate that pitahayas or dragon fruits are nutritious crops. *Selenicereus undatus*—the most cultivated species— displayed variation in sugar content. For fruits from certain plants collected in

Table 3: Previous estimations of betanins (or betacyanins) obtained by solvent extraction and quantification by spectrophotometry. References utilized the name *Hylocereus* (A. Berger) Britton & Rose, the genus that was transferred to *Selenicereus* (Korotkova et al., 2017). *Hylocereus polyrhizus* (F.A.C. Weber) Britton & Rose, is considered a synonym of *S. monacanthus* (Lem.) D.R. Hunt (Korotkova et al., 2017).

Species	Country	Tissue	Betanin (mg/100 g fresh weight)	Source
Hylocereus polyrhizus (F.A.C. Weber) Britton y Rose	Indonesia	Pericarp	73.2	Faridah et al., 2015
Hylocereus polyrhizus (F.A.C. Weber) Britton y Rose	Indonesia	Pericarp	515.2*	Priatni and Pradita, 2015
Hylocereus polyrhizus (F.A.C. Weber) Britton y Rose	Taiwan	Pericarp	13.0	Wu et al., 2006
Hylocereus polyrhizus (F.A.C. Weber) Britton y Rose	Taiwan	Pulp	10.3	Wu et al., 2006
Selenicereus undatus (Haw.) D.R. Hunt	Brazil	Pericarp	101.0	De Mello et al., 2014

the wild or cultivated ones, as well as fruits of the subsp. *luteocarpus*, estimates of glucose and fructose were high. Moreover, the estimates of elevated sugars in wild plants of *S. undatus* suggest that it could be used as a resource for pitahaya breeding. Interestingly, estimates for sugars in *S. ocamponis* were elevated. This pitahaya is cultivated on small plantations and in home gardens on the Pacific slopes of Mexico and has a purple pericarp and pulp. Thus, the potential of this species is enormous for use in the food industry as colorant.

Conclusions

Estimates of sugars and betalains in wild and cultivated *Selenicereus undatus* and *S. ocamponis* plants corroborated the importance of pitahayas as healthy fruits that can provide natural colorants for the food and cosmetic industry. Wild plants of these species represent good sources for breeding programs. *Selenicereus* sp. and *S. ocamponis* are underutilized species with fruits that have an elevated content of betalains and that should be promoted for cultivation.

Author contributions

VS conceived the project, planned, and conducted field-work. AFP processed the samples, extracted and quantified betalains, and did the statistical analyses. The text was prepared by both authors.

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