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Article

A nutraceutical beverage from roselle calyces with different pigmentation

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Abstract

Roselle (*Hibiscus sabdariffa* L.) calyces have abundant phenolic compounds to which multiple therapeutic benefits are attributed. The study aimed to develop a nutraceutical beverage from Hs extracts of genotypes with different pigmentation and phytochemical composition, with sensory characteristics accepted by the consumer and with a high content of antioxidants. Calyces of three cultivars with intense red, light red, and non-pigmentation were used to prepare aqueous extracts, which were mixed in different proportions to achieve beverages with a standardized anthocyanin content (TAC). Four beverage formulations, denoted as B1, B2, B3, and B4, were obtained, which were subjected to global acceptance tests through different sensory variables. The variables determined in the beverages were color, pH, titratable acidity (TA), total soluble phenols (TSPs), TAC, proanthocyanidins (PAs), and antioxidant capacity (AC). The TAC in the beverages ranged from 23 to 24.9 mg anthocyanin per 240 ml portion. The color and TA of the beverages were statistically different according to the objective measurement, but sensory differences were not detected. Beverages B1 and B4, formulated with mixtures of the three cultivars, had the highest TSP content and AC and had the best aroma and flavor. Incorporating pigment-free calyx extracts in the formulation of roselle beverages improves their sensory characteristics and antioxidant content.

Palabras clave:

Hibiscus sabdariffa L., antioxidant capacity, calyces, sensory analysis.

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Introduction

Traditional Mexican medicine attributes therapeutic properties to extracts of roselle [*Hibiscus sabdariffa* L. (Hs)] calyces in conditions such as high blood pressure, kidney stones, and inflammation (González-Stuart, 2011). These properties are associated with calyx polyphenols, among which anthocyanins, phenolic acids, flavonols, and proanthocyanidins predominate (Riaz and Chopra, 2018), the main biological activity related to these compounds is their ability to neutralize or capture free radicals and reduce their oxidative action on cellular components.

Studies on the effectiveness of aqueous roselle extracts in treating conditions are numerous and have been conducted to different extents, including clinical trials (Mckay *et al*., 2009) and laboratory animal trials (El-Shiekh *et al*., 2020). In clinical studies, there is no consensus regarding the therapeutic dose of roselle-based teas or beverages. Nonetheless, the information available so far demonstrates the low toxicity of the extracts used at medium and low doses (Riaz and Chopra, 2018).

The phenolic composition of roselle genotypes' calyces differs according to their pigmentation. Dark-colored calyces have higher anthocyanin content than light-colored calyces (Reyes-Luengas *et al*., 2015), and white calyces contain more flavonols than red calyces (Camelo-Méndez *et al*., 2016). The composition of volatile compounds, which are responsible for the aroma of calyces, also differs between genotypes, with those with light-colored calyces being less aromatic than those with dark-colored calyces (Musa *et al*., 2021).

Published scientific information on differences in the chemical composition of roselle calyces with different pigmentation and information related to therapeutic doses for disease prevention or control can be used to formulate nutraceutical beverages for consumers interested in obtaining health benefits from the consumption of roselle refreshing beverages. The present study aimed to develop a beverage from mixtures of roselle calyx extracts of genotypes with different pigmentation, sensory qualities accepted by consumers, and high antioxidant content.

Materials and methods

Study material

The dehydrated roselle calyces used were from the genotypes Sudán (S) of dark red calyces, Alma Blanca (AB) of white calyces, and Criolla Nayarit (CN) of light red calyces. The first two were grown in Santa María Cortijo, Jamiltepec, in the Costa region of the state of Oaxaca, and the third in Nayarit, in the municipality of Xalisco.

Preparation of aqueous extracts

Two point five grams of calyces (dry base) were weighed, and 100 ml of distilled water was added. The mixture was boiled for 15 min, performing two extraction cycles. The extracts from the first and second extraction were combined, made up to 200 ml with distilled water, and filtered with Whatman 4 paper. Different beverage formulations were made from these extracts. Information on the physical and chemical characterization of the calyces of roselle genotypes used in beverage formulations was published in a previous study (Reyes-Luengas *et al*., 2015).

Preparation of formulations

Four formulations were prepared, which contained at least 21 mg of total anthocyanins/240 ml of beverage (Table 1), the amount of anthocyanins required per day to achieve benefits in aspects such as moderate blood pressure control (McKay *et al*., 2009) or hypercholesterolemia (Gurrola-Díaz *et al*., 2010). The sweetness level was adjusted to 7% with cane sugar. Each beverage formulation was prepared in duplicate.

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Table 1. Formulation of roselle beverages from aqueous extracts of calyces of genotypes with different pigmentation.

Formulation	Sudán (ml)	Alma Blanca (ml)	Criolla Nayarit (ml)	Water (ml)	Total (ml)
B1	51.4	120	68.5	0	240
B2	51.4	0	68.5	120	240
B3	27.4	0	128.5	84	240
B4	27.4	84	128.5	0	240

Physicochemical characterization of formulated beverages

pH. It was measured with a Denver Instrument UB10 potentiometer, calibrated with pH 4 and 7 buffers. Titratable acidity (TA). The AOAC (1984) method for slightly colored solutions was used. Acidity was expressed in milliequivalents of citric acid in % w/v. Color. A Hunter Lab Mini Scan XE Plus (model 45/0-L) colorimeter was used on the CIEL*a*b* scale, with D/65 illuminant, a 10° angle, and a quartz capsule into which 30 mL of the beverage was poured. The Hue and Chroma were calculated based on the parameters a* and b*. The ΔE between each of the different beverages was calculated using the expression $\Delta E = \sqrt{(L_1-L_2)^2 + (a_1-a_2)^2+(b_1-b_2)^2}$ (Wrolstad and Smith, 2010).

Phenolic characterization of formulated beverages

Total anthocyanins (TAC

The TAC was performed as described by Galicia-Flores *et al*. (2008). A standard curve of cyanidin-3-glucoside (Polyphenols, NW) with five curve points (5-25 ppm, R^2= 0.9997) was generated and read on a Perkin-Elmer® lambda 25 spectrophotometer. The TAC was expressed in mg cyanidin-3-glucoside equivalents (CGE)/240 ml beverage.

Total soluble phenols (TSPs

TSPs were analyzed using the Folin-Ciocalteu method, as described by Galicia-Flores *et al*. (2008). Gallic acid was used as a standard to express the results in mg gallic acid equivalents (GAE) per 240 mL of beverage.

Proanthocyanidins (PAs

APs were assessed using Wallace and Giustis (2010) method. A standard curve of catechin (Sigma, MN) was prepared. Results were expressed in mg catechin equivalents (CE) per 240 ml of beverage.

Antioxidant capacity of formulated beverages

DPPH Method

A volume of 2 900 µ of a solution of DPPH (2,2-diphenyl-1-picrylhydrazyl) (Sigma-Aldrich®, Mexico), 100 µM in methanol at 80% v/v were mixed with 100 µl of beverage. After resting 30 min in darkness, absorbance was measured at 515 nm. The reference absorbance was the one obtained by mixing 100 µl of 80% methanol with 2 900 µl of DPPH. The percentage of DPPH reduced was calculated using the formula:

$$\%\,\mathrm{DPPHreduced} = \left[\frac{\mathrm{Abs_r}-\mathrm{Abs_T}}{\mathrm{Abs_r}}\right](100).$$

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Where: Abs_r= reference absorbance of the DPPH solution; Abs_T= absorbance of the formulation at 30 min (Prior *et al*., 2003).

To determine the IC_{50}, dilutions of each formulated beverage were carried out based on the concentration of total soluble phenols (TSPs), and the percentage of reduced DPPH was measured. The concentration of total phenols was plotted against the percentage of reduced DPPH; then, a logarithmic regression was fitted to calculate the IC_{50}. The IC_{50} is the concentration of the extract with which 50% of the DPPH is reduced.

ORAC method

A 20 µl aliquot of each formulation was deposited in the well of a dark 96-well flat-bottom plate along with 5 µl of buffer and 360 µl of 48 nM fluorescein (Sigma, St. Louis, MO). Samples were incubated for 30 s, and initial fluorescence was recorded. The plate was read on a microplate reader (Synergy HT, Biotekinstruments), and the reaction was initiated by adding AAPH (2-2'-Azobis amidinopropane, free radical generator) using the device's auto dispenser (Ou *et al*., 2001).

Samples and control (buffer instead of extract) were analyzed in quadruplicate. The excitation and emission wavelengths were at 485 and 530 nm, respectively. Antioxidant capacity (AC) data were expressed as Trolox equivalents (TE) per 100 ml of beverage (µmol of TE 100 ml^{-1}).

Sensory testing

Discriminatory tests were carried out to determine differences between beverage formulations. For the global acceptability tests, a nine-point hedonic scale was used, where: 1 'I dislike it extremely', 5 'I neither like nor dislike it', and 9 'I like it extremely'. The variables evaluated were color, aroma, acidity, roselle flavor, residual flavor, sweetness, and global acceptance. The evaluation was conducted with 65 panelists.

Statistical analysis

The physical and chemical characterization data of the beverages and the attributes of the sensory evaluation were analyzed under a completely randomized design. The data from the physical and chemical characterization were used to perform a one-way analysis of variance and mean comparison tests (Tukey, $p \leq 0.05$) when appropriate, using the SAS® program version 9.0 (SAS Institute, Inc., 2009). Principal component (PC) analysis of sensory evaluation data was performed with the Unscrambler® program version 9.2 (Camo Process AS, Oslo, Norway). In this case, the means were compared using Duncan's test ($p \leq 0.05$).

Results and discussion

Physicochemical characterization of beverages from calyces of roselle genotypes

The pH differed between the beverages, with the highest corresponding to B2 and the lowest to B3 and B4, which contained a higher proportion of the extract of the CN variety, Table 2. In all cases, it was < 3, which favors the chemical form of the flavylium cation of anthocyanins (Brouillard, 1982), its stability, and its characteristic bright red color. The TA varied between 9.8 and 20.7%, with a statistical difference between the beverages: the highest TA occurred in the beverages with extract of the AB genotype (B1 and B4). The TAC of the beverages was around 24 mg CGE/240 ml of the beverage.

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Table 2. Physical and chemical characterization of the formulations of roselle beverages prepared from cultivars with calyces of different pigmentation.

Beverage	pH	TA	TAC	TSPs	PAs
B1	2.5 ±0.01 b	18.4 ±0.29 b	24.9 ±0.08 a	111.42 ±0.91 a	1.63 ±0.16 a
B2	2.55 ±0 a	9.81 ±0.15 d	23.1 ±0.15 c	69.69 ±0.28 c	1.44 ±0.14 a
B3	2.42 ±0.01c	14.36 ±0.15c	23 ±0.1 c	73 ±0.97 c	0.63 ±0.21 b
B4	2.42 ±0 c	20.7 ±0 a	24.1 ±0.13 b	105.3 ±0.81 b	1.19 ±0.14 ab
LSD	0.02	0.72	0.49	4.64	0.68

LSD= least significant difference; TA= titratable acidity (% w/v); TAC= total anthocyanin content (mg cyanidin-3-glucoside equivalents 240 m^{-1} of beverage); TSPs= total soluble phenols (mg gallic acid equivalents 240 ml^{-1} of beverage); PAs= proanthocyanidins (mg catechin equivalents 240 ml^{-1} of beverage). Equal letters in columns are statistically equal (Tukey, α= 0.05).

The difference in TSPs between the beverages is related to the addition of the extract of the AB variety, with a high content of these compounds (Reyes-Luengas *et al*., 2015). However, all beverages kept a TSP content above 65.55 mg, the daily dose required to achieve beneficial effects (Mckay *et al*., 2009). Regarding PAs, the highest values were in B1 and B2, attributed to the fact that their formulation included a higher proportion of the extract of the S variety, with a higher concentration of these flavonoids than AB and CN (Reyes-Luengas *et al*., 2015).

Color of roselle beverages

Luminosity (L) was statistically different between the four beverages. The low values were related to their dark color, which was higher in B1 and lower in B4. All beverages had a purple-red hue (positive values of a^*). The values of b^* in the beverages were low (< 1), indicative of a slight yellow tint (Table 3).

Table 3. Color parameters and ΔE for beverages formulated from the mixture of extracts of roselle genotypes.

Beverage	L^*	a^*	b^*	Hue (°)	Chroma
B1	2.17d	2.6ab	0.9825a	20.7a	2.78ab
B2	6.17c	2.19b	0.785a	20.6a	2.33b
B3	10.17b	3.36a	0.998a	16.6a	3.51a
B4	14.17a	2.7ab	0.93a	19.2a	2.86ab
LSD	2.71	0.95	0.276	5.98	0.96
		ΔE values between beverages			
ΔE_{B1-B2}	4.0256	ΔE_{B1-B3}	8.0365	ΔE_{B1-B4}	12.001
ΔE_{B2-B3}		4.1737	8.0174	ΔE_{B3-B4}	4.0555

L^*= luminosity (%); LSD= least significant difference. Equal letters in columns are statistically equal (Tukey, α= 0.05).

Hue values correspond to a red-purple hue. No statistical difference was observed for this variable between the beverages analyzed. The color saturation or chroma index showed low values (2.33 - 2.86), which is associated with a high presence of gray tones.

Visual color differences between the four formulated beverages were expressed through ΔE. According to Obon *et al*. (2009), a difference of ΔE between 0 and 1.5 can be considered small and visually imperceptible; from 1.5 to 5, the color difference can be distinguished and is evident for a #E greater than 5. Therefore, it would be difficult for the consumer to notice color differences between the beverages B1 *vs* B2, B3 *vs* B2, and B3 *vs* B4; in contrast, the color differences between the beverages B1 *vs* B3, B1 *vs* B4, and B2 *vs* B4, would be easily noticed.

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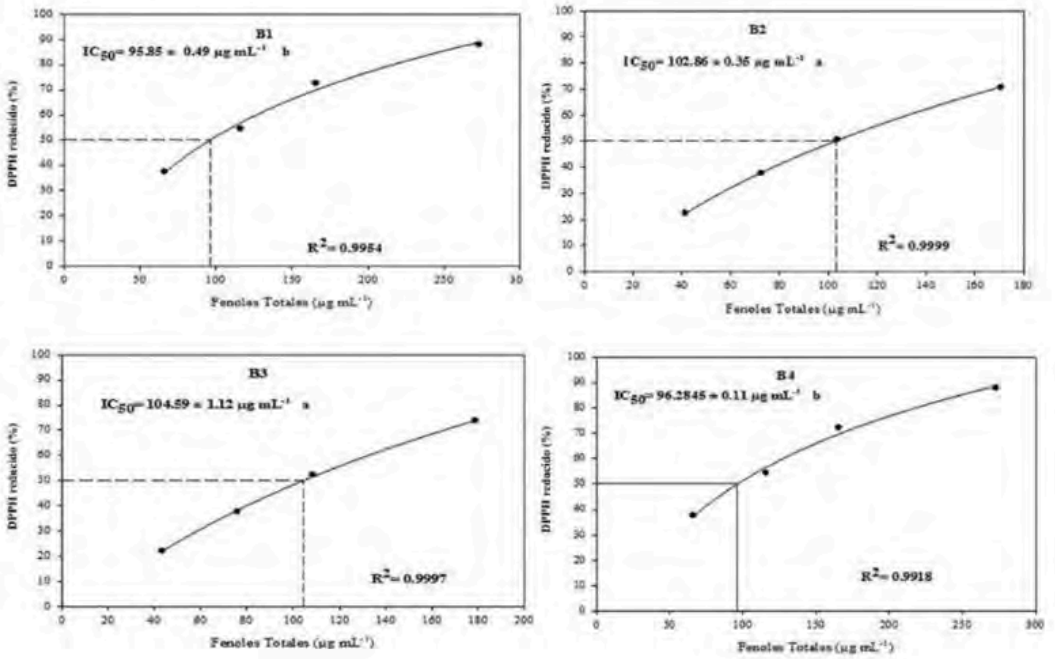
Antioxidant capacity

DPPH method

Antioxidant capacity, as a function of IC_{50} values, was statistically different between beverages. The IC_{50} of B2 and B3 were the same ($p \leq 0.05$) but higher than that of B1 and B4. A higher IC_{50} implies lower antioxidant power (Noreen *et al*., 2017) (Figure 1). The standardization of the concentration of anthocyanins in the beverages caused the IC_{50} values to be similar.

Figure 1. Antioxidant capacity expressed as IC_{50} of roselle beverages. Equal letters in IC_{50} indicate that there are no statistically significant differences (Tukey, α= 0.05).



The lowest IC_{50} values corresponded to B1 and B4 beverages, which contained the extract of the AB genotype (Table 1), which is abundant in phenolic acids and other flavonoids with antioxidant capacity (Reyes-Luengas *et al*., 2015).

ORAC method

There was a statistical difference in antioxidant capacity (AC) between the four beverages (Figure 2). The ones with the highest AC were B1 and B4, with equal AC between them but statistically different from B3. In B1 and B4, the AC values were 1 080 and 1 193 µmoles TE per 100 ml of beverage, respectively.

All beverages had values higher than 621.8 ±1.7 µmol TE per 100 ml, the value reported in roselle water prepared with the traditional procedure used by housewives in Mexico (Sáyago-Ayerdi *et al*., 2007).

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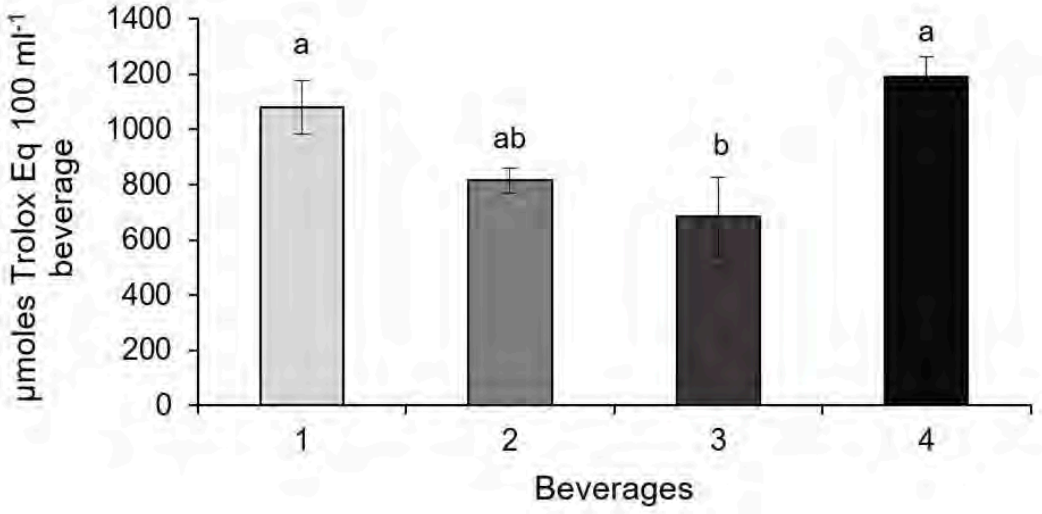
Figure 2. Antioxidant capacity of roselle beverages with the ORAC method. Values with equal letters on the bars indicate that there are no statistically significant differences (Tukey, α= 0.05).



Sensory evaluation

Only the aroma and sweetness attributes were different between the beverages. The beverage with the best roselle aroma was B4, which is attributed to the fact that it incorporates extracts from the three roselle cultivars; in contrast, B2 and B3 beverages had the lowest scores (Table 4), possibly due to the absence of the AB genotype extract in their formulation (Table 1). The characteristic aroma of roselle beverages is attributed to the quantitative balance of their volatile compounds and not to the predominance of any of them (Musa *et al*., 2021).

Table 4. Comparison of means of sensory attributes in roselle beverages formulated from mixtures of calyces extracts from genotypes with different pigmentation.

Attributes	Formulations				LSD
	B1	B2	B3	B4	
Color intensity	6.86 ±1.39† a	6.68 ±1.46 a	6.34 ±1.62 a	6.72 ±1.49 a	0.54
Aroma	5.62 ±1.92 ab	4.92 ±2.15 b	5.12 ±1.92 b	5.95 ±1.76 a	0.69
Acidity	5.48 ±2.09 a	5.58 ±2.24 a	5.78 ±1.82 a	5.55 ±2.06 a	0.84
Sweetness	4.97 ±2.19 b	6.57 ±1.79 a	6.08 ±1.78 a	5.03 ±1.94 b	0.75
Roselle flavor	6.09 ±1.87 a	6.43 ±1.51 a	6.31 ±1.58 a	6.28 ±1.84 a	0.66
Residual flavor	5.4 ±1.86 a	5.85 ±1.8 a	5.97 ±1.73 a	5.71 ±1.94 a	0.67
Global acceptance	6.06 ±1.87 a	6.66 ±1.63 a	6.65 ±1.4 a	6.34 ±1.76 a	0.66

Average values with the same letter by row are statistically equal ($p< 0.05$). LSD= least significant difference.

The panelists perceived the B2 and B3 beverages as the sweetest, even though they all contained 7% w/v of sugar, due to their lower acidity, as they contain more water than the rest. Notably, although the TA difference was more than 10 percentage points between the beverages (Table 2), the panelists did not perceive it. Bechoff *et al*. (2014) sensorily evaluated roselle beverages with differences in acidity up to three times without the panelists identifying them.

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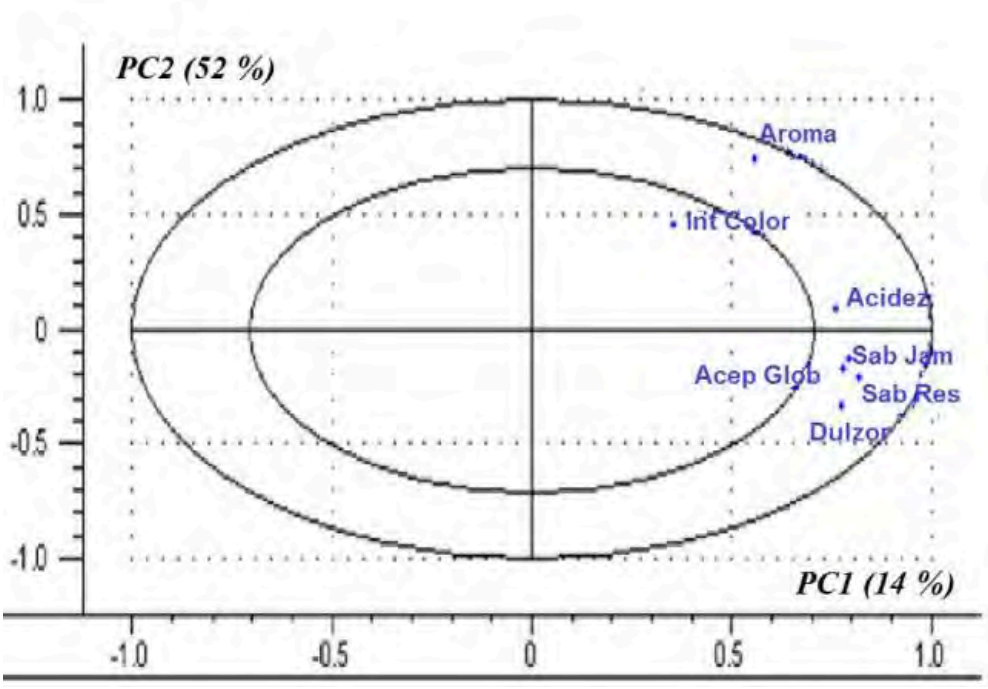
The evaluators did not identify color differences between the beverages evaluated either, which is attributed to the fact that the hue angle, which is the one most associated with visual color perception (Wrolstad and Smith, 2010), was equal among the beverage formulations. In this regard, Bechoff *et al*. (2014) pointed out a lack of correlation between the red color perceived by the panelists and the $L^*a^*b^*$ color parameters.

Principal component analysis of sensory assessment attributes

The ellipses represent 50 to 100% load correlations between the first and second (Figure 3). All the variables showed a high correlation, as they were located in the area of the two ellipses, except for the variable of color intensity (int color), which was located outside the area of the first ellipse, which means that it did not correlate with the rest of the variables. None of the variables analyzed showed opposite locations or negative weight.

Principal component 1 (PC1) was mainly explained by sweetness and acidity, attributes that are located to the right of the ellipse, which means that the sweeter and more acidic the beverage, the better it will be accepted by the panelists, a result that coincides with what was found by Bechoff *et al*. (2014). For principal component 2 (PC2), the representative variables were aroma and color intensity (Int Color). PC1 explained 52% of the total variation in the attributes determined in roselle beverages, while PC2 explained only 14% of this variability (Figure 3 B).

Figure 3. Dispersion of attributes of the sensory evaluation on roselle beverages. Int Color= color intensity; Sab Jam= roselle flavor; Acep Glob= global acceptance; Sab Res= residual flavor.



Conclusions

Mixing aqueous extracts of roselle (*Hibiscus sabdariffa* L.) genotypes with calyces of contrasting pigmentation enhances the beverage's sensory attributes and consumer acceptance. Incorporating the extract of the non-pigmented variety (Alma Blanca) into the mixture favors the beverage's flavor and aroma and increases its antioxidant capacity.

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