Journal of Food Research; Vol. 5, No. 2; 2016
ISSN 1927-0887 E-ISSN 1927-0895
Published by Canadian Center of Science and Education

Antioxidant and Physicochemical Properties of Hibiscus Sabdariffa Extracts from Two Particle Sizes

Sandro Cid-Ortega[1,2] & José Á. Guerrero-Beltrán[1]

[1] Departamento de Ingeniería Química, Alimentos y Ambiental, Universidad de las Américas Puebla, Cholula, Puebla, Mexico

[2] Programa Educativo de Ingeniería en Procesos Alimentarios. Universidad Tecnológica de Izúcar de Matamoros. Izúcar de Matamoros, Puebla, Mexico

Correspondance: José Á. Guerrero-Beltrán, Departamento de Ingeniería Química, Alimentos y Ambiental, Universidad de las Américas Puebla, Cholula, Puebla 72810, Mexico. Tel: 52-222-229-2126. E-mail: angel.guerrero@udlap.mx

Received: December 22, 2015 Accepted: March 21, 2016 Online Published: March 27, 2016

doi:10.5539/jfr.v5n2p98 URL: http://dx.doi.org/10.5539/jfr.v5n2p98

Abstract

The effect of average particle sizes (APS: 0.45 and 1.01 mm), solvent types (ST: distilled water, 2% citric acid solution, and 50% ethanol), and extraction times (ET: 40, 50, and 60 min) on the physicochemical properties, antioxidant capacity, and half maximal effective concentration (EC50) in calyces of Roselle (*Hibiscus sabdariffa*) was analyzed. The extracts obtained with distilled water and 2% citric acid solution had an intense red color which purities (C) were 78.5 ± 2.3 and 79.8 ± 2.2, respectively. The alcoholic extracts showed a dark red color (C = 75.9 ± 1.8). The alcoholic (820.2 ± 73.7 mg Trolox equivalents (TE)/100 g dry calyces) and acid extracts (773.34 ± 53.0 mg TE/100 g dry calyces) showed higher antioxidant capacity than the aqueous extracts (673.24 ± 116.0 mg TE/100 g dry calyces). The EC50 value was better for the alcoholic extracts, followed by the 2% citric acid extracts and finally by the aqueous extracts (13.4 ± 1.0, 14.9 ± 1.0, and 17.2 ± 1.2 mg of extract, respectively). According to results, the best antioxidant properties were achieved using an APS of 0.45 mm, 50:50% ethanol:water ratio, and ETs of 30 or 45 min.

Keywords: *Hibiscus sabdariffa*, Roselle, average particle size, antioxidant capacity, half maximal effective concentration

1. Introduction

In recent years, it has been a great interest for researching about the antioxidant properties of many vegetable products consumed by humans. Many of these products (fruits, vegetables, grains, stems, flowers, to mention few) have characteristic colors, which are due to the presence of the colored chemical compounds that may possess beneficial health characteristics for humans. They might help in reducing degenerative diseases as well as provide beneficial effects on the health and wellbeing of people (Cid-Ortega & Guerrero-Beltrán, 2015). Roselle calyces have a characteristic deep red color, which is mainly due to the presence of anthocyanins. The most common use of Roselle calyces is for obtaining aromatic infusions of intense red color that are traditionally consumed either cool or hot. Roselle extracts are also used as natural pigments for foods and beverages as well as for preparing jams, jellies, and concentrates possessing red color with a characteristic sour taste. Several researchers have pointed out that Roselle extracts may have various therapeutic effects; one of them is the antioxidant capacity, attributed mainly to the content of anthocyanins and phenolic compounds (Tsai, Mcintoshb, Pearceb, Camdenb, & Jordanc, 2002; Tsai & Huang, 2004; Anokwuru, Esiaba, Ajibaye, & Adesuyi, 2011; Amer, El-Sharkawy, Abdel Bar, & Ashour, 2012). It has been reported higher antioxidant capacity in Roselle calyces of red variety than in the white one variety (Christian & Jackson, 2009).

Antioxidants are compounds which may inhibit or decrease the rate of oxidation of other molecules by preventing the initiation and, or propagation of the chain reaction of free radicals. These very reactive radicals can interact with biomolecules causing cell injuries or even death; this may result in developing of chronic disorders such as cancer and cardiovascular or cerebrovascular diseases, among others (Wong, Leong, & Koh, 2006). The use of antioxidants from biomaterials such as plants, instead of synthetic ones, for foods is of great interest because, in addition to act as antioxidants, they may have nutraceutical properties to help in preventing

oxidative damages to cells in the human body (Cid-Ortega & Guerrero-Beltrán, 2015; Mahadevan & Shivali Kamboj, 2009; Carvajal-Zarrabal et al., 2012).

Several researchers have demonstrated the antioxidant capacity of Roselle calyces as well as the extracts from them; however, there is very few information on the effect of the particle size of Roselle calyces on the antioxidant properties in extracts. It has been shown that reducing the particle size increases the amount of bioactive compounds during extraction, such as anthocyanins and phenols (Jokić et al., 2010; Cissé et al., 2012), which would imply a higher antioxidant capacity.

Therefore, the main objective of this work was to determine the influence of the average particle size, type of solvent, and extraction time on the physicochemical properties, antioxidant capacity and half maximal effective concentration in Roselle calyces.

2. Materials and Methods

2.1 Roselle Calyces

Red Creole Roselle calyces (long variety) from Chiautla de Tapia, Puebla, Mexico were used for the analysis. A fine powder (FP) was obtained using a stainless steel Spray Veyco MPV mill model 100 (Mexico); ground powder (GP) was obtained as well using an industrial 12 L JR blender model LM-12 (Mexico). Roselle calyces powders were analyzed for their antioxidant capacity and half maximal effective concentration.

2.2 Average Particle Size

The average particle size (APS) of powders was performed using a Tyler Ro-Tap® RX-812 (Mentor, OH, USA) sifter with different meshes (2, 1.4, 1.0, 0.85, 0.6, 0.5, 0.425, 0.3, 0.25, and 0.125 mm). At the bottom of the column of sieves, a dish to collect fines was placed (Gee & Or, 2002). Each sieving test was performed for 7 min using 50 g of sample; then, powders retained in each sieve were weighed. From this information, the size distribution and cumulative weight curves were plotted as well as the mean diameter, d_{50} (O'Hagan et al., 2005; Guerrero-Beltrán, Jiménez-Munguía, Welti-Chanes, & Barbosa-Cánovas, 2009).

2.3 Preparation of Roselle Extracts

A 2x3x3 factorial design was used, including two average particle sizes (0.45, and 1.01 mm), three types of solvents (distilled water, 2% citric acid, and 50% ethanol), and three extraction times (30, 45, and 60 min). Extractions were performed at 50°C at a ratio Roselle:solvent of 1:10. Mixtures were filtered through Whatman paper No. 4, placed in vials of 40 mL and covered with aluminum foil before analysis.

2.4 Physicochemical Properties in Extracts

2.4.1 Titratable Acidity

It was assessed according to the 942.15 AOAC (1995) method. One mL of extract was placed in a 100 mL glass beaker, 20 mL of distilled water added, thoroughly mixed, and titrated with 0.1 N NaOH until achieve pH 8.3. Results were reported as g of citric acid per 100 mL of extract (% W/V).

2.4.2 Total soluble Solids (TSS)

TSS were determined according to the 932.14 AOAC (1995) method using a hand refractometer (Atago Co. LTD, Tokyo, Japan) model Master-M (range of 0-32°Bx). For correcting reading at 20°C, a set of tables, found in the reference tables of the AOAC methods were used.

2.4.3 Density

It was performed according to the 945.06 AOAC (1995) method. Picnometers of 10 mL were used. Empty (W_1), filled with distilled water (W_2), and filled with sample (W_3) picnometers were weighed. Density (25°C) was calculated according to the following equation:

$$\rho = \left[\frac{W_3 - W_1}{W_2 - W_1}\right] * 1000 * \rho_{H_2O} \quad (1)$$

where ρ (mg/mL) is the density of sample and ρ_{H2O} (g/mL) is the density of water at 25°C.

2.4.4 Color

A Colorgard system05 colorimeter (BYK-Gardner Inc. Silver Spring, Maryland, USA) was used in the transmittance mode. The color of extracts was measured placing 2.5 mL of sample in a 3 mL quartz cell (Konica Minolta Sensing Inc. 2 mm). Color parameters were measured in the *CIELab* scale: *L** (lightness, 0 - 100), *a**

(green to red) and *b** (blue to yellow). From those data, purity (chroma or color saturation, *C*) and hue (*H*) were calculated:

$$C = [a^{*2} + b^{*2}]^{1/2} \quad (2)$$

$$(H = \tan^{-1}[b^*/a^*] \quad (3)$$

2.5 Antioxidant Capacity

The DPPH (1,1-Diphenyl-2-picrylhydrazyl) (Brand-Williams, Cuvelier, & Berset, 1995) method was used with some modifications (Molyneux 2004; Velazquez et al., 2007). One mL of Roselle calyces extract was diluted with ethanol (99.5%) to make 10 mL in a volumetric flask, blended, and then filtered through Whatman paper No. 4. 200 µL of the filtrate were taken and placed in a test tube (covered with aluminum foil) containing 1800 µL of ethanol and 2 mL of DPPH solution (7.9 ± 0.2 mg in 200 mL of ethanol), thoroughly mixed and allowed to stand for 45 min at room temperature (21.6 ± 3.3°C) in the darkness. The absorbance was measured at 517 nm using a Cary 100 UV-visible spectrophotometer (Varian Inc., Palo Alto, CA, USA). The antioxidant capacity was calculated as percent of relative inhibition to the control, using the following equation:

$$I\ (\%) = [1 - (\text{Absorbance of sample/Absorbance of control})]*100 \quad (4)$$

Various standard curves were prepared at various concentrations of Trolox (6-hydroxy-2, 5, 7, 8 tetrametilcromo-2 carboxylic acid 97%): 0, 0.007 ± 0.000, 0.011 ± 0.001, 0.015 ± 0.001, 0.018 ± 0.001, 0.022 ± 0.001, 0.026 ± 0.001, 0.030 ± 0.001 mg. The standard curve (R^2 = 0.985 ± 0.009) was:

$$I\ (\%) = 3395.63 \pm 197.91(1/\text{mg TE})*X\ (\text{mg TE}) - 0.026 \pm 2.250 \quad (5)$$

Results were expressed as Trolox equivalents (TE) per 100 g of Roselle calyces.

2.6 Half Maximal Effective Concentration (EC_{50})

One mL of Roselle calyces extract was diluted with ethanol to make 10 mL in a volumetric flask, mixed, and then filtered through Whatman paper No. 4. Different volumes of this mixture were taken (0, 50, 100, 150, 200, 250, and 300 µL corresponding to 0, 5.0 ± 0.2, 10.0 ± 0.4, 15.0 ± 0.6, 20.0 ± 0.8, 25.0 ± 1.0, and 30.0 ± 1.2 mg of extract, respectively) and placed in test tubes (covered with aluminum foil), and brought to 2 mL with ethanol (99.5%). Then, 2 mL of DPPH solution (7.9 ± 0.2 mg in 200 mL of 99.5% ethanol) were added, mixed and allowed to stand for 45 min at room temperature (22.2 ± 2.6 °C) in the darkness. The absorbance was measured at 517 nm using a Cary 100 UV-visible spectrophotometer (Varian Inc., Palo Alto, CA, USA). The antioxidant capacity was calculated as explained above. Various standard curves (R^2 = 0.921 ± 0.037) were performed in triplicate and results were expressed as mg of Roselle extract:

$$I\ (\%) = 2.56 \pm 0.17(1/\text{mg extract})*X\ (\text{mg extract}) + 11.42 \pm 3.38 \quad (6)$$

2.7 Statistic Analysis

The effect of the average particle size, type of solvent, and extraction time on the physicochemical, antioxidant, and half maximal effective concentration (EC_{50}) properties in Roselle calyces was analyzed by ANOVA. Multivariate analysis and a Tukey's multiple comparison tests were used to compare differences within averages. Differences were considered significant for $p \leq 0.05$.

3. Results and Discussion

3.1 Granulometry

Figure 1 shows the particle size distribution of fine and ground powders of Roselle calyces. The greater weights of fine powder were retained in the sieves of 0.3 mm (24.42 ± 1.98%) and 0.6 mm (28.99 ± 4.11%); the greater weights for the ground powders were retained in the sieves of 1.0 (21.66 ± 03.3%), 1.4 mm (31.76 ± 1.26%), and 2.0 mm (18.80 ± 1.68%). The cumulative weight curves of retained powder are also shown in Fig. 1. From this curve, the median diameter (d_{50}) was determined by interpolation; the d_{50} values were 0.45 ± 0.014 and 1.01 ± 0.031 mm for fine and ground Roselle powders, respectively.

3.2 Physicochemical Properties of Extracts

Table 1 shows the effect of average particle size, type of solvent, and extraction time on the physicochemical properties of Roselle calyces extracts.

3.2.1 Titratable Acidity

The APS showed significant effect ($p > 0.05$) on titratable acidity (Table 1). Extracts obtained with 2% citric acid had higher acidity (3.93 ± 0.27 g/100 mL) than ethanolic (1.96 ± 0.09 g/100 mL) and aqueous (1.98 ± 0.11 g/100 mL) extracts due of the citric acid content in the extraction solution. This was confirmed performing a

comparison of the average acidity of each solvent, subtracting the concentration of citric acid (2%) to acidity values obtained with this solvent. The statistical analysis showed no significant differences ($p > 0.05$) among means of acidity for 50% ethanolic (1.96 ± 0.09 g/100 mL), aqueous (1.98 ± 0.11 g/100 mL), and 2% citric acid (1.90 ± 0.27 g/100 mL) extracts. Conclusively, the higher the extraction time, the lower the percentage of acidity in the extracts obtained with 2% citric acid (Table 1). Abou-Arab et al. (2011) evaluated the physicochemical properties of Roselle extracts obtained with different solvents; they reported an acidity of 11.58 ± 0.02% (citric acid) in acidified ethanol extracts obtained with 1.5 N HCl (85:15), 19.02 ± 1.0% in extracts obtained with 2% citric acid, and 18.85 ± 1.0% in aqueous extracts. These values are much higher than those obtained in this work, which could be due to the extraction method used and the ratio of calyces:solvent (4 g of powder mixed with 100 mL of solvent). The mixture was allowed to stand overnight at 4°C, then filtered; three more successive extractions were performed. Chumsri et al. (2008), on the other hand, reported an acidity of 1.71 ± 0.02% (malic acid) in aqueous extracts using a calyces:water ratio of 1:10 and heating at 50°C for 30 min.

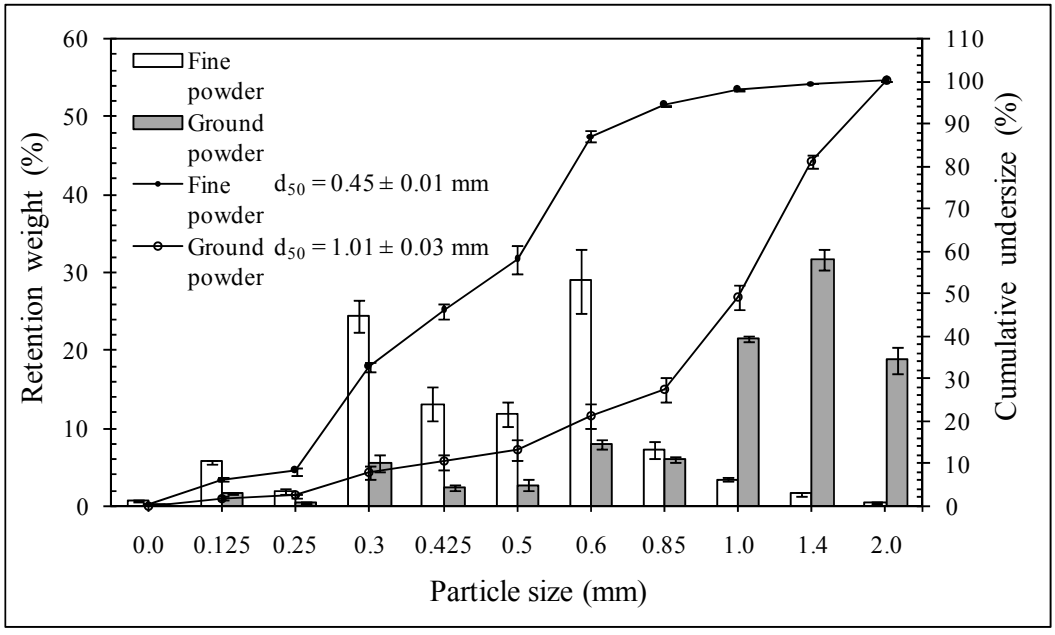

Figure 1. Particle size distribution and cumulative weight curves of fine and ground calyces. Values represent the mean ± standard deviation of three observations (n = 3)

3.2.2 Total Soluble Solids

TSS were higher ($p \leq 0.05$) in alcoholic extracts (14.16 ± 0.62%) than in the other extracts. No significant differences ($p > 0.05$) were observed between TSS in aqueous extract (5.38 ± 0.24%) and those obtained with 2% citric acid (5.18 ± 0.35%). It is noteworthy to say that alcohol was removed using a Büchi rotary evaporator RE 111 (Brinkmann Instruments Inc., Switzerland) at 45°C and 55 cmHg; afterward, water was added for making up the initial volume and TSS were measured. Therefore, TSS decreased due to removal of ethanol. The anterior procedure was done because the 50% ethanol solution showed a TSS amount of 16.2 ± 0.23% at 20°C; the alcoholic extracts, on the other hand, gave an average TSS of 19.5 ± 0.25% at 20°C. The refractive index of ethanol at 20°C is 1.361, equivalent to 18.3°Bx, approximately (Hayes, 1992). TSS of extracts obtained with 2% citric acid solution (total average of 7.2 ± 0.37% at 20°C) were corrected by subtracting TSS of the citric acid solution (1.82 ± 0.04% at 20°C). Furthermore, it was observed that TSS of the alcoholic extracts decreased as time of extraction was prolonged (Table 1) in the extracts obtained using a particle size of 0.45 mm. Abou-Arab, Abu-Aalem, & Abou-Arab (2011) reported a TSS content of 20.0 ± 2.0, 16.0 ± 1.0 and 5.0 ± 0.03% in extracts using 1.5 N HCl (85:15) acidified ethanol, acidified ethanol with 1% citric acid, and distilled water, respectively. These results are similar to those reported in this study for extracts obtained with 50% ethanol, without removing the solvent (19.5 ± 0.25% at 20°C), and water (5.4 ± 0.2%). However, they reported 12.0 ± 1.0% of TSS for extracts obtained with 2% citric acid solution; results that are completely different to results from this study using the same solvent (7.2 ± 0.37% at 20°C). Chumsri, Sirichote, and Itharat (2008) found similar results (5.97 ± 0.08% TSS) to that obtained in this work using a Roselle:water ratio of 1:10 and heating at 50°C for 30 min.

3.2.3 Density

The type of solvent, and in some cases the extraction time, significantly affected ($p \leq 0.05$) density of the extracts. The acid extracts, obtained at different times, showed higher density (1027 ± 0.006 mg/mL) than the aqueous (1020 ± 0.003 mg/mL) and alcoholic (952.1 ± 0.004 mg/mL) extracts. This might be due to the content of citric acid in the solution as well to the type of solvent; each solvent has different density.

Table 1. Effect of average particle size (APS), solvent type (ST), and extraction time (ET) on the physicochemical properties of Roselle calyces extracts

APS (mm)	ST	ET (min)	Density (mg/mL)	Temperature (°C)	TSS[1] (%)	Titratable acidity as citric acid (g/100 mL)
0.45	Ethanol	30	950.7 ± 5.0a	24.3 ± 1.6	14.22 ± 0.7a	1.84 ± 0.04a
	50%	45	950.2 ± 3.0a	23.9 ± 1.9	14.17 ± 0.1ab	1.95 ± 0.10ab
		60	950.4 ± 4.0a	23.7 ± 0.9	13.24 ± 0.5b	1.97 ± 0.02ab
	Distilled	30	1019.8 ± 2.0b	24.1 ± 2.0	5.36 ± 0.2c	2.01 ± 0.10ab
	water	45	1020.5 ± 4.0bc	25.9 ± 2.5	5.40 ± 0.2c	2.00 ± 0.10ab
		60	1019.6 ± 4.0b	26.1 ± 0.9	5.31 ± 0.1c	1.85 ± 0.05a
	Citric acid	30	1027.7 ± 7.0cd	27.3 ± 1.7	5.30 ± 0.3c	4.16 ± 0.25cf
	2%	45	1029.7 ± 6.0d	27.0 ± 2.1	5.14 ± 0.2c	4.01 ± 0.18ce
		60	1022.9 ± 9.0bd	27.7 ± 1.3	5.36 ± 0.2c	3.72 ± 0.23dg
1.01	Ethanol	30	952.2 ± 2.0a	25.2 ± 2.3	14.43 ± 0.1a	1.92 ± 0.10ab
	50%	45	954.5 ± 4.0a	24.7 ± 1.4	14.78 ± 0.5a	1.96 ± 0.03ab
		60	954.3 ± 3.0a	23.1 ± 0.8	14.12 ± 0.5ab	2.09 ± 0.10ab
	Distilled	30	1019.3 ± 2.0b	25.6 ± 2.3	5.46 ± 0.4c	1.95 ± 0.10ab
	water	45	1019.2 ± 4.0b	26.3 ± 1.6	5.28 ± 0.1c	2.12 ± 0.10b
		60	1019.5 ± 3.0b	25.8 ± 2.3	5.46 ± 0.3c	1.94 ± 0.04ab
	Citric acid	30	1025.2 ± 5.0bd	26.2 ± 1.2	5.14 ± 0.3c	4.12 ± 0.22ch
	2%	45	1029.8 ± 5.0d	26.9 ± 1.5	5.10 ± 0.3c	3.97 ± 0.08defh
		60	1026.2 ± 3.0bd	27.6 ± 1.4	5.05 ± 0.6c	3.62 ± 0.16g

Values represent the mean ± standard deviation of three observations. Values bearing different letters in the same column are significantly different ($p \leq 0.05$). [1]TSS: Total soluble solids. Extracts obtained at a ratio Roselle:solvent of 1:10 at 50°C.

3.3 Color Properties

3.3.1 Particle Size Effect

Table 2 shows the effect of particle size, type of solvent, and extraction time on the color properties of Roselle extracts. The average particle size had a significant effect ($p > 0.05$) on all variables, except on hue. Extracts obtained with calyces of an APS of 1.01 mm were lighter (43.6 ± 2.8), showing a tendency to red (61.8 ± 1.0) and yellow (49.0 ± 4.0) colors compared to the L^*, a^*, and b^* values of the extracts obtained with a particle size of 0.45 mm: 41.1 ± 3.4, 60.3 ± 2.2 and 47.9 ± 3.7, respectively. Thus, the extracts obtained with an APS of 1.01 mm had a more intense red color; this is demonstrated with the higher purity (79.0 ± 2.2) compared to that obtained with an APS of 0.45 mm (77.1 ± 2.7).

3.3.2 Type of Solvent Effect

According to the statistical analysis, the color properties of different extracts were significantly affected ($p \leq 0.05$) by the type of solvent. Table 2 shows the effect of the average particle size (APS), type of solvent (TS), and extraction time (ET) on the color parameters of Roselle extracts. In general, the acid ($L^* = 44.0 \pm 2.6$) and aqueous ($L^* = 43.3 \pm 3.0$) extracts were lighter than alcoholic extracts ($L^* = 39.7 \pm 2.8$). While the a^* value (in

the red zone of the color space) was higher for the alcoholic extracts (62.1 ± 1.8) when compared with the aqueous extracts (60.4 ± 1.8) and acid extracts (60.8 ± 1.6); however, no significant differences ($p > 0.05$) were observed within aqueous and acid extracts. The tendency toward the yellow (b^*) color was higher for the acid extracts (51.7 ± 1.6), followed by aqueous extracts (50.2 ± 1.6), and finally, the alcoholic extracts (43.6 ± 1.6). This same tendencies were observed for the hue, which was higher for acid extracts (40.4 ± 0.4), followed by aqueous extracts (39.7 ± 0.4), and finally by alcoholics extracts (35.1 ± 1.2). Purity, on the other hand, was greater for the aqueous (78.5 ± 2.3) and acid (79.8 ± 2.2) extracts compared to the purity of alcoholics extracts (75.9 ± 1.8). This indicates that the extracts obtained with distilled water and 2% citric acid solution had an intense red color, whereas the alcoholic extracts showed a dark red color. The same was observed by Salazar-González, Vergara-Balderas, Ortega-Regules, & Guerrero-Beltrán (2012) in extracts obtained with different solvents (ethanol:water 50:50, ethanol:water 70:30, water, ethanol:1.5 N HCl, 85:15, and 96% ethanol). From this information, the 50:50 ratio ethanol:water extracts had the lowest values in L (17.16 ± 0.03), a (37.76 ± 0.04), b (9.39 ± 0.05) and hue (38.91 ± 0.03) color parameters.

Table 2. Effect of average particle size (APS), solvent type (ST), and extraction time (ET) on the color properties of Roselle calyces extracts

APS (mm)	ST	ET (min)	L^*	a^*	b^*	Hue	Purity
0.45	Ethanol	30	37.8 ± 2.21ad	61.6 ± 1.96acdefg	44.5 ± 1.45a	35.9 ± 0.23a	76.0 ± 2.41ac
	-50%	45	42.8 ± 1.17ac	63.9 ± 0.40bdeg	42.7 ± 2.00a	33.7 ± 1.41b	76.9 ± 0.76 abc
		60	36.0 ± 0.77 d	59.4 ± 0.51cefg	42.8 ± 0.97a	35.8 ± 0.55a	73.2 ± 0.94c
	Distilled	30	41.5 ± 3.65adg	59.4 ± 1.95acefg	49.6 ± 2.25bc	39.9 ± 0.36d	77.4 ± 2.93abc
	water	45	43.3 ± 2.60ag	60.7 ± 1.12abcdefg	50.8 ± 1.04bc	39.9 ± 0.18d	79.1 ± 1.51abd
		60	39.9 ± 3.31adh	57.9 ± 1.90f	48.5 ± 1.62b	39.9 ± 0.03d	75.5 ± 2.50cd
	Citric acid	30	42.9 ± 1.82ai	60.6 ± 0.85abcdefg	51.5 ± 0.68bc	40.4 ± 0.08d	79.5 ± 1.08ade
	(2%))	45	44.9 ± 0.57bcefghi	61.2 ± 0.26abcdefg	51.4 ± 0.49bc	40.0 ± 0.21d	79.9 ± 0.46ade
		60	40.8 ± 3.71adi	58.3 ± 2.80acef	49.8 ± 2.53bc	40.5 ± 0.44d	76.6 ± 3.72ace
1.01	Ethanol	30	42.8 ± 1.17ae	64.0 ± 0.40deg	42.7 ± 2.00a	33.7 ± 1.41cb	76.9 ± 0.76abc
	-50%	45	39.6 ± 0.33adf	62.0 ± 0.36eg	43.9 ± 0.25a	35.3 ± 0.31ab	75.9 ± 0.17ac
		60	39.5 ± 1.82adf	61.8 ± 1.40eg	44.9 ± 1.52a	36.0 ± 0.36a	76.4 ± 2.01abc
	Distilled	30	46.4 ± 1.18bcegi	61.6 ± 0.11abcdefg	50.5 ± 1.05bc	39.3 ± 0.55d	79.6 ± 0.74ade
	water	45	44.9 ± 0.74bcefghi	62.0 ± 0.26 g	52.1 ± 0.30bc	40.0 ± 0.28d	81.0 ± 0.04ae
		60	43.9 ± 1.20afi	60.7 ± 0.20abcdefg	49.7 ± 0.38bc	39.3 ± 0.28d	78.5 ± 0.17ade
	Citric acid	30	43.9 ± 2.75ai	61.1 ± 1.36abcdefg	52.5 ± 1.76b	40.7 ± 0.34d	80.5 ± 2.17ade
	-2%	45	45.7 ± 1.34bcefghi	61.7 ± 0.19abcdeg	52.1 ± 0.53bc	40.2 ± 0.32d	80.8 ± 0.32ae
		60	46.0 ± 1.82bceghi	61.7 ± 0.22abcdeg	53.1 ± 0.60c	40.7 ± 0.41d	81.4 ± 0.27be

Values represent the mean ± standard deviation of three observations. Values bearing different letters in the same column are significantly different ($p \leq 0.05$).

3.3.3 Effect of the Extraction Time

According to the statistical analysis, only the a^* color parameter, hue, and purity were significantly affected ($p \leq 0.05$) by the extraction time. Table 2 shows the effect of the average particle size, type of solvent and extraction time on the color parameters of Roselle calyces extracts. Increasing the extraction time from 45 to 60 min the red color of the extracts decreased from 61.9 ± 1.2 to 60.2 ± 2.2; however, no significant difference ($p > 0.05$) was observed within this extract and the extract obtained at 30 min (61.4 ± 1.8). The same effect was observed for hue and purity, which implies a decrease in red color in the extracts as extraction time increased from 45 to 60 min. Chumsri et al. (2008) reported the same in aqueous extracts obtained at 50°C for 30 to 60 minutes; they

reported *a** average values of 5.14 ± 0.69 and 1.86 ± 1.10, respectively. Ramirez-Rodrigues et al. (2011) reported a decrease of the *a** values (66.73, 66.39, 65.65, and 63.93) as the extraction time increased (2, 4, 8, 16 min, respectively), using a temperature of 90°C. In their study, they used a ratio of Roselle calyces:water of 1:40.

3.4 Antioxidant Capacity of Roselle Calyces

The antioxidant capacity results of Roselle calyces extracts are shown in Table 3. The three independent variables (APS, ST, ET) and their interactions were statistically significant ($p \leq 0.05$). Extracts from an APS of 0.45 mm had higher antioxidant capacity (total average of 790.2 ± 86.3 mg of TE/100 g dry solids (d.s.)), and darker color (Table 2), than those obtained with an APS of 1.01 mm (total average of 721.0 ± 110.4 mg of TE/100 g d.s.). The alcoholic (820.2 ± 73.7 mg of TE/100 g d.s.) and acid (773.34 ± 53.0 mg of TE/100 g d.s.) extracts showed higher ($p \leq 0.05$) antioxidant capacity, and darker color (Table 2), than the aqueous extracts (673.24 ± 116.6 mg of TE/100 g d.s.). A correlation, with negative slope, was observed (Figure 2) between the antioxidant capacity (including all APS and ST) and extraction time (ET); as ET increased, the antioxidant capacity decreased. Therefore, the extraction at 50°C for more than 45 min affects the antioxidant capacity.

Table 3. Effect of average particle size (APS), solvent type (ST), and extraction time (ET) on the antioxidant capacity of Roselle calyces

APS (mm)	ST	ET (min)	Antioxidant capacity (mg TE/100 g of Roselle calyces)
0.45	Ethanol 50%	30	909.61 ± 31.7a
		45	882.34 ± 19.3ac
		60	829.82 ± 61.6ad
	Distilled water	30	735.79 ± 23.0bfg
		45	661.04 ± 55.0fh
		60	792.48 ± 29.6bdi
	Citric acid 2%	30	779.97 ± 27.8bdjk
		45	800.62 ± 66.1bcdj
		60	719.84 ± 72.5ghik
1.01	Ethanol 50%	30	727.30 ± 31.3bfg
		45	763.11 ± 45.4bdg
		60	809.04 ± 32.7bcd
	Distilled water	30	785.30 ± 32.0ghij
		45	562.83 ± 25.7e
		60	502.02 ± 46.5e
	Citric acid 2%	30	800.00 ± 23.0bcdjk
		45	797.34 ± 34.8bdj
		60	742.25 ± 20.8bh

Values bearing different letters in the same column are significantly different ($p \leq 0.05$). Values are mean ± standard deviation of three observations.

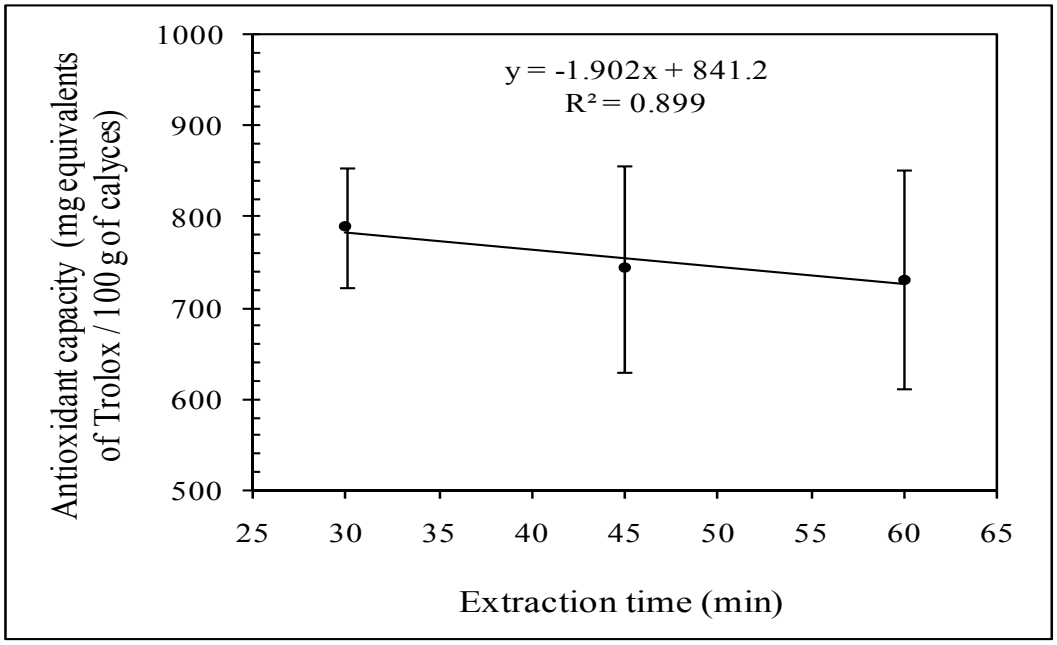

Figure 2. Correlation between the antioxidant capacity and the extraction time at 50°C. Values represent the mean ± standard deviation of eighteen observations (n = 3)

Wong et al. (2006) reported an antioxidant activity of 50 μmol of TE/g of dry Roselle calyces (about 1252 mg of TE/100 g d.s.); the extracts were obtained using 0.5 g of ground calyces and 25 mL of deionised water. The mixture was allowed to stand at room temperature for 1 h in the dark, with occasional agitation. The aqueous extract was obtained by filtering the mixture through Whatman paper No. 1. Salazar-González et al. (2012), who used the same Roselle variety as the one used in this study, reported an antioxidant capacity of 8035 ± 220 μM of TE/100 g (about 2011.1 mg of TE/100 g of calyces) in 50:50 ethanol:water extracts.

3.5 Half Maximal Effective Concentration and DPPH Inhibition

Results of half maximal effective concentration (EC_{50}) and inhibition of DPPH are shown in Table 4. The EC_{50} is defined as the amount of antioxidant needed to decrease the initial concentration of DPPH by 50% (Brand-Williams et al., 1995; Molyneux, 2004); therefore, the lower concentration of extract required to inhibit DPPH by 50%, the better its antioxidant capacity. Extracts from an average particle size of 0.45 mm had the best antioxidant properties because they had lower EC_{50} values (having a total average of 14.8 ± 2.1 mg of extract) compared to those obtained with extracts from particles of 1.01 mm (total average of 15.6 ± 1.7 mg of extract), corresponding to inhibitions of 79.8 ± 3.8 and 77.8 ± 5.8%, respectively. Regarding solvent type, the ethanolic extracts gave the best ($p \leq 0.05$) EC_{50} values (total average of 13.4 ± 1.0 mg of extract), followed by the 2% citric acid extracts (total average of 14.9 ± 1.0 mg of extract), and finally by the aqueous extracts (total average of 17.2 ± 1.2 mg of extract). However, the inhibition was not statically different ($p > 0.05$) for the alcoholic (80.2 ± 2.9%) and acid (82.1 ± 2.1%) extracts. The aqueous extracts showed a 74.1 ± 5.2% of inhibition.

Regarding the extraction time, the lowest EC_{50} value was obtained after 30 (total average of 14.6 ± 1.8 mg of extract) and 45 min (total overage of 15.3 ± 1.2 mg of extract) of extraction. For an extraction of 60 min, the EC_{50} value was 15.6 ± 2.5 mg of extract. The total averages for inhibition were 81.4 ± 3.7, 78.6 ± 2.6, and 76.5 ± 6.6% for 30, 45, and 60 min of extraction, respectively. Furthermore, a correlation was observed (Figure 3) between the percentage of inhibition and EC_{50} with respect to the extraction time; that is, as the EC_{50} increases, the percentage of DPPH inhibition decreases. This confirms the already discussed above, as the extraction time is prolonged the antioxidant capacity may decrease. Chumsri et al. (2008) carried out the extraction process using a ratio of Roselle calyces:water of 1:10 at 50°C for 30 min. They reported an EC_{50} of 44.78 ± 0.49 mg/mL for antioxidant capacity. Similar results were reported by Abou-Arab et al. (2011) obtaining an antioxidant capacity expressed as EC_{50} of 43.18 ± 2.0 mg/mL, using a 2% citric acid solution as the extracting agent. When using distilled water they reported an EC_{50} of 45.64 ± 2.0 mg/mL. Mungole (2011) reported an EC_{50} of 0.13 mg/mL and an 88.03% inhibition in ethanol Roselle extracts. Kumar, Garg and Garg (2012) reported EC_{50} values

of 94.16 ± 1.52 and 46.13 ± 3.37 μg/mL for aqueous and ethanolic (96%) Roselle extracts, respectively.

Table 4. Effect of average particle size (APS), solvent type (ST), and extraction time (ET) on the half maximal efficient concentration (EC_{50}) and DPPH inhibition of Roselle calyces extracts

APS (mm)	ST	ET (min)	Extract (mg)	DPPH Inhibition (%)	EC_{50}[1] (mg extract)	b	m	R^2
0.45	Ethanol	30	28.6±0.10a	84.9±1.7ag	12.81±0.8ad	13.63±1.85	2.84±0.04	0.912 ± 0.01
	50%	45	28.5 ± 0.07a	78.9 ± 1.9bcdeg	13.28 ± 0.6acd	15.09 ± 0.48	2.63 ± 0.08	0.885 ± 0.01
		60	28.5 ± 0.10a	80.9 ± 1.5abceg	12.10 ± 0.3a	17.64 ± 2.12	2.68 ± 0.12	0.849 ± 0.03
	Distilled	30	30.7 ± 0.03bc	79.7 ± 1.6bcdeg	15.72 ± 0.9beh	10.38 ± 1.52	2.52 ± 0.05	0.944 ± 0.02
	water	45	30.5 ± 0.03be	76.4 ± 1.7cdeh	16.60 ± 0.6bik	7.78 ± 0.72	2.55 ± 0.11	0.950 ± 0.01
		60	30.6 ± 0.08bce	72.9 ± 1.2de	18.42 ± 0.9gij	4.60 ± 2.81	2.46 ± 0.05	0.958 ± 0.02
	Citric acid	30	30.9 ± 0.10cd	84.8 ± 1.0ag	13.18 ± 0.8af	14.71 ± 2.57	2.68 ± 0.04	0.897 ± 0.02
	2%	45	31.0 ± 0.05d	79.6 ± 1.1cdi	15.15 ± 0.8bcef	12.29 ± 2.28	2.49 ± 0.03	0.925 ± 0.03
		60	30.8 ± 0.16cdf	80.4 ± 2.1abhi	15.68 ± 0.7bel	9.85 ± 0.90	2.56 ± 0.06	0.943 ± 0.01
1.01	Ethanol	30	28.6 ± 0.09a	80.2 ± 2.8abcdeg	13.74 ± 0.7ae	13.39 ± 1.24	2.67 ± 0.05	0.908 ± 0.01
	50%	45	28.7 ± 0.04a	77.6 ± 1.5bcde	14.86 ± 0.3bcefm	12.56 ± 1.63	2.52 ± 0.09	0.917 ± 0.02
		60	28.6 ± 0.07a	78.7 ± 1.3bcdeg	13.41 ± 0.3afm	15.39 ± 1.22	2.58 ± 0.03	0.880 ± 0.02
	Distilled	30	30.5 ± 0.05be	75.6 ± 1.0deh	17.60 ± 0.5ghil	6.74 ± 2.19	2.46 ± 0.13	0.954 ± 0.01
	water	45	30.6 ± 0.15bfe	76.3 ± 1.7deh	16.43 ± 0.5bijkl	9.27 ± 1.52	2.48 ± 0.06	0.944 ± 0.01
		60	30.6 ± 0.10bfe	64.0 ± 1.8f	18.43 ± 1.0gi	13.02 ± 2.23	2.01 ± 0.01	0.857 ± 0.03
	Citric acid	30	30.9 ± 0.18cdf	83.1 ± 1.2gi	14.71 ± 0.4defk	11.31 ± 1.04	2.63 ± 0.02	0.939 ± 0.01
	2%	45	30.9 ± 0.05cd	82.6 ± 1.2gi	15.57 ± 0.3be	8.50 ± 0.07	2.67 ± 0.06	0.958 ± 0.01
		60	30.7 ± 0.13de	81.9 ± 1.4abi	15.30 ± 0.6bem	9.49 ± 1.22	2.65 ± 0.08	0.951 ± 0.01

Values bearing different letters in the same column are significantly different ($p \leq 0.05$). All values are mean ± standard deviation of three observations. [1]EC_{50} is the amount of antioxidant necessary for reducing the DPPH concentration by 50%.

Mohd-Esa, Shin-Hern, Ismail and Lye-Yee (2010) studied the antioxidant capacity of different parts of Roselle. They reported a 30.8 ± 1.4% inhibition for water extracts and 87.9 ± 0.8% inhibition for 80% ethanol (v/v). The extracts were obtained by stirring one gram of Roselle in one L of solvent for 2 hours at room temperature. Anokwuru et al. (2011) assessed the antioxidant capacity in Roselle calyces extracts using different solvents (methanol, ethanol, acetone, and water). The Roselle calyces were finely ground and 20 g of sample was mixed with 250 mL of solvent and allowed to stand for 72 hours. The filtrate from the mixture was concentrated in an evaporator at 40°C. The methanol extract showed a higher inhibition of the DPPH radical (78 ± 0.25%), followed by the ethanol extract (69 ± 0.46%), the aqueous extract (63 ± 9.97%), and the acetone extract (37 ± 0.01%). Differences in results from this work and other studies are mainly due to the variety of Roselle, as well as the sample:solvent ratio, extracting method and type of solvent. It is noteworthy to say that in the works cited above authors do not mention the average particle size, only alludes to a ground Roselle calyces.

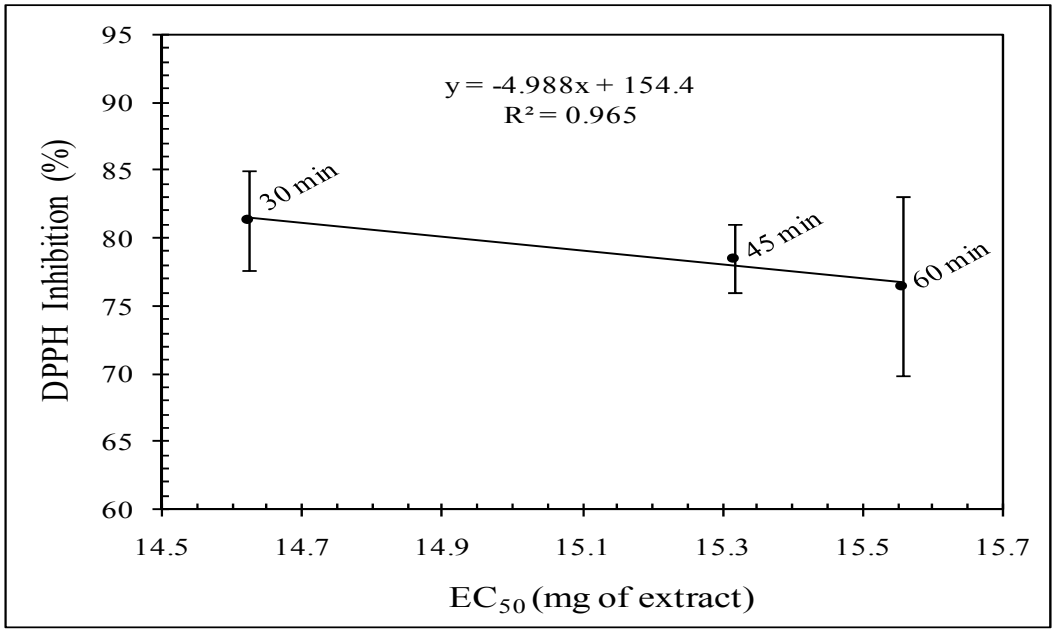

Figure 3. Relationship between the percentage of inhibition and ec_{50} with respect to the extraction time at 50°C. Values represent the mean ± standard deviation of eighteen observations (n = 3)

4. Conclusions

According to the results obtained in this study, the antioxidant activity was significantly influenced by the method for obtaining extracts. The average particle size is an important variable to be considered in the extraction process. The extraction at 50°C in the range of 30 to 45 min did not significantly affect the antioxidant capacity of Roselle calyces extracts. The extracts obtained with these conditions represent an important alternative for antioxidants in processed foods, in addition to providing color and other components with functional properties.

Acknowledgements

Sandro Cid-Ortega thanks to "Programa Educativo de Ingeniería en Procesos Alimentarios" and to Universidad Tecnológica de Izúcar de Matamoros (UTIM), Puebla, México for the scholarship granted to complete his doctoral studies.

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