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Impact of infusion time and ultrasound-assisted extraction on bioactive compounds and antioxidant capacity of foods prepared from seeds and leaves of Ramon tree (*Brosimum alicastrum*)

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Abstract

The objective of the present work was to analyse the concentrations of bioactive compounds, and the antioxidant capacity of three foods prepared from the seeds and leaves of Ramon tree (*Brosimum alicastrum*). We studied the effect of infusion time and ultrasonic extraction on leaves, roasted seeds (coffee substitute), and Ramon flour to optimise the recovery of bioactive components. Infusion time positively affected the concentrations of total phenolic content (TPC), total flavonoid content (TFC), total tannins (TCT), and glycosylated flavonoids (TFG) in the leaves and seeds. The amount of solubilised phenolic compounds increased with increasing infusion time until 2,552 mg of TPC, 54.1 mg of TFC, 75.3 mg of TFG, and 2.40 mg of TCT were obtained per 100 g of leaves. We reported 32.9% greater infusion of toasted seeds in TPC, 87.3% in anthocyanins, 84.6% in TCT, and 91.5% in vitamin C than in leaves. Furthermore, the antioxidant capacity of the seed infusions was 56% greater than that of the leaf infusions. The ultrasonication time was positively correlated with the tannin concentration, which was 43.8% greater in the leaves (5.24 mg/100 g) than in the flour. The neutralising capacity of free radicals determined by 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was 23.6% greater than that of flour (27.93 µmol/g) and leaves (6.48 µmol/g), and was not related to the extraction time. Taken together, these findings showed that Ramon seeds and leaves could be highly functional foods due to their nutritional quality and high content of antioxidant compounds.

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Introduction

Bioactive compounds obtained from plant seeds and leaves have been reported to be highly rich in antioxidants and anti-inflammatory substances, thereby significantly and positively impacting human health (Esmeeta *et al.*, 2022; Dong and Nao, 2023). Ramon tree (*Brosimum alicastrum* Sw.) is one of the dominant tropical trees in the forests of Mexico and Central America. It is an evergreen tree that reaches maturity in approximately 20 years, attaining a height of 30 to 35 m, and a trunk diameter of 1 m or more. The products of this tree have been the primary source of food for the Mayan civilisation (250 to 900 AD). The Mayans recognised the nutritional value of the seeds, productivity, and importance of Ramon tree as a food source for game species (Puleston, 1968; Dussol *et al.*, 2017). However, since 1975, Ramon tree has been listed as an underexploited species with significant potential for attaining food security (Hernández-González *et al.*, 2015).

The fruits of Ramon tree are 1.5 to 2.5 cm in diameter, and weigh approximately 4.5 g. The weight of the seed is 67%. The fruits are yellowish green before maturity, and turn orange or yellow as they ripen (Peters and Pardo-Tejera, 1982). Ground Ramon seeds are incorporated into baked goods such as breads, cakes, and cookies (FDA, 2007). In addition, the roasted seed powder possesses a coffee or dark chocolate flavour that intensifies with further roasting. The fruit's pulp taste is similar to that of tart apricots, and the fruit can be eaten raw or used to prepare jams. The leaves primarily serve as fodder for animal production. In addition, several products have been developed using Ramon tree components for human consumption, and have been marketed, emphasising their medicinal properties (FDA, 2007). The seeds contained 5.53% moisture, 62.6% crude

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protein, 1.31% fat, and 3.27% crude fibre. The polyunsaturated fatty acid content of the seeds is greater than that of canola and olive oil, which are primarily composed of linoleic and linolenic acids, whereas the monounsaturated fatty acid content is lower (Tokpunar, 2010).

Although numerous studies on incorporating pumpkin, amaranth, and legume seeds into several food products have been reported in the literature, little is known about the incorporation of Ramon tree seeds into food for human consumption. A few commercial products have used Ramon seeds as an ingredient. Currently, the United States is witnessing a small but growing demand for Ramon seed powder, which is sold as Mayan nut, Ramon nut, or Ramon seed. The company Guayakí Sustainable Rainforest Products (Sebastopol, CA) produces a coffee substitute called Java Mate, which is composed of yerba mate and roasted Ramon seeds. Similarly, Teeccino (Oxnard, CA) and MontaVida (Tarpon Springs, FL) include Ramon seeds in their herbal coffee alternatives. These products are generally recognized as safe (GRAS), according to the United States Food and Drug Administration (DEN 96524899v1 11/15/2007).

A few studies have analysed the application of Ramon tree products as human food (Gillespie *et al.*, 2004; Yates and Ramírez-Sosa, 2004; Ramírez-Sánchez, 2018). One study has investigated the nutritional characteristics of its flour (Subiria-Cueto *et al.*, 2019), whereas other studies have determined the concentration of phenolic compounds in the seeds, and the methodological efficiency of the extraction of antioxidant compounds (Gullian-Klanian and Terrats Preciat, 2017; Moo-Huchin *et al.*, 2019). Ramon seeds contain higher concentrations of total phenols, including gallic acid, *p*-hydroxybenzoic acid, vanillic acid, caffeic acid, *p*-coumaric acid, and the flavonoid (-)-epicatechin than walnuts, almonds, and peanuts (Ozer, 2016). The radical scavenging activity of 2,2-diphenyl-1-picrylhydrazyl (DPPH) and the inhibitory activity of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) of Ramon seed extracts are greater than those of other fruits, such as walnuts, almonds, and peanuts, and are even greater than those of the commercial antioxidant BHT. Similarly, its iron-reducing antioxidant power (FRAP) is greater than that of peanuts and almonds (Tokpunar, 2010).

The effect of technological processes on the antioxidant components of foods prepared with Ramon seeds and leaves has not been studied in detail. Although roasting does not affect the nutritional quality of components in seeds, it significantly decreases the flavonoid concentration and increases the condensed tannin content of fresh seeds (Quintero-Hilario *et al.*, 2019). In the present work, we assessed the concentration of bioactive compounds and the antioxidant capacity of three products prepared from Ramon seeds and leaves. The present work also assessed the optimum infusion and extraction times needed to achieve greater recovery of bioactive components.

Materials and methods

Sampling

Ramon leaves were collected from a forestry company in Yucatán, Mexico. The leaves were dried at 36°C for 5 d in the laboratory using a convection oven (Fisher Scientific, PA, USA), until the samples reached constant weight. The moisture content was 16.9 ± 1.1%. Subsequently, the dried leaves were ground in a pulveriser mill (VEYCO MPV250, DF, Mexico) using a 0.8 mm sieve until a fine powder was obtained.

The dry seeds were roasted at 90°C for 25 min to make the coffee beans. They were subsequently ground using a 20 mm mesh sieve to obtain a medium-fine dark brown powder.

To obtain the flour, the Ramon seeds were dried in the laboratory at 55°C for 5 d until the samples reached constant weight. The moisture content was 14.2 ± 1.3%. After removing the integument, the seeds were ground through a 0.8 mm mesh sieve to obtain a fine, light brown powder. The flour and pulverised leaves were vacuum-packed and stored at 4°C until further analyses. The nutritional compositions of the Ramon leaf, seed, and flour are presented in Table 1.

Leaf and seed infusions

The effect of infusion time (1, 5, 10, 15, 20, and 25 min) on the concentration and activity of antioxidant compounds in tea prepared from leaves, and coffee obtained from seeds was studied using six treatments performed in quadruplicate.

The leaf infusion was prepared in a glass teapot using a heat-resistant stainless-steel infuser filled with 350 mL of deionised water. Water was first

Table 1. Nutritional composition of leaves, seeds, and flour of Ramon tree (*Brosimum alicastrum*).

Per 100 g contain	Leaf	Seed	Flour
Energy (Kcal)	224	300	312
Carbohydrate (g)	38	63	65
Protein(g)	12	11	10
Fat (g)	2.3	1.4	1.3
Fibre (g)	38	19	15
Calcium (mg)	38	245	183
Sodium (mg)	46	44	20
Zinc (mg)	3	2.3	1.8
Iron (mg)	1.6	7.6	2.8
Vitamin B_1 (mg)	0.04	0.50	0.18
Vitamin B_2 (mg)	1.53	1.22	2.52
Vitamin B_9 (µg)	2,230	1640	970

boiled to 100°C, 15 g of leaf powder was subsequently added, and the time corresponding to each treatment was allowed to rest. The infusion temperature was reduced from 95 to 55°C in 25 min.

The seed infusion was prepared using a French press. Each treatment received 15 g of seed powder, and 170 mL of deionised water was added at 95°C. Pressing was performed once the infusion time of each treatment was finished.

Ultrasound-assisted flour and leaf extracts

The extracts were processed in an ultrasonic processor using the direct sonication method (VCX 130PB, Sonics, Milford, CT, USA) with a 130 W ultrasound probe (1/8" probe) at 20 kHz. Briefly, 2.0 g of dried Ramon leaves and flour was placed in 50 mL glass tubes containing 20 mL of 80% aqueous methanol. To maintain a stable temperature (28 ± 0.5°C), the tubes were placed in a thermostatically recirculating water bath (VWR 117-612; St. Louis, MO, USA). The ultrasonic probe was immersed to a depth of 30 mm in the samples, and the amplitude was set to 80%, corresponding to a sonication power of 74 W/cm^2 (Gullian-Klanian and Terrats Preciat, 2017). Each extract was sonicated for 5, 10, or 15 min at predetermined extraction times. The samples were centrifuged at 1,200 *g* for 15 min at 10°C (Eppendorf, 5804R, Westbury, NY, USA). The sediment was further extracted using the same procedure. After the second extraction, both supernatants were combined, and insoluble materials were filtered through a 0.45 µm membrane to obtain a final volume of 25 mL. Finally, the extracts were placed in dark tubes with screw caps, and stored at -20°C for later use.

Total phenolic content (TPC)

The TPC was determined using the modified method described by Singleton and Rossi (1965). Briefly, 1 mL of Folin-Ciocalteu reagent (diluted 1:10 with distilled water) was added to each tube containing 200 µL of the extract and 800 µL of saturated Na_2CO_3 (75.0 g/L). The tubes were vortexed and allowed to stand at room temperature for 2 h until the blue colour characteristic developed. The absorbance was recorded at 765 nm using a spectrophotometer (Genesys 20 Model 4001/4; Thermo Spectronic, Rochester, NY, USA). The TPC was calculated as units of milligram gallic acid equivalents (GAE) per 100 g using a gallic acid standard curve.

Total flavonoid (TFC) and glycosylated flavonoid (TGF) contents

The TFC was determined using the method described by Zhishen *et al.* (1999). Briefly, 67 µL of each extract was added in triplicate to the wells of a microplate, to which 20 µL of 5% $NaNO_2$ was added. After 5 min, 20 µL of 10% $AlCl_3$ (in 80% methanol) and 133 µL of 1 M NaOH were added. The absorbance was measured at 415 nm using a Multiskan EX microplate spectrophotometer (Thermo Scientific, Rockford, IL, USA).

The TFG content was determined using the method described by De Albuquerque *et al.* (2014). Briefly, 20 µL of $AlCl_3$ (20 g/L ethanolic solution) and 60 µL of $C_2H_3NaO_2$ (50 g/L ethanolic solution) were added to 20 µL of the extract. Subsequently, the mixture was incubated at 20°C for 2.5 h in the dark, after which the absorbance was recorded at 440 nm. The TFC and TGF content were calculated as milligram quercetin equivalents (QEs) per 100 g using the standard quercetin curve.

Total monomeric anthocyanin content (ATC)

The ATC was determined using the differential pH method described by Kuskoski *et al.* (2005). Two mixtures per sample were prepared using buffers with different pH values (pH 1.0, 0.2 N KCl, 0.2 N HCl; pH 4.5, 1 N HCl, 1 M $CH_3COONa \cdot 3H_2O$). Of the extract, 1 mL was added to 4 mL of each buffer. The samples were homogenised and incubated at 4°C for 20 h, after which each sample was vacuum-filtered through a 0.45 µm membrane. The absorbance was measured at 510 and 700 nm (Genesys 20 Model 4001/4; Thermo Spectronic). The absorbance of the sample was calculated using the following formula: Abs = (Abs λvis-max - Abs_{700}) pH 1.0 and (Abs λvis-max - Abs_{700}) pH 4.5. The concentration of monomeric anthocyanins was expressed in terms of cyanidin-3-glucoside and was calculated as monomeric anthocyanin pigment (mg/100 g): $\frac{(\text{A x PM x FD x V x 1000})}{(\varepsilon \; x \; 1 \; x \; M)}$, where PM was the molecular weight of cyanidin-3-glucoside (449.2 g mol^{-1}), FD was the dilution factor, V was the extract volume, ε was the molar extinction coefficient of cyanidin-3-glycoside (29,600), and M was the mass of the sample.

Total condensed tannins (TCT = proanthocyanidins)

The content of TCT was determined using the method described by Butler *et al.* (1982). In total, 500 µL of the extract or sterile deionised water (blank) was added to 3 mL of vanillin diluted to 4% in 60% methanol. The mixture was homogenised by vortexing, after which 1.5 mL of pure HCl was added, and the mixture was incubated at 20°C in complete darkness for 15 min. The absorbance was read at 500 nm (Genesys 20 Model 4001/4; Thermo Spectronic). The amount of total condensed tannins was expressed in mg of (+)-catechin (CAT) per 100 g of sample using the (+)-catechin standard curve. The samples were analysed in triplicate.

Vitamin C

The content of vitamin C was determined using the 2,4-dinitrophenylhydrazine colorimetric method described by Al-Ani *et al.* (2007). Briefly, the samples were homogenised in 6% metaphosphoric acid and centrifuged at 6,000 *g* for 10 min at 4°C. Next, 1.2 mL of the extract was mixed with 1.2 mL of 6% metaphosphoric acid, after which 0.4 mL of the dinitrophenylhydrazine-thiourea-copper sulphate (DTCS) reagent was added to all tubes. The supernatant was filtered through Whatman #1 filter paper, followed by filtration through a 0.45 µm filter. The samples were incubated in a water bath at 37°C for 3 h. Subsequently, the cooled samples were gradually mixed with 2.0 mL of cold sulphuric acid (12 mol/L). The mixture was shaken, and the absorbance at 520 nm was read. The vitamin C content was calculated in milligrams to decilitres (mg/dL) using an ascorbic acid standard curve.

ABTS and DPPH radical scavenging assays

Antioxidant activities were studied by analysing their ability to remove ABTS and DPPH following the methods described by Re *et al.* (1999) and Brand-Williams *et al.* (1995), respectively. All measurements were performed in triplicate.

The radical cation ABTS ($ABTS^+$) was produced by reacting 7 mM ABTS with 2.45 mM potassium persulphate in the dark for 12 to 16 h. This solution was diluted in deionised water to an absorbance of 0.7 at 734 nm. The reaction mixture was mixed with 50 µL of each extract and 150 µL of the $ABTS^+$ solution in the dark. The decrease in the absorbance at 750 nm was measured after 6 min using a spectrophotometer (Multiskan EX, Thermo Scientific).

To conduct the DPPH assay, a mixture of 10 µL of each extract with 190 µL of 0.125 mM DPPH solution was prepared and dissolved in methanol. The decrease in the absorbance of the reaction mixture was measured at 541 nm. The radical scavenging activity of the extracts was calculated as micromoles of Trolox equivalents (TE) per gram of sample (µmol TE/g) using the Trolox standard curve.

Ferric-reducing antioxidant power (FRAP)

The FRAP was determined following the method described by Benzie and Strain (1996). The ferric-reducing antioxidant power (FRAP) reagent was prepared using a mixture of 300 mM acetate buffer (pH 3.6, 10 mM TPTZ (2,4,6-tri(2-pyridyl)-

triazine)) in 40 mM HCl and 20 mM $FeCl_3$. Next, 100 µL of the extracts and 300 µL of deionised water were added to 3 mL of the prepared FRAP reagent. The reaction mixture was incubated at 37°C for 30 min in a water bath, and the absorbance was measured at 593 nm. The reduction activity was calculated as Trolox equivalents (TE) per gram of sample (µM TE/g) using the Trolox standard curve.

Statistical analysis

Statistical analysis was performed using XLSTAT software. One-way analysis of variance (ANOVA) was used to assess significant differences ($p < 0.05$) between mean responses using Duncan's test.

Results and discussion

Leaf and seed infusions

The concentrations of antioxidant compounds in the Ramon leaf and seed infusions are shown in Table 2. The infusion time was strongly and positively correlated with the concentrations of TPC, TFC, TFG, and TCT in the leaf infusions ($p < 0.0001$). The number of phenolic components solubilised and released from cells increased with increasing infusion time, and corresponded to 2552.2 mg of TPC, 54.12 mg of TFC, 75.32 mg of TFG, and 2.40 mg of TCT per 100 g of dry leaf. The ATC reached its maximum value of 0.09 mg/100 g after 20 min of infusion, followed by a decrease, suggesting a negative effect of heat on pigments. The same finding was recorded for vitamin C, where a maximum concentration of 0.26 mg/dL was reached after 10 min of infusion.

The effects of infusion time and temperature on the antioxidant properties and phenolic composition of plant products have been previously reported by other authors. Komes *et al.* (2011) investigated the effect of extraction time and hydrolysis on the content of phenolic compounds, and the antioxidant capacity of six traditional medicinal plants [*Melissa officinalis* L. (lemon balm), *Thymus serpyllum* L. (wild thyme), *Lavandula officinalis* Miller (common lavender), *Rubus fruticosus* L. (blackberry), *Urtica dioica* L. (nettle), and *Olea europea* L. (olive)]. The present work constituted the first study on the impact of infusion time on the antioxidant activity of Ramon tree leaves and seeds. A similar effect has been reported for the leaves of myrtle (*Myrtus communis* L.), an evergreen Mediterranean shrub, in which the content of phenolic acids, flavanol glycosides, and

Table 2. Antioxidant compound concentrations of leaves and roasted seeds of Ramon tree (*Brosimum alicastrum*) at different infusion times ($n = 24$).

	Infusion time (min)						
	1	**5**	**10**	**15**	**20**	**25**	R^2
Leaf							
TPC*	276.2 ± 19.9	489.4 ± 22.6	958.9 ± 70.9	1,252.0 ± 58.6	2,426.8 ± 224.7	2,552.2 ± 88.1	0.998
TFC*	0.00 ± 0.00	0.20 ± 0.13	5.92 ± 0.10	23.72 ± 1.26	45.32 ± 1.66	54.12 ± 2.07	0.988
TGF*	0.00 ± 0.00	4.05 ± 0.83	2.93 ± 0.48	20.25 ± 1.83	68.62 ± 4.42	75.32± 2.05	0.982
ATC**	0.01 ± 0.00	0.04 ± 0.01	0.03 ± 0.00	0.04 ± 0.00	0.09 ± 0.02	0.06 ± 0.02	0.535
TCT*	0.23 ± 0.02	0.29 ± 0.01	0.39 ± 0.03	0.73 ± 0.02	2.26 ± 0.05	2.40 ± 0.15	0.981
Vitamin C	0.03 ± 0.01	0.10 ± 0.05	0.26 ± 0.11	0.13 ± 0.01	0.14 ± 0.01	0.07 ± 0.02	0.407
Seed							
TPC*	448.5 ± 127.8	1,358.7 ± 369.7	1,822.8 ± 157.5	2,415.5 ± 194.7	2,701.7 ± 299.2	3,114.9 ± 143.8	0.998
TFC*	1.26 ± 0.02	11.62 ± 0.59	14.03 ± 0.27	22.4 3 ± 0.62	35.41 ± 0.12	30.14 ± 0.12	0.996
TFG*	2.28 ± 2.28	23.43 ± 0.93	24.22 ± 0.49	32.51 ± 4.01	45.63 ± 16.3	90.81 ± 2.49	0.837
ATC*	0.06 ± 0.02	0.24 ± 0.03	0.28 ± 0.05	0.37 ± 0.02	0.58 ± 0.04	0.54 ± 0.02	0.906
TCT*	1.65 ± 0.10	4.65 ± 0.09	5.81 ± 0.29	7.51 ± 0.37	10.18 ± 0.65	10.94 ± 0.29	0.955
Vitamin C*	0.33 ± 0.06	1.15± 0.12	1.39 ± 0.09	1.77 ± 0.09	1.83 ± 0.12	1.77 ± 0.37	0.705

(*) $ANOVA_{(5,23)}$ (Pr > F, < 0.0001); (**) $ANOVA_{(5,23)}$ (Pr > F, 0.001). Results are expressed as mg/100 g dry matter. TPC = total phenolic content; TFC = total flavonoid content; TGF = glycosylated flavonoids; ATC = total anthocyanins; TCT = total tannins.

flavanols increased significantly with infusion time, reaching 179.6 mg/g TPC and 6.46 mg/g TFC in 15 min (Messaoud *et al.*, 2012). Plant phenolic compounds are a heterogeneous group of products, some of which are soluble in organic solvents, whereas others are glycosides or carboxylic acids, and therefore, soluble in water (Vermerris and Nicholson, 2006). In the case of the Ramon leaf, an increase in the infusion time favoured the solubility and diffusion coefficient of several soluble phenolic compounds within the extracts.

In the roasted seeds, the concentrations of all the antioxidant components increased with the infusion time. Compared to the leaves, the seeds were 32.9% greater in TP, 87.3% greater in ATC, 84.6% greater in TCT, and 91.5% greater in vitamin C (Figure 1). The 25-min infusion resulted in 3,114.9 mg of TPC, 90.9 mg of TFG, and 10.94 mg of TCT. At 20 min, the maximum TF, ATC, and vitamin C concentrations were 35.41 mg/100 g, 0.58 mg/100 g, and 1.83 mg/dL, respectively (Table 2). The content of antioxidant compounds could vary due to the intrinsic characteristics of the plant matrix, weather conditions, and the method used for extraction. Studies have shown that roasting Ramon seeds significantly increases the TCT and TPC contents compared to those of unroasted seeds. The phenolic compounds in coffee beans are reduced by intensive roasting, while their antioxidant capacity is maintained or improved. Twenty-three phenolic compounds, composed mainly of 15 phenolic acids and five polyphenols, have been characterised (Wu *et al.*, 2022). Consistent with the results of the present work, Quintero-Hilario *et al.* (2019) reported values of 1,337.2 mg/100 g of TPC, 44.47 mg/100 g of TFC, and 1874.79 mg/100 g of TCT in seeds roasted at 90°C. for 35 min. Furthermore, the TPC and TFC results were similar to those obtained in the present work, where at 20 min, the infusion resulted in 35.41 mg/100 g of TFC. In the case of TPC, the 5-min extraction resulted in 1,358.7 mg/100 g and doubled at 20 min, reaching 2,701.7 mg/100 g. This suggested a high content of water-soluble TPC in the seeds. The primary difference was found in TCT, where at 25 min of extraction, only 10.94 mg/100 g was obtained. This value was significantly lower than that reported by Quintero-Hilario *et al.* (2019), indicating a lower content of water-soluble proanthocyanins, and more alcohol-soluble molecules in the seeds. Since the authors macerated the roasted seeds with methanol/acetic acid to determine the type of TCT extracted, the variation in the results may be attributed to the difference in the sample preparation methods used to assess the TCT.

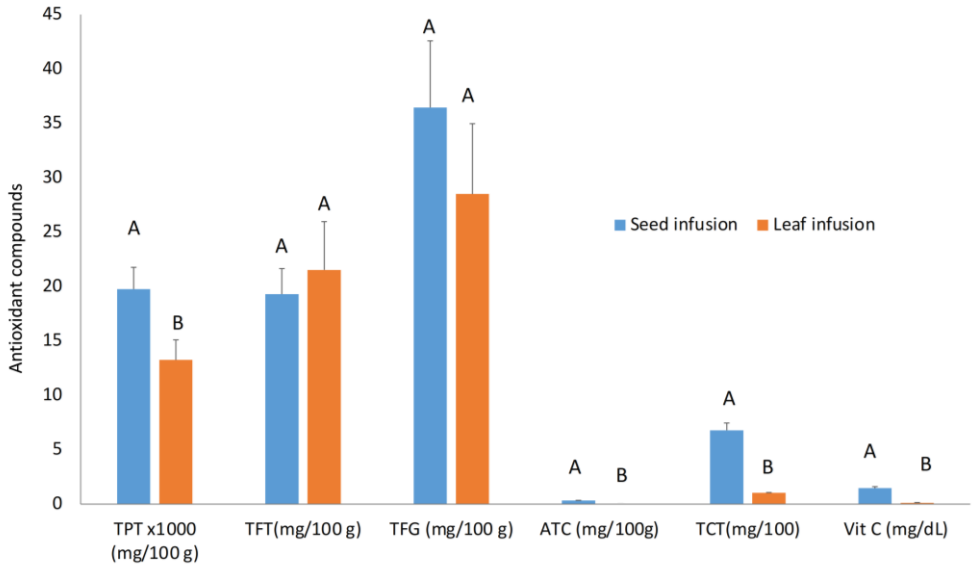

Figure 1. Concentrations of antioxidant compounds in infusions of leaves and roasted seeds of Ramon tree (*Brosimum alicastrum*). TPC = total phenolic content; TFC = total flavonoid content; TGF = glycosylated flavonoids; ATC = total anthocyanins; TCT = total tannins; and VitC (mg/dL) = vitamin C (ascorbic acid). Means denoted with different uppercase letters are significantly different between treatments (ANOVA $_{(5,23)}$; $p < 0.05$).

Antioxidant capacity of leaf and roasted seed infusions

The ability of the extracts to capture free radicals was analysed using ABTS and DPPH assays. The FRAP test is based on the reduction of ferric tripyridyltriazine [Fe(III)-TPTZ] to ferrous tripyridyltriazine [Fe(II)-TPTZ] (Prenzler *et al.*, 2021). The antioxidant capacity was greater after seed infusion than after leaf infusion. The FRAP test resulted in more significant results. The antioxidant capacity of the seeds was 56% greater than that of the leaves (Figure 2).

No significant differences were detected between the leaf and roasted seed infusions regarding the uptake of free radicals by DPPH. In the seed infusion, DPPH reached a maximum concentration of 9.71 µmol/g at 10 min, and a minimum concentration of 9.37 µmol/g at 25 min. The concentration in the leaves reached its maximum at 5 min (9.62 µmol/g), decreasing by 5% up to 20 min (Table 3). Its behaviour was similar to that of the myrtle leaf infusion, whereas the infusion time increased from 5 to 15 min, and the DPPH uptake capacity decreased by 20.5% (Messaoud *et al.*, 2012).

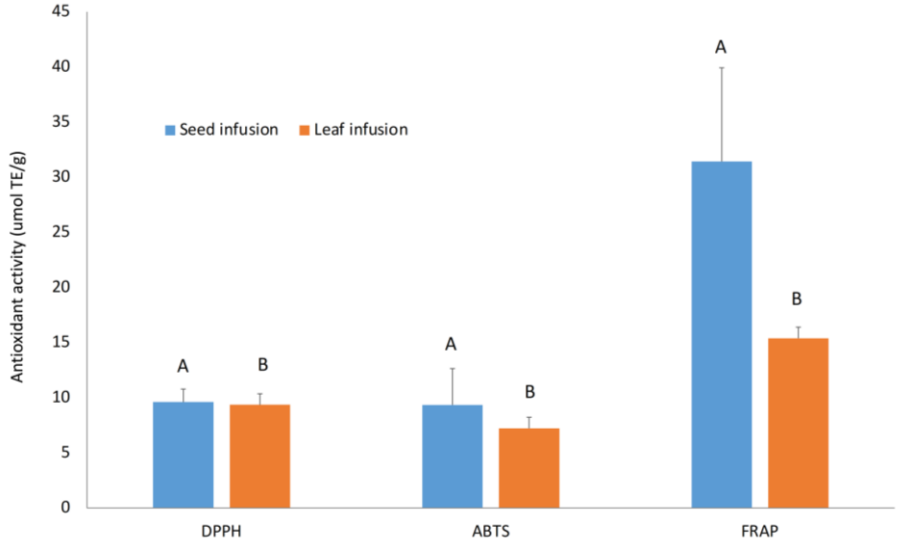

Figure 2. Antioxidant activities of leaf and roasted seed infusions of Ramon tree (*Brosimum alicastrum*). DPPH = 2,2-diphenyl-1-picrylhydrazyl assay; ABTS = 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation assays; and FRAP = ferric-reducing antioxidant power assay. Means denoted with different uppercase letters are significantly different between treatments (ANOVA $_{(5,23)}$; $p < 0.05$).

Table 3. Antioxidant activity of leaves and roasted seeds of Ramon tree (*Brosimum alicastrum*) at different infusion times (n = 24).

	Infusion time (min)						
	1	**5**	**10**	**15**	**20**	**25**	R^2
				Leaf (µmol TE/g)			
DPPH*	9.34 ± 0.00	9.62 ± 0.02	9.47 ± 0.03	9.42 ± 0.04	9.15 ± 0.02	9.16 ± 0.01	0.920
ABTS*	0.77 ± 0.15	5.69 ± 0.69	8.80 ± 0.18	9.21 ± 0.18	9.15 ± 0.15	9.75 ± 0.19	0.962
FRAP**	6.05 ± 1.08	8.21 ± 0.97	13.56 ± 1.64	19.65 ± 3.37	20.64 ± 3.29	24.29 ± 4.75	0.642
				Seed (µmol TE/g)			
DPPH*	9.65 ± 0.00	9.71 ± 0.02	9.71 ± 0.00	9.66 ± 0.01	9.60 ± 0.01	9.37 ± 0.08	0.789
ABTS*	9.60 ± 0.14	9.85 ± 0.04	9.90 ± 0.04	9.82 ± 0.08	8.88 ± 0.65	8.06 ± 0.46	0.576
FRAP*	68.7 ± 8.70	48.4 ± 14.13	44.9 ± 5.73	22.9 ± 4.20	22.1 ± 0.29	5.97 ± 0.83	0.724

(*) ANOVA $_{(5,23)}$ (Pr > F, < 0.0001); (**) ANOVA $_{(5,23)}$ (Pr > F, 0.001). Results are expressed as µmol TE/g (Trolox/g dry matter). DPPH = 2,2-diphenyl-1-picrylhydrazyl assay; ABTS = 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radical cation assays; and FRAP = ferric-reducing antioxidant power assay.

The ABTS assay revealed that the leaves were more sensitive to infusion time than the seeds, increasing proportionally from 1 to 10 min (0.77 - 8.80 µmol/g). Afterward, it remained constant, reaching a maximum concentration of 9.75 µmol/g at 25 min. The effect of the infusion was not evident in the seeds, where the ABTS value remained constant from 1 to 15 min (9.60 - 9.82 µmol/g), and subsequently started decreasing until it reached 8.06 µmol/g. These values were lower than those reported by Quintero-Hilario *et al.* (2019) who reported that roasting the seeds increased the antioxidant activity due to the formation of melanoidin coupled with the presence of tannins. The TCT of the Ramon seeds in the study by Quintero-Hilario *et al.* (2019) was significantly greater than that in the present work.

The FRAP of the leaves increased exponentially from 1 min (6.05 µmol/g) to a maximum of 24.3 µmol/g at 25 min of infusion. Although the seeds reached a high reducing power of Fe(III)-TPTZ in the 1st min of infusion (68.7 µmol/g), its value decreased exponentially to a minimum of 5.97 µmol/g at 25 min. Ozer (2016) reported that the ethanolic extraction of the Ramon seeds resulted in a FRAP value of 80.8 µmol/g, which was higher than our aqueous extractions. According to Ozer (2016), these values were higher than those of peanuts (16.1 µmol/g) and almonds (15.2 µmol/g), but lower than that of walnuts (226 µmol/g).

Ultrasound-assisted extraction of Ramon flour and leaves

The ultrasonic extraction of the leaves and Ramon seed meal resulted in higher TFC, ATC, and TCT values than infusions. Unlike infusion, which is a solution of solute in water, ultrasonic extraction applies sound energy to agitate the particles in a sample, resulting in cavitation. Cavitation generates cycles of expansion and contraction of the exposed material, producing microchannels that separate and interrupt the cells and disintegrate them (Rodrigues and Fernandes, 2017). This process favours the penetration of the solvent, thus increasing the extraction rate (Espada-Bellido *et al.*, 2017).

The TPC after ultrasonic extraction was lower than that after infusion, indicating that several phenolic components responded better to passive stripping with aqueous solutions than to the methanol/water mixture. These results contrasted those obtained by Moo-Huchin *et al.* (2019) who reported that an ethanol/water mixture (1:1, v/v) was the most efficient extraction solvent for recovering higher concentrations of phenolic compounds with greater antioxidant power. However, we used a combination of a solvent/water mixture and ultrasound, which could have affected the extraction of TPCs. According to Goskonda *et al.* (2002), acoustic cavitation causes the formation of free radicals, structurally changing certain phenolic compounds. The average TPC of the Ramon flour was 434.3 ±11.7 mg/100 g, within the range reported by Moo-Huchin *et al.* (2019). No significant difference was reported in the TPC (411.0 ± 0.77 mg/100 g) or TFC (11.64 ± 0.86 mg/100 g) values of the flour with increasing ultrasonic extraction time (Table 4). However, in the Ramon leaves, ultrasonic extraction time affected the TPC recovery, reaching its maximum at 15 min (411.0 ± 7.7 mg/100 g).

In flour, the extraction time was positively correlated with TCT, reaching a value of 3.38 ± 0.20 mg/100 g. This concentration was 43.8% lower than that in the leaves, which resulted in an average value of 5.24 ± 0.15 mg/100 g. TCTs can form complexes with proteins; proline, for example, increases the affinity and the formation of bonds between the hydroxyl groups of tannins and peptide bonds of proteins (Adamczyk *et al.*, 2017). The presence of high-molecular-weight proteins in the flour probably affects the retention of TCT because it promotes the formation of more complex bonds. In the leaves, a high concentration of TCT directly determines the total amount of ATC (Figure 3). Condensed tannins are derived from flavan-3-ol or proanthocyanin units, and are hydrolysed into anthocyanidins in acidic media at elevated temperatures.

Flour-processing conditions probably affect the availability of ATC. The maximum concentration of ATC in the flour was obtained after 5 min of extraction (0.03 ± 0.00 mg/100 g). However, an increase in the extraction time did not result in a significant recovery of the compounds (Table 4). Among flavonoids, anthocyanins represent the most critical group of water-soluble pigments. They are highly susceptible to changes in pH, temperature, and redox potential during flour production. ATC are highly stable at acidic pH; however, their stability decreases as the pH approaches neutral. ATC become highly unstable at pH values higher than 7

Table 4. Concentration and antioxidant activity of leaves and seed meal of Ramon tree (*Brosimum alicastrum*) at different ultrasonic extraction times (n = 54).

	Ultrasonic extraction time (min)					
	5	**10**	**15**	**5**	**10**	**15**
	Leaf			**Seed**		
	Antioxidant compounds (µM TE/g)					
TPC*	368.2 ± 6.76[a]	388.71 ± 6.36[b]	410.95 ± 7.73[c]	419.44 ± 10.67	436.27 ± 13.63	447.65 ± 11.00
TFC	49.59 ± 2.39	52.44 ± 2.19	54.56 ± 2.12	10.02 ± 0.71	13.11 ± 0.67	11.80 ± 1.17
ATC*	0.12 ± 0.02[a]	0.14 ± 0.01[a]	0.07 ± 0.02[b]	0.02 ± 0.00[a]	0.03 ± 0.00[a]	0.01 ± 0.00[b]
TCT*	5.19 ± 0.14	5.14 ± 0.16	5.39 ± 017	2.56 ± 0.24[a]	2.50 ± 0.25[a]	3.38 ± 0.20[b]
	Antioxidant capacity (µmol TE/g)					
DPPH*	29.07 ± 2.22[a]	42.13 ± 2.84[b]	38.34 ± 4.66[b]	9.32 ± 0.59	9.82 ± 0.83	9.10 ± 0.81
ABTS	6.59 ± 0.23	6.27 ± 0.28	6.59 ± 0.26	26.85 ± 0.97	28.98 ± 0.82	27.96 ± 1.06
FRAP*	99.70 ± 11.24[a]	93.81 ± 14.96[a]	150.81 ± 18.56[b]	6.97 ± 0.71	7.86 ± 0.92	10.70 ± 1.40

(*) $ANOVA_{(2,51)}$ (Pr > F, < 0.0001). TPC = total phenolic content; TFC = total flavonoid content; ATC = total anthocyanins; TCT = total tannins; DPPH = 2,2-diphenyl-1-picrylhydrazyl assay; ABTS = 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radical cation assays; and FRAP = ferric-reducing antioxidant power assay.

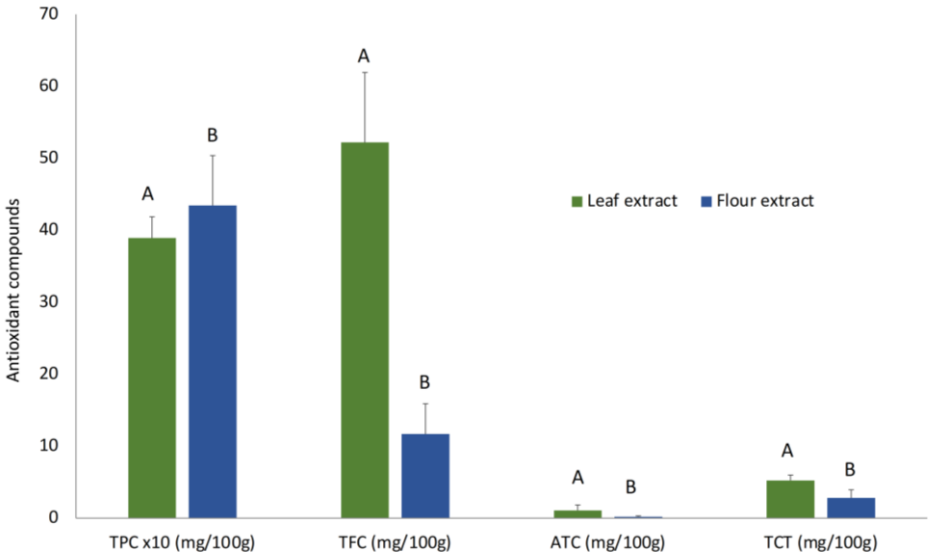

Figure 3. Concentrations of antioxidant compounds in ultrasound-assisted extraction of leaves and seed meal of Ramon tree (*Brosimum alicastrum*). TPC = total phenolic content; TFC = total flavonoid content; ATC = total anthocyanins; and TCT = total tannins. Means denoted with different uppercase letters are significantly different between treatments ($ANOVA_{(2,51)}$; $p < 0.05$).

(Hutchings, 1999). In addition, simple ATC molecules are esterified to one or more sugars, resulting in the loss of glycosylating sugars and ring opening with increasing temperature and the consequent production of colourless chalcones (Timberlake, 1980). Conditions favouring the aerobic oxidation of ascorbic acid worsen the stability and cause significant losses of anthocyanins (Sondheimer and Kertesz, 1953).

Antioxidant capacity of leaves and flour after ultrasonic extraction

The neutralising capacity of free radicals estimated by ABTS was 23.6% greater in the flour (27.93 ± 0.95 µmol/g) than in the leaves (6.48 ± 0.26 µmol/g). It did not depend on the extraction time. In contrast, FRAP and DPPH were found to have antioxidant effects on the leaves. Similar to that in the leaves, FRAP peaked in the flour after 15 min.

However, the antioxidant effect was 92.6% greater in the leaves (150.81 ± 18.56 µmol/g) than in the flour (10.70 ± 1.40 µmol/g). The leaves showed a 30% greater DPPH capacity (40.24 ± 3.8 µmol/g) than did the flour (9.41 ± 0.74 µmol/g), and this capacity was dependent on the extraction time, with 10 min as the time of the maximum antioxidant activity (Figure 4).

The antioxidant activity of plant material is determined by interactions between different bioactive compounds. Plants undergo several reaction mechanisms to delay or inhibit the oxidative deterioration caused by free radicals. Therefore, the *in vitro* antioxidant activity of complex extracts is assessed using different complementary methods to evaluate the diversity of reaction mechanisms. Hence, these methods are not specific to a compound or a class of phenolic compounds (Floegel *et al.*, 2011).

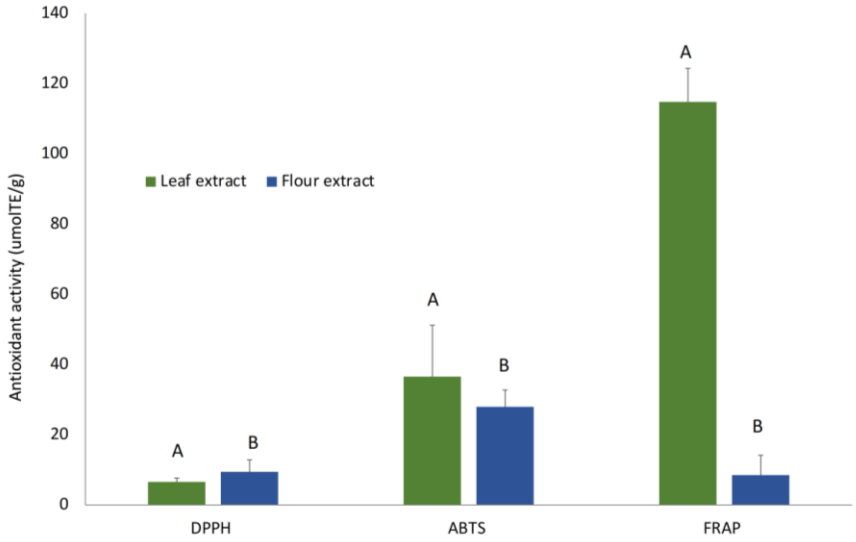

Figure 4. Antioxidant activities of ultrasound-assisted extraction of leaves and seed meal of Ramon tree (*Brosimum alicastrum*). DPPH = 2,2-diphenyl-1-picrylhydrazyl assay; ABTS = 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation assays; and FRAP = ferric-reducing antioxidant power assay. Means denoted with different uppercase letters are significantly different between treatments (ANOVA $_{(2,51)}$; $p < 0.05$).

Correlations between antioxidant capacity and concentration of phenolic compounds

In agreement with the findings of other authors (Babbar *et al.*, 2014; Bhuyan *et al.*, 2016), our results showed significant linear relationships between the TPC of leaf infusions and the antioxidant activity of leaf and roasted Ramon seed infusions in an infusion time-dependent manner.

After 10 min of infusion, the ABTS capacity of the roasted seeds significantly correlated with the TPC (0.80) and TFC (0.90). Similar findings were obtained for FRAP, where at 10 min, R = 0.83 for the TPC and R = 0.96 for the TFC. In the case of leaf infusion, ABTS was significantly correlated with TPC (0.80) and TFC (0.83) at 10 min. The strongest relationship between FRAP and TPC was observed at 5 min (0.80), and that between FRAP and TFC was observed at 20 min (0.60). The DPPH assay was the least sensitive indicator of antioxidant activity. The DPPH assay showed that the leaf infusion reached a significant TPC value (0.80) in 25 min. The seed infusion at 10 min reached a maximum range (0.40) with both TPC and TFC. High conversion values in the infusions confirmed that TPC were the most critical metabolites contributing to antioxidant activity in an infusion time-dependent manner.

The extraction process using energy and solvents can extract different antioxidant compounds (simple and polymerised forms) using infusions, implying the presence of different species of radicals involved in other trials. In ultrasound extractions, the correlation of TPC with antioxidant capacity was less significant than that for infusions. After 20 min of extraction, the TPC of flour was significantly correlated with the DPPH radical scavenging ability (0.73). The ABTS activity correlated significantly (0.44) with the TPC after 10 min of extraction.

Conclusion

The present work investigated the impact of infusion time on the bioactive content and antioxidant activity of leaves and seeds of *B. alicastrum*. The products derived from *B. alicastrum* seeds and leaves are often consumed as functional foods due to their high nutritional quality and high content of antioxidant components. Commonly, leaf infusions are prepared with a 5-min infusion, during which a high concentration of antioxidant compounds are solubilised. The concentration of phenolic compounds in the products derived from *B. alicastrum* leaves and seeds increased with increasing infusion time, and these compounds are important sources of compounds with antioxidant activity. After 20 min of rest, the injection resulted in an optimal consumption temperature (60°C), thereby increasing the concentrations of TPC, TFC, TGF, ATC, and TCT by 79.9, 99.6, 94.1, 88.9, and 87.2%, respectively. For roasted seeds as a coffee substitute, pressing after 5 min resulted in high concentrations of TPC, TFC, and TGF, including ATC and vitamin C. We do not recommend waiting for 10 min to perform pressing because it does not significantly increase the concentrations of antioxidants.

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