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TOTAL PHENOLIC CONTENT AND ANTIOXIDANT POTENTIAL OF 'ORA-PRO-NOBIS' LEAVES: AN *IN VITRO* COMPARATIVE STUDY BETWEEN *PERESKIA ACULEATA* MILLER AND *PERESKIA GRANDIFOLIA* HAW

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ABSTRACT

The ora-pro-nobis is an unconventional vegetable belonging to the genus *Pereskia* and native to South America. In Brazil, the two species *P. aculeata* and *P. grandifolia* are native to the Atlantic Forest and their leaves are widely used for the preparation of different regional dishes. The aim of the present work was to evaluate, in a comparative fashion, the total phenolic contents and antioxidant potentials of *P. aculeata* and *P. grandifolia* leaves. For this purpose, the efficiency of three extraction media, namely 70% ethanol in water, water at room temperature and water at 50 ºC, were used for obtaining the interest compounds. Furthermore, the antioxidant capabilities of the prepared extracts were assessed using a set of four different *in vitro* chemical methods. The results obtained in this work indicate that the ora-pro-nobis leaves (*P. aculeata* and *P. grandifolia*) are sources of phenolics with promising antioxidant activity. The hydroethanolic extraction was the most suitable for extracting bioactive compounds from the leaves of both species. In addition, it was found that the extracts of *P. aculeata* had not only a higher phenolic content but also superior antioxidant capacities, assessed by 4 different methods (DPPH, ABTS, FRAP and ORAC), than the extracts of *P. grandifolia*.

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INTRODUCTION

Non-conventional leaf vegetables have already had a large presence in the Brazilian diet, but little by little, they have been forgotten and devalued due to the migration of the rural population to large centers and the change in eating habits. However, these greens are of great importance due to their high nutritional and therapeutic values, besides easy cultivation since they are mostly native plants (Viana *et al*., 2015; Pinela, Carvalho and Ferreira, 2017). *Pereskia aculeata* Miller and *Pereskia grandifolia* Haw., popularly known as 'ora-pro-nobis' (Lat. pray-for-us) or poor man´s meat, in reference to its outstanding protein content, are examples of non-conventional green leafs (Zem *et al*., 2017).

Belonging to the Cactaceae family, they are native to South America. Both can be easily confused, especially when they are not in bloom, as the distinction can be made by the color of the flower and the fruit. Normally, *P. aculeata* has yellowish flowers and fruits, whereas *P. grandifolia* presents longer leaves as well as purple flowers and fruits (Souza *et al*., 2016a) (Figure 1). In the city of Diamantina, in the State of Minas Gerais, Brazil, a study found that a large part of the population regularly consumes ora-pro-nobis (de Almeida and Correa, 2012). *Pereskia* spp. leaves are traditionally used in the preparation of savoury dishes such as salads, soups, omelets and pies, whereas the leaf flour figures as an enriching ingredient in the manufacture of breads and pastas. Furthermore, the leaf mucilage can replace eggs in food formulations, which is interesting for the public with food

allergies. Likewise, the fruits are employed in the production of juices, liquors and desserts, and the seeds can be germinated to produce edible shoots (Francelin *et al*., 2018, Garcia *et al*., 2019). In the past few years, sundry *in-vitro* and *in-vivo* pharmacological studies have evidenced the anti-inflammatory and wound-healing (Pinto *et al*., 2015; Pinto *et al*., 2016), anti-obesity (de Almeida *et al*., 2016), antioxidant (Sousa *et al*., 2014), antiproliferative and antimicrobial (Souza *et al*., 2016b) effects of ora-pro-nobis extracts, and, not least, confirmed their absence of toxicity (Silva *et al*., 2017). Recently, our group (Garcia *et al*., 2019) reported promising biological activities and the phenolic profile of *P. aculeata* leaves. Nevertheless, and despite the aforementioned reports, to our best knowledge, comparative studies on *Pereskia* species remain scarce. Considering the above, our aim was to comparatively evaluate the total phenolic content and antioxidant potential of *Pereskia aculeata* and *Pereskia grandifolia* leaves. For this purpose, the efficiency of three extraction media, namely 70% ethanol in water, water at room temperature and water at 50 ºC, were studied for obtaining the interest compounds. Furthermore, the antioxidant capabilities of the prepared extracts were assessed using a set of 4 different *in vitro* chemical methods.

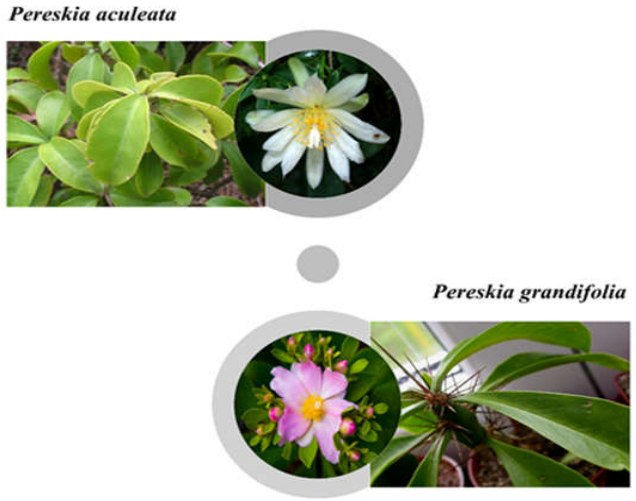

Figure 1. Morphological differences of leaves and flowers of *P. aculeata* and *P. grandifolia*

MATERIAL AND METHODS

Plant material: Organic dried leaves of *Pereskia aculeata*, in the form of a flour, were acquired from a reliable commercial source ('Ora Pro Nobis Brasil, Joinville', Santa Catarina, Brazil). *Pereskia grandifolia* leaves were collected at the State University of Maringá, and dried in an oven at 45 ° C for 24 h before being turned into flour by spraying it in a ball mill.

Extract preparation: Three different extraction media were tested: deionized water at room temperature, hot deionized water (50 ° C) and a hydroethanolic solution (70% ethanol in deionized water). The extract was prepared using a ratio flour/extractor solution of 1:20, that is, to 1 g of flour 20 mL of extractor medium was added. The vials were sealed and shaken for 2 h at 130 rpm at room temperature and protected from light. The procedure was repeated three times. The extracts were combined and centrifuged at 1792*g* for 15 min. Only the supernatant from the hydroethanolic extraction was evaporated at 35 °C to remove ethanol. Lastly, the materials were lyophilized (FreeZone 4.5 model 7750031, Labconco, Kansas City, MO, USA) and stored in a freezer at −20 ° C until further analyses.

Determination of total phenolic content (TPC): TPC values were estimated by the Folin-Ciocalteu method (Singleton and Rossi, 1965). Briefly, 2.0 mL of a properly diluted sample was added to 300 µL of sodium carbonate (1.9 M Na_2CO_3) and 100 µL of Folin reagent (1 M). The mixture was left to stand for 1 hour in the dark and the absorbance determined at 725 nm in a spectrophotometer (Shimadzu). Gallic acid was used as a standard to construct a calibration curve. Analyses were performed in triplicates and results were expressed in µg of GAE) per mg extract.

Determination of antioxidant activity via several methods: Four chemical *in-vitro* methods were applied to assess the antioxidant activity of the ora-pro-nobis extracts: (1) reduction of the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH, Sigma-Aldrich, St. Louis, MO, USA); (2) reduction power of the ferric ion (FRAP); (3) reduction of the 2,2-azino-bis (3-ethylbenzothiazoline-6-sulphonate) cation (ABTS, Sigma-Aldrich, St. Louis, MO, USA), and (4) oxygen radical absorbance capacity (ORAC). The free radical scavenging activity (DPPH) was evaluated as previously described (Soares *et al*., 2009), with some modifications. Initially, a stock solution of DPPH was prepared by dissolving 24 mg of DPPH in 100 mL of methanol and stored at -20 °C until use. Then, the DPPH working solution was prepared: 10 mL of the DPPH stock solution was added to 45 mL of methanol. Following, 150 µL of the sample and 2850 µL of the DPPH working solution were added to a tube. Reaction took place for 1 hour at room temperature in the absence of light. As a negative control of the reaction, distilled water was used instead of the sample. Then, absorbance was determined at 515 nm. A 0.02% butyl hydroxy toluene (BHT) solution was used as a positive control. To calculate the percentage of DPPH discoloration, the following equation was used: $[(Abs_{Control} - Abs_{Sample})/Abs_{Control}] \times 100$. Results were presented in IC_{50}, which is the amount of extract that promotes a 50% reduction in the oxidation of the DPPH substrate. The FRAP assay was performed as described by Pulido *et al*. (2000). To prepare the FRAP solution, 25 mL of acetate buffer (300 mmol/L) were mixed with 2.5 mL of a TPTZ solution (2,4,6-Tris 2-pyridyl-s-triazine) (10 mmol/L) and 2.5 mL of the ferric chloride solution (20 mmol/L). To the 900 µL of the FRAP reagent in an assay tube, 90 µL of distilled water were added plus 30 µL of sample at different concentrations. After 30 min of reaction at 37 ° C in the dark, reading was done at 595 nm. Results were expressed in µM of Trolox equivalents (TE) per mg of extract. For the ABTS method the protocol of Soares *et al*. (2009) was adopted with some modifications. Initially, a solution of 7.4 mM ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonate) and a 2.6 mM persulfate solution were prepared separately. These solutions were mixed in equal volumes and allowed to react for 12 hours at room temperature in the dark to obtain the working solution of $ABTS^{\bullet +}$. To 1.0 mL of this working solution 59 mL of methanol were added. For the reaction, 150 µL of sample and 2850 µL of $ABTS^{\bullet +}$ were combined in a tube. The mixture was kept for 2 h in the dark and the absorbance was read at 734 nm. Water was used as a negative reaction control and BHT 0.02% was employed as a positive control. The percentage of sequestration of the ABTS ·+ radical was calculated using the equation: $[(Abs_{Control} - Abs_{Sample})/Abs_{Control}] \times 100$. Results were given as IC_{50}. The ORAC method was

carried out as described by Ou *et al*. (2002), with adaptations. In a 96-well plate, 25 µL of sample or potassium phosphate buffer (for blank) and 150 µL of fluorescein were aliquoted. The plate was pre-incubated in a fluorimeter at 37 ºC for 15 minutes under shaking. Soon after a volume of 25 µL of AAPH (2,2'-azobis (2-amidinopropane)dihydrochloride) was added and the plate was put back in the device and maintained there for 70 minutes at 37 ºC. Readings were taken every 2 minutes at the wavelengths of 485 nm and 520 nm. Results were expressed in µM of Trolox equivalents (TE) per mg of extract.

Statistical analysis: All results were expressed as mean values plus standard deviations (SD) as an outcome of the three repetitions of the assays. For comparing the arithmetic means the t test or analysis of variance (ANOVA) were applied according to context. ANOVA was followed by Tukey's post-hoc testing using the software Prisma 7.0 (GraphPad), with a significance level of 5% ($p \leq 0.05$).

RESULTS AND DISCUSSION

The yields of extraction were expressed as percentage and are displayed in Table 1. Yield values ranged between 25.0 and 37.7%. The hydroethanolic solution allowed higher extraction yields than water (whether cold or hot), for both *Pereskia* species. Sousa *et al*. (2014) found less expressive results than ours for both aqueous and hydroethanolic extracts of *P. aculeata* leaves, respectively 17% and 16%. The extraction efficiency depends mainly on the selection of the extraction media. The most important factor for choosing an extraction medium is the polarity of the target compound, that is, the employed medium must have the same polarity as the solute of interest (Altemimi *et al*., 2017). According to Dent *et al*. (2013), hydroethanolic solutions are suitable media for the extraction of phenolic molecules due to the different polarities of the bioactive constituents and their acceptability as a food grade solvent.

Table 1. Extraction yields of *P. aculeata* and *P. grandifolia* leaves

	Hydroethanolic extract (%)	Cold aqueous extract (%)	Hot aqueous extract (50º C) (%)
P. aculeata	$32.1 \pm 4.5^{a,1}$	$25.0 \pm 2.1^{b,1}$	$27.2 \pm 2.4^{b,1}$
P. grandifolia	$37.7 \pm 3.6^{a,1}$	$26.6 \pm 1.7^{b,1}$	$28.1 \pm 3.1^{b,1}$

The data represent the mean ± standard deviation (n = 3). Values signed with the same letter in the same line do not differ statistically ($p \geq 0.05$) according to ANOVA. Values signed with the same number in the same column do not differ statistically ($p \geq 0.05$) according to the t test

Table 2. Total phenolic contents (TPC) of *P. aculeata* and *P. grandifolia* leaves

	Hydroethanolic extract (µg GAE/mg)	Cold aqueous extract (µg GAE/mg)	Hot aqueous extract (50º C) (µg GAE/mg)
P.aculeata	$84.07 \pm 1.65^{a,1}$	$46.45 \pm 1.14^{b,1}$	$44.72 \pm 2.87^{b,1}$
P.grandifolia	$49.43 \pm 0.55^{a,2}$	$41.84 \pm 1.87^{a,1}$	$31.56 \pm 1.73^{b,2}$

TPC values are expressed in µg equivalents of gallic acid equivalents per mg of extract. The data represent the mean ± standard deviation (n = 3). Values signed with the same letter on the same line do not differ statistically ($p \geq 0.05$) according to ANOVA. Values signed with the same number in the same column do not differ statistically ($p \geq 0.05$) according to the t test.

Phenolic phytocomponents are largely responsible for the antioxidant activity of plant matrices (Corrêa *et al*., 2019). For this reason, the total phenolic content (TPC) of the ora-pro-nobis extracts were evaluated and the results are shown in Table 2. The hydroalcoholic extraction was the most efficient, allowing TPC values of 84.07 and 49.43 µg GAE/mg for *P. aculeta* and *P. grandifolia*, respectively. Sousa *et al*. (2014) reported a similar result (95.6 µg GAE/mg of extract) when also investigating a 70% ethanol-water extract of *P. aculeata* leaves. On the other hand, Sim *et al*. (2010) found a less expressive TPC value (35.79 mg GAE/g) than ours in their study on aqueous leaf extracts of *P. grandifolia*. Furthermore, Souza *et al*. (2016b) verified that the extraction of *P. aculeata* leaves using methanol was considerably less effective, yielding a TPC value of 15.04 mg GAE/g extract. Four different antioxidant assays were used to estimate the antioxidant activities of the ora-pro-nobis extracts, with results displayed in Tables 3 and 4. The application of more than two techniques for evaluating the antioxidant potential of plant extracts is fundamental, as antioxidant molecules act by distinctive mechanisms, each possessing its specific target within the reaction matrix (Corrêa *et al*, 2014; Gonçalves *et al*., 2019). Hence, heterogeneous chemical reactivities result in variant degrees of antioxidant action in the chemical assays (Correa *et al*., 2017). To our best knowledge, this is the first report presenting compararisons of antioxidant capabilities of *Pereskia* spp. using such an ample set of methods. Regarding the DPPH test, the hydroethanolic extraction allowed the lowest IC_{50} values for both species studied, hence the highest antioxidant capacities among samples (Tables 3 and 4). The *P. aculeate* hydroethanolic extract, for instance, had an IC_{50} value 3-fold lower (IC_{50}= 73.30 µg/mL) than those of the corresponding aqueous extracts. Among the water extractions, cold extraction showed the best results in the DPPH method. According to Andreo *et al*. (2006), organic solvents and elevated temperatures facilitate the extraction of antioxidant molecules. However, in this work, the use of water at 50º C did not increase the extraction of bioactive substances from ora-pro-nobis.

Table 3. Antioxidant potential of *Pereskia aculeata* leaf extracts assessed via four different *in-vitro* methods

	Hydroethanolic extract	Cold aqueous extract	Hot aqueous extract (50º C)
DPPH IC_{50} µg/mL	73.30 ± 6.4^{a}	374.94 ± 3.1^{b}	242.69 ± 11.3^{c}
ABTS IC_{50} µg/mL	32.57 ± 2.2^{a}	60.21 ± 6.4^{b}	66.29 ± 0.1^{b}
FRAP µmol TE/mg	476.15 ± 15.2^{a}	116.23 ± 8.4^{b}	173.92 ± 8.4^{c}
ORAC µmol TE/mg	8183.00 ± 70.3^{a}	4524.97 ± 205.8^{b}	4381.26 ± 563.1^{b}

DPPH and ABTS results are expressed in IC_{50} values: sample concentration providing 50% of antioxidant activity. FRAP and ORAC results are expressed in µmol of Trolox equivalents per mg of extract. The data represent the mean ± standard deviation (n = 3). Values signed with the same letter on the same line do not differ statistically ($p \geq 0.05$) according to the Tukey test.

Table 4. Antioxidant potential of *Pereskia grandifolia* leaf extracts assessed via four different *in-vitro* methods

	Hydroethanolic extract	Cold aqueous extract	Hot aqueous extract (50º C)
DPPH IC_{50} µg/mL	207.27 ± 4.68^{a}	279.27 ± 0.96^{b}	321.77 ± 4.39^{c}
ABTS IC_{50} µg/mL	49.09 ± 4.25^{a}	56.36 ± 1.46^{a}	54.25 ± 2.15^{a}
FRAP µmol TE/mg	224.26 ± 16.5^{a}	159.83 ± 17.07^{b}	171.73 ± 5.93^{b}
ORAC µmol TE/mg	5436.3 ± 385.7^{a}	2762.3 ± 237.8^{b}	2522.8 ± 377.00^{b}

DPPH and ABTS results are expressed in IC_{50} values: sample concentration providing 50% of antioxidant activity. FRAP and ORAC results are expressed in µmol of Trolox equivalents per mg of extract. The data represent the mean ± standard deviation (n = 3). Values signed with the same letter on the same line do not differ statistically ($p \geq 0.05$) according to the Tukey test.

The same trends were observed for the ABTS trial results, although with milder discrepancies amidst samples. Likewise, for the FRAP and ORAC methods, the hydroalcoholic extracts of *Pereskia* spp. presented the highest values of µmol Trolox equivalents/mg of extract, hence best antioxidant potentials among the assessed extracts. So much that, for the ORAC assay the IC_{50} values of the hydroethanolic extracts of both species were 2-fold higher than those of the aqueous extracts (up to 8183.00 µmol TE/mg for *P. aculeata*). Such greater antioxidant capacities verified for the hydroethanolic extracts of *Pereskia* spp. in all four antioxidant methods are likely related to their superior TPC values (Table 2). Almost all the existing reports on the *in vitro* antioxidant properties of *Pereskia* species were produced using solely the DPPH method. Souza *et al*. (2016) reported a low scavenging activity of the DPPH radical for a methanolic extract of *P. aculeata*, with an IC_{50} value of 7.09 mg/mL. Sim *et al*. (2010) found a less expressive DPPH activity than that found in our study (IC_{50}=5 mg/mL) for the aqueous extract of *P. grandifolia* leaves. Correspondingly, Hassanbaglou *et al*. (2012) verified lower activities for ethyl acetate (IC_{50}=168 µg/ml), hexane (IC_{50}=244 µg/ml), methanol (IC_{50}=278 µg/ml) and ethanol (IC_{50}=540 µg/ml) leaf extracts of *Pereskia bleo* (Kunth) DC., using the same method.

Conclusion

The results obtained in this work indicate that the ora-pro-nobis leaves (*P. aculeata* and *P. grandifolia*) are sources of phenolics with promising antioxidant activity. The hydroethanolic extraction was the most suitable for extracting bioactive compounds from the leaves of both species. In addition, we found that the extracts of *P. aculeata* had not only a higher phenolic content but also superior antioxidant capacities assessed by 4 different methods (DPPH, ABTS, FRAP and ORAC) than the extracts of *P. grandifolia*.

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