

**Determination of the phenolic, antioxidant and antimicrobial potential of leaf extracts of
Pereskia grandifolia Haw**

**Determinação do potencial fenólico, antioxidante e antimicrobiano de extratos foliares
de *Pereskia grandifolia* Haw**

**Determinación del potencial fenólico, antioxidante y antimicrobiano de extractos de
hojas de *Pereskia grandifolia* Haw**

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Abstract

Research on Unconventional Food Plants (UFP) for the presence of bioactive and antimicrobial compounds is scarce and the expansion of knowledge regarding their properties becomes necessary. In this study, four different types of extracts (aqueous and hydroalcoholic obtained by reflux and sonication) from ora-pro-nóbis (*Pereskia grandifolia* Haw) were investigated for the presence of phenolic compounds, antioxidants, and antibacterials. The extracts of *Pereskia grandifolia* Haw obtained by reflux were more efficient than sonication because they extracted a greater amount of phenolic compounds, antioxidants, flavones, and flavonols. In relation to the solvent, it was detected that the hydroalcoholic allowed a greater quantification of total flavonoids (flavones and flavonols, flavanones and di-hydroflavonols) and antioxidants (measured by the methods of DPPH, molybdate and chelating power). When water was used as a solvent extractor, larger amounts of total phenolic compounds and antioxidants (measured by DPPH and chelating power) were observed. All extracts presented inhibitory activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa*, and aqueous extracts obtained by reflux and sonication being the largest inhibition halos. However, when the minimum bactericidal concentration was determined, only the hydroalcoholic extract obtained by reflux showed an effective result at the tested concentrations. The characterization and quantification of the phenolic, antioxidant and antibacterial compounds of *P. grandifolia* Haw provide important information to increase the use of this plant, as an alternative source of compounds beneficial to health, and with potential antimicrobial activity.

Keywords: UFP; Ora-pro-nóbis; Bioactive compounds; Spectrophotometry; Pathogens.

Resumo

Pesquisas em Plantas Alimentícias Não Convencionais (PANC) quanto à presença de compostos bioativos e antimicrobianos são escassas e a ampliação do conhecimento a respeito

de suas propriedades torna-se necessária. Neste estudo, quatro tipos diferentes de extratos (aquosos e hidroalcoólicos obtidos por refluxo e sonicação) de ora-pro-nóbis (*Pereskia grandifolia* Haw) foram investigados quanto à presença de compostos fenólicos, antioxidantes e antibacterianos. Os extratos de *Pereskia grandifolia* Haw obtidos por refluxo foram mais eficientes do que a sonicação, pois extraíram uma quantidade maior de compostos fenólicos, antioxidantes, flavonas e flavonóis. Em relação ao solvente, detectou-se que o hidroalcoólico permitiu uma maior quantificação de flavonóides totais (flavonas e flavonóis, flavanonas e dihidroflavonóis) e antioxidantes (medidos pelos métodos de DPPH, molibdato e poder quelante). Quando a água foi usada como solvente extrator, maiores quantidades de compostos fenólicos totais e antioxidantes (medidos pelo DPPH e poder quelante) foram observados. Todos os extratos apresentaram atividade inibitória contra *Staphylococcus aureus* e *Pseudomonas aeruginosa*, sendo os extratos aquosos obtidos por refluxo e sonicação os que apresentaram maiores halos de inibição. Porém, quando a concentração mínima bactericida foi determinada, apenas o extrato hidroalcoólico obtido por refluxo apresentou resultado efetivo nas concentrações testadas. A caracterização e quantificação dos compostos fenólicos, antioxidantes e antibacterianos de *Pereskia grandifolia* Haw fornecem informações importantes para aumentar o uso dessa planta, como fonte alternativa de compostos benéficos à saúde, e com potencial antimicrobiano.

Palavras-chave: PANC; Ora-pro-nóbis; Compostos bioativos; Espectrofotometria; Patógenos.

Resumen

La investigación en Plantas Alimenticias No Convencionales (PANC) sobre la presencia de compuestos bioactivos y antimicrobianos es escasa y es necesaria la ampliación del conocimiento sobre sus propiedades. En este estudio, se investigaron cuatro tipos diferentes de extractos (acuoso e hidroalcohólico obtenidos por reflujo y sonicación) de ora-pro-nóbis (*Pereskia grandifolia* Haw) en busca de la presencia de compuestos fenólicos, antioxidantes y antibacterianos. Los extractos de *Pereskia grandifolia* Haw obtenidos por reflujo fueron más eficientes que la sonicación, ya que extrajeron una mayor cantidad de compuestos fenólicos, antioxidantes, flavonas y flavonoles. En cuanto al solvente, se encontró que el hidroalcohólico permitió una mayor cuantificación de flavonoides totales (flavonas y flavonoles, flavanonas y dihidroflavonoles) y antioxidantes (medidos por los métodos de DPPH, molibdato y poder quelante). Cuando se utilizó agua como disolvente extrator, se observaron mayores cantidades de compuestos fenólicos totales y antioxidantes (medidos por DPPH y poder

quelante). Todos los extractos mostraron actividad inhibidora frente a *Staphylococcus aureus* y *Pseudomonas aeruginosa*, siendo los extractos acuosos obtenidos por reflujo y sonicación los que presentaron los mayores halos de inhibición. Sin embargo, cuando se determinó la concentración bactericida mínima, solo el extracto hidroalcohólico obtenido por reflujo mostró un resultado efectivo en las concentraciones ensayadas. La caracterización y cuantificación de los compuestos fenólicos, antioxidantes y antibacterianos de *Pereskia grandifolia* Haw aportan información importante para incrementar el uso de esta planta, como fuente alternativa de compuestos beneficiosos para la salud y con potencial antimicrobiano.

Palabras clave: PANC; Ora-pro-nóbis; Compuestos bioactivos; Espectrofotometría; Patógenos.

1. Introduction

Brazil is one of the most biodiverse countries in the world with a large number of plants, used for different purposes such as food, pharmacological, environmental and others (Clement, 1999; Leal et al., 2018). Some species of plants are well known and their uses are traditional. This is the case of food plants which have one or more parts or products that can be used in human food (Prescott-Allen R & Prescott-Allen, 1990). However, there are a large number of species that are not explored and have restricted or regional use. This group of underutilized plants has received great attention, both for the expansion of monocultures and the use in food, as by scientists, for biological and medicinal properties. Authors propose the use of the expression “unconventional food plants- UFP” (*Plantas Alimentícias Não-Convencionais* - PANC, in Portuguese). This term generally refers to plants that have no market value and are produced on small scales, native and exotic plants, and plants that have an unusual method of processing (Kinupp & Lorenzi, 2014).

Among the classes of unconventional food plants are the ora-pro-nóbis, popular name of the species *P. aculeata* Mill. and *P. grandifolia* Haw. This is a rustic plant known in Brazilian popular medicine for its therapeutic properties, besides preventive, palliative or curative (Kazama et al., 2012). Its nutritional potential is due to the presence of lipids, proteins, amino acids, minerals (such as calcium, iron, magnesium, potassium, and zinc) and dietary fiber (Souza, 2014; Vargas, 2017).

Pereskia grandifolia Haw has different uses as medicinal, edible and ornamental. In addition, the leaves of this plant are use in treatments for cancer, hypertension, diabetes and inflammatory diseases (Harlev et al., 2012; Abdelwahab, 2013; Sharif et al., 2013). This

species has bioactive molecules that act as sequestrants of reactive oxygen species (ROS), thus preventing and / or reducing oxidative damage to DNA and consequently the initiation of some chronic diseases (Diplock at al., 1988).

Despite the benefits mentioned above, showing nutritional and ethnopharmacological relevance of ora-pro-nóbis leaves, information on their phytochemical constitution remains limited. Many studies have focused on the chemical evaluation related to the profile of amino acids and phytosterols, (Pinto et al., 2015; Souza at al., 2016). In this context, it is necessary to evaluate the possibility of a complete and efficient use of *P. grandifolia* Haw and to prove the functional properties of plant extracts through chemical composition and *in vitro* tests focused on antioxidant and antimicrobial action. Natural bioactive extracts with antioxidant activities can be used as substitutes for synthetic additives (Garcia et al., 2019). Likewise, the antimicrobial effects of some phytochemicals can inhibit the growth of pathogenic microorganisms (Corrêa et al., 2018). On the other hand, it is known that the efficiency of plant extracts depends on the species used, the concentration of the active compound present in the plant, the part of the plant (leaf, stem, seed), the extraction methods and the stability of the components (Kamel, 2000; Brugalli, 2003).

According to the above, the objective of the work was to perform a spectrophotometric characterization of the presence of phenolic compounds in the extracts of *Pekeskia grandifolia* Haw, as well as to evaluate the antioxidant and antimicrobial actions of these extracts, which were obtain by different methods.

2. Materials and Methods

2.1 Collection and identification of the Botanical Material

Plants of *P. grandifolia* Haw were collected in the Garden of Medicinal Plants at the Department of Agriculture of the University of Lavras (DAG/UFLA) (21°14'07 "S, 44° 58'22" W, 879 m altitude). The leaves were identified in the Department of Biology/UFLA, and their exsicta deposited in the EPAMIG BH.1 Herbarium under registration number PAMG58224.

2.2 Preparation of extracts

The aqueous and hydroalcoholic extracts (70%) of *P. grandifolia* Haw were prepared. For the preparation, fresh leaves were used, which were collected and washed with distilled water, then dried naturally (in the shade). The leaves were then shaved to a size of 0.5 cm².

The extracts were prepared by: a) aqueous reflux; b) aqueous sonication; c) hydroalcoholic reflux d) hydroalcoholic sonication.

To obtain pure extracts, 5% (w/v) of the vegetal material (fresh leaves) were crushed using a porcelain capsule and mortar. The extract obtained by refluxing was produced by heating for two hours using a modified Clevenger apparatus. The preprepared raw material was heated for 30 minutes while maintaining a closed system boiling to reduce possible volatilization losses. The extract obtained by sonication was prepared in an ultrasonic bath (model NI1204, 50Hz) in water during a 30 minutes cycle. After the extractive procedures, they were filtered on filter paper and then stored at -20 °C until the tests were performed.

2.3 Determination of Phenolic Compounds of *Pereskia grandifolia* Haw extracts

2.3.1 Quantification of Total Phenols

Quantification of the total phenolic compounds was determined by the colorimetric method using the *Folin-Ciocalteu* reagent described by Slinkard & Singleton (1997). The calibration curve prepared with gallic acid. The reactions were carried out in glass tubes. 20µL of extracts, 125mL of Na₂CO₃ and 100mL of ethanolic solution were used *Folin-Ciocalteu* 10% (v/v). Subsequently, 250µL was transferred to the 96 well microplate, reacting for 120 minutes in the dark at room temperature and reading in triplicate in a spectrophotometer at 760nm. The results were expressed as milligram equivalents in gallic acid per gram of dry extract (mg.GAE.G⁻¹).

2.3.2 Quantification of flavonoids (flavones and total flavonols)

The content of total flavonoids in extracts was determine by the method of Ahn et al. (2004). Briefly, 100 µL of 2% aluminum chloride (AlCl₃) methanolic solution was mix with the same volume of sample solution (100 µL of each extract). After 60 minutes at room temperature, the absorbances of the samples were carried out at 420 nm, against a blank [100 µL solvent (water or 70% ethanol) + 100 µL AlCl₃ solution]. Total flavonoid content was determined using a standard quercetin curve with five-point concentrations (0.125, 0.0625, 0.0312, 0.0156, and 0.0078 µg / mL). $y = 16.944x - 0.0351$, where y is the absorbance and x is the concentration; R₂ = 0.9999). Total flavonoid content was expressed in milligrams of quercetin per gram of dry extract (mg.EQ.g⁻¹).

2.3.3 Quantification of total dihydroflavonoids

Quantification of total dihydroflavonoids was performed according to Popova, Bankova & Butovska (2004). 500µL of 2,4-dinitrophenylhydrazine methanolic solution (DNP) was mixed with 200µL of sample solution or standard and heated at 50°C for 50 minutes. After cooling to room temperature, 2mL of 10% KOH in methanol (v/v) was added to the reagent solution (sample + DNP). Then 50µL of the reagent solution (sample + DNP + KOH) were diluted in 500µL of methanol and centrifuged at 3.000rpm for 10 minutes. A volume of 250µL of each sample was transferred to the wells of the microplate. The absorbances were read at 486 nm, against a blank [200 µL solvent (70% water/ethanol) + 500 µL DNP + 2000 µL 1% KOH]. The total content of dihydro flavonoids was determined using a standard naringenin curve with six points of concentrations (3, 1.5, 0.75, 0.375, 0.1875 and 0.0937 µg/mL). $Y = 0.7118x - 0.0611$, where y is the absorbance and x is the concentration; $R^2 = 0.9999$. The results were expressed as milligram equivalents in naringenin per g of dry extract (mg.EN.g^{-1}).

2.4 Determination of the antioxidant activity of *Pereskia grandifolia* Haw extracts

2.4.1 Total antioxidant capacity (TAC)

The total antioxidant capacity was measured based on the ammonium molybdate reduction method described by Prieto, Pineda & Aguilar (2004). For this, 100µL of the extracts were mixed with 3mL of the reagent solution (0.6M sulfuric acid, sodium phosphate 28mM and 4mM ammonium molybdate). After reaction for 90 minutes at 95 °C, the samples were cooled to room temperature and their absorbances measured at 695nm. The assay was performed in triplicate and the results expressed as milligram equivalents in ascorbic acid per gram of dry weight of the sample (mg.EAG.g^{-1}).

2.4.2 Free radical scavenging activity (DPPH)

The activity of free radical capture by DPPH was performed according to the method proposed by Brand-Williams, Cuvelier & Berset (1995). Different concentrations of the samples (3.12 - 0.19 mg/mL) were added to 270 µL of 0.2 mM DPPH methanolic solution. The solutions were incubated at room temperature in the dark for 60 minutes and the

spectrophotometric readings carried out at 517nm. BHT was used as a positive control, while methanol was used as a negative control. The activity of free radical capture by DPPH was expressed by the percentage of inhibition (50%), calculated by the formula: $IC_{50} (\%) = (A_0 - A_1) / A_0$, where A_0 is the absorbance of the negative control and A_1 the absorbance of samples. The activity of elimination of free radicals was expressed as IC 50, corresponding to the extract concentration capable of inhibiting 50% of the DPPH radicals.

2.4.3 Determination of the chelating power

The degree of chelation of the iron ions II by the extracts was evaluated according to Miguel et al. (2018). In aliquots of 100 μ L of the extracts 30 μ L of $FeCl_2 \cdot 4H_2O$ (2mM) was added. Then 40 μ L ferrozine (5mM) was added, the reactive solutions were incubated for 10 minutes at 25 °C and absorbance read at 562nm. The percentage of inhibition of complex formation ferrozine - Fe^{2+} was determined using the formula: $[(A_0 - A_1) / A_0 * 100]$ where A_0 is the absorbance of the Fe-ferrozine complex²⁺, and A_1 the absorbance of the samples analyzed. The results were expressed as IC 50, corresponding to the concentration effective for the chelation of 50% of iron ions of II. EDTA were used as positive control and the samples were analyzed in triplicates.

2.5 Evaluation of the antimicrobial activity of *Pereskia grandifolia* Haw extracts

The antimicrobial activity of *P. grandifolia* Haw extracts was evaluated by antibiotic susceptibility testing, antimicrobial activity using agar diffusion method, and determination of minimum bactericidal concentration (MBC).

2.5.1 Microorganisms and obtainment of the inoculum

Strains used in the experiment were *Staphylococcus aureus* GL 4133, *Staphylococcus aureus* GL5674, provided by Embrapa Dairy Cattle headquartered in Juiz de Fora/MG, and *Pseudomonas aeruginosa* ATCC 25853 provided by the Oswaldo Cruz Foundation (Fiocruz). Stock cultures were stored in freezing medium (glycerol: 15 mL; bacteriological peptone: 0.5 g; yeast extract: 0.3 g; NaCl: 0.5 g; distilled water: 100 mL) and kept frozen during the period of the experiment.

The reactivation of the strains was performed inoculating 100 µL of culture into tubes containing 10 mL of BHI broth (Brain Heart Infusion Broth) with incubation at 37°C/24h under aerobic conditions. The final concentration reached in culture was 10⁸ CFU/g, using the McFarland scale.

2.5.2 Sensitivity to antibiotics

Antimicrobial susceptibility testing was performed using the agar diffusion method according to recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2018). The microorganisms were reactivated in BHI broth at 37°C/24 h and the standardized inoculum with cell density corresponding to 10⁸ CFU / mL, by McFarland Scale.

An aliquot of the culture was spread on TSA (Trypticase Soy Agar) and discs containing the following antibiotics were then added for the Gram-positive and Gram-negative strains: cephalothin (30mg), gentamicin (10mg) and enrofloxacin (5mg). The plates were then incubated at 37 °C/24 hours and after that period the inhibition halos were measured around the discs.

2.5.3 The antimicrobial activity of *Pereskia grandifolia* Haw extracts

The antimicrobial activity of the extracts of *P. grandifolia* Haw was performed using agar diffusion method following recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2017). The microorganisms were reactivated in BHI broth at 37 °C/24 h and the standardized inoculum with cell density corresponding to 10⁸ CFU/ mL, by McFarland Scale. Surface scattering was performed on TSA agar and subsequently, the discs containing the following extracts (5% w/v) were added from fresh leaves of *P. grandifolia* Haw: aqueous sonication extract, aqueous reflux, aqueous (maceration) and hydroalcoholic (maceration). Ethanol (70%) was used as negative control. The plates were then incubated at 37 °C/24 h and, after that period, the inhibition halos were measured around the discs. The methodology was done in triplicate and three replicates.

2.5.4 Determination of minimal bactericidal concentrations (MBC) of the extracts

The minimum bactericidal concentrations (MBC) of the extracts were determined employing the broth microdilution technique in polystyrene 96 well plates according to Clinical and Laboratory Standards Institute (CLSI, 2018) with adaptations.

The different extracts were diluted to obtain concentrations of 0.0781, 0.1562, 0.3125, 0.625, 1.25, 2.5 (v/v); 150 μ L aliquots of the extract solutions were added to the wells containing 150 μ L TSB and diluted at the concentrations proposed and 10 μ L of standard cultures were inoculated at 10^8 CFU/mL. Alcohol (70%) was used as the negative control. The microplates were sealed and incubated at 37 °C for 24 hours. After this period, absorbance reading was performed in Tecan's Elisa Reader (D0 600 nm). The experiment was performed in triplicate and three replicates.

2.6 Statistical

Statistical analysis of the data was performed through analysis of variance ANOVA, where the results were considered significant when $p < 0.05$ and Student's t-test, with significance level $\alpha = 0.05$. Statistical evaluation of the results was done through Statistical Software.

3. Results and Discussion

3.1 Quantification of phenolic compounds from *Pereskia grandifolia* Haw extracts

The results obtained in the quantifications of phenolic compounds of extracts of *P. grandifolia* Haw are presented in Table 1.

Table 1. Phenolic constituents of different extracts of *Pereskia grandifolia* Haw(1).

| Extraction methods | Total phenolic (mg.EAG.g ⁻¹) ⁽²⁾ | Flavones and flavonols total (mg.EQ.g ⁻¹) ⁽³⁾ | Flavanones and dihydro flavonols total (mg.EN.g ⁻¹) ⁽⁴⁾ |
|--|---|--|--|
| Aqueous reflux | 9.88 ± 4.25 ^a | 0.26 ± 2.58 ^b | 4.04 ± 2.16 ^c |
| Aqueous sonication | 0.63 ± 0.47 ^d | 0.06 ± 0.11 ^d | 1.70 ± 0, 75 ^d |
| Hydroalcoholic reflux ⁽⁵⁾ | 6.51 ± 1.05 ^b | 0.57 ± 0.11 ^a | 11.8 ± 1.20 ^b |
| Hydroalcoholic sonication ⁽⁵⁾ | 4.97 ± 2.58 ^c | 0.13 ± 0, 11 ^c | 15.27 3.26 ^a |

(1) Mean and standard deviation followed by the same letters in the column did not differ statistically from each other at the $P < 0.05$ level according to the Scott-Knott test; ⁽²⁾ Expressed in milligrams of gallic acid per gram of fresh leaf; ⁽³⁾ Expressed in milligram equivalent of quercetin per gram of fresh leaf; ⁽⁴⁾ Expressed in mg equivalents of naringenin/g of fresh leaf; ⁽⁵⁾ Obtained with 70% etanol. Source: Authors.

As a reference, the averages obtained in the quantification of total phenolic compounds showed the predominance of these substances in the extracts obtained, where they presented values between 0.63 and 9.88 mg EAG.g⁻¹ of dry plant. It was observed that the recovery of the phenols was affected by the extraction method (P<0.05), these results being similar to those reported by Scherer & Godoy (2014). The reflux method was the most efficient and water the most effective solvent (9.88 mg.EAG.g⁻¹), followed by the hydroalcoholic solvent (6.5 mg.EAG.g⁻¹). The sonication provided a reduction in the quantification of these substances, being the aqueous solvent of lower extractive efficiency (0.63 mg.EAG.g⁻¹). It was verify that the reflux extraction was the most effective method to obtain these molecules because it extracted a greater quantity of phenolic compounds. This finding was evidenced by Tasioula-Margari & Tsabolatidou (2015) who explained that the extraction of soxhlet (equivalent to reflux) is the technique most use to isolate phenols from solid matrices. This is because, during the heating process, the intracellular evaporation of the water can alter the lignocellulosic structure, besides promoting the protein denaturation, resulting in greater availability of active compounds in the matrix (Lemos et al., 2012).

Another factor that can directly influence the potentiation of the extraction of bioactive substances is the choice of the solvent. Sim et al. (2010) when using ethyl acetate and hexane to extract phenolic compounds present in leaves of *P. grandifolia* Haw found respectively 45.99 and 19.08 mg.EAG.g⁻¹ of phenols results superior to those found in this research. Hayouni et al. (2007) explain that the influence of different solvents on the extraction of total phenolic compounds is a result of differences in polarity and solubility among the different phenolic components present in the plants. However, due to the low toxicity and abundance, water and hydroalcoholic solvents are preferred when the objective is to obtain antioxidant products for the food industry (Gómez-Plaza, Miñano & López-Roca, 2006).

The optimization, identification, and maximization of the most effective way to extract phenolic compounds is important since these elements may be associated with the plant protection system, which, in the face of stress situations (biotic and abiotic), stimulate the development of secondary metabolites (Lemos et al., 2012). These, in turn, have been referr to as having multiple biological effects, including antioxidant activity characterized by the elimination or impediment of free radical formation (Boulanouar et al., 2013). The superiority of phenolic compounds present in *P. grandifolia* Haw in relation to other UFPs is demonstrated in a study published by Viana et al. (2015) that investigated the presence of total phenols in “type I azedinha”, “type II azedinha”, “bertalha”, “beldraga”, “caruru” and “peixinho”, and found respectively 0.23, 0.35, 0.25, 0.40, 0.56 and 0.77 mg.GAE.g⁻¹ in them.

Regarding to the quantification of flavones and total flavonols, these ranged from 0.06 to 0.57 mg.EQ.g⁻¹ values, the lowest values found for the extracts obtained by sonication and the highest values for those obtained by reflux. The hydroalcoholic extract obtained by reflux showed the highest efficacy (0.57 mg.EQ.g⁻¹), therefore, the methodology, as well as the solvent, had a direct influence on the final result. The temperature used in the reflux technique was also a significant factor in the recovery of these constituents in *P. grandifolia* Haw and since sonication did not use it, the extraction obtained by this method obtained an inferior result (P <0.05). Shi et al.(2003) explain that heating can soften the plant tissue and weaken the interactions between phenol and phenol-protein polysaccharides, therefore, more polyphenols migrate into the solvent. These results are within the standards found by other authors (Moreira et al., 2008; Kalogeropoulos et al., 2009), where total flavonoid values are lower than total phenolics.

Regarding the influence of the solvent used, Spagolla et al. (2019) found that, in the alcoholic extraction in blueberry, greater phenolics and total flavonoids were obtain in the 80:20 (v/v) methanol/water mixture and the 60:40 (v/v) ethanol/water mixture. Park et al. (1998) showed that for ethanolic extracts of propolis it was possible to extract a greater amount of flavonoids in the alcoholic concentrations between 60 and 80% and the ethanolic extracts at 70 and 80% presented great antioxidant activity. According to Mokrani & Madani (2016), the type of solvent, temperature and pH affect the amount of phenolic compounds to be extracted, however, there is no data in the literature that relates the three parameters studied, so there is no ideal condition for the extraction.

Concerning the quantification of total flavanones and dihydroflavonols, it was verify that the hydroalcoholic extract obtained by sonication was the most efficient, followed by the hydroalcoholic reflux method, whereas the aqueous sonic extraction was the least efficient. According to the results, it was demonstrate that the choice of solvent (hydroalcoholic) was a preponderant factor in the extractive efficiency of these molecules. Dai & Manper (2010) explain that polar solvents are often use for extracting polyphenols from plant materials and ethanol is commonly used because of their safety for human consumption. According to Vizzoto and Pereira (2011) the water is not considered an effective solvent and results in the extract with a high impurity (due to the presence of organic acids, sugars, and soluble proteins), and may interfere in the quantification of the compounds.

The evaluation of different classes of flavonoids in the extract is of great importance for *Pereksia grandifolia* Haw, as there are still no reports of identification of these substances in this plant. The increase in the consumption of these substances is desired due to the

antioxidant action and due to the presence of aromatic hydroxyl groups that have therapeutic activity as an anti-carcinogenic effect, anti-inflammatory and antiviral (Choi & Kim, 2003; Vessala, Hrmmatia & Vasei, 2003; Zuanazzi & Montanha, 2004).

3.2 Screening of the total antioxidant capacity of *Pereskia grandifolia* Haw extracts

In this work the antioxidant activity was evaluated by molybdate method (TAC), DPPH and chelating power (Table 2) emphasizing that the lower the results found, higher the antioxidant activity of material (except for TAC) (Choi & Kim, 2003).

Regarding to the total antioxidant capacity (TAC) determined by the molybdate method, which is based on the competence of the compound to generate the reduction of ammonium molybdate, it can be observed in Table 2 that the procedure employing hydroalcoholic reflux showed the highest capacity followed by aqueous refluxing. The extracts obtained by aqueous sonication and hydroalcoholic were statistically similar ($P < 0.05$), however, they received inferior results proving that the ultrasonic vibration is not effective in extracting these substances.

Table 2. Antioxidant activity of different extracts of *Pereskia grandifolia* Haw⁽¹⁾.

| Extraction Methods | Total antioxidant | DPPH | Chelating |
|--|---|--------------------------|--------------------------------|
| | capacity (mg.EAG.g-1) ⁽²⁾ | (IC50) ⁽³⁾ | Power (IC50) ⁽³⁾ |
| Aqueous reflux | 161 ± 0,50 ^b | 0,21 ± 0,35 ^c | 1,0 ± 0,69 ^b |
| Aqueous sonication | 0,34 ± 0,24 ^c | 2,85 ± 0,02 ^a | 1,66 ± 0,04 ^a |
| Hydroalcoholic reflux ⁽⁴⁾ | 0,36 ± 0,13 ^c | 0,93 ± 0,14 ^b | 2,19 ± 0,30 ^a |
| Hydroalcoholic sonication ⁽⁴⁾ | 2,01 ± 0,69 ^a | 0,43 ± 0,01 ^c | 0,87 ± 0,04 ^b |

(1) Mean and standard deviation followed by the same letters in the column do not differ statistically from each other at the $P < 0.05$ level according to the Scott-Knott test; ⁽²⁾ Expressed in milligrams equivalent in gallic acid per gram of dry weight of the sample; ⁽³⁾ Corresponding to the concentration of extract capable of sequestering/inhibiting 50% of the DPPH/iron ions; ⁽⁴⁾ Obtained with 70% ethanol. Source: Authors.

The antioxidant activity was also evaluate by the DPPH radical sequestration method, which is based on the capture of the roots by compounds present in the extract. In this respect, it was observed that extractions obtained by reflux were the most effective and no significant difference was detected among the solvents ($P < 0.05$), proving that this variable does not influence this parameter. This result can be explain because during the heating (used in the

reflux) there is rupture of the cell wall of the vegetable, thus, a greater amount of antioxidant actives is extracted. Another factor that contributes to the stability of the extract at high temperature is that the plant under study presents thermostability (Carvalho et al., 2009).

Similar results were also found when quantifying the chelating power of the samples, which obtained extractive maximization with the reflux method without significant influence ($p < 0.005$) of the solvent used. In this condition, the chelation of Fe^{2+} ions was superior to those obtained by aqueous and hydroalcoholic sonication, both also similar ($p < 0.005$). Different results were found by Souza (2014) who analyzed the influence of extractive solvent on leaves of *P. grandifolia* Haw for antioxidant activity and found that in the 80% acetone extract higher antioxidant activity was observed, followed by the extract in 70% ethanol and water. Vargas (2017) explains that the three solvents analyzed in such work are polar, but acetone is constituted by a larger carbon chain, decreasing the polarity of the solvent. Thus, a more comprehensive amount of substances responsible for the antioxidant potential could be extracted, including compounds of medium and high polarity, which corroborates with the data obtained in this work.

The chemical complexity of extracts, consisting of dozens of compounds with different functional groups, polarity and chemical behavior, can lead to contradictory results depending on the test used. Therefore, the adoption of more than one trial to evaluate the antioxidant potential of extracts is a more informative and indispensable tool (Oztürk, 2012). Moreover, several methods and solvents have been used for the extraction of bioactive compounds from plant materials. Finding a single method that is suitable for bioactive extraction is important, but it is not a simple due to the diversity of chemical structures and sensitivity variations of the compounds to the extraction conditions (Morzelle, 2012). There is no solvent system that is suitable for extracting all bioactive compounds from a matrix, so the diversity of results is justifiable.

3.3 Sensitivity to antibiotics

The sensitivity of *S. aureus* and *P. aeruginosa* strains to cephalothin, gentamicin and enrofloxacin antibiotics is shown in Table 3.

Table 3. Sensitivity of strains of *Staphylococcus aureus* and *Pseudomonas aeruginosa* by different antibiotics observed by inhibition halos (mm).

| Microorganism | Antibiotics | Inhibition Halo (mm) | Sensitivity (mm) |
|---|--------------------|----------------------|-------------------|
| <i>Staphylococcus aureus</i> GL 5674 | Cephalothin (30mg) | 23 | S _≥ 18 |
| | Gentamicin (10mg) | 20 | S _≥ 15 |
| | Enrofloxacin (5mg) | 35 | S _≥ 18 |
| <i>Staphylococcus aureus</i> GL 4133 | Cephalothin (30mg) | 35 | S _≥ 18 |
| | Gentamicin (10mg) | 17 | S _≥ 15 |
| | Enrofloxacin (5mg) | 30 | S _≥ 18 |
| <i>Pseudomonas aeruginosa</i> ATCC 25853 | Cephalothin (30mg) | 48 | S _≥ 18 |
| | Gentamicin (10mg) | 30 | S _≥ 15 |
| | Enrofloxacin (5mg) | 5 | S _≥ 17 |

Source: Authors.

The results for the sensitivity of *S. aureus* and *P. aeruginosa* to antibiotics were analyzed based on data published by Clinical and Laboratory Standards Institute (2017) using standards for antimicrobial susceptibility testing. For the antibiotic cefalotin (30mg) strains of *S. aureus* are considered sensitive when the inhibition halo is ≥ 18 . For gentamicin (10mg) the inhibition halo should be ≥ 15 and for enrofloxacin ≥ 18 . *P. aeruginosa* strains should present halo values ≥ 21 for cephalothin (30mg), ≥ 15 for gentamicin (10mg) and ≥ 17 for enrofloxacin (5mg).

In this study it can be said that strains of *S. aureus* evaluated are sensitive to all the antibiotics used, and the strain of *P. aeruginosa* is not sensitive only to the antibiotic enrofloxacin (5mg).

The category "sensitive (S)" means that infection by a particular strain can be treated appropriately with the dose of antimicrobial agent recommended for this type of infection and infecting species, except when contraindicated. The strains can also be classified as "intermediate (I)" or "resistant (R)", I when a higher dosage of the medicament is desirable for controlling the microorganism, and R when the microorganism is not inhibited by the systemic concentrations of the antimicrobial agent normally achievable with the usual treatments (CLSI, 2017).

Although antimicrobials with a single mechanism of action have been effective in recent years, bacterial resistance to antibiotics is currently one of the most relevant public

health problems, since many bacteria previously susceptible to the antibiotics have become resistant over time. Thus, studies are stimulated in the search for new agents that may be antimicrobial and that minimize bacterial resistance, most of them using plants used in folk medicine. In this context, the antibacterial activity of extracts of *P. grandifolia* Haw were evaluated against strains of *Staphylococcus aureus* and *Pseudomonas aeruginosa*, and the results can be compared to those obtained by the conventional antibiotics used above.

3.4 Biological activities of *Pereskia grandifolia* Haw extracts

The antimicrobial activity of extracts of *P. grandifolia* Haw was evaluated on strains of *S. aureus* and *P. aeruginosa*. The results obtained refer to the inhibition halos formed for each extract, and are highlighted in Table 4.

Table 4. Antibacterial action of the different extracts of *Pereskia grandifolia* Haw against *P. aeruginosa* and *S. aureus* ⁽¹⁾

| Extracts | Inhibition halo diameter (mm) | | |
|-----------------------------|--------------------------------------|--------------------------------------|--|
| | <i>Staphylococcus aureus</i> GL 4133 | <i>Staphylococcus aureus</i> GL 5674 | <i>Pseudomonas aeruginosa</i> ATCC 25853 |
| Aqueous (maceration) | 8 ^a | 6 ^a | 5 ^a |
| Aqueous reflux | 10 ^b | 12 ^c | 5 ^a |
| Aqueous sonication | 15 ^c | 10 ^b | 11 ^b |
| Ethanol (maceration) | 8 ^a | 6 ^a | 5 ^a |

(1)Averages followed by the same letters in the column do not differ statistically. Source: Authors.

The aqueous extract of maceration was chosen since in popular culture, macerated leaves or water-based infusions are used in the treatment of diseases and the extract in ethanol (70%), since the use of this solvent is reported as efficient extraction of phenolic compounds (Jayaprakasha et al., 2001). In addition, extracts of aqueous reflux and aqueous sonication were chosen to the antibacterial test. The agar diffusion test using paper disc was performed for all extracts and for ethanol (70%) as negative control, which did not show a growth of inhibition halo, thus proving that ethanol (70%) did not act as antimicrobial and its use did not interfere in the results.

Although the values of the inhibition halos presented above do not classify the microorganisms as "sensitive" to the extracts (Table 3), it can be said that there was inhibitory activity. Among the extracts, aqueous extract obtained by sonication was highlighted with inhibition halo of 15 mm for *S. aureus* GL 4133, 10 mm for *S. aureus* GL 5674 and 11 mm for *P. aeruginosa*; followed by the aqueous extract obtained by reflux, which presented inhibition halos of 10 mm for *S. aureus* GL 4133 and 12 mm for *S. aureus* GL 5674. Both extracts obtained by maceration showed smaller inhibitory activity.

These results may be associated to the extraction method. In the reflux extraction method, the bioactive compounds are extract by repeated washing with an organic solvent, in this study, ethanol. In ultrasonic bath extraction, sonication produces mechanical, chemical, and physical effects. Both methods result in the rupture of the biological membranes facilitating the release of the bioactive compounds of the plant, as well as intensifying the penetration of the solvent into the cellular material, increasing the transfer of these compounds to liquid (Cárcel et al., 2012; Costa et al., 2013). Maceration is a technique that does not lead to the total exhaustion of the vegetable due to the saturation of the solvent and/or the establishment of a balance between solvent and the interior of the cell, thus limiting the extraction of bioactive compounds (Marques & Vigo, 2009).

Among the strains, inhibition halos were lower for *P. aeruginosa*, which is Gram-negative. According to Taheri (2013), Gram-negative bacteria exhibit higher resistance to active compounds when compared to Gram-positive bacteria that are more sensitive to plant infusion and essential oil of herbs. This resistance is attribute to the presence of a complex unified outer septum present in Gram-negative bacteria reducing the rate of penetration of antimicrobial compounds (Farzaneh & Carvalho, 2015).

Some studies have evaluated the antimicrobial action of different species of *P. grandifolia* Haw. However, without many effective results. Turra et al., (2007) analyzed the antibacterial susceptibility of leaf extracts of *P. grandifolia* Haw (alcoholic, hexane, dichloromethane, chloroformic, ethyl acetate and methanolic extracts). None of the extracts evaluated showed activity against the tested strains of *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis* and *Pseudomonas aeruginosa*. The same was observe by Santos et al. (2010) using extracts of *Pereskia aculeata* against the cariogenic bacteria *Enterococcus faecalis*, *Streptococcus mutans* and *Lactobacillus casei*.

Zareisedehizadehet et al. (2014) evaluated the *in vitro* antibacterial properties of *Pereskia bleo*. The methanolic and hexane extracts demonstrated efficiency against *Salmonella* sp and *Pseudomonas aeruginosa*. In addition, *Pereskia bleo* extract obtained with

dichloromethane solvent showed a promising antibacterial effect against *Staphylococcus aureus* that is resistant to the antibiotic methicillin.

Rodrigues (2016) evaluated the antimicrobial action of aqueous and hydroalcoholic extracts of *Pereskia aculeata* Mill on different bacteria, including *Staphylococcus aureus* ATCC 25923 and *Pseudomonas aeruginosa* ATCC 10145, however, did not observe inhibition halo growth for any of them. Vargas (2017) evaluated the antimicrobial activity of *Pereskia aculeata* Mill against *Escherichia coli*, *S. aureus*, *Candida albicans* and *Candida tropicalis*. The results showed that only the extract obtained with petroleum ether presented action on *S. aureus*.

Although there are few papers in the literature with extracts of *P. grandifolia* Haw and corroborating with the results found in this study, the inhibitory action of the analyzed extracts serves as inspiration for this plant to be studied in depth. Other extracts can be produced using various methods, and / or evaluated on other bacteria, isolated and / or combined with already consolidated antimicrobials.

3.5 Determination of minimum bactericidal concentrations of extracts (MBC)

In addition to the antimicrobial potential of extracts of plants from *P. grandifolia* Haw, the minimum bactericidal concentration (MBC) was determined for the studied microorganisms. The data obtained are show in Table 5.

Two extracts were select for the determination of MBC, both obtained by the same extraction method: the aqueous reflux extract, because it showed an inhibitory action evidenced by the formation of the inhibition halo, and the hydroalcoholic reflux extract, because it presented good antioxidant activity. Ethanol (70%) was use as a negative control.

Table 5. Minimal bactericidal concentrations (%) of different extracts of *Pereskia grandifolia* Haw on strains of *S. aureus* and *P. aeruginosa*.

| Extract | Microorganisms | | |
|------------------------------|-------------------------------------|--------------------------------------|--|
| | <i>Staphylococcus aureus</i> GL4133 | <i>Staphylococcus aureus</i> GL 5674 | <i>Pseudomonas aeruginosa</i> ATCC 25853 |
| Aqueous reflux | > 2,5% | > 2,5% | > 2,5% |
| Hydroalcoholic reflux | 1,25% | 0,63% | 0,63% |
| Ethanol (70%) | > 2,5% | > 2,5% | > 2,5% |

Source: Authors.

The aqueous reflux extract did not inhibit the growth of any strain studied at the concentrations tested (maximum 2.5%). For the hydroalcoholic reflux extract, the MBC for *S. aureus* GL 4133 was 1.25%, for *S. aureus* GL 5674 of 0.625% and for *P. aeruginosa* of 0.625%.

According to Jayasena & Jo (2013), the phenolic compounds are the main responsible for the antimicrobial activity, to increase the permeability of the cellular membranes, leading to the loss of important constituents for the maintenance of the cellular metabolism.

Taking into account the analysis of phenolic constituents, presented in Table 1, the two extracts (aqueous reflux and hydroalcoholic reflux) presented satisfactory total phenolic contents, the aqueous reflux extract being the one with the highest content. In this sense, the result for MBC analysis is otherwise (the aqueous reflux extract did not show MBC).

This result can be explain by the existence of different groups of chemical components present in plant extracts, besides phenolics. Thus, the antibacterial action of the extract depends on the bioavailability of the chemical component to the bacterial cell and is not attribut only to a specific mechanism, but to several targets in the cell (Nazzaro, Fratianni & Martino,2013), which may differ between species of microorganisms and/or strains of the same species.

In general, the study elucidated the presence of bioactive compounds in the leaves of *P. grandifolia* Haw. In other hands, it was possible to observe the antimicrobial action of some extracts. However, not all extracts were evaluated for antimicrobial action. Thus, further

studies in this context should be conducted in order to study the antimicrobial action of leaf extracts obtained by different extraction methods. Furthermore, different species of the plant should be studied, as well as the antimicrobial action against other pathogenic microorganisms.

4. Conclusion

It was possible to verify the presence of phenolic compounds, antioxidants, favones and flavonols in large amounts when the extract was obtain by reflux. In addition, there was a difference in the quantification of total flavonoids and antioxidants between the extracts obtained by different solvents; the hydroalcoholic extract showed these components in greater quantity. All extracts showed inhibitory activity against the tested bacteria, however, only the hydroalcoholic extract obtained by reflux was bactericidal at the tested concentrations. The information found in this work shows a complex chemical composition of the extracts, proves that the methodology of obtaining influences the chemical profile and highlights the antimicrobial potential of leaf extracts of *P. grandifolia* Haw, encouraging new research in this area and potentiating the use of unconventional food plants for different uses.

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