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Phenolic compounds and antioxidant activity of extracts of pequi peel (*Caryocar brasiliense* Camb.)

[1]Monteiro, S.S., [2]da Silva, R.R., [3]da S. Martins, S.C., [4]Barin, J. S. and [4*]da Rosa, C. S.

[1]*Nutrition Course, Institute of Health and Biotechnology, Federal University of Amazonas – UFAM, Amazonas, Brazil*
[2]*Federal University of Santa Maria – UFSM, Rio Grande do Sul, Brazil*
[3]*Pharmacy Course, Health Sciences, Centro Universitário Franciscano – Unifra, Rio Grande do Sul, Brazil*
[4]*Department of Science and Food Technology, Centre for Rural Sciences, Federal University of Santa Maria – UFSM, Rio Grande do Sul, Brazil*

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Abstract

Given the need for new natural antioxidants to retard lipid oxidation in foods (e.g. meat products), fresh research into new sources of these compounds is required. The pequi (*Caryocar brasilense* Camb.) is a fruit that is native to the cerrado biome and it is known and consumed mainly by the people who inhabit this region. It has a peel which is rich in phenolic compounds that can be used as a natural antioxidant. This study aimed to chemically characterise pequi peel and to determine the content of β – carotene and polyphenols. The antioxidant capacity of different extracts of pequi peel obtained by focused microwave radiation and conventional shaking were also evaluated. The extracts were obtained using the solvents 80% ethanol and water, for 20 minutes at a temperature of 70°C. The chemical composition was determined by analysis of moisture, lipids, protein and dietary fibre. The antioxidant activity was assessed by evaluation of the potential for scavenging free radicals using the model 2,2 - diphenyl picryl hydrazyl (DPPH) method, and by β-carotene/linoleic acid assay. The amount of polyphenols was determined by the Folin-Ciocalteau method. The data showed that pequi peel is rich in water, carbohydrates and dietary fibre. The content of β -carotene was 20.71 ± 1.65 μg/100g. The extracts that showed the best results in terms of polyphenols and antioxidant activity were the 80% hydroethanolic extract and the aqueous extract, respectively, both of which subjected to extraction by shaking. These results indicate that the extracts had a high polyphenol content and high antioxidant activity.

Introduction

Lipid oxidation is a reaction which limits the shelf life of many foods and causes reduced quality in the product. These changes in quality can be perceived by alterations in properties such as flavour, colour and texture. These changes can trigger various secondary reactions, with the formation of free radicals which, as well as forming compounds that give an "off" flavour, lead to other reactions that may affect the security and stability of the product, resulting in nutrient losses and promoting successive oxidative reactions, with the production of potentially toxic compounds (Gardini, 2001).

In Brazil, the National Agency for Sanitary Surveillance (ANVISA) defines antioxidants as substances that slow the onset of oxidative modification in food (Brasil, 1997). Antioxidants can be divided into synthetic antioxidants, such as butylated hydroxyanisole (BHA), and butylated hydroxytoluene (BHT), or natural antioxidants, such as bioactive phenolic substances. To protect lipids and to avoid sensory and apparent deterioration, food manufacturers have made use of various food additives that have antioxidant properties. However, consumer awareness about the health risks posed by synthetic additives has stimulated studies on the use of natural additives, or alternative methods for extending shelf life, as well as increased safety and prevention against damage from lipid oxidation (Georgantelis *et al*., 2007).

The use of natural antioxidants has the advantage of immediate acceptance by the consumer, and their use is not regulated by law. Natural antioxidants may have modes of action that have not yet been fully elucidated; they usually act as acceptors of free radicals, as chelating or sequesters of singlet oxygen,

*Corresponding author.
Email: *claudiasr37@yahoo.com.br*
Tel: +55 55 3220 8254

or as deactivators of pro-oxidant metals (Ghiretti *et al*., 1997).

Pequi is the popular name of *Caryocar brasiliense* Camb. It belongs to the Caryocaceae family and it is also known as pikui, pequiá, amêndoa de espinho, grão de cavalo, or amêndoa in Brazil. Pequi trees are found in warm regions, such as the north and centre-west regions of Brazil and the fruit is typical of the Brazilian cerrado (Gonçalves *et al*., 2011). The fruit is a drupe composed of 76.7% peel, 21.6% of seed (kernels) and 1.7% of fruitlets (pyrenes that have not completed their physiological development) (Vera *et al.*, 2005). According to Marques (2001), the peel is composed of two layers: a thin, leathery, greyish-green colour (exocarp); and another that is thicker and fleshy, yellowish- white in colour (outer mesocarp). Although the peel has a high content of dietary fibre (Barbosa; Amante, 2002), it is little used, which creates an environmental problem when it is disposed of.

Because it is rich in carotenoids, polyphenols and vitamin C, pequi has compounds that protect the lipid fraction of oxidation; the fruit is native to regions of high incidence of solar radiation, which can promote the formation of free radicals (Almeida; Silva, 1994; Rodrigues-Amaya, 1997; Lima, 2008). In their study on the antioxidant activity of fruits from the cerrado, Roesler *et al.* (2007) found that the extracts which showed the highest phenolic content were the ethanolic and aqueous extracts of pequi peel.

The present study aimed to determine the content of β-carotene, as well as to quantify the polyphenols and antioxidant potential of extracts of pequi peel, which were obtained by focused microwave radiation and conventional shaking.

Materials and Methods

Raw material

The fruits were purchased from Grande Sertão Cooperative, located in the city of Montes Claros, Mina Gerais, in January 2012 and were transported in styrofoam boxes by plane. Upon receipt, they were selected by the absence of defects, pests and diseases, had their surfaces washed with mild detergent to remove dirt, and were rinsed under running water. Sanitisation was then performed with 200 $mg.L^{-1}$ sodium hypochlorite for 20 min and then the fruits were manually cut in the diametral direction with stainless steel knives and the peel was separated from the pyrenes. The peel was then subjected to bleaching in water at 75°C for 6 minutes, and immediately immersed in iced water, vacuum packed and stored at -18°C until use.

For analysis, the peel was put into a forced-air oven at 55°C for 48 hours. Then, the sample was ground in an analytical mill cooled to 4°C (Quimis, model Q298A21, Brazil) with the aid of an ultra-thermostated bath (Solab, model SL-152/10) and then standardised using 60 grain size mesh (0.25 mm).

Chemical composition

The moisture determination was made by drying in an oven at 105°C to constant weight and the fixed mineral residue was incinerated in a muffle furnace at 550°C. Proteins were determined by the Kjeldahl method. Total lipids were obtained by extraction of the ethereal fraction in a Soxhlet apparatus. The total dietary fibre fraction was determined by the enzymatic gravimetric method (method 985.28). Carbohydrates were determined by difference (AOAC, 1998).

Determination of β-carotene

For the determination of β-carotene, the methodology described by Nagata and Yamashima (1992) was used, with adaptions. One gram of powdered pequi peel was weighed and placed in a test tube. Then, 10 mL of acetone-hexane (4:6 v/v) were added and the solution was homogenised for 5 minutes. Subsequently, the solution was filtered with filter paper into a 100 mL volumetric flask and completed to volume with acetone-hexane (4:6 v/v). Before conducting the reading in the spectrophotometer, the supernatant was filtered and the equipment was calibrated with acetone-hexane (4:6 v/v). Readings were taken at 453, 505 and 663 nm.

Afterwards, the calculation of the concentrations of β-carotene was performed according to equation 1 and the results were multiplied by 1000 to be expressed in μg/100mL:

$$\beta\text{-carotene (mg/100mL)} = 0.216A_{663} - 0.304A_{505} + 0.452A_{453}$$

Equation (1)

Obtaining extracts

To obtain the extracts, two previously-tested different temperatures, 50 and 70°C, were used and after preliminary testing to determine polyphenols , the temperature of 70°C was chosen in order to extract a higher polyphenol content. The extraction time was set at 20 minutes in accordance with the study by Viera (2012).

Extraction by shaking

The extraction by shaking was performed in the laboratory of the Department of Food Science and Technology at the Federal University of Santa Maria,

RS. The methodology used was as described by Lima (2008), with modifications. The extract was prepared from the previously-ground pequi peel, which was weighed (1 g) in a beaker, and 50 mL of solvent (aqueous or 80% hydroethanolic) was added. These mixtures were then taken to an ultra-thermostated bath (Solab, model SL-152/10) and subjected to constant shaking using a shaker (Marconi MA-039) for 20 min at 70°C. Subsequently, the extracts were filtered through filter paper and centrifuged at 3000 rpm for 20 minutes. The supernatants were stored in amber vials and stored in a freezer (-18°C) until analysis.

Extraction assisted by focused microwave radiation

The microwave-assisted extractions were conducted in the laboratories of the Industrial and Environmental Chemistry sector of the Department of Chemistry at the Federal University of Santa Maria, RS. For the extractions assisted by focused microwave, the procedures for sample digestion described by Costa, Korn and Castro (2006) were used, with modifications. The extractions were performed using a microwave oven with focused radiation, with two cavities equipped with glass jars of a capacity of 180 mL (Star System 2, 800 W, CEM, Matthews, N.C., USA).

Initially, the ground pequi peel was weighed (1 g) and transferred to glass vials; 50 mL of solvent (aqueous or 80% hydroethanolic) were added and subjected to microwave irradiation time of 20 minutes at 70ºC. In this system of irradiation using microwave there is no possibility of shaking the solution during the extraction process.

After the completion of extraction, the extracts were filtered through filter paper and centrifuged at 3000 rpm for 20 minutes. Subsequently, the supernatants were placed in amber vials and stored in a freezer (-18°C) until analysis. After each extraction, decontamination of the apparatus was performed with 10 mL of ethanol per 10 minutes.

Determination of polyphenols

For the determination of polyphenols, the Folin-Ciocalteau method, as described by Singleton, Orthofer and Lamuela- Raventos (1999) was used, with modifications. The extract was diluted in solvent (aqueous or 80% hydroethanolic) in a volumetric flask. Subsequently, an aliquot (0.2 mL) of the solution was mixed with 1 mL of 0.2N Folin-Ciocalteau reagent (diluted 1:10 v/v), followed by shaking and the addition of 0.8 mL of 7.5% (w/v) carbonate sodium solution (Na_2CO_3). After incubation at room temperature (25°C) for 2 hours in the dark, absorbance was measured at 765 nm on a spectrophotometer (Biospectro, model SP-220). The results of the concentration of polyphenols were expressed as gallic acid equivalents (mg GAE.Kg^{-1}), calculated by a calibration curve $Y = 0.0158\ x + 0.003$, $R^2 = 0.9901$ for the aqueous extract; and $Y = 0.0173\ x + 0.1558$, $R^2 = 0.9900$ for the 80 % hydroethanolic extract , where Y is the absorbance and x is the concentration, constructed with concentrations ranging from 0 to 70 mg.Kg^{-1}.

In vitro *antioxidant activity*

DPPH• radical method

The methodology used was as described by Brand-Williams, Cuvelier, and Berset (1995), with adaptations. This method is based on the sequestration capacity of the radical 2,2-diphenyl-1-picryl-hydrazyl (DPPH). The technique consisted of incubating, for thirty minutes, 2 mL of a hydroethanolic solution (80% v/v) of 0.1 mM DPPH with 2 mL of solutions containing different concentrations of each extract of pequi peel, and BHT standard at levels ranging from 0.1 to 2000 μg/mL.

The control solution consisted of 0.1 mM DPPH in the corresponding solvent (aqueous or 80% hydroethanolic) and white solution of only solvent. After incubation, readings of the samples were performed using a spectrophotometer (Biospectro, model SP-220) at a wavelength of 517 nm. The percentage of antioxidant activity (AA%) was calculated as the percentage of uptake of DPPH, according to equation 2:

$$AA\% = 100 - \{[(Absorbance.\ sample - Absorbance.\ white) \times 100] \div Absorbance.\ control\}$$

Equation (2)

After evaluating the range of optimal concentration, the concentration required to capture 50% of the DPPH free radical was calculated (IC_{50}).

β-carotene/linoleic acid system assay - formation of peroxides

The analyses were performed according to the method described by Miller (1971), with adaptations based on the oxidation (discolouration) of β -carotene in a particular emulsion. To form the emulsion, the following were added together: 20 μL of β-carotene solution dissolved in chloroform (20 mg/mL) 40 μL of linoleic acid, 530 mg of Tween 40 emulsifier, and 1 mL of chloroform to complete the emulsification. The emulsion was evaporated on a rotary evaporator at 40°C for 10 minutes to complete the evaporation

of the chloroform. After evaporation, 100 mL of previously- oxygenated (for 30 minutes) distilled water was added under constant shaking. The emulsified solution was clear, with absorbance in the range of optical density between 0.6 to 0.7 at 470 nm.

An amount of 0.2 mL of each extract of pequi peel, at concentrations of 20, 200 and 2000 ppm, was added to 5 mL of the emulsified solution. The reading was taken immediately at zero time using a spectrophotometer (Pró-Análise , model UV-1 100) and afterwards the tubes were placed in a water bath at 50°C and absorbance readings were performed every 15 minutes for 2 hours. The method above was also followed for the control but replacing the sample by the respective solvent (distilled water or 80% ethanol).

The results were expressed as a percentage of the inhibition of oxidation, which was calculated in relation to the decrease in absorbance of the control, according to equation 3:

$$\%I = [(Ac - Aam) \div Ac] \times 100 \quad \text{Equation (3)}$$

Where, Ac = initial absorbance – final absorbance (control) and Aam = initial absorbance – final absorbance (sample).

Statistical analysis

The analyses were conducted in triplicate. Results were expressed as mean ± standard deviation and subjected to analysis of variance (ANOVA). The means were compared using Tukey's test, with a significance level of 95% ($p<0.05$). The results were analysed using the SPSS programme, version 19.0.

Results and Discussion

Chemical composition and β-carotene

The results of the chemical composition of the pequi peel are shown in Table 1. It can be seen that the peel is important source of dietary fibre (6.52%) and 100 g of peel provides 30 kcal. The results show that 100 g of pequi peel can provide 1.5% of the daily calorific needs of an adult, based on a 2000 calorie diet, and 26% of dietary fibre needs, based on a recommendation of 25 g daily (Brasil, 2003). This demonstrates that the use of pequi peel may make it feasible to add nutritional value to other products, given that it would increase dietary fibre content without incurring large losses to energy, as the aim is to reduce calorific intake. Although pequi peel cannot be eaten in its fresh state, the results of this study suggest the importance of obtaining other uses for its consumption.

Table 1. Chemical composition of pequi peel (*Caryocar brasiliense* Camb.) in full base (g/100g of sample)

Constituents	Peel (g/100g of sample)
Moisture	85.79 ± 0.0046
Ash	0.36 ± 0.0004
Protein	0.59 ± 0.0203
Lipids	0.15 ± 0.0190
Dietary Fibre	6.52 ± 0.8093
Soluble Fibre	1.47 ± 0.1585
Insoluble Fibre	5.05 ± 0.8760
Non-fibrous carbohydrates	6.59
Total Energy (kcal/100g)	30.07

Values expressed as mean ± standard deviation, n = 3

Table 2. Polyphenol content expressed as gallic acid equivalent (g GAE.Kg^{-1} dm*) of extracts of pequi peel (*Caryocar brasiliense* Camb.)

Extracts	Polyphenols
Aqueous, focused microwave	$11.36^{d} \pm 4.45$
Aqueous, extraction by shaking	$72.18^{c} \pm 4.96$
80% hydroethanolic, focused microwave	$75.83^{b} \pm 0.82$
80% hydroethanolic, extraction by shaking	$78.58^{a} \pm 1.13$

Values expressed as mean ± standard deviation, n = 4. Different letters in the same column indicate significant difference ($p < 0.05$) by Tukey's test. *dm = dry mass.

Thus, the addition of dietary fibre into products may contribute to the development of fortified foods or functional foods, which are currently in high demand (Ramos; Oliveira, 2002; Sousa; Souza Neto; Maia, 2003). The β-carotene content in the pequi peel was 20.71 ± 1.65 µg/100g lower than found in the pulp in other research (Oliveira *et al.*, 2006;. Oliveira et al., 2008;. Gonçalves *et al.*, 2011). Pequi is characterised by large amounts of carotenoids, including β-carotene, which is present in small amounts. The importance of carotenoids goes beyond their role as a pigment; while some are precursors of vitamin A, others have antioxidant action and are considered to be functional foods (Bender, 2005).

Polyphenols

The results of the concentration of polyphenols of the aqueous and hydroethanolic extracts obtained by extraction assisted by focused microwave and shaking are shown in Table 2. The values found for the phenolic compounds in the extracts of pequi peel showed significant differences for both the type of solvent as well as for the extraction method used, with the 80% hydroethanolic extract obtained by shaking having the highest polyphenol content (78.58 g GAE/Kg).

The aqueous extract obtained by using focused microwave showed the lowest concentration of phenolics, probably because of the absence of shaking

Table 3. Maximum antioxidant activity and inhibitory concentration (IC_{50}) of the extracts of pequi peel (*Caryocar brasiliense* Camb.), and BHT, using the free radical DPPH

Extracts	Aa_{max}(%)	IC_{50} in µg/mL
Aqueous, focused microwave	$89.78^{b} \pm 0.40$	$69.16^{a} \pm 4.79$
Aqueous, extraction by shaking	$90.51^{b} \pm 1.37$	$51.02^{b} \pm 0.04$
80% hydroethanolic, focused microwave	$92.82^{ab} \pm 1.29$	$58.36^{b} \pm 0.37$
80% hydroethanolic, extraction by shaking	$93.90^{ab} \pm 1.10$	$37.96^{c} \pm 0.19$
BHT	$97.35^{a} \pm 1.81$	$14.69^{d} \pm 1.39$

Values expressed as mean ± standard deviation, n = 6. Different letters in the same column indicate significant difference ($p < 0.05$), by Tukey's test.

Table 4. Antioxidant activity of extracts of pequi peel (*Caryocar brasiliens*e Camb.) and of BHT by the β-carotene/linoleic acid method

Extracts	% of oxygen inhibition Concentration (ppm)		
	20	200	2000
Aqueous, focused microwave	$46.36^{c} \pm 1.56$	$65.34^{c} \pm 2.19$	$83.89^{b} \pm 0.94$
Aqueous, extraction by shaking	$77.70^{a} \pm 0.94$	$84.77^{b} \pm 2.19$	$97.13^{a} \pm 0.31$
80% hydroethanolic, focused microwave	$39.34^{d} \pm 2.05$	$44.51^{d} \pm 2.93$	$57.56^{c} \pm 2.05$
80% hydroethanolic, extraction by shaking	$53.21^{b} \pm 1.17$	$65.22^{c} \pm 0.59$	$78.67^{b} \pm 1.46$
BHT*	$38.30^{d} \pm 0.59$	$97.31^{a} \pm 0.88$	ne**

Values expressed as mean ± standard deviation, n = 4. Different letters in the same column indicate significant difference ($p < 0:05$) by Tukey's test.* The concentration of BHT used was 0.4 and 4 ppm for comparison with the different extracts of pequi peel. ** ne = not evaluated.

of the solution during the extraction process, since the equipment used does not allow such a process. Thus, the extraction process may be limited because the microwave irradiation is stopped when the solvent temperature reaches 70°C. For samples with small particle size and more fragile consistency, the lack of shaking may not have much influence; however, for samples of peel with high levels of fibre, such as pequi, the influence may be greater.

Comparing the extraction assisted by focused microwave with the method using shaking it is possible to see a significant difference between the concentration of polyphenols in both solvents; there was lower extraction for focused microwave extraction, which contradicts the results of other studies showing a more efficient extraction of phenolic compounds through the use of focused microwave radiation (Hayat *et al*., 2009; Ballard *et al.*, 2010.). Sun *et al.* (2007) and Vieira (2012) used extraction assisted by focused microwave and they observed an increase in phenolic content in asparagus and propolis. Thus, it can be inferred that for the pequi samples, a longer extraction, or the development of a system for shaking the solution during the extraction process may be necessary to increase the efficiency of the process.

Differences in the methodologies used to obtain extracts make it difficult to compare results. Research indicates that many factors can interfere in the content of polyphenols in the extracts, such as plant growth, soil, preparation of the plant for extraction, the extraction process, and the methodology used to identify these compounds (Madsen; Bertelsen, 1995). It is noteworthy that, for industrial purposes, the method of shaking is cheaper, it does not require skilled labour, and it is the method that is most common in academic research.

Roesler *et al.* (2007) determined the polyphenols in ethanolic and aqueous extracts of the peel, pulp and seed of fruits of the Brazilian cerrado, using the Folin-Ciocalteau method and they found values higher than those obtained in the present study for ethanolic and aqueous extracts of pequi peel, which were 209.37 and 208.42 g GAE.kg^{-1}, respectively. Bernardes *et al.* (2011) determined the polyphenol content in the peel and pulp of plums, oranges, kiwi fruit and apples and confirmed that the peel of all these fruits presented phenol contents significantly ($p < 0.05$) higher than for the pulp, except for apple, which did not present significant difference.

In vitro *antioxidant activity*

A common way of expressing the results of this method is to calculate the IC_{50}; the smaller the IC_{50} presented by the extract, the smaller the amount of it that would be required to reduce 50% of the DPPH radical, and the greater its antioxidant activity (Lima, 2008). As can be seen in Table 3, all the extracts showed activity in scavenging the DPPH free radical. The best result was for the 80% hydroethanolic extract by shaking, with IC_{50} equal to 37.96 mg/mL, but with lower BHT. Regarding antioxidant activity,

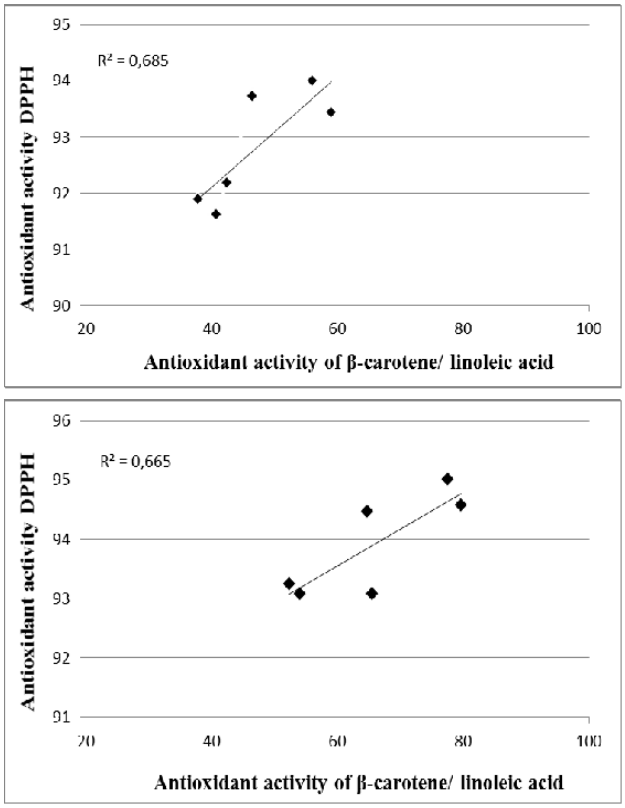

Figure 1. A: Correlation between the methodologies of antioxidant activity of β-carotene/ linoleic acid and of DPPH for the 80% hydroethanolic extract using focused microwave, for pequi peel (*Caryocar brasiliense* Camb.). B: Correlation between the methodologies of antioxidant activity of β-carotene/ linoleic acid and of DPPH for the 80% hydroethanolic extract with shaking, for pequi peel (*Caryocar brasiliense* Camb.)

the evaluated extracts showed values higher than 90% of scavenging free radicals, except for the aqueous extract that used focused microwave.

Roesler *et al.* (2007), evaluated the ethanolic and aqueous extract of pequi peel and found IC_{50} equal to 9.44 and 17.98 $\mu g.mL^{-1}$, respectively, concluding that the extracts had high antioxidant power. The relationship between the concentration of polyphenols and the ability to scavenge free radicals from the 80% hydroethanolic pequi peel extracts obtained by shaking appears to be quite significant, given that these extracts were those with the highest concentration of polyphenols and antioxidant activity.

The antioxidant activity and the BHT of the extracts of pequi peel analysed using the β-carotene/ linoleic acid method is shown in Table 4. It can be seen that the extracts showed activity in combating the peroxides that were formed; the aqueous extract that was shaken showed a higher activity ($p < 0.05$) than the others. It was also observed that antioxidant activity increased with increasing concentrations. The aqueous extract under shaking showed high antioxidant capacity (20 ppm = 77.70%, 200 ppm = 84.77% and 2000 ppm = 97.13%). These data are corroborated by several authors who have studied different food matrices in which the antioxidant activity of aqueous extracts were superior to other extracts (Vidal *et al*., 2006; Jardini; Mancini Filho, 2007). Melo *et al*. (2011) worked with 80% hydroethanolic and aqueous extracts using ultrasound in organic residues and they obtained antioxidant activity values by the method of auto-oxidation of the β-carotene/ linoleic acid system, which showed no significant difference between Verdejo white grape extracts and the ethanolic extract of Isabel red grapes.

Comparison and correlation of antioxidant activity

The correlation between the antioxidant activity of the 80% hydroethanolic extract obtained by focused microwave, with the 80% hydroethanolic extract obtained by shaking, between the methodologies of β-carotene/ linoleic acid and that of the assay of capture of DPPH radicals, was positive, with an R^2 of 0.685 and an R^2 of 0.665, respectively (Figures 1A and 1B). This demonstrates that, despite the differences in the action mechanisms of these methodologies, the extracts were moderately effective in capturing isolated radicals, as in the prevention of the oxidation of the linoleic acid. It is important to conduct different tests to assess antioxidant activity in order to obtain more precise answers about the interaction of the compounds present in samples with different radicals generated during the reaction (Robards *et al*., 1999).

Conclusion

Pequi peel is basically composed of water, carbohydrates and dietary fibre, which can be added to other products, making it feasible to use this waste to provide a healthier diet for individuals. The content of β-carotene in the peel was lower than that present in the pulp. The two methods used for extraction of phenolics were effective, but the conventional method by shaking extracted a higher phenolic content than the focused microwave method.

In general, in the evaluation of in vitro antioxidant activity of extracts of pequi peel, the 80% hydroethanolic extract using the shaking method showed better antioxidant activity through the DPPH method. Using the method of testing in the β-carotene/ linoleic acid system, the extract that showed the highest percentage of inhibition of oxidation, i.e. antioxidant activity, was the aqueous extract that was also subjected to shaking.

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