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Analytical Methods

Antioxidant activity of *Caryocar brasiliense* (pequi) and characterisation of components by electrospray ionization mass spectrometry

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Abstract

The *Caryocar brasiliense* known commonly as pequi is a tropical fruit of Brazilian Cerrado and is considered an important option of income and food for the populations living in this biome. Our previous study indicated that *C. brasiliense* had high total phenol content (209 g as gallic acid equivalent kg^{-1}) and excellent scavenging activity against 2,2-diphenyl-1-picrylhydrazyl radical (IC_{50} of 9.44 μg ml^{-1}). In this study, we evaluated the highly efficient antioxidant activity of *C. brasiliense* using the biological relevant method of chemically induced lipid peroxidation. The half inhibition concentration did not exceed 0.8 μg ml^{-1}. In addition, polar components of pequi ethanolic extract were investigated by direct infusion electrospray ionization mass spectrometry (ESI-MS). The technique revealed the presence of important bioactive components widely reported as potent antioxidants such as gallic acid, quinic acid, quercetin, and quercetin 3-*O*-arabinose possibly explaining its higher antioxidant activity. This is the first report on the composition by ESI-MS of pequi extract demonstrating excellent antioxidant activity.

Keywords: Pequi; Electrospray ionization mass spectrometry; Total phenols; Antioxidant activity; TBA

1. Introduction

Searching for an economically viable and environmentally friend application of Brazilian Cerrado natural resources is highly important and equally urgent in order to avoid the total destruction of natural vegetation and Cerrado biodiversity. The Cerrado, the second largest biome in South America after the Amazon rainforest, had not suffered human interference until 40 years ago. Nowadays, the Brazilian Cerrado biome is challenged by two different realities: the great possibility of food production, being considered one of the most important agriculture celery of the world and the extremely rich biodiversity (Proença, Oliveira, & Silva, 2000). It occupies almost 25% of Brazil and its very rich flora, which is just beginning to be studied, is estimated to be comprised of about 1000 species of trees, 3000 species of herbs and shrubs, and about 500 of climbers (Mendonça et al., 1998). In the last 30 years, progressive mechanization with improved techniques for clearing and fertilizing the soil have contributed to the accelerated destruction of the natural vegetation, and it is estimated that 40% of the Cerrado biome has already been deforested (Ratter, Ribeiro, & Bridgewater, 1997). Within the framework of our research on natural resources of Cerrado, investigations were conducted on to *Caryocar brasiliense* (pequi). The fruits of pequi (*C. brasiliense*) are an important option of income and food for the populations living in the Cerrado regions of Brazil. Its fruits are widely consumed "in natura" as well as to prepare juice, ice cream, liquor, jelly and specially the

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traditional dishes famous throughout Brazil. The pulp of the pequi has good quantity of edible oil, vitamin A and proteins. On the other side, the nut has also oil, which is applied in cosmetic products such as soaps and skin emulsions (Silva & Tassara, 2001). From the peel and leaf it is extracted a high quality yellow color that is used for dying fibers. Regarding the folk medicine, the pulp oil is traditionally used as tonic agent against asthma, influenza, cold and bronchopulmonary diseases (Almeida, Proença, Sano, & Ribeiro, 1994). There are very few scientific studies examining the relationship between the biological activities and the ethnobotanical uses by traditional herbal medicine in order to validate them. Recently, Roesler, Catharino, Malta, Eberlim, and Pastore (2007a) and Roesler, Malta, Carrasco, Barata, and Sousa (2007b) demonstrated the scavenging activity of pequi extracts against free radical DPPH. The antifungal activity against *Cryptococcus neoformans* of different parts of *C. brasiliense* had been investigated by Passos et al. (2002). Oliveira and Gilbert (1968) mentioned that ethanolic extracts form leaves could have antitumor properties. In recent years, growing efforts have been made in the fields pharmaceuticals and foodstuffs to study so called antioxidants, especially those of natural origin. Oxidation is a metabolic process that leads to energy production necessary for essential cell activities. However, metabolism of oxygen in living cells also leads to the unavoidable production of oxygen-derived free radicals, commonly known as reactive oxygen species (ROS) (Adegoke et al., 1998; Mccord, 1994). In the absence of adequate endogenous antioxidant defenses, the propagation of free radical-producing events can lead to the co-oxidation of nucleophilic cellular constituents and the reaction of secondary lipid autoxidation products with nucleophilic macromolecules such as membrane constituents, enzymes and DNA (Yuan & Kitts, 1996). Therefore the excess free radicals circulating in the body oxidize the low density lipoproteins (LDL), making them potentially lethal, the excess free radicals can also accelerate ageing processes and have been linked to other very serious pathologies, such as brain stroke, diabetes mellitus, rheumatoid arthritis, Parknson's disease, Alzheimer's disease and cancer (Ames, Gold, & Wilet, 1995; Ames, Shigenaga, & Hagen, 1993; Christen, 2000; Diaz, Frei, & Keaney, 1997; Esterbauer, Gebicki, Puhl, & Jurens, 1992; Esterbauer, Puhl, Dieber-Rothender, Waeg, & Rabl, 1991; Lang & Lozano, 1998). The importance of the antioxidants constituents of plant materials in the maintenance of health and protection against heart diseases and cancer is also raising interest among scientists, food manufacturers and consumers as the trend for the future is toward functional food with specific health effects (Loliger, 1991). Typical compounds that possess antioxidant activity include phenols, phenolic acids and their derivatives, flavonoids, tocols, phospholipids, amino acids and peptides, phytic acid, ascorbic acid, pigments, and sterols (Xing & White, 1996). Many phenols exert powerful antioxidant effects in vitro, inhibiting lipid peroxidation by acting as chain breaking peroxyl radicals scavengers. Phenols with two adjacent –OH groups, or other chelating structures, can also bind transition metal ions in forms poorly active in promoting free radical reacting. Phenols can also directly scavenge ROS, such as OH, ONOOH and HOCl (Halliwell & Gutteridge, 2000). Electrospray (ESI) is a soft and wide-ranging ionization technique that has revolutionized the way the molecules are ionized and transferred to mass spectrometers (MS) for mass and structural characterization (Fenn, Mann, Meng, Wong, & Whitehouse, 1989). ESI has therefore greatly expanded the applicability of mass spectrometry to a variety of new classes of molecules with thermal instability, high polarity and mass (Catharino et al., 2005). These unique features of direct infusion ESI-MS have recently been applied for fingerprinting of complex mixtures such as Cerrado fruit araticum (Roesler et al., 2007a, 2007b), bee propolis (Sawaya et al., 2004), beer (Araujo et al., 2005), wine (Catharino et al., 2006; Cooper & Marshall, 2001), whisky (Møller, Catharino, & Eberlin, 2005) and vegetable oil (Catharino et al., 2005; Wu, Rodgers, & Marshall, 2004).

The aim of the present work was to prepare different extracts from pequi fruits and evaluate the antioxidant activity of this fruit by using the in vitro model lipid peroxidation considering rat liver microsomes as an oxidative system. In addition, the present work explores the ability of the fast and versatile electrospray ionization mass spectrometry (ESI-MS) technique with direct infusion to characterize the extracts of pequi, which demonstrated high antioxidant activity.

2. Material and methods

2.1. Reagents and standards

Solvents and reagents were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA).

2.2. Plant material

Pequi fruits (*C. brasiliense*) were obtained from Erlow Farm, Km 07 Br 070 Goiânia Brazil, in the center west of Brazil. Fruit harvests were conducted twice and special care was taken to avoid damaged fruits. Fruits were transported to the University of Campinas (UNICAMP) and stored at 5 °C until used which was usually within 2 months.

2.3. Ethanolic extraction

Undamaged fruits were selected and peel, pulp and seeds were manually separated. About 100 g of each part of the fruit (pulp, peel and seeds) were cut in small parts, mixed and extracted twice with 300 mL of aqueous ethanol (5:95, v/v, water:ethanol) using a household mixer for 20 min. The extracts were filtered through cotton membrane and the residue was re-extracted under the same conditions. The residues were kept for further evaluations. The

resultant material was subjected to vacuum rotary evaporation at 40 °C to remove ethanol. The concentrated ethanolic extracts were lyophilized and stored at −18 °C in amber glass bottles until used.

2.4. Preparation of rat liver microsomes

Male wistar rats receiving normal diets and weighing 200–300 g were killed by decapitation after overnight starvation. Livers were removed and homogenized (1:2 w/v) in ice-cold phosphate buffer (100 mM, pH 7.4) containing 1 mM EDTA and 1.15% KCl. The homogenate was centrifuged (10,000*g*, 20 min, 4 °C), and the supernatant was collected and centrifuged (105,000*g*, 60 min, 4 °C). To be stored, the pellet was suspended (0.5 mg liver/mL) in ice-cold phosphate buffer (100 mM, pH 7.4) containing 0.1 mM EDTA and 20% glycerol (Omura & Sato, 1964). Microsomal protein concentrations were determined by the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951) with bovine serum albumin used as standard.

2.5. Antioxidant activity

Thiobarbituric acid reacts with malondialdehyde (MDA) to form a diadduct, a pink chromogen, which can be detected spectrophotometrically at 532 nm (Van der Sluis, Dekker, Verkerk, & Jongen, 2000). It was optimized to be able to use microtiter plates and ELISA reader, which makes it possible to analyze large numbers of samples in run. Microsomes were thawed on ice and were diluted with Tris–HCl buffer (50 mM, pH 7.4) containing 150 mM KCl to 1 mg/mL protein and centrifuged (100,000*g*, 60 min, 4 °C). The pellet was ressuspended with 1 ml of the Tris buffer and diluted to the concentration needed (final concentration 0.5 mg/mL protein unless otherwise stated) (Omura & Sato, 1964, Singh, Chidambar Murthy, & Jayaprakasha, 2002; Van der Sluis et al., 2000).

The microsomes (aliquots of 240 μL) were pre incubated in a 48-well plate for 5 min at 37 °C. Samples of 30 mL of different concentration of extracts dissolved in ethanol or blank (corresponding with the solvent ethanol) were added. Lipid peroxidation (LPO) was induced by adding 15 μL of ascorbic acid (4 mM) and 15 μL of $FeCl_3$ (0.2 mM). After incubation for 60 min at 37 °C the reaction was stopped by addition of 0.5 mL of 0.83% thiobarbituric acid dissolved in TCA–HCl (16.8% w/v trichloroacetic acid in 0.125 N HCl). LPO was assessed by measuring thiobarbituric acid reactive species (TBARS) after the plates were heated for 15 min at 80 °C and subsequent centrifugation (2500 rpm, 15 min). A 250 μL sample of each incubation was transferred to 96-well plates, and absorption was read at 540 nm (color) vs 620 nm (turbidity correction) by ELISA reader (Singh et al., 2002; Van der Sluis et al., 2000). Final concentration of test materials in the cuvettes were between 1.0 and 50.0 ug/mL.

2.6. Calculations

The percentage of inhibition produced by a sample at a given concentration can be calculated from the absorbance readings. The percentage of inhibition is expressed as the inhibition of lipid peroxidation of that sample compared to the lipid peroxidation in a blank

$$\% \text{ Inhibition} = ((A_{\text{blank}} - A_{\text{sample}})/A_{\text{blank}}) \times 100$$

where A_{blank} is the absorbance of the blank ($A_{540} - A_{620}$) and A_{sample} is the absorbance of the sample ($A_{540} - A_{620}$).

2.7. Electrospray ionization mass spectrometry

For fingerprinting ESI-MS analysis a hybrid high-resolution and high-accuracy (5 ppm) Micromass Q-TOF mass spectrometer (Micromass, Manchester, UK) was used. The general conditions were: source temperature of 100 °C, capillary voltage of 3.0 kV and cone voltage of 40 V. For measurements in the negative ion mode, ESI(−)-MS, 10.0 μL of concentrated NH_4OH were added to the sample mixture having a total volume of 1000 μl yielding 0.1% as final concentration. For measurements in the positive ion mode ESI(+)-MS, 10.0 μL of concentrated formic acid were added giving a final concentration of 0.1%. ESI-MS was preformed by direct infusion with a flow rate of 10 μL min^{-1} using a syringe pump (Harvard Apparatus). Mass spectra were acquired and accumulated over 60 s and spectra were scanned in the range between 50 and 1000 *m/z*. Structural analysis of single ions in the mass spectra from pulp, peel and seeds extracts was performed by ESI-MS/MS. The ion with the *m/z* of interest was selected and submitted to 15–45 eV collisions with argon in the collision quadrupole. The collision gas pressure was optimized to produce extensive fragmentation of the ion under investigation. The compounds were identified by comparison of their ESI-MS/MS fragmentation spectra with literature whenever possible. The equipment was calibrated with a solution of Phosphoric acid, permitting a resolution of less than 20 ppm. The compounds whose fragmentation spectra were not found in literature were tentatively identified based on their high-resolution mass.

3. Results and discussion

The pequi fruit is a very important income of the regional population and it has been used as food complement, for vegetable oil production (food and cosmetics applications) and also for tradition medicine. The pequi most consumed part is the pulp that has approximately 76% of oil (dry matter), 3% of proteins, 14% of fiber and 11% of other carbohydrates (Marx, Andrade, & Maia, 1997). On the other hand, the endocarp (seed) has 6.76% of carbohydrates, 1.02% of proteins and 10% de lipids (Silva, Silva, Junqueira, & Andrade, 1994). The pulp + seed represent just 25% of the fruit. Although the epicarp (peel) represents 75% of the fruit, there are few studies on this part probably

because it is a residue dismissed by regional population (Roesler et al., 2007a, 2007b).

3.1. Inhibition of lipid peroxidation

The rat liver microsomes was chosen as an oxidative system because it is close to the in vivo situation where both an aqueous phase and a lipid phase are present. The extent of lipid peroxidation after chemical induction by radical formation is monitored by the thiobarbituric acid (TBA) test. Fifty percent inhibition of lipid peroxidation of microsomes to TBARS by hydroxyl radicals, generated by $FeCl_3$ system, requires 0.78 μg mL^{-1} of peel ethanolic extract and 33.84 μg mL^{-1} of pulp + seed etanolic extract. For comparative purposes, the IC_{50} for gallic acid and quercetin were also measured. The IC_{50} achieved for gallic acid was 1.01 μg mL^{-1} or 5.94 μM and for quercetin 1.18 μg mL^{-1} or 3.5 μM. Van der Sluis et al. (2000) reported about the same order of antioxidant potency for catechin and quercetin using the same method. Thus pequi peel ethanolic extract was highly efficient antioxidant using the biological relevant model of iron chemically induced microsome peroxidation by scavenging peroxy radical. The IC_{50} results of the pequi lipid peroxidation inhibition potential are shown in Table 1. Regression analysis of lipid peroxidation inhibition results showed a high dependence of pequi extracts concentration as demonstrated in Figs. 1 and 2. The best adjustment correlation was obtained by using quadratic model ($R^2 = 0.99$ and 0.97 for pequi peel and seed + pulp, respectively). The excellent performance of pequi peel extract as natural antioxidant was previous achieved with the ability to scavenge stable radical DPPH (Roesler et al., 2007a, 2007b). As expected, the DPPH and TBA assays did not presented the same correlation tendency or absolute IC_{50} results mainly due to the different reaction medium conditions (hydrophilic and hydrophobic), different substrates (chemical radical and biological membranes) and different product monitored of each assay. Antioxidant activity and mechanisms are system-dependent and can vary with radical substrates targets, solvent, antioxidant phase localization, etc. Owing to the complexity of the oxidation–antioxidation processes, no single method is capable of providing a comprehensive picture of the antioxidant profile of a studied sample (Koleva, Beek, Linssen, & Evstatieva, 2002). Integrating results from multiple assays with different mechanisms is important to elucidate differences in reactivity between compounds, as well as changes in reaction rates and mechanisms with solvent, environment and antioxidant concentration. According to Schaich, Fisher, and King (1993), for example, curcumins scavenge radicals rapidly in lipids but when water is present metal complexation dominates. Pequi peel ethanolic extract had high antioxidant activity (smallest IC_{50} and highest slope value for the correlation between activity and extract concentration) whatever the oxidative substrates or assay chosen thus indicating the presence of compounds with potent antioxidant activity in pequi peel with so high activity as other known antioxidant plant products.

Table 1
Half-inhibition concentration (IC_{50}) of pequi extracts and its components against lipid peroxidation

	Inhibition of iron-induced rat liver microsomal lipid peroxidation			
	Experiments data		Kweon et al. (2001)	Van der Sluis et al. (2000)
	IC_{50} (μg mL^{-1})	IC50 (μMol)	IC_{50} (μMol)	IC_{50} (μMol)
Ethanolic extracts				
Caryocar brasilense peel	0.78 ± 0.1			
Caryocar brasilense (pulp + seed)	33.84 ± 4.2			
Quinic acid derivatives				
3-*O*-(3′-Methylcaffeoyl) quinic acid			29.8 ± 0.07	
5-*O*-Caffeoyl-4-methylquinic acid			19.2 ± 0.22	
3-*O*-Caffeoyl-1-methylquinic acid			14.6 ± 0.37	
Quercetin		3.5 ± 0.2		9.7 ± 1.9
Gallic acid		5.9 ± 0.3		

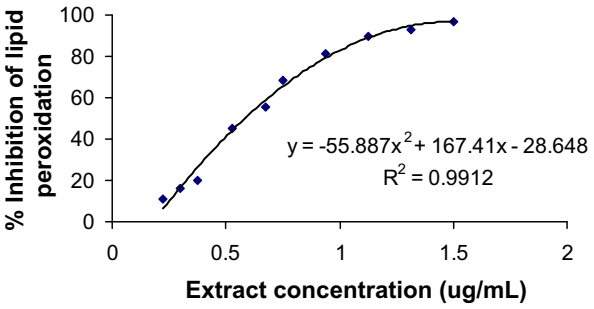

Fig. 1. Inhibition of lipid peroxidation by ethanolic pequi peel extract.

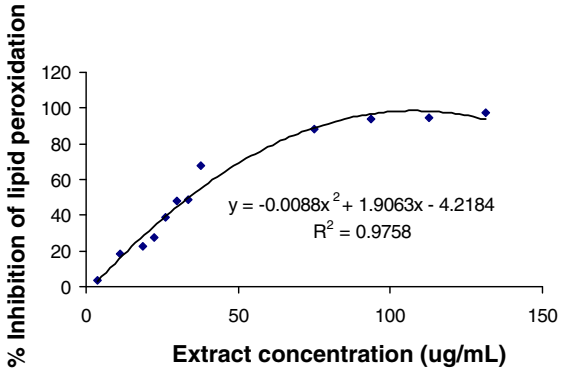

Fig. 2. Inhibition of lipid peroxidation by ethanolic pequi (pulp + seed) extract.

On the other hand, pequi seed + peel ethanolic extract demonstrated better potential to inhibit lipid peroxidation than to scavenge stable radical DPPH as shown in Tables 1 and 2. Considering that the lipid peroxidation substrate (microsomes) contains both an aqueous phase and a lipid phase and DPPH radical substrate is just hydrophilic, it may be possible that pequi seed + peel ethanolic extract is more efficient when lipid phase is present.

3.2. ESI-MS fingerprints

Electrospray ionization (ESI) mass spectrometry (MS) has recently become one of the most powerful analytical approaches for the study of polar, hydrophilic or thermolabile compounds and has therefore found widespread application (Gaskell, 1997; Yamashita & Fenn, 1984). The pequi extracts were analyzed by direct insertion ESI-MS both in the negative and positive ion modes. However, ESI(−)-MS fingerprints produce by far the most characteristic data; hence only ESI(−)-MS data will be presented and discussed. ESI(−)-MS provides a very sensitive and selective method for the identification of polar organic compounds with acidic sites, including therefore the major bioactive phenolic, organic acids and sugars components expected to be found in tropical fruits. De-protonated forms of the compounds of interest were then selected and dissociated and their ESI-MS/MS were compared to literature.

The investigation by direct infusion electrospray ionization mass spectrometry (ESI-MS) provided important information of biactive components of pequi peel ethanolic extract which are widely reported as potent antioxidants probably explaining the antioxidant activity of the extracts (Kim et al., 2006; Kweon, Hwang, & Sung, 2001; Roche, Dufour, Mora, & Dangles, 2005). The major phenolic components identified for pequi ethanolic peel extract by ESI-MS fingerprints were: *p*-hydro benzoic acid of *m/z* 137, gallic acid of *m/z* 169, quinic acid of *m/z* 191, quercetin of *m/z* 301 and quercetin 3-*O*-arabinose of *m/z* 433 (Fig. 3) by comparison from data found in the literature (Bastos et al., 2007; Moller, Catharino, & Eberlin, 2007). As reported in Table 2, the phenolic compounds found in pequi peel ethanolic extract (quercetin and gallic acid) were also tested and demonstrated high antioxidant activity in the same bioassay system. The results of half inhibition concentration IC_{50} against lipid peroxidation for gallic acid

Table 2
Compounds identified in pequi peel ethanolic extract using ESI(−)-MS/MS

Compound	$[M-H]^{-1}$ *m/z*	MS/MS ions *m/z*
ESI-MS ions (m/z)		
Gallic acid	169	125,79
Quinic acid	191	173, 127, 111, 93, 85
Quercetin	301	179, 151, 121, 107
Quercetin 3-*O*-arabinose	433	301

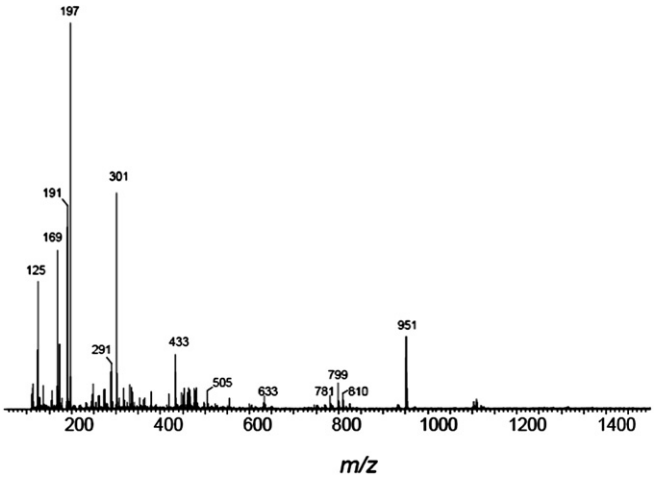

Fig. 3. ESI-MS fingerprints of pequi peel ethanolic extract.

was 1.01 μg mL^{-1} or 5.94 μM and for quercetin 1.18 μg mL^{-1} or 3.5 μM. According to Van der Sluis et al. (2000), the IC_{50} result of quercetin against lipid peroxidation was 9.7 μM. Considering the same bioassay system, Kweon et al. (2001) reported that the IC_{50} of quinic acid derivatives are in the range of 14.6–29.8 μM. Regarding DPPH assay system, the IC_{50} results for pequi compounds achieved by ESI-MS were previous reported by Roesler et al. (2007a, 2007b), Kim et al. (2006), Saleem, Kim, Jin, and Lee, (2004), Barrato et al. (2003), Van der Sluis et al. (2000). The correlation analysis between total phenol content of pequi different extracts and scavenging activity against DPPH radical showed a remarkable correlation ($R^2 = 0.9792$) which clearly demonstrates the a strong relation between these phenolic compounds and antioxidant activity.

The phenolic compounds act by scavenging free radicals and quenching the lipid peroxides. The hydroxyl and phenoxy groups of phenolic compounds donate their electron to the free radicals and neutralize them, forming phenolic radical and quinine methide intermediate, which is excreted via bile (Pan, Spencer, & Leary, 1999). An epidemiological study in the Netherlands (the Zutphen study) suggested an inverse relation of the incidence of coronary heart disease and stroke in elderly men with the dietary intake of flavonoids (especially quercetin) (Keli, 1996). In addition, lipid peroxidation may be prevented at the initiation stage by free radical scavengers, while the chain propagation reaction can be intercepted by peroxy-radical scavengers such as phenolic antioxidants (Takahama, 1983). Hence the phenolic compounds achieved in pequi peel ethanolic extract probably are the responsible for its antioxidant activity. In conclusion, pequi peel ethanolic extract showed excellent free radical scavenging activity and high potential to inhibit lipid peroxidation. It is likely that the rich composition of the pequi peel extract particularly the presence of phenolic components such as gallic acid, quinic acid, quercetin, and quercetin 3-*O*-arabinose possibly explain its higher antioxidant activity. Thus pequi peel may serve

important pharmaceutical, cosmetic or food complement roles, linked to its antioxidant behavior, as those related to prevention of diseases induced by oxidation stress. In addition, pequi peel can be considered a cost effective natural antioxidant source since pequi peel is actually considered a residue from pequi fruit applications. In vivo models are needed to evaluate the possibility of pequi extract as a natural source for the development of a dietary supplement due to its high antioxidant activity.

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