Effect of extraction conditions on total polyphenols content and in vitro antioxidant activity of Kat cucumer (*Parmentiera aculeata*)

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Abstract

Phenolic compounds are widely ubiquitous in nature. They are responsible for the proper functioning of plants and can be used to treat cardiovascular disorders and prevent some cancers. The present study focused on the Kat cucumber (Parmentiera aculeata), a fruit native to the state of Yucatan (Mexico) whose phenolic content and antioxidant activity had not been studied before. This work determined the amount of total phenols in *P. aculeata* using the Folin-Ciocalteu method, and the antioxidant activity using the free radical 2,2-diphenyl-1-picrylhydrazyl (3-ethylbenzothiazoline)-6 and 2,2'-azino-bis ammonium (DPPH) sulfonate (ABTS) methods. The extractions were performed under three different temperature conditions (20 °C, 50 °C and 70/50 °C), two acidic conditions and different volumes of solvent. The highest amount of total phenols (98.34 mg equivalent of gallic acid/100 g fruit weight) was obtained with an extraction temperature of 70/50 °C, a solvent volume of 40 ml and no acidification. The highest inhibition of antioxidant activity was obtained with the ABTS method, reaching 35.84 % of free radical scavenging.

Key words: Antioxidants, extraction, polyphenols, thermal processing

Introduction

Oxidation, a process that is constantly occurring within the human body, involves the transfer of electrons from one atom to another. However, when the transfer is only partial, one of the atoms is left with a single electron, generating what is known as a free radical, a very oxidative species that reacts rapidly with nearby cells, causing damage to them. An excess of oxidative species, due to internal and/or external sources, is known as oxidative stress, and it is associated with the appearance of many diseases such as arteriosclerosis, cancer, acute and chronic kidney failure, diabetes and hypertension, among others (Young and Woodside 2001). All aerobic organisms have defense mechanisms that allow damaged cells to be removed or repaired. Antioxidant compounds ingested with food contribute to these processes. There is a growing interest in the study of certain fruits and vegetables with antioxidant properties because their consumption can help prevent certain chronic diseases by reducing the imbalance that can be created in the organism between the oxidants that are produced by metabolism and the systems that exist to neutralize them. For this reason, it has been reported that increasing the amount of antioxidant substances in the body by consuming them in the diet can be a healthpromoting measure. Of course, the antioxidant capacity of different fruits and vegetables is influenced by different parameters such as plant variety, geographical area and even daylight hours. It is therefore necessary to evaluate local fruit varieties described as antioxidant sources in order to determine their true antioxidant potential.

Polyphenols are considered the most important antioxidant dietary compounds, containing hydroxyl functional groups on aromatic rings. They are a large family that comprises a very varied set of molecules, from the simplest ones (such as an aromatic ring with one or more hydroxyl groups), to the most complex (such as high molecular weight polymers). These compounds have not only been attributed antioxidant properties, but also antiinflammatory and antimicrobial activity (Chen et al. 2015), and they have been associated with the prevention of different chronic diseases related to oxidative stress (Barberan 2003).

The benefits provided by fruits and vegetables arise from certain components such as vitamins (A, C, E, and some folate). dietary fiber and non-essential phytochemicals. These phytochemicals include phenolic compounds of great importance due to their ability to neutralize free radicals and other biological effects that are still under study (Bravo 1998). It has been shown that the Vitamin C in apples accounts for only 0.4 % of their antioxidant capacity, suggesting that the complex blend of phytochemicals found in fruits and vegetables exert their beneficial health effects primarily through synergistic and additive effects. Polyphenols are the most abundant antioxidants in our diet; the average daily intake is around 1 g, 10 times higher than the intake of vitamin C, 100 times higher than the intake of vitamin E and 500 times higher than the intake of carotenes. Fruit polyphenols include a wide range of compounds with antioxidant activity such as hydroxycinnamates, gallic acid derivatives, flavones and anthocyanins. The composition of phenols varies widely among different fruits and vegetables, different types of soil, stages of maturity and even fertilization stages (Waterman and Mole 1994). The presence of different tissues in the same fruit can also be a source of variation (Eberhardt et al. 2000). One of the ways to assess the beneficial effect of a fruit is determining the amount of total phenols.

The extraction process is important for the isolation and identification of phenolic compounds. The extraction capacity depends on the solvent, the nature and preparation of the material to be extracted, the chemical structure of the phenolic compounds, temperature, extraction time, solid-liquid ratio, extraction method used and the presence of interfering substances (Bucić-Kojić et al. 2011). The amount of phenols is estimated using the Folin Ciocalteu method, which involves a reaction between the yellow Folin Ciocalteu reagent and the phenolic groups. This reaction yields a blue-colored complex with a maximum absorption at 725 nm. The results are usually expressed as mg equivalent of gallic acid/100 g of the sample.

The content of antioxidant compounds in fruits and vegetables and, therefore, their associated antioxidant capacity, can be affected by physiological factors such as ripening, and by technological factors such as storage and processing conditions (Helyes and Lugasi 2006). It is thus necessary to have adequate methods for determining the antioxidant capacity of these products and to evaluate the behavior of this capacity in the different post-harvest processes.

The total antioxidant capacity of a sample depends on the synergistic interactions between different compounds, and on the specific mode of action of each of them. Thus, it is necessary to combine several methods to correctly evaluate the antioxidant capacity of a sample.

Regarding extraction, it is necessary to combine at least two solvent mixtures with different polarities to facilitate the extraction of all antioxidant compounds. Compared to the use of a single solvent, the successive use of methanol/water (50:50, v/v) at pH 2, and acetone/water (70:30, v/v) has yielded the best results in the extraction of antioxidants from various plant products.

In recent years, several methods have been developed to evaluate the antioxidant capacity of food products based on different factors, such as metal reduction (FRAP), peroxyl radical scavenging capacity (ORAC, TRAP), hydroxyl radical scavenging capacity (deoxyriribose test), the scavenging capacity of radicals derived from certain organic molecules (ABTS, DPPH), the quantification of products generated during lipid peroxidation (TBARs, oxidation of LDLs), etc (Sanchez 2002, Aruoma 2003). Among the fastest, most operationally simple and most reproducible methods are the DPPH and ABTS methods. The ABTS method consists of the generation of an ABTS⁺ radical by the reaction between ABTS and potassium persulfate, which produces a greenish-blue chromophore with maximum absorption at wavelengths of 415, 645, 734 and 815 nm. The presence of antioxidants is associated with a decrease in the absorbance of the ABTS⁺⁺ radical. The results are usually expressed as umol of Trolox/g material analyzed. This method can be used with a wide range of pH values and is used for both aqueous and organic systems. The 1,1-diphenyl-2-picrylhydrazyl (DPPH) molecule is known as a stable free radical due to the delocalization of an unpaired electron over the whole molecule; this prevents the dimerization of the molecule, in contrast to freest radicals. The delocalization of the electron also intensifies the typical deep violet color of the radical, which it absorbs in methanol at 517 nm. When the DPPH solution reacts with the antioxidant substrate, which donates a hydrogen atom, the violet color fades. The change of color is monitored spectrophotometrically and is

used to determine the parameters associated with antioxidant properties.

The Kat cucumber is the fruit of a tree named Parmentiera aculeata (Kunth) Seem that belongs to the Bignoniaceae family. It is commonly known as cuajilote, guajilote, cuachilote or tree cucumber. The fruit is a linearoblong, yellowish-green berry, up to 20 cm long and 5 cm in diameter, slightly curved, with a wrinkled surface and numerous longitudinal grooves (Fig. 1). The nectary ring, persistent and accrescent, is located at the base of the fruit. It has a firm and fleshy shell and a fibrous, succulent and bittersweet pulp embedded with numerous seeds (Niembro et al. 2010). Its external color (shell color) can be green, dark yellow or light brown when the fruit is ripe. The pulp has a yellow pastel cream color when ripe (Paredes et al. 2007). The fruit is used as fodder for livestock. The aqueous extract from the flowers, the fruit, the bark and roots of this plant are used in traditional medicine as a remedy to cure kidney stones due to their diuretic properties, and to treat symptoms such as asthma and cough (Niembro, et al. 2010). This fruit is widely distributed in Mexico, from southern Tamaulipas and San Luis Potosí to the Yucatan Peninsula on the Gulf side, and from the center of Sinaloa to Chiapas on the Pacific side. In Yucatan, this fruit is produced in backyards and its consumption is linked to local culinary traditions. There is little or no information about its bioactive components (especially phenols) and about its antioxidant potential. Given the current consumption trends focusing on healthy foods, more information about the nutritional properties of this fruit would allow to consider its potential contribution to a healthy diet. Thus, the objective of the present study was to extract and quantify the amount of polyphenols in the fruit of the Kat cucumber, and to determine its antioxidant capacity.



Figure 1: Kat cucumber (Parmentiera aculeata (Kunth) Seem)

Materials and methods

Raw materials

Kat cucumber fruits were collected at a local orchard in the municipality of Baca, in the state of Yucatan (Mexico). The fruits were selected according to their ripeness, color, size, and absence of lesions and insects.

Preparation of samples

The collected fruits were washed with drinking water and left to dry. Both ends of the fruit were trimmed and the rest was cut into equal parts. When cut, the fruit darkened immediately. There are different techniques to prevent the oxidation processes that cause the darkening of the tissues. In the present study, a polyvinylpyrrolidone solution (0.5 %) was used to prevent or reduce oxidative stress and to prevent triggering the series of events that lead to fruit oxidation. This solution was used during the homogenization of the pulp (Azofeifa 2009).

Assessment of physico-chemical characteristics.

Titratable acidity

This characteristic was evaluated according to the Official methods of analysis (AOAC 2000), titrating an aliquot of the pulp with a NaOH 0.1 N solution, using phenolphthalein as an indicator. The analysis was performed in triplicate. The results are expressed as the percentage of citric acid present in the sample.

Total soluble solids (TSS)

The TSS value (degree Brix or °Brix) was determined by manually extracting juice from the cucumber, taking a drop and carrying out measurements using an Abbe-type manual refractometer, with three repetitions for each measurement on three different Kat cucumbers chosen at random.

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To quantify the content of ascorbic acid, an aliquot of the sample was titrated with a 2,6-dichloroindophenol solution until the pink color persisted for more than 5 seconds. The 2,6-dichloroindiphenol solution was standardized with an ascorbic acid solution. The results are expressed as mg of ascorbic acid/100 g of fruit weight.

Extraction of polyphenols

The extraction procedure was performed using two successive treatments of methanol/water (50:50, v/v) followed by acetone/water (70:30, v/v), and methanol/water (50:50, v/v) acidified to pH 2 followed by acetone/water (70:30, v/v) (Restrepo et al. 2009).

Five grams of previously homogenized sample were weighed; 10 ml of methanol/water (50:50, v/v) were added and the mixture was stirred for 1 h in the dark. After this time, it was centrifuged for 10 minutes at 4500 rpm. The supernatant was stored in the dark at 4 °C and the residue was subjected to a second extraction, stirring again for 1 h with 10 ml of acetone/water (70:30, v/v), following the procedure described above. The obtained supernatants were mixed and stored under refrigeration and in the dark until further use.

In addition to the volume of 10 ml of extraction solution, other volumes of the initial extraction solution (methanol/water; 50:50, v/v) were evaluated: 20 ml, 30 ml, 40 ml and 50 ml, with a second extraction using 20 ml, 30 ml, 40 mL and 50 mL of acetone/water (70:30, v/v), respectively. The same volumes of methanol/water (50:50, v/v) were also evaluated adjusting the solution to pH 2 with 0.1N hydrochloric acid, with a second extraction using the aforementioned volumes of acetone/water (70:30 v/v). The content of phenols in the supernatants was determined directly from aliquots of them (Jimenez et al. 2001).

Determination of the appropriate extraction temperature

Different extraction temperatures were evaluated. Five grams of sample were weighed and extractions were carried out as mentioned above, using methanol/water (50:50, v/v) at 20 °C and acetone/water (70:30, v/v) at 20 °C; methanol/water (50:50, v/v) at 50 °C and acetone/water (70:30, v/v) at 50 °C; methanol/water (50:50, v/v) at 70 °C and acetone/water (70:30. v/v) at 50 °C. The same procedure, along with the described temperature conditions, was performed using methanol/water (50:50, v/v) acidified to pH 2.

Total polyphenol content

The concentration of total phenols was measured using the Folin-Ciocalteu colorimetric method (Singleton and Rossi 1965). The quantification was carried out by plotting a calibration curve of gallic acid at concentrations of 0.05-0.5 mg/ml. The results are expressed as equivalent milligrams of gallic acid (mg EAG/100 g of fruit weight).

Determination of antioxidant capacity

ABTS method (2,2'-azino-bis (3-ethylbenzothiazoline)-6 ammonium sulfonate)

This method evaluates the ability to trap radicals present in the medium. The blue-green cationic radical ABTS⁺⁺, a stable compound that is soluble in methanol, is generated by the interaction of ABTS with potassium persulfate. The method consists in evaluating the antioxidant capacity of the sample as a function of its ability to reduce the concentration of the ABTS⁺⁺ radical (Re et al. 1999). In the present study, the absorbance of 2.97 ml of cationic radical, mixed with 0.03 ml of sample, was measured at 734 nm. The results are expressed as percentage of inhibition.

Where:

% of inhibition = (initial absorbance - final absorbance)/initial absorbance) * 100

DPPH method (2,2-diphenyl-1-picrilhydracil)

This work used the technique described by Brand-Williams et al. (1995), which consists in measuring the absorbance of the DPPH[•] radical (0.1 mM) dissolved in methanol (80 %) at 517 nm. When the DPPH solution is mixed with a substance that can donate a hydrogen atom (antioxidant), it reduces the DPPH[•] radical, causing it to loss its purple color, a reaction that is stoichiometric with respect to the number of electrons captured by the radical, which turns from purple to pale yellow (Molyneux 2004). For this purpose, 3.9 ml of the DPPH[•] radical was mixed with 0.1 ml of sample, allowing the mixture to stand for half an hour and then measuring absorbance. The results are reported as percentage (%) of inhibition.

Statistical analysis

The experiments were carried out in triplicate. The average and standard deviation values of the response variables were determined. The experiments were carried out according to a completely randomized design. An analysis of variance (ANOVA) was performed and Fisher's least significant difference method (LSD) was applied at a significance level of 5 %. All statistical analyzes were carried out using the Statgraphics Centurion XVI program.

Results and discussion

Physicochemical characteristics

°Brix values were 7.96 % \pm 0.956, indicating that the sweetness of the fruit is low. The titratable acidity, expressed as the percentage of citric acid was 0.26 % \pm 0.0095, while the maturity index (IM) was 30.6. The results obtained are similar to those reported by Paredes et al.

(2007) (°Brix 8 %, acidity 0.25, IM 27), who mention that these conditions indicate that the fruit is organoleptically suitable for consumption.

of vitamin C in the Kat cucumber, although relatively low, gives this fruit some antioxidant properties. The vitamin C content was 9.14 ± 0.343 mg of ascorbic

acid/100 g of fruit, higher than the values found in green apple (6 mg), banana (3.1 mg) and coconut (3.1 mg)

Quantification of polyphenols

Table 1: Polyphenol content for different volumes and extraction temperatures
Total phenols (mg EAG/100g)

Volume (ml)	T: 20°C	T: 50°C	T: 70°C and 50°C	
10	27.25 ± 2.72ª	35.58 ± 0.381ª	73.96 ± 0.108 ^a	
20	30.98 ± 1.742^{ab}	48.95 ± 0.326 ^b	78.68 ± 6.751 ^a	
30	32.75 ± 1.306 ^{ab}	50.72 ± 1.306 ^b	89.60 ± 1.306 ^a	
40	35.83 ± 0.435 ^b	55.87 ± 0.871 ^b	98.34 ± 3.266 ^{ab}	
50	11.85 ± 2.994°	38.25 ± 5.444ª	57.04 ± 8.983°	
Different letters in the energy achieves indicate simultiment differences $(n < 0.05)$				

Different letters in the same column indicate significant differences ($p \le 0.05$).

Table 1 shows the total content of phenols obtained with different volumes and extraction temperatures. The results indicate that the concentration of phenols increases as the volume of solution increases up to 40 ml; however, when the volume reaches 50 ml, any more increases in the solution volume cause a significant decrease in the concentration of phenols for the three different temperatures. The highest content of polyphenols was obtained with a volume of 40 ml of methanol/water solution (50:50, v/v) with a second extraction using acetone:water (70:30, v/v). Although in all cases the highest concentration of total phenols was associated with a solution volume of 40 ml, the analysis of means showed that there were no significant differences (p≤0.05) between the phenol concentration values found with 20, 30 and 40 ml, but the results obtained with these volumes were significantly different than those obtained with volumes of 10 and 50 ml. The plot of means (Fig. 2) show that the highest content of polyphenols was obtained by performing the first extraction at 70 °C and the second extraction at 50 °C.

(Shrivas et al. 2005). This result indicates that the content



Figure 2: Analysis of means of the concentration of polyphenols extracted from Kat cucumber at different temperatures

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Volume (ml)	T: 20 °C	T: 50 °C	T: 70 °C and 50 °C
10	38.74 ± 6.26 ^a	34.44 ± 1,742 ^a	34.59 ± 0.762 ^a
20	37.19 ± 0.54 ª	42.41 ± 1,415 ^a	45.57 ± 0.435 ^b
30	31.28 ± 2,123 ^a	56.44 ± 0.490 ^b	46.96 ± 2,123 ^b
40	28.64 ± 8.058 ^a	48.90 ± 3.484 ^{ab}	54.34 ± 0.653 °
50	11.85 ± 2.994 ^b	28.45 ± 5.989 ^a	27.64 ± 2,450 ^d
Dif	ferent letters in the same column	indicate significant differences	s (p≤0.05)

The results of Table 2 show that the concentration of polyphenols extracted at 20 °C was significantly lower than the concentrations found at temperatures of 50 °C and 70/50 °C. The results also indicate that the acidification of the solvent to pH2 was did not improve the extraction of polyphenols from Kat cucumber samples. The greatest concentration of polyphenols was obtained when the extractions were carried out at a temperature of 50 °C and a solvent volume of 30 ml. The plot of means (Fig. 3) shows no significant differences ($p \le 0.05$) between the concentration of polyphenols at 50 °C and at 70/50 °C.

Means and 95% Fisher LSD



Figure 3: Analysis of means of the concentration of polyphenols extracted from Kat cucumber at different temperatures, with acidification of the solvent system

The highest concentrations of polyphenols were obtained when the extraction was performed without acidification of the solvent system (Fig. 4). This may be because the acidic environment favors the extraction of free anthocyanins over other phenolic compounds, which could indicate that the Kat cucumber does not contain anthocyanins.

Means and 95% Fisher LSD



Figure 4: Plot of means of the extraction conditions, with and without acidification of the solvent system

Extracting polyphenols with solvents is the most widely used procedure for the recovery of phenolic compounds from plant material. The extraction process is important for the isolation and identification of phenolic compounds. The extraction capacity depends on the solvent, the nature and preparation of the material to be extracted, the chemical structure of the phenolic compounds, temperature, extraction time, solid-liquid ratio, extraction method used and possible presence of interfering substances (Bucić-Kojić et al. 2011).

The molecular chemical properties of each solvent have different effects on extraction selectivity. Water and

methanol are polar solvents in which different polar and ionic solutes are soluble, including proteins, carbohydrates and mineral salts. These solvents increase the solubility of organic material, which has less polarity. In contrast, dipolar solvents, such as acetone, have a relatively intermediate polarity and solubilize solutes with similar relative polarity. The addition of a certain amount of water to methanol, as well as to acetone, increases the dielectric constant of the solvent mixture, improving its extraction capacity (Beltrán et al. 2013). It should be noted that the solubility in water and alcohol of phenolic compounds is generally higher in the case of diphenolic and polyphenolic compounds, which means that these types of compounds may be present in the Kat cucumber.

The use of high temperatures and polar solvents for the aqueous extraction of phenolic compounds helps obtain a higher concentration of total phenols, mainly due to the number of -OH groups present in phenolic compounds, since the presence of these groups increases the solubility in water of phenolic compounds and facilitates their extraction (Vermerris and Nicholson 2008). It has been reported that phenols, having more than one -OH group, are more soluble in water. High temperatures also play a very important role in the extraction of phenolic compounds because they lead to an increase in the diffusion and solubility coefficients of phenolic compounds by promoting the release of polyphenols through the breakdown of certain cellular structures (AI-Farsi and Lee 2008). This occurs because thermal energy increases molecular vibration and therefore the division and separation of compounds, which favors the breakdown of intermolecular forces and some molecular bonds (Duque and Morales 2005). Furthermore, an increase in temperature can contribute to the activation of enzymes that participate in the decomposition of complex compounds that form hydrogen bonds with phenolic compounds, facilitating the extraction of the latter (Muñoz et al. 2015).

The extraction time and temperature are important parameters that should be optimized to minimize the energy cost of the extraction process, taking into account that the phenolic compounds of interest may start to degrade at certain temperatures.

Free radical inhibition percentage

The antioxidant properties of the Kat cucumber were determined using the DPPH and ABTS methods. In vitro

antioxidant assays usually rely on a free radical scavenger and are relatively simple to perform. The DPPH method is faster, simpler and cheaper than other free radical scavenging assays (Hseu et al. 2008), while the ABTS discoloration assay is suitable for use with hydrophilic and lipophilic antioxidants (Alam et al. 2012).

The Kat cucumber showed antioxidant capacity with both the ABTS and DPPH methods. The results of both methods showed a good correlation, with a correlation coefficient (r) = 0.9073, which indicates that both these methods can be used to determine the antioxidant capacity of the fruits. Dudonné et al. (2009) reported a strongly positive correlation between ABTS and DPPH results (r = 0.906) when studying aqueous plant extracts.

Phenolic components generally show significant scavenging activity against the DPPH radical (Sekeroglu et al. 2012), which is why the antioxidant activity of the Kat cucumber is evaluated using the DPPH method, which, together with ABTS, is one of the most widely used assays for the evaluation of antioxidant activity. Floegel et al. (2011) compared both methods in the evaluation of antioxidant activity results of both. In the present study, the ABTS assay yielded a higher value of radical scavenging activity than DPPH (35.84 % and 31.05 % respectively), which suggests that polyphenols make an important contribution to the antioxidant capacity of the Kat cucumber.

Table 3 shows that the highest radical scavenging capacity (35.84 %) of the Kat cucumber was obtained with the ABTS method; it was a higher value than the one obtained for green prickly pear with the DPPH method (34.20 %) (Repo and Encina 2008).

Table 3: Percentage of free radical scavenging in the Kat cucumber according to ABTS and DPPH

Method	% of free radical scavenging		
DPPH	31.05 ± 0. 689		
ABTS	35.84 ± 0.943		

In the present study, the DPPH method indicates a lower percentage of free radical scavenging compared to the ABTS method. This is explained by the low selectivity of the ABTS method, which reacts to any aromatic hydroxy compound, independently of its actual antioxidant potential (Roginsky and Lissi 2005). Thus, it should be kept in mind that the DPPH method is more selective than the ABTS method and, unlike it, does not react to flavonoids with no hydroxyl groups in the B-ring or to aromatic acids containing a single hydroxyl group (Roginsky and Lissi, 2005). This is why there is so much variation in antioxidant capacity measurements. Other studies use different quantities of reagents and sample material, and the handling of the samples and the measurement of their properties vary too. This makes it difficult to compare the results obtained by different studies.

Conclusions

The results of the present study show that the highest concentration of polyphenols (98.34 \pm 3.266 mg EAG/100 g) is obtained by performing the extraction using a mixture of methanol:water (50:50) followed by a second extraction with acetone:water (70:30) at high temperatures (70 and 50 °C, respectively) without acidification of the solution to pH 2. The results show that the Kat cucumber has bioactive compounds, such as vitamin C and polyphenols, and inhibits free radicals. These properties allow to recommend the consumption of the Kat cucumber as part of a healthy diet

The biological activity of polyphenols is related to their antioxidant character, which is explained by their ability to chelate metals, inhibit the activity of the enzyme lipoxygenase and act as free radical scavengers. Various international organizations recommend а daily consumption of at least five servings of fruit or vegetables to ensure an adequate intake of antioxidants in order to prevent diseases associated with oxidative stress. According to Vasco et al. (2008) and Zapata et al. (2014), fruits can be classified into three categories according to their phenolic content: low (<100mg EAG/100 g), medium (100-500 mg EAG/100 g) and high (>500 mg EAG/100 g). According to this classification, the Kat cucumber has a low phenolic content, lower than papaya (134.1 mg EAG/100 g), but higher than banana (84.8 mg EAG/100 g), passion fruit (39.1 mg EAG/100 g), peach (30.5 mg EAG/100 g) and prickly pear (52 mg EAG/100 g).

Further studies are required on the composition, properties and compounds of this fruit to fully determine the benefits of its use in the food and/or pharmaceutical industries.

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