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Antioxidant activity of fifteen seeds from fruit processing residues by different methods

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ABSTRACT. This study identified and quantified five phenolic compounds, and evaluated the antioxidant capacity *in vitro* of fifteen native and exotic Brazilian fruit seeds that are typically discarded as waste. The contents of phenolic compounds were determined by ultra-performance liquid chromatography coupled with tandem mass spectrometry, and the antioxidant capacity was determined by oxygen radical absorbance capacity (ORAC) and 2,2-diphenyl-1-picrylhydrazyl (DPPH•) antioxidant assays. The results showed the antioxidant activity of *Campomanesia pubescens, Hovenia dulcis Thunberg* and *Syzygium jambos (L.) Alston* in the ORAC assay, and *Hymenaea stigonocarpa, Hovenia dulcis Thunberg* and *Campomanesia pubescens* in the DPPH• assay. Among the fifteen samples, four were highlighted regarding phenolic compound analyzes: *Hovenia dulcis Thunberg* (5.723 μg g^{-1}) for gallic acid and myricetin (111.057 μg g^{-1}), *Passiflora edulis* (1.208 μg g^{-1}) for chlorogenic acid, *Annona atemoya* (1.0580 μg g^{-1}) for vanillic acid, and *Campomanesia pubescens* (0.420 μg g^{-1}) for ferulic acid. Therefore, these fruit seeds can be used as alternative sources of natural antioxidants.

Keywords: gallic acid; ferulic acid; myricetin; UPLC-MS/MS; DPPH•; ORAC.

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Introduction

Fruit cultivation is one of the most prominent sectors in the Brazilian agribusiness. Brazil is the third largest producer of fruits worldwide, at 38.36 m tons, after China and India and has an arable area of 2.2 m hectares distributed throughout the country (Food and Agriculture Organization of the United Nations [FAO], 2015). The fruit processing sector of Brazil consumed an estimated 23.8 m tons in 2013 (Reetz, 2015).

Among the residues generated from fruit processing, the seeds are usually discarded. However, they contain significant amounts of compounds with antioxidant capacity, such as phenolic compounds. Thus, quantitative and qualitative identification of these antioxidants can enable the use of this residue and decrease food industry wastes (O'Shea, Arendt, & Gallagher, 2012; Bataglion, Silva, Eberlin, & Koolen, 2015).

Phenolic compounds are antioxidants that act primarily as free radical terminators, reacting with high-energy particles, known as free radicals and turning them into thermodynamically more stable compounds, by donating hydrogen atoms or electrons or chelating metal cations. These characteristics of free radical neutralization, act during the initial and propagative phases of free radical-induced oxidation (Shahidi & Naczk, 2003; Tlili et al., 2015).

Phenolic compounds possess an aromatic ring bearing one or more hydroxyl groups and their structures may range from simple phenolic molecules to highly polymerized structures. Several classes of phenolic compounds have been identified. Approximately two-thirds of the antioxidant compounds found in fruit belong to the flavonoid class. The remaining one-third is composed of phenolic acids; other existent antioxidants are less prominent but all are derived from the secondary metabolism of plants (Liu, 2004).

The flavonoids can be divided into flavonols (quercetin, kaempferol, and myricetin), flavones (luteolin and apigenin), flavanols (catechin, epicatechin, epigallocatechin, epicatechingallate, and epigallocatechingallate), flavanones (naringenin), anthocyanidins, and isoflavonoids (genistein). The phenolic acids can be divided into two categories, the ones derived from benzoic acids (hydroxybenzoic acids) and those derived from cinnamic acids (hydroxycinnamic acids) (Liu, 2004).

The consumption of fruits is an effective strategy to increase the ingestion of antioxidants and decrease oxidative stress, which can lead to a decrease in the development of chronic diseases, such as cancer and cardiovascular diseases (Song et al., 2010).

This study aimed to evaluate their *in vitro* antioxidant activity using the 2,2-diphenyl-1-picrylhydrazyl (DPPH•) and oxygen radical absorbance capacity (ORAC) assays and determine five phenolic compounds in seeds of 15 native and exotic fruits by ultra-performance liquid chromatography-electrospray ionization tandem mass spectrometry (UPLC-ESI-MS/MS)

Material and methods

Chemicals and materials

Gallic, Chlorogenic and Ferulic acids, Myricetin, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 2,2-diphenyl-1-picrylhydrazyl (DPPH•), 2,2-azobis-2-methyl-propanimidamide dihydrochloride (AAPH) and HPLC-grade methanol were supplied by Sigma–Aldrich® (state of São Paulo, Brazil). Vanillic acid and fluorescein were obtained from Fluka® (state of São Paulo, Brazil). Formic acid was obtained from Tedia® (state of Rio de Janeiro, Brazil). Ultrapure water was obtained from a Milli-Q ultrapure water purification system (Millipore, Bedford, MA, USA).

Samples

Fifteen types of fruit (Table 1) were obtained from Maringá (23º 25' S, 51º 57' W), Paraná, Brazil. The seeds were manually separated from the peels and pulps and then dried at ambient pressure and temperature (25°C) for 4 hours. After, they were triturated, then passed through a sieve (80 mm) and subsequently stored under vacuum at -18°C.

Extraction procedure

The extraction was performed according to Santos et al. (2011). Two grams of each seed powder was combined with methanol (1:10, w v^{-1}) by magnetic stirring at 25°C for 2 hours. Subsequently, the extracts were centrifuged at 4000 rpm (Sanyo, Harrier 18/80) for 10 min and the solvent was evaporated at 35ºC in a rotary evaporator under vacuum.

DPPH• assay

An aliquot (25 µL) of the sample extract was added to 2 mL of a DPPH• solution (6.25×10^{-5} mol L^{-1} in methanol). The solution was kept in the dark at room temperature for 30 min and then the absorbance was measured at 517 nm. Methanolic solutions of Trolox, with concentrations ranging from 100 to 1500 µmol mL^{-1}, were used to construct a calibration curve. The results were expressed as Trolox equivalents per gram of sample (µmol TE g^{-1}), as described by (Brand-Williams, Cuvelier, & Berset, 1995) with modifications by Ma et al. (2011).

Table 1. List of fifteen seeds of fruits.

	Popular name*	Scientific name	Origin
1	Abiu	*Lucuma caimito*	Native
2	Abricó	*Dovyalis abyssinica Warb*	Exotic
3	Atemóia	*Annona atemoya*	Exotic
4	Cereja	*Eugenia involucrata*	Native
5	Coquinho	*Syagrus romanzoffiana*	Native
6	Gabiroba	*Campomanesia pubescens*	Native
7	Jambo amarelo	*Syzygium jambos (L.) Alston*	Exotic
8	Jambolão	*Syzygium cumini L.*	Exotic
9	Jatobá-do-Cerrado	*Hymenaea stigonocarpa*	Native
10	Maracujá	*Passiflora edulis*	Exotic
11	Papaia	*Carica papaya*	Exotic
12	Pitanga	*Eugenia calycina*	Native
13	Sete capotes	*Campomanesia guazumifolia*	Native
14	Uva-Japão	*Hovenia dulcis Thunberg*	Exotic
15	Uvaia	*Eugenia pyriformis Cambess.*	Native

*Popular name in Brazil. **2020 Brazilian flora in construction.

ORAC assay

The oxygen radical absorbance capacity (ORAC) values were determined according to the method of Zulueta, Esteve, and Frígola (2009). The samples were diluted with phosphate buffer solution and 25 µL of this solution was transferred to a 96-well microplate. Fluorescein solution (150 µL, 40 nM) was added to each well and the microplate was then heated at 37ºC for 5 min. After, 25 µL of AAPH (100 mM) was added and the fluorescence was recorded immediately at an excitation wavelength of 485 nm and an emission wavelength of 535 nm every minute for 30 min in a Victor™ fluorometer (Perkin Elmer Wallac, USA). The ORAC values were expressed as µmol TE g^{-1} of the sample.

UPLC-ESI-MS/MS analysis

The phenolic compounds were analyzed using a UPLC system (Waters, Milford, MA). Analyses were performed in negative mode. Chromatographic separation was performed using a Waters Acquity® UPLC BEH C18 (2.1 x 50 mm, 1.7 µm particle size) column at a flow rate of 0.210 mL min^{-1}. The column was maintained at 30 ± 1°C and the sample injection volume was 1.5 µL. The mobile phase was composed of solvent A (water acidified with 0.1% formic acid) and solvent B (methanol). The following gradient was used and the organic solvent (B) percentage was changed linearly as follows: 0 min, 30; 0.33 min, 60; 1 min, 60; 1.33 min, 50; 2.33 min, 50; 2.67 min, 30; and 4 min, 30% (Rotta, Haminiuk, Maldaner, & Visentainer, 2017)

The ESI Xevo Acquity® (Waters, Milford, MA) source parameters were as follows: 3.0 kV capillary voltage, 50 L $hour^{-1}$ cone gas flow, 700 L h^{-1}desolvation gas flow, 550°C desolvation temperature, and 130°C source temperature. The mass spectrometer was operated in MS/MS mode using multiple reaction monitoring (MRM) and a 30 ms dwell time. Table 2 summarizes the acquisition window definition, precursor and product ions, and the MS parameters selected. Quantitative analysis was performed by the external calibration method, at five concentration levels between 10–500 µg kg^{-1}. Data were processed using MassLynx™ 4.1 software and the results were expressed as µg per 100 g^{-1} of the sample.

Statistical analysis

The results were reported as mean ± standard deviation (SD). Data were analyzed by one-way analysis of variance (ANOVA) with Statistica v. 8.0 (2008). Mean values were compared by Tukey's test (5% probability).

Results and discussion

The ORAC and DPPH• tests were chosen to evaluate the in vitro antioxidant activity and the results are shown in Table 3.

The ORAC assay is a direct method that consists in measuring the decrease of fluorescein (from a synthetic reagent), as a result of oxidative damage caused by peroxyl radicals (ROO•) (hydrogen atom transfer - HAT). The ORAC method also measured the ability of antioxidants to protect proteins from oxidative damage. The antioxidant capacity of foods depends on many factors, like colloidal properties of the substrates, oxidation conditions and the location of antioxidants in different phases (Zulueta et al., 2009). The seed fruits showed a diverse range of ORAC activity values, with *Campomanesia pubescens*, *Hovenia dulcis Thunberg* and *Syzygium jambos* (L.) *Alston* having the highest values.

Table 2. Selected ion transitions and optimized parameters for phenolic compounds analysis by UPLC-ESI-MS/MS.

Compounds	Tr (min)	Transition (MRM) (H^-)	Cone Voltage (V)	Collision energy (V)
Gallic acid	0.72	168.9 > 124.9[a]	34	14
		168.9 >78.9[b]		23
Chlorogenic acid	0.96	353 > 191[a]	28	23
		353 > 126.9[b]		34
Vanillic acid	1.40	166.9 > 151.9[a]	30	15
		166.9 > 107.8[b]		19
Ferulic acid	2.19	192.9 > 178[a]	28	13
		192.9 > 134[b]		17
Myricetin	2.60	317 > 178.9[a]	43	19
		317 > 150.9[b]		15

[a]First transition used for quantitation. [b]Second transition used for identification.

Table 3. Antioxidant activity in methanolic extracts of seed fruits natives and exotics based on fresh weight (FW)[a].

Seeds	ORAC/(μmol TE g^{-1})	DPPH•/(μmol TE g^{-1})
Annona atemoya	$46.14^{e,f,g}$±1.25	4.82^{c}±0.32
Campomanesia guazumifolia	79.98^{d}±1.54	$124.58^{b,c}$±19.21
Campomanesia pubescens	289.92^{a}±8.29	$525.47^{a,b,c}$±36.32
Carica papaya	$40.49^{h,i}$±0.76	4.06^{c}±0.19
Dovyalis abyssinica Warb	13.22^{l}±0.16	1.77^{c}±0.03
Eugenia calycina	54.34^{e}±2.15	1.22^{c}±0.15
Eugenia involucrata	14.99^{l}±0.26	1.94^{c}±0.05
Eugenia pyriformis Cambess	$52.27^{e,f}$±1.71	$320.72^{a,b,c}$±11.32
Hovenia dulcis Thunberg	136.79^{b}±2.90	$634.72^{a,b}$±40.81
Hymenaea stigonocarpa	75.74^{d}±1.49	1305.72^{a}±207.32
Lucuma caimito	44.28^{e}±2.02	6.45^{c}±0.40
Passiflora edulis	$41.67^{g,h,i}$±0.30	$156.732^{b,c}$±27.37
Syagrus romanzoffiana	$28.87^{j,k}$±0.52	9.19^{c}±0.73
Syzygium cumini L.	$35.49^{i,j}$±0.59	$434.96^{a,b,c}$±14.77
Syzygium jambos (L.) Alston	112.06^{c}±4.56	$489.62^{a,b,c}$±74.88

[a]Results are expressed as mean ± standard deviation of four replicates. Means followed by different small letters in the same column are significantly different by Tukey test at 5% probability. TE: Trolox equivalents; DPPH•: 1,1-diphenyl-2-picrylhydrazyl; ORAC: Oxygen radical antioxidant capacity.

The DPPH• assay is an indirect method of electron transfer, characterized by a redox reaction between the oxidant and antioxidant. It is stable because it contains three aromatic rings, with resonance effect, which are needed for stabilizing the DPPH• radical electronic charge. The DPPH• radical stabilization is also attributed to a shift of the unpaired electron in the DPPH•, between the three NO_2 groups and the two nitrogen atoms, since all of them are atoms/groups that allow the shifting of electrons (Martinez, Valek, Rešetić, & Ružić, 2006).

One of the mechanisms of the indirect method is based on the transfer of electrons (TE). For Karadag, Ozcelik, and Saner (2009), it is difficult to differentiate TE and HAT reaction mechanisms for the DPPH• assays, and according to Huang, Ou, and Prior (2005), the principal mechanism for DPPH• assays is the hydrogen atom transfer through marginal reaction. Besides, the HAT between hydrogen and DPPH-H may lead to the formation of DPPH•.

Campomanesia pubescens (Myrtaceae), is native to the Brazilian cerrado. The pulp and peel of this fruit are consumed fresh or used to produce juices and jellies. The leaves and stems are used in popular medicines, to combat infections of the urinary tract and diarrhea. Although their seeds are discarded, they have gallic acid (3,4,5-trihydroxybenzoic acid), ferulic acid and myricetin, phenolic compounds which were found in seeds of *Campomanesia pubescens* during this work (Silva, Cardoso, Fante, Rosell, & Boas, 2013).

In traditional Chinese medicine, the fruits of *Hovenia dulcis Thunberg* (Rhamnaceae), are used for their hypoglycemic effects (Jeong-Sang, Chang-Soo, & Jong-Bang, 2005). Fang, Lin, Chan, Lin, and Lin (2007) revealed the ethanol extract of this fruit decreased liver injury by controlling the enzymes, glutamate oxaloacetatetransaminase (GOT) and glutamate pyruvate transaminase (GPT), in mice. Park, Kim, Rehman, Na, and Yoo, (2015) identified the flavonoids, ampelopsin, taxifolin, myricetin and quercetin, in the fruit extract of *Hovenia dulcis Thunberg*, which were different to the phenolic compounds found in the seed extract in the current study.

Syzygium jambos (L.) *Alston* (Myrtaceae) is known for its anti-inflammatory, analgesic and antimicrobial activity (Ávila-Peña, Peña, Quintero, & Suárez-Roca, 2007). These medicinal attributes are associated with all parts of the plant. In particular, the seeds are used to treat diabetes, diarrhea and dysentery (Sharma, Kishore, Hussein, & Lall, 2013).

The fruits of *Hymenaea stigonocarpa* (Fabaceae), exhibit anti-inflammatory and antioxidant effects in intestinal diseases of rats (Orsi, Seito & Di Stasi, 2014). These effects can be explained by the presence of the flavonoid, myricetin, present in the composition of the seeds, as identified in this study.

These findings corroborate those found by Ayala-Zavala et al. (2011), which reported that the content of functional compounds in various tissues of fruits is located preferentially in the peel and seeds. In Table 4, the analyses were carried out using UPLC-ESI-MS/MS. The most abundant phenolic compounds present in the fruit seeds were hydroxybenzoic acids (vanillic acid and gallic acid), hydroxycinnamic acids (chlorogenic acid and ferulic acid) and the flavonol, myricetin.

Table 4. Quantification of phenolic compounds methanol extracts in fruit seed and nuts in expressed in μg g^{-1} dry weight.

Seeds	Gallic acid	Chlorogenic acid	Vanillic acid	Ferulic acid	Myricetin
Annona atemoya	nd	$0.107^{a,b}$±0.022	1.080^{a}±0.034	0.208^{c}±0.027	0.108^{a}±0.018
Campomanesia guazumifolia	393.005^{e}±23.770	nd	nd	nd	0.847^{a}±0.014
Campomanesia pubescens	11.783^{a}±0.539	nd	nd	0.420^{d}±0.032	0.799^{a}±0.040
Carica papaya	nd	nd	24.13^{b}±3.36	$0.16^{b,c}$±0.01	nd
Dovyalis abyssinica Warb	0.135^{a}±0.01	nd	nd	nd	nd
Eugenia calycina	99.042^{b}±1.158	0.039^{a}±0.000	nd	nd	0.700^{a}±0.014
Eugenia involucrata	nd	$0.202^{a,b}$±0.036	0.765^{a} ±0.042	0.105^{b}±0.004	nd
Eugenia pyriformis Cambess	150.830^{c}±8.117	1.056^{c}±0.057	nd	0.114^{b}±0.007	0.151^{a}±0.002
Hovenia dulcis Thunberg	5.723^{a}±0.538	nd	nd	nd	111.057^{e}±1.293
Hymenaea stigonocarpa	9.593^{a}±0.508	nd	nd	nd	4.326^{b}±0.171
Lucuma caimito	nd	0.061^{a}±0.009	nd	0.002^{a}± 0.000	nd
Passiflora edulis	0.667^{a}±0.052	1.208^{c}±0.019	nd	nd	nd
Syagrus romanzoffiana	nd	nd	nd	0.116^{b}±0.009	0.101^{a}±0.000
Syzygium cumini L.	0.574^{a}±0.000	nd	nd	nd	nd
Syzygium jambos (L.) Alston	0.574^{a}±0.000	nd	nd	nd	nd

[a]Results are expressed as mean ± standard deviation of four replicates in μg g^{-1}. Means followed by different small letters in the same column are significantly different by Tukey test at 5% probability. nd: not detected.

The composition and concentration of plant phytochemicals or bioactive compounds may change according to the cultivation, location, and parts of the fruit. Also, the location and sunlight exposure of a single cultivar, the agricultural practices, fruit maturation stage and post-harvest handling can all considerably affect the phytochemical composition of the fruit. Thus, the same fruit may present a diverse bioactive composition of varied concentrations (Ornelas-Paz, Yahia, & Gardea, 2008; Sancho, Yahia, & González-Aguilar, 2011).

According to Rocha et al. (2011), the phenolic compounds are generally associated with the environmental adaptation and resistance mechanism in plants. These factors can influence the flavor, technological characteristics, as well as the nutritional potential of fruits. Similar processes occur in the seeds, which contain a variety of nutrients.

Gallic acid was among the predominant phenolic acids analyzed in the seeds (ten samples showed this compound). Gallic acid has attracted attention due to its capacity to abstract free radicals, which contributes to its antioxidant, immunomodulatory and anti-inflammatory activities (Murota & Terao, 2003) as well as its ability to induce cell apoptosis in cancer cells (Nam, Rho, Shin, & Son, 2016). According to Fu et al. (2011), who studied the total antioxidant activity and phenolic content of 62 fruit, gallic acid and quercetin were the predominant phenolic compounds found in the analyzed fruit. Clifford (1999) performed biological studies that showed high antioxidant capacity and anti-carcinogenic properties in vitro of chlorogenic acid. In the current study, six seeds had chlorogenic acid in their composition and *Passiflora edulis* had the highest concentration of this hydroxycinnamic acid.

Chlorogenic acid exhibits anti-diabetic and antilipemic activities. It also attenuates the gene expression of peroxisome proliferator-activated receptor-α in the regulation of liver function in rats (Ong, Hsu, & Tan, 2013; Wan et al., 2013) and contributes to the maintenance of the gastric epithelium in vitro (Bhatt, Rawat, Badhani, & Rawal, 2017). Other phenolic compounds identified in the current study have also been associated with important bioactive properties (Huang et al., 2012) showed that vanillic acid has anti-inflammatory activity in mice and ferulic acid has activity in suppressing platelet aggregation (Arai et al., 2000; Yang et al., 2016) reported the consumption of flavonoids (such as myricetin) was inversely related to the total plasma cholesterol and the total concentration of low-density lipoprotein cholesterol in humans.

Conclusion

Considering these results, the typically discarded seeds of the studied fruits, showed different antioxidant compounds and can be an alternative source of antioxidants. New products can potentially be developed from the flour or bran prepared from these seeds.

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