



Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

Bioassay-guided isolation of proanthocyanidins with antioxidant activity from peanut (*Arachis hypogaea*) skin by combination of chromatography techniques



Tatiane L.C. Oldoni^{a,*}, Priscilla S. Melo^b, Adna P. Massarioli^b, Ivani A.M. Moreno^b, Rosângela M.N. Bezerra^c, Pedro L. Rosalen^d, Gil V.J. da Silva^e, Andréa M. Nascimento^f, Severino M. Alencar^b

^a Department of Chemistry, Federal Technological University of Paraná (UTFPR), 85501-970 Pato Branco, PR, Brazil

^b Department of Agri-Food Industry, Food and Nutrition, "Luiz de Queiroz" College of Agriculture, University of Sao Paulo (USP), 13418-900 Piracicaba, SP, Brazil

^c Faculty of Applied Sciences, Piracicaba Dental School, University of Campinas (UNICAMP), 13484-350 Piracicaba, SP, Brazil

^d Department of Physiological Sciences, University of Campinas (UNICAMP), 13414-903 Piracicaba, SP, Brazil

^e Department of Chemistry, FFCLRP, University of São Paulo (USP), 14040-901 Ribeirão Preto, SP, Brazil

^f Institute of Biological and Exact Sciences, Federal University of Ouro Preto (UFOP), 35400-000 Ouro Preto, MG, Brazil

ARTICLE INFO

Article history:

Received 24 June 2014

Received in revised form 10 May 2015

Accepted 4 July 2015

Available online 6 July 2015

Chemical compounds studied in this article:

(+)-Catechin (PubChem CID: 9064)

p-Coumaric acid (PubChem CID: 637542)

(-)-Epicatechin (PubChem CID: 72276)

Epigallocatechin (PubChem CID: 72277)

Ferulic acid (PubChem CID: 445858)

Gallic acid (PubChem CID: 370)

Quercetin (PubChem CID: 5280343)

Proanthocyanidin A1 (PubChem CID: 9872976)

Proanthocyanidin A2 (PubChem CID: 124025)

Keywords:

Purification

By-product

Chromatography

Antioxidant activity

Phenolic compounds

ABSTRACT

Purification and bioassay-guided fractionation were employed to isolate proanthocyanidins with antioxidant activity from peanut skin (*Arachis hypogaea* Runner 886). The crude extract was prepared with acetone (60% v/v) and purified using chromatographic methods, including a semipreparative HPLC technique. As a result, two proanthocyanidins were isolated and identified using NMR, epicatechin-(2 β \rightarrow O \rightarrow 7, 4 β \rightarrow 8)-catechin (proanthocyanidin A1) and epicatechin-(β \rightarrow 2 O \rightarrow 7, 4 β \rightarrow 8)-epicatechin (proanthocyanidin A2). Despite the structural similarity, differences were observed in their antioxidant activity. Proanthocyanidin A1 proved to be more active, with EC₅₀ value for DPPH radical scavenging of 18.25 μ g/mL and reduction of Fe³⁺-TPTZ complex of 7.59 mmol/g, higher than that of synthetic antioxidant BHT. This compound evaluated by ABTS^{•+} was similar to that of natural quercetin. Therefore, peanut skin is an important source of bioactive compounds that may be used as a mild antioxidant for food preservation.

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1. Introduction

Proanthocyanidins have nutritional and biological interest because they are considered potent antioxidants (Manach, Mazur, & Scalbert, 2005) due to the presence of a B-ring catechol

group (dihydroxylated B-ring) capable of readily donating hydrogen (electron) to stabilize a radical species (Williams, Spencer, & Rice-Evans, 2004). Furthermore, because of the various o-dihydroxy groups present in structures with high molecular weight, procyanidins have a high ability to complex metal ions, such as Fe (III), Cu (II) and Al (III), as well as proteins.

Proanthocyanidins are well-known for their potential benefits to human health and for presenting valuable biological activities,

* Corresponding author at: CP 591, 85501-970 Pato Branco, PR, Brazil.

E-mail address: tatianeloldoni@utfpr.edu.br (T.L.C. Oldoni).

such as antimicrobial, antioxidant, anticancer, antifungal, anti-allergic, anti-inflammatory, and vasodilatory actions (Pearson, Schmitz, Lazarus, & Keen, 2001; Pizzolitto et al., 2013; Zhang, Liu, Han, & Wei, 2013).

While the kernel of the peanut is a prized commodity, the skin is a low value by-product of peanut processing with a commercial value of approximately US\$ 12–20 per tones (Sobolev & Cole, 2004). Skins have been used for animal feed or burned for energy. However, recent research has shown that skin has a high content of phenolic compounds as flavonoids, phenolic acids, procyanidins dimmers and oligomers (Ballard & Mallikarjunan, 2009; Ballard, Mallikarjunan, Zhou, & O'Keefe, 2010; Lou et al., 2004; Sarnoski, Johnson, Reed, Tanko, & O'Keefe, 2012; Tsujita, Shintani, & Sato, 2014; Yu, Ahmedna, & Goktepe, 2005; Yu, Ahmedna, Goktepe, & Dai, 2006) that makes it a product with potential benefits for human health and valuable biological activities (Awad, Chan, Downie, & Fink, 2000; Yu, Ahmedna, & Goktepe, 2010).

Lou et al. (2004) and Sarnoski et al. (2012) isolated proanthocyanidins from peanut skin and identified them. The compounds were tested for antioxidant activity. However, the authors did not use bioassay-guided fractionation, and the compounds were isolated at random. Thus, it is not possible to sure the bioactive compounds were, in fact, isolated.

The bioassay-guided fractionation technique has been employed by many researchers to study natural products (Baldé et al., 2010; Bargougui et al., 2014; Campos, Azevedo, Filho, Perez, & Braga, 2013; Ding, Ding, Zhang, & Luo, 2013; Oldoni et al., 2011; Teke et al., 2011). The main reason to choose this technique is the rationalization process used to isolate biologically active substances from complex natural extracts.

Although few studies have been carried out with peanut skin, it is possible this low cost product is potentially rich in compounds with functional and biological activities, such as phenolic compounds. Therefore, the aim of the present study was to evaluate the antioxidant activity of crude extract and fractions of peanut skin and, subsequently, isolate proanthocyanidins using the bioassay-guided fractionation technique.

2. Materials and methods

2.1. Samples and standards

The peanut skin samples (*Arachis hypogaea* Runner 886), supplied by CAP Agroindustrial (Dumont, SP, Brazil) in 2010 and 2011, were obtained as a by-product of the blanching process of peanuts. The skin samples were lyophilized, weighed, and stored at -18°C until analysis.

Silica gel 60 and 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) were purchased from Merck (Darmstadt, Germany); butylated hydroxytoluene (BHT), and butylated hydroxyanisole (BHA) standards from Synth (Diadema, SP, Brazil); Amberlite XAD-2[®] resin, 2-2'-azino-di-(3-ethylbenzthiazoline sulfonic acid) (ABTS), 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) analytical standards quercetin, (+)-catechin, (–)-epicatechin, gallic acid, ferulic acid, *p*-coumaric acid and epigallocatechin from (Sigma Aldrich, St. Louis, MO, USA); and Sephadex LH-20 from Amersham Pharmacia (Uppsala, Sweden). All the solvents used for chromatography were of high performance liquid chromatography (HPLC) grade and all the other chemicals were of analytical-reagent grade.

2.2. Extraction and isolation bioguided of bioactive compounds

A representative sample of peanut skin (23 g) was extracted with a mixture of acetone: water (60:40), acidified to pH 1.5 with 0.1 mol/L HCl, in a thermostated bath at 70°C for 30 min. The

extract was centrifuged at $5000\times g$ for 15 min, filtered, and concentrated in a rotary evaporator at 36°C until the acetone had evaporated completely. The sample was lyophilized and the concentrated solid, i.e., the acetone extract of peanut skin (AEPS) was used for purification and subsequent analyses. The AEPS was further purified using Amberlite XAD-2[®] resin, resulting in the methanolic fraction (Met-fr) and the aqueous fraction (Aqu-fr).

The active Met-fr was separated on a Sephadex LH-20 column (5 cm \times 30 cm), using the technique of gel filtration (hydroxypropylated, cross-linked dextran), and eluted with methanol, yielding 123 subfractions. The subfractions obtained were monitored by thin layer chromatography (TLC) using the anisaldehyde reagent (4-methoxy-benzaldehyde), and acetic acid as a developing reagent, followed by incubation at 100°C for 5 min. Fluorescent substances were viewed under ultraviolet (UV) light at 254 nm and 366 nm (Alencar et al., 2007).

Subfractions that were similar in color and Rf were regrouped into 18 subfractions. These subfractions were tested and evaluated for their antioxidant activity and chemical profile using HPLC (Francisco & Resurreccion, 2009a). Subfraction 10 showed the highest activities using the DPPH[•] and ABTS^{•+} radical scavenging methods. Therefore, it was purified by semipreparative HPLC using an Agilent Prep-ODS (H) column (250 mm \times 20 mm). The mobile phases were composed of water (solvent A) and acetonitrile (solvent B) at a constant flow rate of 6 mL/min. The gradient started with 20% solvent B to 30% B in 30 min, 55% B in 35 min, and 20% B in 40 min. The column was maintained at a constant temperature of 30°C . The peaks of eluted compounds were collected in a fraction collector coupled to a liquid chromatography system, tested for antioxidant activity using the DPPH[•] and ABTS^{•+} radical scavenging methods and ferric reducing antioxidant power (FRAP), and also analyzed by nuclear magnetic resonance (NMR) for elucidation of their chemical structures.

2.3. Nuclear magnetic resonance (NMR)

NMR spectra, obtained in CD_3OD using tetramethylsilane (TMS) as internal standard, were recorded in a Bruker DRX 500 spectrometer, operating at 500.13 MHz for ^1H and 125.76 MHz for ^{13}C . The data were obtained by 1D and 2D NMR experiments (^1H – ^1H COSY, HMQC and HMBC). The chemical shifts are expressed in δ (parts per million) and the coupling constants (J) in Hz (Freimund, Sauter, Käppeli, & Dutler, 2003).

All the compounds, already described in the literature, were identified by comparison of their spectral data (nuclear magnetic resonance – NMR) with reported values.

Epicatechin-(2 β \rightarrow O \rightarrow 7, 4 β \rightarrow 8)-catechin (**A1**): ^1H and ^{13}C NMR data were in agreement with the reported literature values (Lou et al., 2004).

Epicatechin-(β \rightarrow 2 O \rightarrow 7, 4 β \rightarrow 8)-epicatechin (**A2**): ^1H and ^{13}C NMR data were in agreement with the reported literature values (Lou et al., 2004).

2.4. Antioxidant activity using the 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) free radical scavenging method

DPPH free radical scavenging activity was measured as described by Moraes de Souza, Oldoni, Regitano-d'Arce and Alencar (2008). The reaction medium consisted of 0.5 mL of the extract or purified fractions, 3.0 mL of ethanol, and 0.3 mL of 0.5 mmol/L DPPH[•] solution in ethanol. The mixture was incubated in the absence of light at room temperature for 45 min. Subsequently, the absorbance was measured using a spectrophotometer (UV mini-1240, Shimadzu Co., Kyoto, Japan) at 517 nm. The anti-radical activity was calculated using the following equation:

$$\text{Antioxidant activity (\%)} = 100 - [(Aa - Ab) \times 100 / Ac]$$

where:

Aa = absorbance of the sample; Ab = absorbance of the blank (prepared by replacing the volume of DPPH[•] solution with an equal volume of ethanol); Ac = absorbance of the control (prepared by replacing the volume of extract with an equal volume of ethanol).

To determine the antioxidant activity of the isolated compounds, the analysis of the DPPH scavenging activity was performed in a 96-well polystyrene microplate. Aliquots of 170 μL of the solution, 60 μL of the DPPH[•] radical solution in ethanol, and 50 μL of the isolated compounds were added to each microplate cavity. After incubation for 45 min at room temperature, the absorbance was measured at 517 nm with a spectrophotometer. The antioxidant activity was determined as previously described.

The concentrations of the samples (AEPS, purified fractions, and isolated compounds) responsible for a 50% decrease in the DPPH free radical initial activity (EC_{50} , $\mu\text{g}/\text{mL}$) were calculated by linear regression of the antioxidant activity, measured at various concentrations of each sample. All analyzes were carried out in triplicate.

2.5. Antioxidant activity using the 2,2'-azino-di-(3-ethylbenzthiazoline sulfonic acid) (ABTS) method

The antioxidant activity by the ABTS method was performed according to Re et al. (1999). The ABTS^{•+} radical was formed by the reaction of 7 mmol/L ABTS with 140 mmol/L potassium persulfate, incubated at 25 °C in the dark for 12–16 h. The radical was diluted with ethanol to obtain the absorbance value of 0.700 ± 0.200 at 734 nm. Under dark conditions, 3.0 mL of the ABTS^{•+} radical solution was added to 30 μL of each dilution of AEPS, purified fractions, and isolated compounds and the absorbance was read at 734 nm in a spectrophotometer after 6 min against ethanol as blank. Trolox was used as reference at concentrations ranging from 100 to 2000 $\mu\text{mol}/\text{L}$ and the results of the

antioxidant activity were expressed as mmol of trolox equivalent antioxidant capacity (TEAC)/g of peanut skin. All analyzes were carried out in triplicate.

2.6. Antioxidant activity using the ferric reducing antioxidant power (FRAP) method

The determination of antioxidant activity using the FRAP method was performed as described by Kukić et al. (2008). FRAP reagent was prepared at the moment of analysis by mixing 25 mL of 300 mmol/L acetate buffer (pH 3.6), 2.5 mL of 10 mmol/L 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) in 40 mmol/L HCl, and 2.5 mL of 20 mmol/L FeCl_3 in aqueous solution. An aliquot of 100 μL of each extract was added to 3 mL of FRAP reagent and incubated at 37 °C in water bath for 30 min. The absorbance was measured, aqueous solutions of ferrous sulfate (100–2000 $\mu\text{mol}/\text{L}$) were used for calibration, and the results were expressed as mmol of Fe^{2+}/g of lyophilized sample. All analyzes were carried out in triplicate.

2.7. Statistical analysis

A randomized design was used for all experiments. Data were analyzed by ANOVA using the Statistical Analysis System (SAS) software. Mean separation was performed using the Tukey's test at the 95% confidence interval.

3. Results and discussion

In this study, the antioxidant activities of AEPS, purified fractions, and isolated compounds obtained from the peanut skin were investigated. The activity-directed purification (Fig. 1) of the crude extract using several chromatographic separations resulted in the isolation of two compounds with high antioxidant activity, 10 mg of epicatechin-(2 β → O → 7, 4 β → 8)-catechin (**A1**) and 2 mg of epicatechin-(β → O → 7, 4 β → 8)-epicatechin (**A2**) (Fig. 2). The

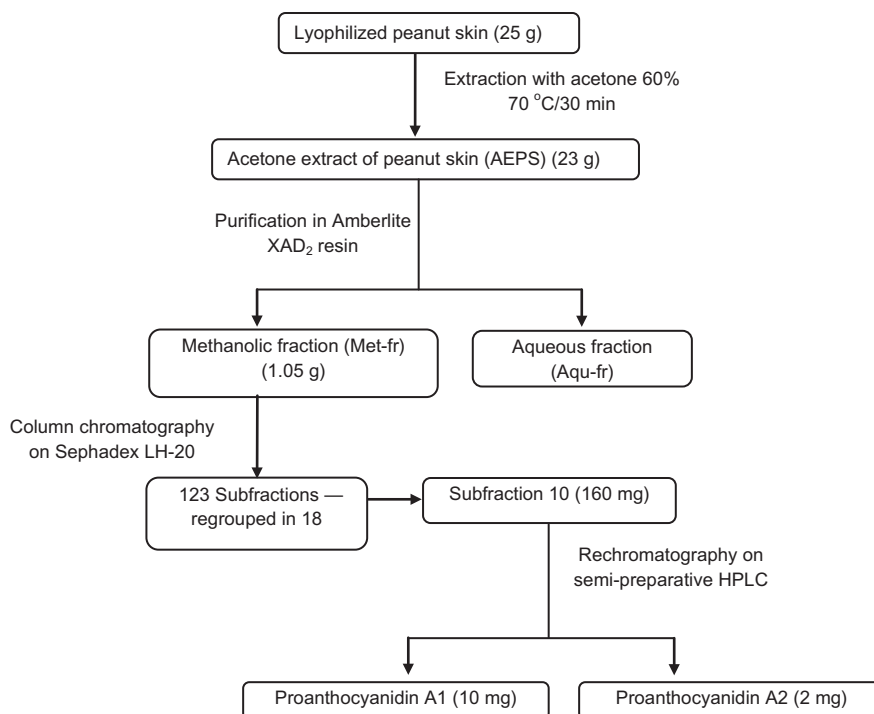


Fig. 1. Procedure for bioassay-guided isolation of compounds from peanut skin.

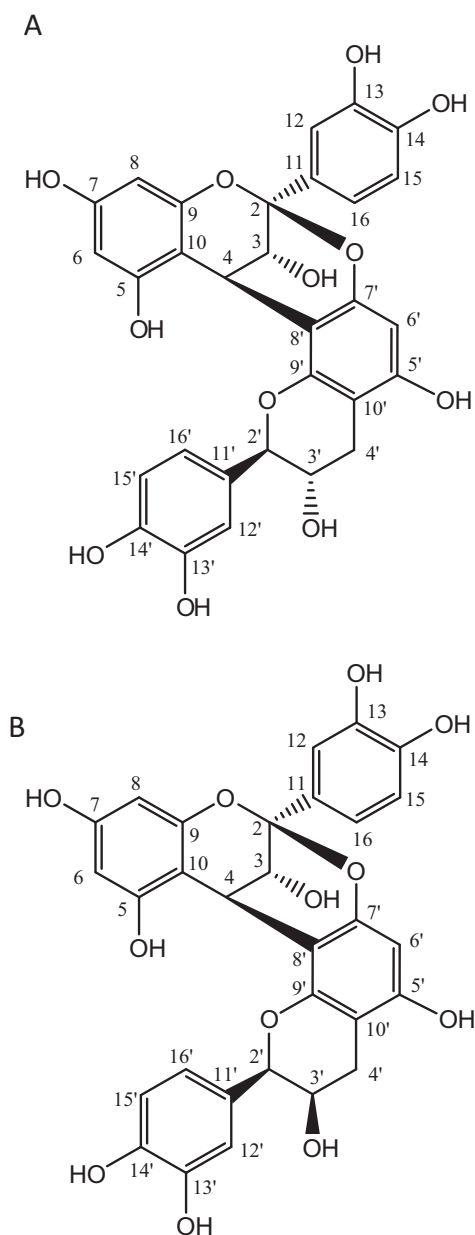


Fig. 2. Structures of the bioactive compounds isolated from peanut skin: (A) epicatechin-(2 β \rightarrow O \rightarrow 7, 4 β \rightarrow 8)-catechin (proanthocyanidin A1), (B) epicatechin-(2 β \rightarrow O \rightarrow 7, 4 β \rightarrow 8)-epicatechin (proanthocyanidin A2).

tests for detection of antioxidant activity were monitored throughout the fractionation and isolation steps.

The structures of the compounds were identified by their spectroscopic data (^1H NMR, ^{13}C NMR) measurements and comparison with published values (Lou et al., 2004).

3.1. Bioassay-guided for antioxidant activity

The first step in the process of isolation of active compounds was the purification with Amberlite XAD-2[®] resin. The Met-fr and Aqu-fr were evaluated for their antioxidant activity using the DPPH $^{\cdot}$ and ABTS $^{\cdot+}$ radical scavenging methods (Table 1) and chemical profile by HPLC-DAD (Fig. 3).

The results of antioxidant activity, expressed as radical scavenging of DPPH $^{\cdot}$ and ABTS $^{\cdot+}$, showed that Met-fr has higher activity than AEPS, while Aqu-fr displayed the lowest activity (Table 1).

These results indicate that the purification process using Amberlite XAD-2[®] resin was efficient, because it resulted in an increase in antioxidant capacity of Met-fr compared with AEPS.

The results found herein for the antioxidant activity of peanut skin are higher than those reported by Francisco and Resurreccion (2009b) for peanut skin from different regions and processed under different temperatures, which ranged from 0.62 to 2.56 mmol of TEAC/g of peanut skin.

Although the Met-fr elicited a more potent antioxidant effect than the Aqu-fr, the HPLC-DAD profiles registered for the fractions were similar, with variation in the intensities of peaks (Fig. 3).

In view of the higher antioxidant activity, chemical profile and amount of the met-fr available, this was purified on a column of Sephadex LH-20. Elution of Met-fr allowed the collection of 123 subfractions, which were analyzed by TLC.

Among the subfractions evaluated, subfraction 10 showed the highest antioxidant activity and a HPLC-DAD positive profile (Fig. 3) for chemical isolation indicating a mixture with UV spectra compatible with phenolic compounds. The major constituents were collected providing compounds with high purity (proanthocyanidins A1 and A2) (Fig. 3).

3.2. Antioxidant activity of isolated compounds

The results of antioxidant activity, expressed as EC₅₀, indicated that the isolation and purification procedures were adequate. A decrease in EC₅₀ value was observed during the purification process (Table 2). The best activities were obtained for Met-fr, subfraction 10, and proanthocyanidin A1, with EC₅₀ values of 16.10, 15.65, and 18.25 $\mu\text{g}/\text{mL}$, respectively. Compared with the synthetic antioxidant BHA (EC₅₀ = 25 $\mu\text{g}/\text{mL}$) (Yuan, Bone, & Carrington, 2005), the two compounds isolated in this study and their extracts are very promising, since they have a great potential for applications in food and pharmaceutical industries.

To the best of our knowledge, this is the first time that bioactive proanthocyanidins have been isolated from peanut skin using the bioassay-guided fractionation technique, and that their antioxidant activity and structure elucidated.

Although Lou et al. (1999) isolated proanthocyanidins A1 and A2 from the water soluble fraction of peanut skin, the authors did not determine their antioxidant activity. Despite isolating other constituents from peanut skin and evaluating them for their antioxidant activity using the EC₅₀ test, Lou et al. (2004) did not use bioassay-guided isolation. Appeldoorn et al. (2009) also isolated procyanidins A-type dimers and B-type dimers from peanut skin and grape seeds, respectively. However, because the authors did not evaluate bioactivity, they could not determine whether these compounds were responsible for the antioxidant activity. The bioassay-guided fractionation technique ensures that the compounds responsible for biological activity are isolated.

The standards quercetin, catechin, and epicatechin were also tested in our study, and their antioxidant activities (radical ABTS method, Fig. 4) were compared with the AEPS, purified fractions and isolated compounds. The highest activity was found for proanthocyanidin A1 (6.54 mmol TEAC/g), very similar to the standards quercetin, catechin, and epicatechin (7.52, 7.33, and 7.50 mmol TEAC/g, respectively). The mixture of proanthocyanidin A1 + proanthocyanidin A2 (1:1 mix in the same proportion) and proanthocyanidin A2 alone had the lowest values for antioxidant activity.

The isolated compounds proanthocyanidin A1 and A2 are diastereoisomers and the only difference between them is the stereochemistry of the $-\text{OH}$ group at the 3' position. Despite their structural similarity, these compounds exhibited different physical, chemical, and biological features. Proanthocyanidin A1 proved to be more bioactive than proanthocyanidin A2, and the

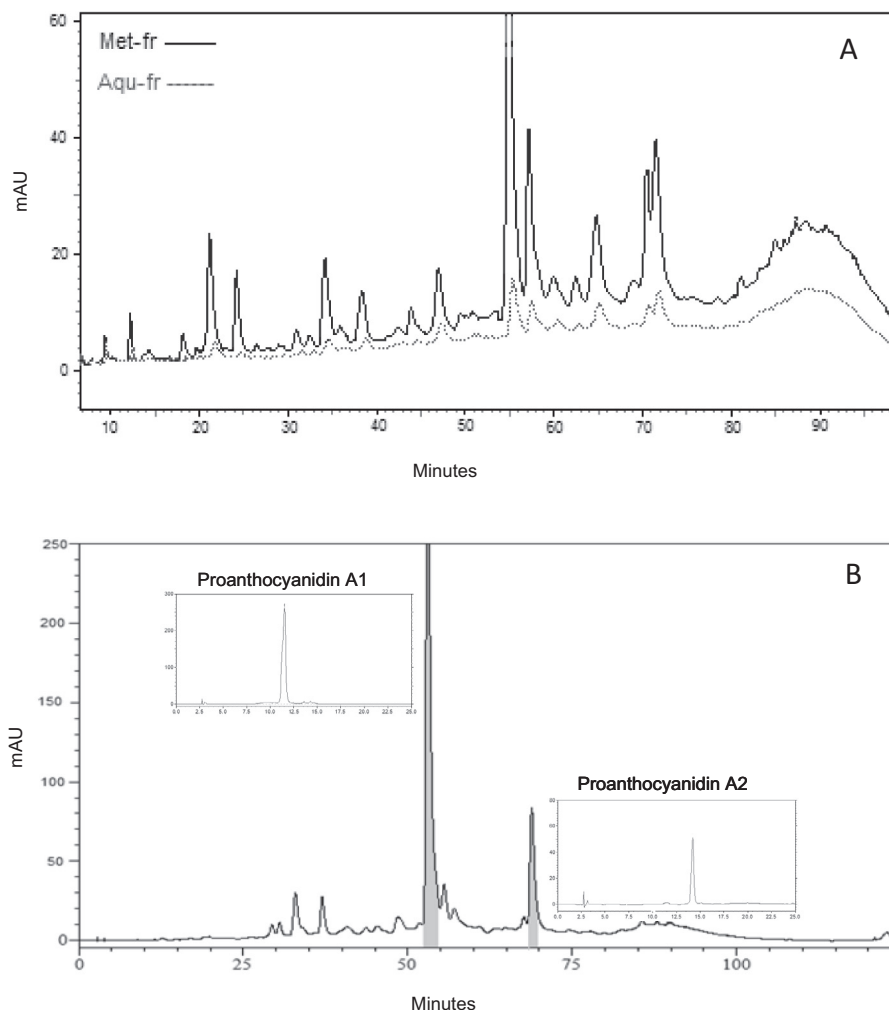


Fig. 3. RP-HPLC profile registered for peanut skin fractions and isolated compounds. (A) Methanolic and aqueous fractions; (B) subfraction 10 and collection of proanthocyanidin A1 and proanthocyanidin A2 using semipreparative RP-HPLC.

Table 1

Antioxidant activity of the acetone extract of peanut skin (AEPS) and its methanolic (Met-fr) and aqueous (Aqu-fr) fractions using the ABTS and DPPH methods.^a

Sample	Antioxidant activity	
	ABTS (mmol TEAC/g)	DPPH (%)
AEPS	4.4 ^b ± 0.10	55.9 ^a ± 2.55
Met-fr	5.5 ^a ± 0.50	60.0 ^a ± 1.44
Aqu-fr	3.2 ^c ± 0.15	41.3 ^b ± 1.80

Each value is expressed as mean (triplicate) ± standard deviation (SD). The same letters in the column are not significantly different at the 0.05 level. The samples were tested at the concentration of 50 µg/mL.

antioxidant activity of an equimolar mixture of both compounds was considerably less, which may be due to the reduced activity showed by the latter.

The isolated compounds were also evaluated for their antioxidant activity by the FRAP method. The activities obtained for the AEPS, Met-fr, subfraction 10, and proanthocyanidin A1 were 7.75, 7.33, 7.55, and 7.59 mmol Fe²⁺/g, respectively (Fig. 4). The values found for the standards, epicatechin and catechin, were 21.3 and 20.9 mmol Fe²⁺/g, respectively. Comparing the results found in this study using the FRAP method with the synthetic standard BHT, which presents antioxidant activity of 1.59 mmol Fe²⁺/g (Borneo,

Table 2

Antioxidant activity of the acetone extract of peanut skin (AEPS), its methanolic fraction (Met-fr), subfraction 10, and isolated compounds (proanthocyanidin A1 and proanthocyanidin A2) using the DPPH method, expressed as EC₅₀.

Sample	Antioxidant activity – DPPH
	EC ₅₀ (µg/mL)
AEPS	20.62 ^b ± 0.05
Met-fr	16.10 ^d ± 0.21
Subfraction 10	15.65 ^e ± 0.07
Proanthocyanidin A1	18.25 ^c ± 0.01
Proanthocyanidin A2	31.10 ^a ± 0.05

Each value is expressed as mean (triplicate) ± standard deviation (SD). The same letters in the column are not significantly different at the 0.05 level.

León, Aguirre, Ribotta, & Cantero, 2009) and with natural antioxidants tested, the isolated compounds show great potential as antioxidants in the food and pharmaceutical industries.

Given that studies on antioxidant activity using the FRAP method are scarce, this discussion focuses on compounds isolated from other matrices. Using the FRAP method Cerezo, Cuevas, Winterhalter, Garcia-Parrilla, and Troncoso (2010) evaluated the antioxidant activity of compounds isolated from strawberry and found values ranging from 2.75 to 7.67 mmol Fe²⁺/g. The isolated

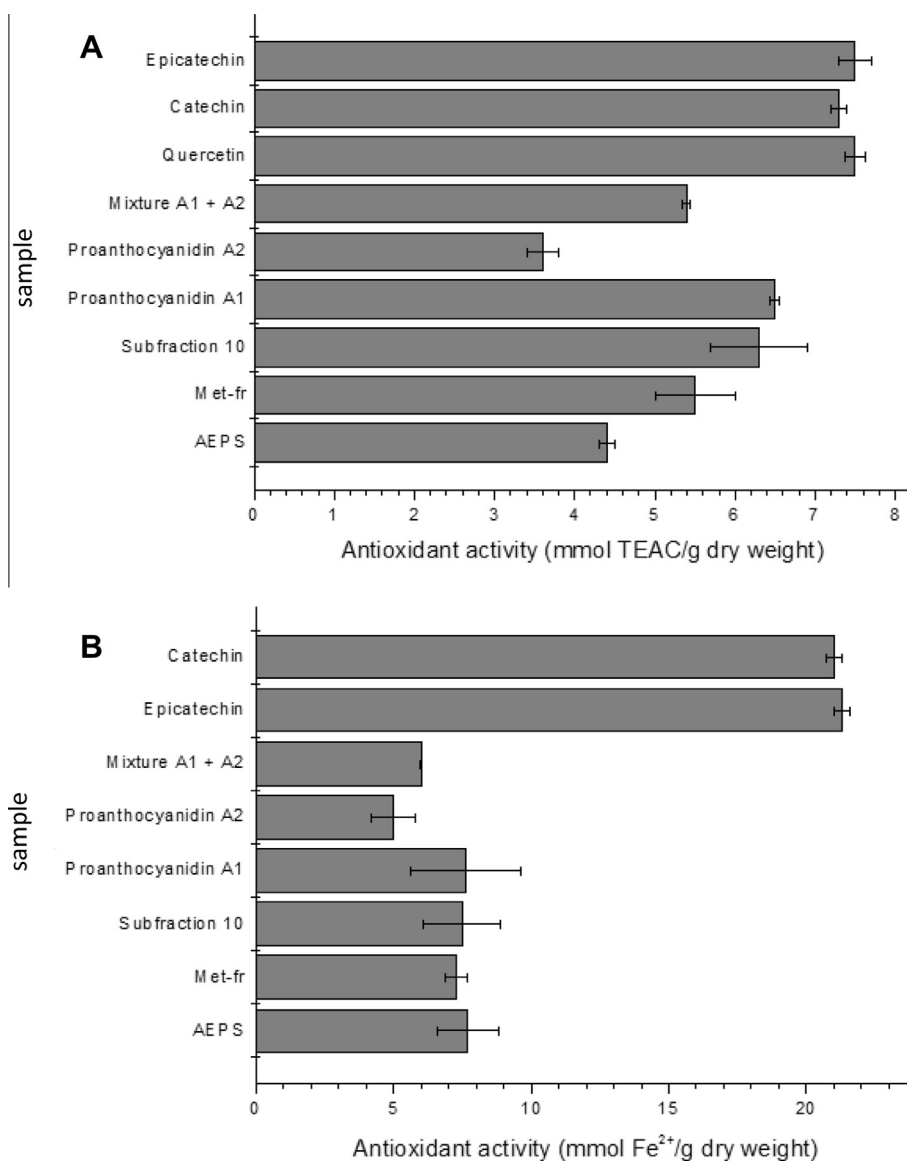


Fig. 4. Antioxidant activity of the acetone extract of peanut skin (AEPS), its methanolic fraction (Met-fr), subfraction 10, isolated compounds from peanut skin and natural standards using the ABTS (A) and FRAP (B) methods (results expressed as mean value \pm standard deviation, $n = 3$).

compounds were identified as anthocyanins and the most active one was pelargonidin-3-glucoside.

4. Conclusions

From the present work, we can conclude that the antioxidant activity presented by AEPS indicates high potential. To the best of our knowledge, this is the first report describing the bioassay-guided isolation of active compounds from peanut skin. The process isolated compounds with higher activities than AEPS, proving that the isolation process was performed properly. The antioxidant activities of proanthocyanidin A1 and A2 were considerably higher than the synthetic antioxidants BHA and BHT, and activities near to potent natural antioxidants, such as quercetin and epicatechin. The proanthocyanidin A1 is a potent antioxidant with no synergistic effect with its A2 isomer.

On the whole, it is interesting to note that a by-product from Brazilian peanut skin is a valuable source of natural bioactive molecules and has properties that suggest applications in the food and pharmaceutical industries. We suggest that, *in vivo*

studies should be carried out to verify the effectiveness of these antioxidant compounds in biological systems.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

The authors are thankful to State of São Paulo Research Foundation (FAPESP – Brazil) (Proc. Nos. 200855492-7 and 200907944-9) for the financial support and to Direction of Research and Post Graduation (DIRPPG) of the Federal Technological University of Parana (UTFPR).

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