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Full Length Research Paper

Antioxidant activities and phenolic compounds of raw and cooked Brazilian pinhão (*Araucaria angustifolia*) seeds

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In this study, the antioxidant properties and the polyphenolic contents of raw and cooked pinhão seeds were evaluated. Four methods were used to evaluate the antioxidant activity: 1,1-diphenyl-2-picrylhydrazyl free radical scavenging (DPPH), 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid scavenging (ABTS), ferrous ions chelating activity and lipid peroxidation inhibition (LPO). The EC_{50} values (extract concentration producing 50% antioxidant effect), in µg/ml extract for raw and cooked extracts yielded respectively: DPPH, 870.7±30.8 and 112.6±5.4; ABTS, 170.7±7.8 and 52.1±2.1; ferrous ions chelating activity, 1,719±84.8 and 761±77.1; and LPO, 55.9±4.7 and 53.8±4.5. The improvement in the antioxidant activity in the cooked seeds can be attributed to the migration of polyphenolic compounds from coat to seed during cooking (5.92 ± 0.09 µg/mg and 24.06 ± 1.3 µg/mg in the raw and cooked seed extracts, respectively). Both raw and cooked seeds presented elevated amounts of catechin, 17.49±0.06 and 21.08±0.09 mg/100 g of seed, respectively.

Key words: Antioxidant activity, *Araucaria angustifolia*, catechin, food processing, *p*inhão seed, polyphenolics.

INTRODUCTION

Araucaria angustifolia is a native conifer from South America, growing in southern and southeastern Brazil and Northeastern Argentina (Conforti and Lupano, 2008). Natural populations or plantations of *A. angustifolia* in Brazil are distributed primarily in the southernmost states of Paraná, Santa Catarina and Rio Grande do Sul. The seed of *A. angustifolia*, named pinhão, is a seasonal product that is produced in the period from April to August (Cladera-Olivera et al., 2012). In the past, pinhão has constituted an important source of carbohydrates for south Brazilian natives. Nowadays, pinhão is consumed usually after cooking in water, or baking, and posterior dehulling. Also, the seeds are used to prepare a kind of flour employed in regional dishes. It presents low contents of lipids (less than 2%) and proteins (less than 3%). Starch is the most important nutrient (around 36%) of pinhão seed (Cordenunsi et al., 2004). The literature about the nutritional aspects of the pinhão seed is very scarce. Some studies have characterized its starch as resistant starch, that is the starch that is resistant to enzyme digestion, passing though the small intestine and reaching the large bowel where it may be fermented by the colonic microflora (Bello-Pérez et al., 2006; Conforti and Lupano, 2008; Cordenunsi et al., 2004). Concerning proteins, two major lectins from the pinhão seed have been evaluated (Datta et al., 1991, 1993) and one polyphenol oxidase was recently purified and characterized (Daroit et al., 2010).

The pinhão seeds are also considered to be a source of dietary fibre, magnesium and copper (Cordenunsi et al., 2004). The pinhão seed is a large and oblong seed

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Figure 1. *Araucaria angustifolia* seed (pinhão).

(Figure 1). It has a very resistant coat and a thin internal membrane, both of which are rich in polyphenolic compounds especially tannins. During cooking, part of the coat polyphenolic compounds migrates to the seed. For this reason, the cooked seed has a brown color and an astringent taste (Cordenunsi et al., 2004). The polyphenolic compounds are secondary plant metabolites important to human health because they have potential beneficial roles in cardiovascular disease and cancer, possibly associated with their antioxidant properties (Gamez et al., 1998). Condensed and hydrolysable tannins of relatively high molecular weight have also shown to be effective antioxidants with greater activity than simple phenols (Hagerman et al., 1998). It is generally believed that antioxidants are able to scavenge free radicals and reactive oxygen species and that they can be extremely important in inhibiting oxidative mechanisms that lead to degenerative diseases (Gamez et al., 1998).

There has been a worldwide trend towards the use of wild plants due to their phytochemicals, of which phenolics are the most important (Brewer 2011). The presence of flavonol quercetin was described in the cooked pinhão seed probably due to the migration of this molecule from the coat during cooking in water (Cordenunsi et al., 2004). Until now, however, no efforts have been done to evaluate more properly the antioxidant properties of the pinhão seed. Taking this into consideration, the objectives of this study were to compare the contents in phenolics as well as the antioxidant activities of raw and cooked in water Brazilian pinhão seeds. We determined the antioxidant activities by measuring the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging ability, 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) cation scavenging ability, β-carotene-linoleic acid assay, free radical scavenging activities and ferrous ion chelating ability. The composition of phenolic compounds was also analyzed.

MATERIALS AND METHODS

The pinhão seeds used in this study were purchased from a local market (Maringá, PR, Brazil) in June 2010. The pinhão seeds used in this work had a mean weight of 8.5 ± 1.5 g, and a mean size of 6.7 ± 1.2 cm. DPPH, ABTS, β-carotene, linoleic acid, ferrous chloride, Folin-Ciocalteu's phenol reagent and the standards gallic acid, protocatechuic acid, p-hydroxybenzoic acid, ferulic acid, sinapic acid, quercetin and catechin, were purchased from Sigma Chemical Co. (St Louis, Mo., U.S.A.). All other chemicals were of analytical grade.

Preparation of samples and extraction of phenolic compounds

The seeds were washed with tap water and dried at room temperature for 24 h, selected and stored at -20°C in plastic bags until use. Two samples of 500 g each were used. The first one was the raw group (raw seeds). The second sample was cooked in water for 30 min in a pressure pot. The coats of the seeds were removed and the seeds were dried at 40°C until constant weight. After drying, the raw seeds and the cooked seeds were milled until a fine powder was obtained, and stored under refrigeration until use. The raw and cooked seed powders (100 g) were used for extraction by stirring with 100 ml of 70% ethanol (in water) at room temperature and at 140 rpm for 3 h and filtered through Whatman filter paper number 1. The extractions were repeated three times. No increases in yield were achieved by further extractions. The combined filtrates were concentrated with a rotary vacuum evaporator at 40°C to eliminate ethanol and finally freeze-dried. The freeze-dried powders were stored in freezer until use.

Determination of total phenolic and flavonoid contents

Total phenolic contents were determined by Folin-Ciocalteu assay (Singleton and Rossi, 1965) and expressed as gallic acid equivalents. The determination of flavonoids and proanthocyanidins were done by means of colorimetric assays (Alothman et al., 2009; Sun et al., 1998) and expressed as catechin equivalents.

Analysis of phenolic compounds by high performance liquid chromatography

A high liquid performance chromatography (HPLC) system (Shimadzu, Tokyo) with a LC-20AT Shimadzu system controller, Shimadzu SPD-20 A UV-Vis detector, equipped with a reversed-phase Shimpak C18 column (4.6 × 250 mm), was used for the analysis of phenolics compounds. All samples in triplicate were filtered through a 0.22-µm filter unit (Millex® -GV, Molsheim, France) before injection. The absorbance of each sample solution was measured at 280 and 370 nm. The mobile phase was water with glacial acetic acid, adjusted to pH 2.25 (solvent A) and acetonitrile 100% (solvent B). A linear gradient system, starting with 15% B, increasing to 90% in 20 min and reducing at 50% in 21 min, at a flow rate of 0.5 ml/min were used. The temperature was kept at 40°C, and the injection volume was 20 µL.

Identification of the peaks of the investigated compounds was carried out by comparison of their retention times with those obtained by injecting standards in the same conditions, as well as by spiking the samples with stock standard solutions. The concentrations of the identified compounds in the extract samples were calculated by means of the regression parameters obtained from calibration curves. All calibration curves using gallic acid, catechin and quercetin showed high degrees of linearity (r^2 >0.99). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

Evaluation of antioxidant properties

Four methods were used to evaluate the antioxidant properties. The 1,1-diphenyl-2-picryl-hydrazyl (DPPH) assay was done as previously described (Soares et al., 2009), with some modifications. The stock solution was prepared by dissolving 24 mg DPPH in 100 ml methanol and then stored at −20°C until use. The working solution was obtained by mixing 10 ml stock solution with 45 ml methanol to obtain an absorbance of 1.1 ± 0.02 units at 515 nm. A volume of 150 µL of each extract was allowed to react with 2,850 µL of the DPPH solution, vigorously shaken and maintained for 1 h at room temperature in the dark. Distilled water was used instead of extract as a control. Then the absorbance was measured at 515 nm. The capability to scavenge the DPPH radical was calculated using the following equation:

$$\text{DPPH scavenging activity}\,(\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

Where, $A_{control}$ was the absorbance of the reaction in the presence of water and A_{sample} is the absorbance of the reaction in the presence of the extract. The extract concentration producing 50% inhibition (EC_{50}) was calculated from the graph of the DPPH scavenging effect against the extract concentration. Gallic acid, catechin and quercetin were used as standards.

For the 2,2'-azino-bis 3-ethylbenzthiazoline-6-sulphonic acid (ABTS) assay, the procedure followed the method previously described with some modifications (Soares et al., 2009). The stock solutions included 7.4 mM $ABTS^{\bullet+}$solution and 2.6 mM potassium persulfate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in the dark. The solution was then diluted by mixing 1.0 ml $ABTS^{\bullet+}$ solution with 60 ml of methanol to obtain an absorbance of 1.1 at 734 nm. A fresh $ABTS^{\bullet+}$ solution was prepared for each assay. A volume of 150 µL of each extract was allowed to react with 2,850 µL of the $ABTS^{\bullet+}$ solution for 2 h in the dark. Then the absorbance at 734 nm was measured. Distilled water was used instead of pinhão seed extracts as a control. The capability to scavenge the ABTS radical was calculated using the following equation:

$$\text{ABTS scavenging activity}\,(\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

Where, $A_{control}$ is the absorbance of the reaction in the presence of water and A_{sample} is the absorbance of the reaction in the presence of the extract. The extract concentration producing 50% inhibition (EC_{50}) was calculated from the graph of the ABTS scavenging effect against the extract concentration. Gallic acid, catechin and quercetin were used as standards.

The ferrous ion chelating ability of the extracts was determined as previously described with some modifications (Soares et al., 2009). A sample (0.7 ml) of each extract was diluted in 0.7 ml of distilled water and mixed with 0.175 ml of $FeCl_2$ (0.5 mM) and the absorbance (A_0) was measured at 550 nm. Afterward, the reaction was initiated by the addition of 0.175 ml ferrozine (0.5 mM). The mixture was shaken vigorously for 1 min and left standing at room temperature for 20 min when the absorbance (A_1) was measured at 550 nm. The percentage of inhibition of the ferrozine–Fe^{2+} complex formation was calculated as follows:

$$\text{chelating ability}\,(\%) = \frac{A_o - A_1}{A_o} \times 100$$

A lower absorbance indicates higher chelating ability. The extract concentration producing 50% chelating ability (EC_{50}) was calculated from the graph of antioxidant activity percentage against the extract concentration. Gallic acid, catechin and quercetin were used as standards.

The antioxidant activity of extracts was finally evaluated by the capability to inhibit the lipid peroxidation through the β-carotene–linoleate model system (Soares et al., 2009). First, β-carotene (0.2 mg) was dissolved in 1.0 ml of chloroform. Next, 0.02 ml of linoleic acid plus 0.2 ml of Tween 80 was added and the mixture was left standing at room temperature for 15 min. After evaporation of chloroform, 50 ml of oxygenated distilled water was added and the mixture was shaken to form an emulsion (β-carotene–linoleic acid emulsion). Aliquots of 3.0 ml of this emulsion were transferred into test tubes containing 0.2 ml of different concentrations of extracts. The tubes were shaken and incubated at 50°C in a water bath. As soon as the emulsion was added to each tube, the zero time absorbance (A_0) was measured at 470 nm. A second absorbance (A_1) was measured after 120 min. A blank, without β-carotene was prepared for background subtraction. Distilled water was used instead of pinhão seed extracts as a control. Lipid peroxidation (LPO) inhibition was calculated using the following equation:

$$\text{LPO inhibition}\,(\%) = \frac{A_o - A_1}{A_o} \times 100$$

The assays were carried out in triplicate and the results expressed as mean values ± standard deviations. The extract concentration producing 50% antioxidant activity (EC_{50}) was calculated from the graph of antioxidant activity percentage against the extract concentration. Gallic acid, catechin and quercetin were used as standards.

Statistical analyses

All analyses were performed in triplicate. The data were expressed as means ± standard deviations and t test were carried out to assess for any significant differences between the means. Differences between means at the 5% ($p \leq 0.05$) level were considered significant.

RESULTS AND DISCUSSION

Extraction yields, total phenolics and flavonoids

There are no satisfactory solvent extraction systems that would be suitable for the isolation of all classes of antioxidant compounds. However, in studies where the objective was to characterize mainly phenolics, alcohols (methanol and ethanol), acidified or not, acetone and mixtures of alcohol–water and acetone-water have been frequently used (Sulaiman et al., 2011). We tried several combinations of these solvents and in all cases, the extraction yields for raw and cooked seeds were around 5.0 - 6.0%. For this reason, and considering price and safety of the extraction solution, 70% ethanol in water was used throughout. The extraction yields (around 5%) of raw and cooked seeds were similar to those found for other foods rich in complex carbohydrates. Methanol was used as extraction solution with extraction yields ranging

Table 1. Total phenolic and flavonoid contents of raw and cooked pinhão seed.

Compounds	Raw seed	Cooked seed
Total phenolics		
µg/mg extract	5.92 ± 0.09[a]	24.06 ± 1.30[b]
mg/100 g seed	296.00 ± 4.50[a]	1,203.00 ± 65.00[b]
Flavonoids		
µg/mg extract	1.16 ± 0.02[a]	11.89 ± 0.26[b]
mg/100 g seed	58.00 ± 1.00[a]	594.50 ± 13.00[b]
Proanthocyanidins (condensed tannins)		
µg/mg extract	0.45 ± 0.02[a]	40.70 ± 0.59[b]
mg/100 g seed	22.50 ± 1.00[a]	2,035.00 ± 29.50[b]

Means with different letters in each line are significantly different ($p \leq 0.05$).

Table 2. Quantification by HPLC of the main phenolics in the raw and cooked pinhão seed.

Phenolic compounds	Raw seed	Cooked seed
Gallic acid		
µg/mg extract	0.105±0,001[a]	0.241±0,017[b]
mg/100 g seed	0.358±0.002[a]	0.821±0.058[b]
Catechin		
µg/mg extract	6.180±0,021[a]	7.960±0,034[a]
mg/100 g seed	17.487±0.057[a]	21.081±0.090[a]
Quercetin		
µg/mg extract	0.030±0.001[a]	0,361±0,002[b]
mg/100 g seed	0.068±0.005[a]	0.692±0.005[b]

Means with different letters in each line are significantly different (P≤0.05).

from 3.82 to 7.44 for cereals such as wheat, oat, rye and barley (Zielinski and Kozlowska, 2000). Rocha-Guzmán et al. (2007) obtained an extraction yield of 6.0 - 6.8% for several types of beans using acetone as the extraction solution and 7.0 - 8.7% using methanol.

Three methods were used to quantify the phenolic compounds in the raw and cooked seed extracts (Table 1). All of them revealed a considerable increase in the polyphenolics contents in the seeds after cooking. These results reveal that pinhão seeds contain elevated amounts of polyphenolics, 296.00 ± 4.50 mg/100 g of raw seed and 1,203 ± 65.00 mg/100 g of cooked seed. Since the seeds were cooked with the coats, it seems natural to associate the improvement in the polyphenolics contents with the migration of these compounds from the coats to the seeds. The three main phenolic compounds identified in the raw and cooked seed extracts by HPLC analysis were quercetin, catechin, and gallic acid (Table 2). The amounts of catechin in the seed extracts were 6.180 ± 0.021 µg/mg extract (raw seed) and 7.960 ± 0.034 µg/mg extract (cooked seed). These results reveal that the pinhão seed contains elevated amounts of catechin: 17.49 ± 0.06 mg/100 g of raw seed and 21.08 ± 0.08 mg/100 g of cooked seed. Consequently, the amounts of catechin in the pinhão seed are comparable those found in catechin rich foods such as raw apples (9.0 mg/100 g), apricots (11.0 mg/100 g), grapes (17.6 mg/100 g) and blackberries (18.7 mg/100 g) (Han et al., 2007, USDA 2003).

One important observation of this work is the fact of the gallic acid and quercetin contents in the cooked seed were at least twice and ten times superior to those found in the raw seed, respectively. Considering that the pinhão seed coat is rich in tannins, the migration of such compounds during cooking was certainly facilitated by the fact that several tannins are thermo-labile and that small phenolics can be produced by their partial degradation/hydrolysis (Henriquez et al., 2008).

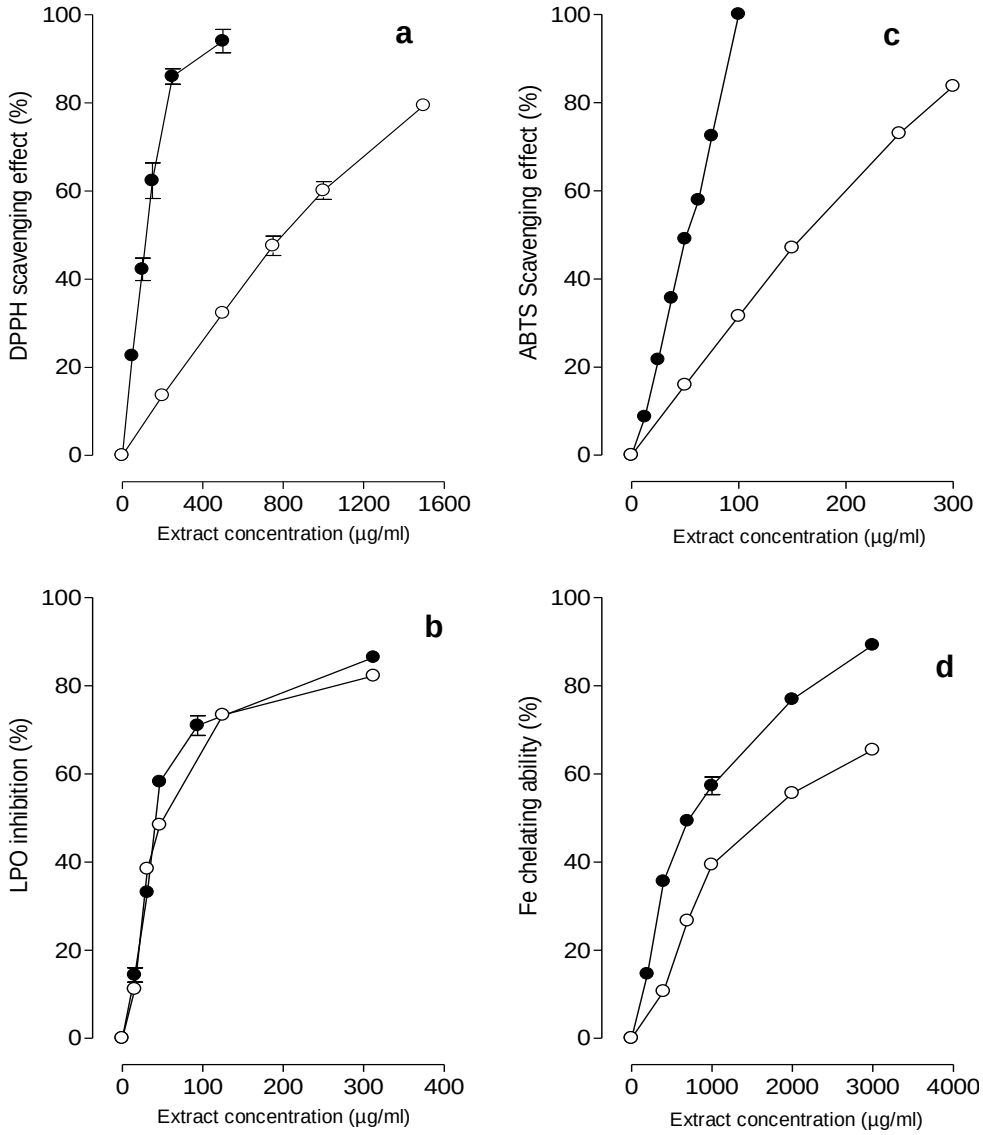

Figure 2. Antioxidant activities of hydroalcoholic extracts from pinhão (*Araucaria angustifolia*) seed. (○) raw seed; (●) cooked seed.

Antioxidant properties of the raw and cooked pinhão seed extracts

For a better understanding of the antioxidant properties of the raw and cooked pinhão seed extracts, four different chemical *in vitro* assays were done, each one based on a different antioxidant mechanism. The antiradical properties were evaluated using both the DPPH and $ABTS^{\cdot+}$ scavenging assays. Further, we also measured the ferrous ion chelation activity and the capability of inhibiting lipid oxidation (autoxidation of the linoleic acid system). Gallic acid, quercetin and catechin were assayed for their antioxidant properties in parallel, considering that these phenolics were identified in the extracts.

DPPH is one of the most used synthetic radicals to evaluate antiradical properties of bioactive compounds and food extracts. It is more stable than common natural radicals (hydroxyl and superoxide radicals) and it is unaffected by certain side reactions, such as metal-ion chelation and enzyme inhibition. In this work, DPPH scavenging properties were evaluated by testing at least six different concentrations for each extract in repeated experiments at least in triplicate (Figure 2A). Results were reported as the concentration required obtaining 50% radical activity inhibition. Higher antiradical activity, thus, corresponds to lower EC_{50} values (Table 3). Similar to what was observed for the total phenolics contents, cooking of the seed had a dramatic effect on the DPPH scavenging activity; the value of EC_{50} changed from

Table 3. EC_{50} values of pinhão seed extracts and phenolics standards.

Parameter	DPPH scavenging (µg/ml)[a]	ABTS scavenging (µg/ml)[a]	LPO inhibition (µg/ml)[b]	Chelating ability (µg/ml)[b]
Pinhão seed extracts				
Raw	870.7±30.8[a]	170.7±7.8[a]	55.9±4.7[a]	1,719±84.8[a]
Cooked	112.6±5.4[b]	52.1±2.1[b]	53.8±4.5[a]	761±77.1[b]
Phenolics Standards				
Gallic acid	1.42±0.02	0.65 ± 0.03	75.6 ± 5.30	>2,000
Quercetin	2.92±0.01	2.26±0.01	4.72±0.26	-
Catechin	2.85±0.05	1.37±0.02	17.62±0.89	-

EC_{50} values were obtained by interpolation from linear or non linear regression analysis ([a]linear regression; [b]non-linear regression). Each value is expressed as mean ± standard deviation (n = 3). nd = not detected EC_{50} value: the effective concentration at which DPPH• radicals or $ABTS^{\bullet+}$ cation radicals were scavenged by 50%; ferrous ions were chelated by 50%; β-carotene-linoleic acid assay (LPO inhibition) was inhibited by 50%. Means with different letters within a row are significantly different ($P \leq 0.05$).

870.7 ± 30.8 µg/ml (raw seed) to 112.6±5.4 µg/ml (cooked seed). It seems likely that migration of antioxidant molecules from coat to seed occurred during cooking. It is important to note that condensed and hydrolysable tannins of high molecular weight have shown to be more effective as DPPH scavengers than simple phenols (Hagerman et al., 1998; Rocha-Guzmán et al., 2007).

The basis of the $ABTS^{\cdot+}$ scavenging assay is to monitor the decay of the radical cation $ABTS^{+}$ produced by the oxidation of ABTS caused by the addition of antioxidants. In analogy to the DPPH assay, the results were expressed as EC_{50} values, higher antiradical activity corresponding thus to lower EC_{50} values (Table 3). For each extract and standard molecule, at least six concentrations and at least three different experiments were performed (Figure 2B). Here also, the cooking of the seed had a dramatic effect on the ABTS scavenging activity which ranged from 170.7±7.8 µg/ml (raw seed) to 52.1±2.1 µg/ml (cooked seed). In our analysis, thus, both the DPPH and the ABTS methods can be considered to be equivalent for evaluating the antioxidant properties of raw and cooked pinhão seeds. This does not always happen, as reported in a recent study in which both methods were also used to evaluate the antioxidant properties of a variety of plant foods (Floegel et al., 2011). In this study, a better correlation between the contents of both phenolics and flavonoids and the antioxidant activity was obtained with the ABTS assay.

Lipid oxidative damage has been recognized to be of fundamental importance because of its numerous biological and nutritional implications: deterioration of flavor and aroma of food, decay of nutritional and safety qualities, cellular damage related to carcinogenesis, premature aging and other diseases (Kumaran and Karunakaran, 2006). Figure 2C shows the antioxidant activity of the pinhão seed extracts as measured by the bleaching of the β-carotene–linoleate system. The free radical linoleic acid attacks the highly unsaturated β-carotene, and the presence of different antioxidants can hinder the extent of β-carotene-bleaching by neutralizing the linoleate free radical and other free radicals formed in the system. The absorbance decreased rapidly in samples without antioxidant, whereas in the presence of an antioxidant the color was retained for a long time (Kumaran and Karunakaran, 2006). Again, results were reported as the concentration required obtaining 50% LPO inhibition (Table 3). Unlike the DPPH and ABTS assays, the EC_{50} values for LPO inhibition assay of the raw and cooked pinhão seed extracts were very similar ($p \leq 0.05$). Obviously the migration of phenolic compounds from the coat to the seed during cooking did not affect the LPO inhibition. This discrepancy is, however, difficult to explain. A possible reason could be that the seed contents in those antioxidants that are more intensively involved in the LPO inhibition were not significantly changed by the cooking process. These compounds were not identified in the present study and this is a point that remains to be solved by further studies.

In this work, the ferrous ion chelation activity of the pinhão seed extracts was evaluated using the ferrozine method. At least, five different concentrations for each extract were tested in these experiments (Figure 2D). The EC_{50} values were calculated by non-linear regression analysis (Table 3). When compared to the antiradical activity, the iron chelation capacity of the pinhão seed extracts were relatively weak, ranging from 1719 ± 84.8 µg/ml (raw seed) to 761±77.1 µg/ml (cooked in water seed). It is well-known that the tannins have elevated capability to chelate metal ions (Diaz et al., 2010). On the other hand, it has been reported that compounds with structures containing two or more of the following functional groups –OH, –SH, –COOH, $–PO_3H_2$, –C=O, $–NR_2$, –S– and –O– in a favorable structure-function configuration can show metal chelation activity (Lindsay, 1996). For this reason, the weak ferrous chelation activity found in the raw and cooked pinhão seed extracts can be unrelated to their phenolic contents.

It is important to point out that no phenolic compounds used in this work, gallic acid, catechin and quercetin presented ferrous ion chelation activity (Table 3). Metal-mediated formation of free radicals may cause modifications in the DNA bases, enhanced lipid peroxidation, and changes in calcium and sulfhydryl homeostasis. Because of its high reactivity, iron is one of the most important lipid oxidation pro-oxidants, particularly in its ferrous state. So, the effective Fe^{2+} chelators may afford protection against oxidative damage by inhibiting reactive oxygen species (ROS) production and lipid peroxidation (Liyana-Pathirana and Shahidi, 2007).

Conclusion

In the present work, the antioxidant properties of a traditional edible seed of southern Brazil were described for the first time. Pinhão seeds (raw and cooked) appear to be a catechin-rich food. Additionally, cooking of the pinhão seeds in water strongly promoted migration of gallic acid and quercetin compounds from coat to seed and greatly improved their antioxidant properties.

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