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Identification and quantification of antioxidant compounds in cowpea[1]

Identificação e quantificação de compostos antioxidantes em feijão-caupi

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ABSTRACT - Dietary antioxidant compounds have been widely studied because of their ability to delay or inhibit oxidative damage, which allows them to play an important role in the prevention of diseases and the promotion of health. The identification and characterization of such compounds are required before their use in humans. This study aimed to identify and quantify antioxidant compounds in the cowpea cultivar, BRS Tumucumaque, and the cowpea strain, Pingo de Ouro 1-2, in view of their potential use in the development of new products with potent antioxidant activity. Here, we report the antioxidant activity and the phenolic compound content of the aforementioned cowpeas. The antioxidant extracts were analyzed by HPLC in a Shimadzu LC-20AT chromatograph model equipped with a manual injector using standard solutions of pure phenolic compounds, including gallic acid, quercetin, caffeic acid, chlorogenic acid, ferulic acid, *p*-coumaric acid, catechin, and epicatechin. Gallic acid was the phenolic compound with the highest level in both BRS Tumucumaque and Pingo de Ouro 1-2 (45.4 ± 2.66 and 93.4 ± 1.25 mg/100 g, respectively). Moreover, we identified and quantified catechin (5.67 ± 0.34 and 6.48 ± 0.51 mg/100 g, respectively), epicatechin (8.67 ± 0.47 and 2.95 ± 0.17 mg/100 g, respectively), ferulic acid (11.1 ± 1.42 and 13.8 ± 0.55 mg/100 g, respectively), and chlorogenic acid (2.39 ± 0.24 and 0.59 ± 0.28 mg/100 g, respectively). In contrast, caffeic acid was only identified in BRS Tumucumaque and quantified at 27.8 ± 2.99 mg/100 g. We conclude that *Vigna unguiculata* demonstrates functional potential, as both the strain and the cultivar contain antioxidant compounds that help in disease prevention and health maintenance.

Key words: Phenolics. Bioactive. *Vigna unguiculata*.

RESUMO - O estudo de compostos antioxidantes em alimentos tem sido bastante difundido na atualidade, tendo em vista a magnitude da atuação destes auxiliando na prevenção de doenças e promoção da saúde, devido ao retardamento ou inibição dos danos oxidativos, sendo de suma importância a identificação destes antioxidantes para utilização em seres humanos. Este estudo teve por finalidade identificar e quantificar compostos antioxidantes em uma cultivar e em uma linhagem de feijão-caupi, tendo em vista sua potencial utilização na elaboração de novos produtos e funcionalidade dos antioxidantes. Foram estudados a cultivar de feijão-caupi BRS Tumucumaque e a linhagemPingo de Ouro 1-2. Os extratos foram analisados por CLAE em equipamento modelo LC-20AT, com injetor manual. Para as análises foram preparadas soluções padrões com os ácidos fenólicos puros, tais como: ácido gálico, quercetina, ácido cafeico, ácido clorogênico, ácido ferúlico, ácido p-cumárico, catequina e epicatequina. O ácido gálico foi o composto fenólico com maiores teores (45,4 ± 2,66 e 93,4 ± 1,25 mg/100 g) na cultivar BRS Tumucumaque e na linhagem Pingo de Ouro 1-2, respectivamente. Foram identificados também catequina (5,57 ± 0,34 e 6,48 ± 0,51 mg/100 g), epicatequina (8,67 ± 0,47 e 2,95 ± 0,17 mg/100 g), ácido ferúlico (11,1 ± 1,42 e 13,8 ± 0,55 mg/100 g), ácido clorogênico (2,39 ± 0,24 e 0,59 ± 0,28 mg/100 g) em concentrações importantes na cultivar BRS Tumucumaque e na linhagem Pingo de Ouro 1-2, respectivamente, sendo que o ácido cafeico (27,8 ± 2,99 mg/100 g) só foi identificado na cultivar BRS Tumucumaque. Concluiu-se que a linhagem e a cultivar estudadas apresentaram compostos antioxidantes que auxiliam na prevenção de doenças e manutenção da saúde, comprovando o potencial funcional do feijão-caupi.

Palavras-chave: Fenólicos. Bioativos. *Vigna unguiculata.*

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INTRODUCTION

Antioxidants are substances that can retard or inhibit oxidative damage by preventing the onset or propagation of chain oxidation reactions. Thus, they can ameliorate the damage caused by free radicals, preventing the onset of diseases associated with such damage (HALLIWELL, 1996).

Phenolic compounds are antioxidants containing an aromatic ring with one or more hydroxyl substituents, among which their functional groups can be found. These compounds can be divided into two major groups, namely flavonoids and phenolic acids. They act as antioxidants not only as a result of their ability to donate hydrogen or electrons but also because of their stable intermediate radicals that can prevent the oxidation of various food ingredients, particularly fatty acids and oils (SOARES *et al.*, 2008).

According to their mode of action, antioxidants can be classified into primary and secondary. Primary antioxidants act by interrupting the reaction chain by converting free radicals into thermodynamically stable products through the donation of hydrogen, and/or by reacting with free radicals, forming a lipid-antioxidant complex that can react with another free radical. Secondary antioxidants delay the first stage (initiation) of autoxidation by various mechanisms that include metal complexation, oxygen sequestration, and decomposition of hydroperoxides, and result in the formation of non-radical species, the absorption of ultraviolet radiation, or the deactivation of singlet oxygen (ANGELO; JORGE, 2007).

After studying fruits, vegetables, and pulps, Hasssimoto, Genovese and Lajolo (2005) concluded that the total antioxidant activity is not the product of one or another compound alone but results from the interaction between different compounds having synergistic and antagonistic effects.

Cowpea (*Vigna unguiculata* L. Walp), also called "feijão-de-corda" (string bean), green bean, brown bean, stick bean, or "Macassar" bean (name varies based on the region of the country), stands out from other legumes because of its easy adaptation to the tropical ecosystem and its nutritional value (FROTA *et al.*, 2010).

Cowpea beans play an important role in human nutrition as they are a source of proteins, carbohydrates, dietary fibers, vitamins of the B complex, minerals, polyphenols, and a small quantity of lipids (CAVALCANTI *et al.*, 2016; OLUWATOSIN, 1998; PEREIRA *et al.*, 2014). They are a significant dietary component in developing countries in Africa, Latin America, and Asia, and constitute a valuable low-cost source of protein. The protein content of cowpea beans is higher than that of other legumes such as peas and pods, which makes them an important source of plant proteins for rural and urban populations. They also display antioxidant properties because of containing polyphenols, which are compounds with antioxidant activity (CARVALHO *et al.*, 2012; KALPANADEVI; MOHAN, 2013; NDERITU *et al.*, 2013).

Clinical and epidemiological studies have reported that cereal, fruit, and legume phenolic antioxidants are the main contributors to the significantly lower incidence of chronic non-communicable diseases in populations whose diets are high in the consumption of these foods (ROESLER *et al.*, 2007). Cowpea has great potential to help reduce the incidence of these diseases.

Therefore, it is of fundamental importance to identify the phenolic compounds present in cowpea cultivars, with a view to selecting those with the greatest potential for use in biological trials and clinical studies, and whose consumption may provide health benefits through disease prevention.

MATERIALS AND METHODS

Both the cowpea cultivar, BRS Tumucumaque, and the cowpea strain, Pingo de Ouro 1-2, were provided by Embrapa Meio Norte (Embrapa Mid-North), an ecoregional research center of the Brazilian Agricultural Research Corporation located in Teresina (PI, Brazil), a city with the following coordinates: altitude, 72 meters; latitude, 5°5' South; longitude, 42°48' West.

To prepare the standard stock solution, 0.125 g of gallic acid (vacuum dried) was dissolved in a 25-mL flask with deionized water. Aliquots of 0 (0 ppm), 1 mL (50 ppm), 2 mL (100 ppm), 3 mL (150 ppm), 5 mL (250 ppm), and 10 mL (500 ppm) were placed into 100 mL volumetric flasks and filled to the mark with deionized water. Another 2 mL of deionized water was added to each flask and 100-µL aliquots were transferred with an automatic pipette to 10-mL volumetric flasks. Half a milliliter of Folin-Ciocalteu reagent was added and stirred. After 5 minutes, 1.5 mL of 20 % w/v sodium carbonate solution was added, the flask was shaken, and the volume was adjusted to 10 mL with deionized water. After 2 hours in the dark at 24 °C, absorbance at 765 nm was measured using 10 mm cuvettes and a plot of gallic acid concentration (in mg/L) versus absorbance was created.

Phenolic extracts were obtained according to the methodology of Rufino *et al.* (2010). The sample was preheated in a ventilated oven at 60 °C for one hour and then comminuted. To obtain the acetonic extract (A), 0.2 g of the

sample was added to 8 mL of 80 % acetone, submitted to ultrasonic treatment for 1 hour, and then centrifuged at 4,000 g for 15 minutes. The supernatant was collected and diluted to 10 mL with deionized water. Two milliliters of deionized water was added to a 10-mL flask, 100 µL of the sample was transferred with an automatic pipette to a 10 mL volumetric flask, and 0.5 ml of Folin-Ciocalteu reagent was added to each flask and vigorously stirred. After 5 minutes, 1.5 mL of 20 % w/v sodium carbonate was added, shaken, and diluted with deionized water to 10 mL. After 2 hours at room temperature, the absorbance at 765 nm was measured in a 10 mm cuvette (SINGLETON; ROSSI JUNIOR, 1965).

The $ABTS^+$ radical solution was prepared by mixing 5 mL of the ABTS solution, which was made by dissolving 0.0384 g of the ABTS reagent in 10 mL of deionized water, and 5 mL of 2.45 mM potassium persulfate solution, which was prepared by dissolving 0.0331 g of potassium persulfate in 25 mL of deionized water. After the mix had been homogenized, it was transferred to an amber flask and kept for 16 hours at room temperature in darkness.

After the activation of the $ABTS^+$ radical, a 1-mL aliquot was diluted in 50 mL of ethanol. Absorbance was measured in 10 mm cuvettes at 734 nm and adjusted to 0.700 by adding radical to reduce or ethanol to increase its value, respectively. Thereafter, a 2.9-mL aliquot was pipetted into a cuvette, to which 60 µL of the extract was immediately added and the absorbance was measured after 7 minutes. The results were expressed in µM TEAC (Trolox equivalent antioxidant capacity) per 100 g of sample, calculated from the absorbance of each extract using a standard curve of Trolox (a standard antioxidant). The method was performed according to Re *et al.* (1999).

We dissolved 0.0394 g of the DPPH radical in 10 mL of methanol. The solution was diluted 1:100 with 80 % v/v methanol solution, its absorbance was adjusted to 0.800, and 2.9 mL was pipetted into a 10 mm cuvette. After the initial absorbance was measured, 100 µL of the extract was added and left for 30 minutes in the dark. After the incubation, the absorbance was measured again and compared to the one of the standard antioxidants, Trolox. The results were expressed in µM TEAC/100 g of sample, according to Kim *et al.* (2006).

The total anthocyanin content was determined using the pH differential method (GIUSTI; WROLSTAD, 2001). Briefly, 550 µL of the diluted sample was transferred to a test tube. Five milliliters of potassium chloride (0.025 M KCl, pH 1.0) was added, homogenized, and stored for 10 minutes in the absence of light. The same procedure was repeated using 550 µL of the diluted sample and 5 mL of sodium acetate (CH_3COONa 0.4 M, pH 4.5). Absorbance (A) was calculated from the following equation:

$$A = (A_{max\ vis} - A_{700nm})_{pH\ 1.0} - (A_{max\ vis} - A_{700nm})_{pH\ 4.5}$$

where $A_{max\ vis}$ is the absorbance at the maximum absorption wavelength in the visual spectrum (420 nm) and A_{700nm} is the absorbance at 700 nm. The concentration of monomeric pigments was calculated by the following equation:

$$Monomeric\ anthocyanins\ (mg/100\ g) = (A \times MW \times DF \times 100)/(\varepsilon \times 1)$$

where MW is the molecular weight, DF is the dilution factor, ε is the molar absorptivity, and 1 is for a standard 1 cm path length. We used the MW and ε values of cyanidin-3-glucoside (MW = 449.2 g mol^{-1}, ε = 26,900 L cm^{-1} mol^{-1}), thus our results corresponded to cyanidin-3-glycoside equivalents.

Extracts were analyzed by HPLC according to Pereira *et al.* (2004) and Tiberti *et al.* (2007). Analyses of phenolic compounds were carried out using an LC-20AT Shimadzu HPLC instrument equipped with an SIL-20AC manual injector, a CBM-20^a controller, and an SPD-M20A diode array detector (Shimadzu, Kyoto, Japan). The column used was Shim-Pack VP-ODS-2 (25 cm × 0.5 cm, 5 µm particle size; Shimadzu) and a C18 stationary phase.

Eluent A was 0.1 % (v/v) trifluoroacetic acid (TFA) in deionized water and eluent B was acetonitrile. Prior to the start of the analysis, the system was equilibrated with a mixture of 9.5:0.5 (A:B). The gradient was as follows: 5 % B (10 minutes), 5–100 % B (40 minutes), 100 % B (5 minutes), and 5 % B (5 minutes). During the analysis, the temperature was maintained at 35 °C and the eluent flow rate at 1 mL min^{-1}. Detection was performed at 190– 400 nm.

To validate the method, the following parameters were used: the limit of detection (LD), calculated as the minimum concentration able to provide a chromatographic signal three times higher than the noise signal; the limit of quantification (LQ), calculated as the minimum concentration capable of providing a chromatographic signal five times greater than the noise signal; the repeatability of the areas of the peaks of the phenolic compounds, through the analysis of ten replicates of the samples; and recovery tests (AOAC, 2005).

Standard solutions were prepared from various pure phenolic acids (gallic acid, naringin, quercetin, rutin, caffeic acid, chlorogenic acid, rosmarinic acid, ferulic acid, *p*-coumaric acid, catechin, and epicatechin) by diluting 1 mg of each standard acid in mobile phase (0.1 % TFA in Mili-q water) to 10 mL and then filtering through 0.22 µm filters. After 2 hours of evaporation, the samples were resuspended in 1 mL of the mobile phase, filtered, and then injected into the chromatograph. Analyses were

performed in quadruplicate and lasted 60 minutes for each sample. Results were expressed in mg/g of dry matter.

A database was developed. The determinations were performed in triplicate or quadruplicate (for those performed by HPLC), and the obtained data were presented as means ± standard deviation.

RESULTS AND DISCUSSION

Bioactive compounds are naturally produced by plants and have been applied in the food and pharmaceutical industry because of their beneficial effects on humans (KHANG *et al.*, 2016).

Table 1 shows the content of total phenolic compounds, flavonoids, and anthocyanins in the BRS Tumucumaque cowpea cultivar and the Pingo de Ouro 1-2 strain, expressed on a dry basis, in the acetonic extract (averages of three determinations).

BRS Tumucumaque contained 199 ± 1.98 mg GAE (gallic acid equivalent)/100 g. Marathe *et al.* (2011) analyzed various legumes that included, among others, common beans, cowpeas, chickpeas, soybeans, and peas, and classified them into three different groups according to their phenolic compound content (low: <100, medium: 100–200, high: >200 mg GAE/100 g). Based on their classification system and the results of the present study, BRS Tumucumaque (177.07 ± 0.78 mg GAE/100 g) can be classified in the moderate phenolic content group and Pingo de Ouro 1-2 (437 mg GAE/100 g) in the high content group.

After studying the flours of four cultivars of raw cowpea consumed in Pakistan, Zia-Ul-Haq *et al.* (2013) reported levels of phenolic compounds (1,190-1,620 mg GAE/100 g) higher than those observed in the current study. In contrast, Khang *et al*. (2016) obtained from white cowpea a phenolic content (19.46 mg GAE/100 g) lower than the one observed in the present work. A number of factors can affect the phenolic compound content in legumes, e.g., genetic and environmental factors. Moreover, measurements may vary depending on the extraction method, e.g., solvent type (KHANG *et al*., 2016).

With respect to flavonoids, BRS Tumucumaque had the highest content (45.8 mg GAE/100 g). According to Behling *et al.* (2004), the daily intake of flavonoids is about 44 mg from cereals, 79 mg from potatoes, 45 mg from grains and nuts, and 162 mg from other vegetables and herbs. Wang *et al.* (2008) analyzed 40 legume accessions of selected legumes, and the two studied cowpea accessions showed high levels of total flavonoids (2004: 441.9 μg/g, 2005: 252.9 μg/g).

In our study, we did not detect anthocyanins in the BRS-Tumucumaque cowpea cultivar, whereas the Pingo de Ouro 1-2 strain presented a total anthocyanin content of 27.8 mg/100 g, which is higher than that obtained by Andrade and Ogliari (2010) for the cowpea cultivar BRS Marataoã (2.20 mg/100 g). The present study confirms the statements of Akond *et al.* (2011) on the direct relationship between the bean tegument color and the amount of anthocyanins, since BRS-Tumucumaque is brown in color.

Similar results were reported by Huber (2012) who studied common beans of the cultivars BRS9435-comet (brown) and Xamego (black), as well as the G-2358 (white) strain. The highest tannin content was observed in the Xamego crude cultivar (11.21 mg catechin/g), while such compounds were not detected in the G-2358 strain.

Our results regarding the antioxidant activity of the cowpea cultivar BRS Tumucumaque and the cowpea strain Pingo de Ouro, as determined by the DPPH and ABTS free radical capture methods, are shown in Table 2.

Xu and Chang (2012) analyzed the health benefits related to the antioxidant activity of 13 legumes consumed in the United States, including cowpea beans and common beans, and reported that the antioxidant activity, as assessed by the DPPH method, ranged from 107 μmol TEAC/100 g in yellow soybean to 1,940 μmol TEAC/100 g in black beans. The results obtained in the present study by the DPPH method were higher than those observed by those authors in yellow peas (358 μmol TEAC/100 g), chickpeas (294 μmol TEAC/100 g),

Table 1 - Total phenolic, flavonoid, and total anthocyanin content in the cowpea cultivar, BRS Tumucumaque, and the cowpea strain, Pingo de Ouro 1-2

Bioactive compound	BRS Tumucumaque cultivar (mg/100 g)	Pingo de Ouro, 1-2 strain (mg/100 g)
Total phenolics	177 ± 0.78*	437 ± 2.48*
Flavonoids	45.8 ± 0.31*	6.44 ± 0.15*
Anthocyanins	ND	27.8 ± 4.81**

*gallic acid equivalent (GAE); ** cyanidin-3-glucoside equivalent; ND – not detected; Values are mean ± standard deviation (SD), *n* = 3 determinations

Table 2 - Total antioxidant activity in the cowpea cultivar, BRS-Tumucumaque, and the cowpea strain, Pingo de ouro 1-2, as determined by DPPH and ABTS

Method	Cultivar/Lineage	Mean ± SD (µmol TEAC*/g)
DPPH	BRS Tumucumaque	552 ± 2.89
	Pingo de Ouro	666 ± 2.28
ABTS	BRS Tumucumaque	557 ± 2.65
	Pingo de Ouro	602 ± 1.25

*TEAC- Trolox Equivalent Antioxidant Capacity

green peas (277 µmol TEAC/100 g), and yellow soybeans (107 µmol TEAC/100 g), but lower than that obtained in cowpeas (707 µmol TEAC/100 g).

Deng *et al.* (2013) investigated cowpea samples commercialized in China. They reported values higher than ours, both for the antioxidant capacity (ABTS: 1727-2312 µmol TEAC/100 g, FRAP: 1357-1924 µmol Fe (II)/100 g) and the phenolic content (717-939 mg GAE/100 g). In contrast, Oboh (2006), who evaluated the ability of raw cowpea samples (two white and three brown tegument cultivars) to sequester the DPPH free radical, obtained percentages of free radical inhibition in the range of 5.5-29.9%, much lower than the 40-50% we observed in our study.

Figures 1, 2, 3, and 4 show the chromatograms of the phenolic compounds of the BRS- Tumucumaque cultivar and the Pingo de Ouro 1-2 strain. The results of the determination are displayed in Table 3. Catechin, epicatechin, gallic acid, ferulic acid, chlorogenic acid, and quercetin were present in both plants, while BRS Tumucumaque also contained caffeic acid. Gallic acid was the most, and quercetin the least, abundant phenolic compound.

According to the Family Budgets Survey (Pesquisa de Orçamentos Familiares, POF) of the Brazilian Institute of Geography and Statistics (Instituto Brasileiro de Geografia e Estatística, IBGE) for 2008-2009, beans (including cowpeas), with their high per capita consumption rates, were one of the foods that contributed the most to the intake of phenolic compounds by the Brazilian population (IBGE, 2010).

Khang *et al.* (2016) reported the presence of caffeic acid in white cowpea at 7.08 ± 6.6 mg/100 g, which is lower that the content we found in BRS Tumucumaque (27.8 ± 2.991 mg/100 g). They also reported a ferulic acid content of 1.82 ± 0.8 mg/100 g), which is lower than the ones obtained in our study for BRS Tumucumaque (11.1 ± 1.429 mg/100 g) and Pingo de Ouro 1-2 (13.8 ± 0.554 mg/100 g). They also identified synapic acid (9.96 ± 7.4 mg/100 g), *p*-coumaric acid (8.18 ± 0.65 mg/100 g), benzoic acid (19.95 ± 36.4 mg/100 g), ellagic acid (4.83 ± 6.5 mg/100 g), cinnamic acid (1.25 ± 3.2 mg/100 g), and syringic acid (3.04 ± 5.3 mg/100 g).

In a study carried out by Nderitu *et al.* (2013), red and cream cowpeas were found to contain ferulic acid, *p*-hydroxybenzoic acid, and *p*-coumaric acid. Moreover,

Figure 1 - Chromatogram of the Pingo de Ouro 1-2 strain (sample 1) at 279 nm

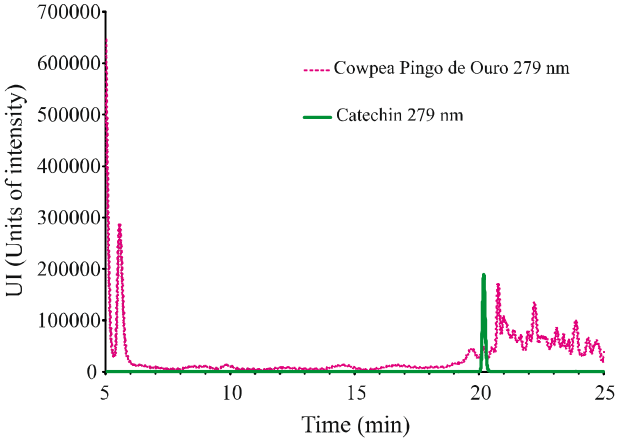

Figure 2 - Chromatogram of the Pingo de Ouro 1-2 strain (sample 2) at 279 nm

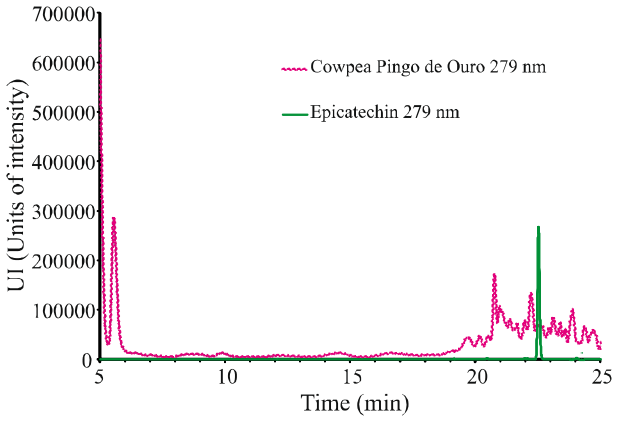

Figure 3 - Chromatogram of the cowpea cultivar BRS Tumucumaque (sample 1) at 279 nm

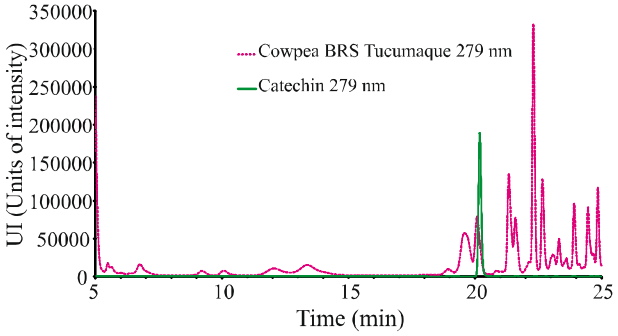

Figure 4 - Chromatogram of the cowpea cultivar BRS Tumucumaque (sample 2) at 279 nm

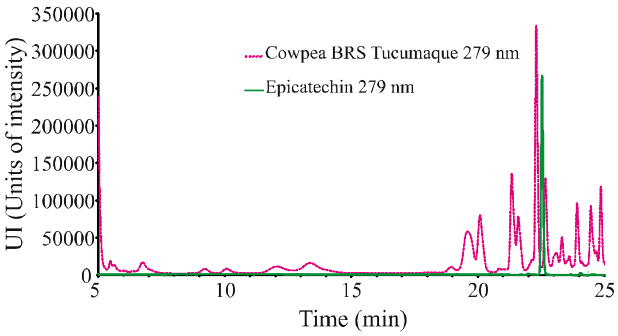

Table 3 - Identification and quantification of phenolic compounds in the acetonic extracts of the cowpea cultivar, BRS Tumucumaque, and the Pingo de Ouro 1-2 strain

Phenolic compound	BRS Tumucumaque (mg/100 g)	Pingo de Ouro 1-2 strain (mg/100 g)
Catechin	5.57 ± 0.341	6.48 ± 0.512
Epicatechin	8.67 ± 0.473	2.95 ± 0.171
Gallic acid	45.4 ± 2.667	93.4 ± 1.250
Ferulic acid	11.1 ± 1.429	13.8 ± 0.554
Chlorogenic acid	2.39 ± 0.247	0.59 ± 0.283
Caffeic acid	27.8 ± 2.991	-
Quercetin	0.01 ± 0.000	0.01 ± 0.000

Values are means ± standard deviation (SD), n = 4 determinations

they performed a simulated gastrointestinal digestion and observed that the enzyme digests of these cowpeas could inhibit DNA damage induced by free radicals, suggesting that the phenolic compounds present in cowpea maintain their radical-sequestering activity after gastrointestinal digestion.

CONCLUSION

Cowpea has clear functional potential as both the BRS Tumucumaque cultivar and the Pingo de Ouro 1-2 strain contain antioxidant compounds in amounts that may allow them to contribute to disease prevention and health maintenance.

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