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In vitro bioaccessibility of phenolic compounds and antioxidant activity in biofortified cowpea cultivars[1]

Bioacessibilidade *in vitro* de compostos fenólicos e atividade antioxidante em cultivares biofortificadas de feijão-caupi

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ABSTRACT - The present work aimed to evaluate the contents of total phenolic compounds, total flavonoids and antioxidant activity, as well as to identify and quantify phenolic acids before and after simulated gastrointestinal digestion in vitro, in raw and cooked cowpea grains, of biofortified cultivars BRS Aracê and BRS Tumucumaque. The raw grains were analyzed as flour and the cooked grains were analyzed after maceration, before and after cooking and in the stages of the digestive process. The contents of total phenolic compounds were analyzed by the spectrophotometric method, using the reagent Folin-Ciocalteu, and total flavonoids using a spectrophotometric method with quercetin as standard. The antioxidant activity was evaluated using the free radical capture method ABTS (2,2'-azino-bis (3-ethylbenzothiazolino-6-sulfonic acid) and FRAP (Ferric Reducing Antioxidant Power). Eight phenolic acids were investigated, and the identification and quantification was performed by high performance liquid chromatography (HPLC), the content of total phenolic compounds and the antioxidant activity were reduced during cooking, but increased with simulated digestion in vitro, due to the release of bound forms. action of digestive enzymes, there was a difference in the behavior of the raw and cooked cultivars.The phenolic acids suffered degradation under gastrointestinal conditions, but the cultivars analyzed maintained compounds with relevant bioactivity (raw grain - gallic, caffeic and p-cumáric acids; cooked grain - acids and caffeine) and antioxidant activity, which can help protect against chronic non-communicable diseases, demonstrating that cowpea is a common food bioaccessible natural antioxidants.

Key words: *Vigna unguiculata*. Thermal processing. Bioactive compounds. In vitro digestion.

RESUMO - O presente trabalho objetivou avaliar os teores de compostos fenólicos totais, flavonoides totais e atividade antioxidante, bem como identificar e quantificar os ácidos fenólicos antes e após a digestão gastrointestinal simulada *in vitro,* em grãos crus e cozidos de feijão-caupi, das cultivares biofortificadas BRS Aracê e BRS Tumucumaque. Os grãos crus foram analisados na forma de farinha e os grãos cozidos foram analisados após maceração, antes e após a cocção e nas fases do processo digestivo. Analisou-se os conteúdos de compostos fenólicos totais pelo método espectrofotométrico, utilizando o reagente *Folin-Ciocalteu*, e flavonoides totais utilizando método espectrofotométrico com a quercetina como padrão. Avaliou-se a atividade antioxidante pelo método de captura dos radicais livres ABTS (ácido 2,2'-azino-bis (3-etilbenzotiazolino-6-sulfônico) e FRAP (*Ferric Reducing Antioxidant Power*). Pesquisaram-se oito ácidos fenólicos, e a identificação e quantificação foi realizada por cromatografia líquida de alta eficiência (CLAE). O conteúdo de compostos fenólicos totais e a atividade antioxidante foram reduzidos durante a cocção, mas aumentaram com a digestão simulada *in vitro*, devido à liberação de formas ligadas. Após a ação das enzimas digestivas, houve diferença no comportamento das cultivares cruas e cozidas. Os ácidos fenólicos sofreram degradação sob condições gastrointestinais, mas as cultivares analisadas mantiveram compostos com relevante bioatividade (grão cru – ácidos gálico, cafeico e p-cumárico; grão cozido – ácidos gálico e cafeico) e atividade antioxidante, que podem auxiliar na proteção contra doenças crônicas não transmissíveis, demonstrando que feijão-caupi é um alimento fonte de antioxidantes naturais bioacessíveis.

Palavras-chave: *Vigna unguiculata*. Processamento térmico. Compostos bioativos. Digestão *in vitro*.

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INTRODUCTION

A diet rich in legumes has been associated with a lower incidence of oxidative stress and chronic non-communicable diseases (NCDs), such as diabetes, obesity, and cardiovascular and kidney diseases. The protective effects of legumes can be partially related to the presence of total phenolic compounds (TPC), which can eliminate free radicals (FR), thus protecting biomolecules such as lipids, proteins, and DNA from damage caused by oxidative stress (NDERITU *et al*., 2013; SANCHO; PAVAN; PASTORE, 2015).

The potential protective action has incited the interest of the scientific community, and this ability has been identified in cowpeas by the presence of bioactive compounds such as phenolic acids, flavonols, flavan-3-ols, anthocyanins, and condensed tannins, and also by the anti-inflammatory and antioxidant (AA) activity on free radicals *in vitro* and *in vivo* (DENG *et al*., 2013; MOREIRA-ARAÚJO *et al*., 2017; SOMBIÉ *et al*., 2018). These compounds are concentrated in the tegument of the grains and are responsible for most of the color of the cowpea seeds (SOMBIÉ *et al*., 2018).

Several studies have shown that digestion induces significant changes in phenolic compounds in different foods, leading to changes in antioxidant activity; however, little is known about the changes brought about by gastrointestinal digestion (GD) in cowpea (*Vigna unguiculata*) phenolics (NDERITU *et al*., 2013). Although interest in this subject has recently increased, there are no studies to date that address the content of bioactive compounds such as the biofortified cultivars of this legume, (e.g., BRS Aracê and BRS Tumucumaque) and their protection against free radicals at each stage of the digestive process.

The use of biofortified cultivars is relevant both for the food industry and for the population, as it increases the content of certain nutrients in the products (LOUREIRO *et al.*, 2018), contributing to the achievement of the daily requirement and meeting the interests of consumers for foods with higher nutritional value (HOBBS *et al*., 2014). However, nutrient bioaccessibility of food must be considered, including in biofortified products, as it can change when linked to the food matrix.

For Lucas-González *et al*. (2018), the term bioaccessibility refers to the amount of the compound released from the food matrix, solubilized in the aqueous phase (chyme), and available for absorption into the systemic circulation through the intestinal wall. Therefore, the bioactivity of phenolic compounds depends on their bioaccessibility during the digestive process, where they are metabolized by electrolytic fluids, as well as gastric, intestinal, pancreatic, and hepatic enzymes, and microbiota.

GD *in vitro* has frequently been used to simulate gastrointestinal conditions, as it can be considered relatively simple when compared to the *in vivo* model, in addition to being safe and free of ethical restrictions (SANCHO; PAVAN; PASTORE, 2015). The accessible fractions of the phenolic compounds in the diet during cooking and digestion and their potential bioaccessibility are important determinants of their potential health benefits. The aim of the present study was to evaluate the content of total phenolic compounds, total flavonoids, and antioxidant activity, as well as to identify and quantify phenolic acids before and after simulated gastrointestinal digestion *in vitro*, in raw and cooked cowpea beans, from BRS Aracê and BRS Tumucumaque biofortified cultivars.

MATERIAL AND METHODS

Two genetically improved cowpea cultivars were analyzed: BRS Aracê and BRS Tumucumaque, were supplied by the Genetic Resources and Breeding Sector (Embrapa Meio-Norte, Teresina-PI, Brazil) and kept in the Laboratory of Bromatology and Food Biochemistry (Department of Nutrition/Federal University of Piauí, Teresina-PI, Brazil) at a temperature of 8 ºC, until analysis. The raw cowpea was crushed in a cyclone mill (TE-651/2-TECNAL) until a homogeneous powder was formed (0.5 mesh). Cowpea was cooked in a bean:water ratio of 1:3 (w/v) in a domestic pressure cooker of 2 L capacity for 13 minutes at a temperature of 121 ºC (BARROS *et al*., 2017). The cooked grains and the respective cooking broth were macerated, and stored under refrigeration (± 8 ºC) until subsequent analysis.

The simulation of *in vitro* GD of 1.5 g of the samples, diluted in Milli-Q water (1:4, m.v-1) was performed in four stages: oral, gastric, duodenal (MINEKUS *et al*., 2014) with simulation of the enzymatic action of the colonic microbiota (FOGLIANO *et al*., 2011).The solution was centrifuged for 40 minutes after each step at 2173.5 x g, and filtered by a quantitative filter paper. The supernatant was collected and its volume measured before storing at -20 ºC. For all stages of digestion, white was prepared to avoid overestimation in the quantification of the bioactive compounds analyzed.

Initially, raw and cooked cowpea bean extracts were prepared, according to the methodology of Rufino *et al*. (2010), using a mixture of solvents: methanol (50%), acetone (70%) and Milli-Q water in a 2:2:1 ratio. After *in vitro* digestion, the filtrate was collected from each stage of digestion for the analysis of total bioactive compounds and antioxidant activity.

The TPC concentrations were determined using the Folin-Ciocalteau reagent with absorbance measurement at 765 nm using a spectrophotometer (BEL, Model 1102, Milan, Italy). The results are expressed as milligrams

of gallic acid equivalents (GAE) per 100 g of dry sample. The concentration of TPC was calculated from a standard curve constructed using gallic acid standards (SINGLETON; ROSSI, 1965).

The method described by González-Aguilar *et al.* (2007), was used to evaluate the concentration of total flavonoids, with absorbance measured at 425 nm. Different concentrations of quercetin (0-100 mg/L) were used to construct a standard curve, and the results are expressed as milligrams of quercetin equivalents (QE) per 100 g of dry sample.

The identification and quantification of phenolic compounds were performed by high performance liquid chromatography (HPLC), according to the methods of Pereira *et al*. (2004) e Tiberti *et al*. (2007).

The phenolic acid standards used were solubilized in pure methanol. The mobile phases used were filtered through HAWP and HVWP membranes of aqueous and organic solvents, respectively (0.45 mm pore size, Millipore Corporation, Milford, MA, USA), with the aid of a vacuum pump. Before the samples were injected into the chromatograph, they were filtered in filters for syringes with 0.45 µm pore and 33 mm diameter (Millipore Corporation, Milford, MA, USA).

The phenolic compounds were analyzed using a LC-20 AT high-performance liquid chromatograph (Shimadzu Corporation, Japan). The separation was performed using a Shimadzu GVP-ODS pre-column (10 mm × 4.6 mm) in line with a Shim-pack VP-ODS column (150 × 4.6 mm i.d., 5-µm particle size) (Sigma-Aldrich, St. Louis, MO, USA) equipped with a UV-Vis SPD-20A detector. The flow rate was maintained at 0.7 $mL \cdot min^{-1}$ and the column temperature was maintained at 40 °C, with an injection volume of 10 µL. The gradient of the mobile phase was composed of (A) methanol with 1% acetic acid and (B) 1% acetic acid: from 0-1 min, 10% A; 1-5 min 15% A; 5-10 min, 20% A; 10-15 min, 25% A; 15-25 min, 30 % A; 25-30 min, 70 % A; 30-40 min, 80% A; 40-50 min, 10% A. The total run time was 50 min. The compounds were detected at 280 (i.e., gallic acid, epicatechin, and ellagic acid), 320 (i.e., caffeic, p-coumaric, chlorogenic, and ferulic acids), and 360 nm (i.e., quercetin). The peaks were identified by comparison with the retention time of standards, and the quantification of the compounds was based on the areas of the respective peaks detected using the LabSolutions acquisition software version 5.57 SP1 Copyright (Shimadzu Corporation). The column calibration was performed by injecting the standards in triplicate at nine different concentrations (i.e., 0.014; 0.056; 0.225; 0.45; 7.81; 15.62; 31.25; 62.5, and 120.0 $\mu g \cdot mL^{-1}$). The levels of phenolic compounds were expressed as $\mu g \cdot mL^{-1}$.

The ABTS free radical capture method was conducted according to Re *et al*., (1999). The absorbance was measured in a spectrophotometer (BEL, Model 1102, Monza, Milan, Italy) at 734 nm. A standard curve was constructed using Trolox at different concentrations (0-100 mg/L) as a reference. The results are expressed as µmol of Trolox Equivalent Antioxidant Activity (TEAC) per 100 g of dry sample.

To evaluate the antioxidant activity using the FRAP (ferric reducing antioxidant potential) technique, the method described by Benzie and Strain (1993) was used, with modifications by Arnous, Makris and Kefalas (2002). Absorbance was measured in a spectrophotometer (BEL, Model 1102, Monza, Milan, Italy) at 620 nm. A standard curve was constructed using Trolox at different concentrations (0-100 mg/L) as a reference. The averages were calculated according to the standard curve, and the results are expressed as µmol of TEAC per 100 g of dry sample.

The bioavailability index (BI) was used to evaluate changes in bioactive compounds due to GD and calculated according to the equation BI (%) = 100* B/C (ORTEGA *et al*., 2011). B is the phenolic content, flavonoids or antioxidant activity as measured by the ABTS and FRAP assays, and C is the amount of these compounds before digestion, expressed in the same units.

Para avaliar as alterações nos compostos bioativos, ao longo da digestão gastrointestinal *in vitro*, os índices de bioacessibilidade (IB) foram calculados de acordo com a Equação: IB (%) = 100. B/C (ORTEGA *et al*., 2011). Onde B é o teor de compostos fenólicos, flavonoides e proantocianidinas totais ou atividade antioxidante pelos ensaios ABTS e FRAP, quantificados no sobrenadante após processo de digestão, e C, a quantidade desses compostos antes da digestão, expressa nas mesmas unidades. All analysis results were expressed on a dry basis.

Data analysis was performed using the Statistical Package for the Social Sciences Program - SPSS, Version 17.0. The results are shown as means and standard deviations. Before starting the statistical analyses, the Kolmogorov-Smirnov non-parametric normality test was applied to test for normal distribution of the data. Subsequently, Student's T-test was used to verify the differences between the averages of raw and cooked grains, and the types of cultivars, while analysis of variance (ANOVA), and Tukey multiple comparisons test were used to identify differences between concentrations of the phenolic compounds, with significance set at $p < 0.05$, and a confidence interval (CI) of 95% (HILBE; ROBINSON, 2013).

RESULT AND DISCUSSION

The effects of simulated gastrointestinal digestion on the release of total phenolic and flavonoid compounds from the raw and cooked grains of the cultivar BRS Aracê (Table 1) and BRS Tumucumaque (Table 2) were differentiated according to each stage of gastrointestinal digestion, with the exception of the colonic phase, in which there was a significant reduction ($p<0.05$) in the content of these compounds for both raw and cooked grains of both cultivars.

Table 1 - Content of phenolic compounds and total flavonoids before and after gastrointestinal digestion *in vitro* considering each stage, in raw and cooked grains of the cultivar BRS Aracê

Digestion steps	Total phenolics (mg GAE.100^{-1} g)		Total flavonoids (mg QE.100^{-1} g)	
	Uncooked grain	Cooked grain	Uncooked grain	Cooked grain
Before digestion	227.98 ± 4.12 ^{a}A	126.58 ± 0.00 aA	42.82 ± 1.01 ^{a}A	22.45 ± 1.18 bA
Oral	226.70 ± 2.27 ^{a}A	115.37 ± 0.00 bB	38.43 ± 0.00 aB	20.08 ± 0.00 bB
Gastric	328.67 ± 0.00 aB	87.31 ± 0.00 bC	147.90 ± 0.00 aC	78.37 ± 0.00 bC
Duodenal	367.40 ± 0.16 ^{a}C	107.81 ± 5.23 bD	115.06 ± 0.16 ^{a}D	56.81 ± 0.00 bD
Colonic	34.00 ± 0.00 aD	19.52 ± 0.00 bE	29.81 ± 1.09^{a}E	21.30 ± 0.00 bAE
BI (%)				
Oral	99.4	91.1	89.7	89.4
Gastric	144.2	69.0	345.4	349.1
Duodenal	161.2	85.2	268.7	253.1
Colonic	14.9	15.4	69.6	94.9

Legend: Results are expressed as mean ± standard deviation. BI: Refers to the Bioaccessibility Index, calculated from the equation BI (%) = 100. B/C (see Materials and Methods). GAE: gallic acid equivalents. QE: Quercetin equivalents The same lowercase superscripted letters between the raw and cooked bean types showed no significant difference between the averages according to the Student's t-test ($p<0.05$, 95% confidence interval [CI]). The same uppercase letters between the phases imply no significant difference between the averages according to the one-way ANOVA test ($p<0.05$, 95% CI)

Table 2 - Content of phenolic compounds and total flavonoids before and after gastrointestinal digestion *in vitro* considering each stage, in raw and cooked grains of the cultivar BRS Tumucumaque

Digestion steps	Total phenolics (mg GAE.100^{-1} g)		Total flavonoids (mg QE.100^{-1} g)	
	Uncooked grain	Cooked grain	Uncooked grain	Uncooked grain
Before digestion	297.23 ± 4.24 ^{a}A	167.15 ± 6.94 bA	59.36 ± 2.03 ^{a}A	43.97 ± 0.67 bA
Oral	281.80 ± 2.27 ^{a}B	154.68 ± 0.00 bB	50.36 ± 1.37 ^{a}B	39.18 ± 0.00 bB
Gastric	277.18 ± 4.55 ^{a}C	88.86 ± 2.19 bC	140.17 ± 2.72 ^{a}C	84.16 ± 0.00 bC
Duodenal	312.24 ± 0.00 aD	93.00 ± 5.23 bD	83.62 ± 2.59 ^{a}D	50.15 ± 0.00 bD
Colonic	32.39 ± 2.27 ^{a}E	16.30 ± 2.27 bE	26.58 ± 0.00 aE	21.30 ± 0.00 bE
BI (%)				
Oral	94.8	92.5	84.8	89.1
Gastric	93.2	53.2	236.1	191.4
Duodenal	105.0	55.6	140.9	114.0
Colonic	10.9	9.7	44.8	48.4

Legend: Results are expressed as mean ± standard deviation. BI: Refers to the Bioaccessibility Index, calculated from the equation BI (%) = 100. B/C (see Materials and Methods). GAE: gallic acid equivalents. QE: Quercetin equivalents The same lowercase superscripted letters between the raw and cooked bean types showed no significant difference between the averages according to the Student's t-test ($p<0.05$, 95% CI). The same uppercase letters between the phases imply no significant difference between the averages according to the one-way ANOVA test ($p<0.05$, 95% CI)

For both cultivars, cooking promoted a reduction in TPC content before digestion. Previous studies (BARROS *et al*., 2017; CAVALCANTE *et al*., 2017) have shown that the reduction of these compounds when cooked relates to their ability to form complexes with proteins and carbohydrates, which makes their extraction difficult as oxidation can occur during the cooking process.

Considering the BI, the conditions employed in simulated gastrointestinal digestion made phenolic compounds and total flavonoids more accessible for absorption through the intestinal barrier for further cell use. The results of the present study are consistent with those reported in other research (CHEN *et al*., 2015; PEREZ-HERNANDEZ *et al*., 2016) simulating gastrointestinal digestion *in vitro* in common bean cultivars and in other cowpea cultivars. DG *in vitro* affected the content of TPC and antioxidant activity in studies by Hachibamba *et al*. (2013), and Mtolo, Gerrano and Mellem (2017) where the content of phenolic compounds and the free radical scavenging activity of cowpea increased with simulated enzymatic digestion. However, this study differs from that of Faller, Fialho and Liu (2012) in the evaluation of feijoada, a common dish in Brazil that combines different species of grains and legumes, where there was no significant difference ($p<0.05$) in the content of phenolics and total flavonoids.

As detailed in Tables 1 and 2, there was a difference in the behavior of the raw and cooked cultivars after digestion. There showed an increase in the content of these compounds after GD (raw grains) up to the duodenal phase, a result consistent with similar studies (HACHIBAMBA *et al*., 2013; MTOLLO; GERRANO; MELLEM, 2017). However, Nderitu *et al*. (2013) obtained opposite results, observing a reduction in some types of phenolic and flavonoid acids after GD. In addition, those authors concluded that even after digestion, total cowpea flavonoids inhibited radical-induced DNA damage and may reduce the risk of oxidative stress-related health issues.

However, there was a significant reduction ($p <0.05$) in the content of total flavonoid compounds when passing from the gastric to the duodenal phase (raw and cooked grains). Since they are highly sensitive to alkaline conditions, they may have been degraded or cleaved in the duodenum with the formation of new chemical compounds or other food components, such as minerals, proteins, fibers, and sugars. These may then have formed complexes with flavonoids (CHEN *et al*., 2016), or were transformed into unknown or undetected structural forms, resulting in decreased bioaccessibility (HACHIBAMBA *et al*., 2013).

Table 3 shows the levels of phenolic compounds identified in the grains of the cultivar BRS Aracê before and after digestion. For this cultivar before digestion, five phenolic acids were identified in the raw grain, which were superior to the results verified by Moreira-Araújo *et al*. (2017), who only identified chlorogenic (0.59 mg/100 g) and ferulic (13.8 mg/100 g) acids, and in contrast to the present study, these authors did not identify caffeic acid in the raw grains of cowpea genotype Pingo de Ouro 1-2.

Table 3 - Phenolic compounds identified and respective levels before and after gastrointestinal digestion *in vitro* in the duodenal phase, in raw and cooked grains of the biofortified cowpea cultivar BRS Aracê

Grains	Compounds	mg.100g^{-1}		BI (%)
		Before digestion in vitro	After digestion in vitro	
Uncooked	Gallic	47.05 ± 0.60 ^{a}A	32.72 ± 1.41 bA	69.5
	Chlorogenic	3.43 ± 0.04 ^{a}B	1.16 ± 0.10 bB	33.8
	Caffeic	26.35 ± 0.91 ^{a}C	14.07 ± 0.64 bC	53.4
	p-coumaric	1.41 ± 0.03 ^{a}D	2.65 ± 0.07 bD	187.9
	Ferulic	23.64 ± 0.42 ^{a}E	4.91 ± 0.74 bE	20.8
Cooked	Gallic	40.2 ± 0.50 ^{a}A	21.77 ± 0.78 bA	54.1
	Chlorogenic	-	-	-
	Caffeic	20.80 ± 0.55 ^{a}B	12.53 ± 1.11 bB	60.2
	p-coumaric	-	-	-
	Ferulic	21.68 ± 0.32 ^{a}BC	2.40 ± 0.25 bC	11.1

Legend: Results are expressed as mean ± standard deviation. BI: Refers to the Bioaccessibility Index, calculated from the equation BI (%) = 100. B/C (see Materials and Methods). The same lowercase superscripted letters between the bio-accessibility before and after signifies no significant difference between the averages according to Student's t-test ($p<0.05$, 95% CI). The same uppercase letters between the compounds before and after imply no significant difference between the averages according to the one-way Test ANOVA (Post Hoc Multiple comparisons, the Tukey test, $p<0.05$, 95% CI)

Table 4 shows the levels of phenolic compounds identified in the grains of the cultivar BRS Tumucumaque before and after digestion. Before digestion, five phenolic acids were identified in the raw grain, which is consistent with Moreira-Araújo *et al*. (2017), who identified gallic (45.4 mg/100 g), chlorogenic (2.39 mg/100 g) caffeic (27.8 mg/100 g) and ferulic (11.1 mg/100 g) acids, in addition to catechin (5.57 mg/100 g) and epicatechin (8.67 mg/100 g) in the raw grains of the same cultivar analyzed in the present study.

The different levels of phenolic acids obtained for each cultivar were likely related to the genotype. After cooking, there was a reduction in the content of phenolic acids in both cultivars, which was consistent with the TPC content, possibly due to the complexation of these with other substances or losses due to oxidation at high temperatures.

Gallic acid was present in greater quantities in raw and cooked extracts before and after simulated digestion (Tables 3 and 4). According to Nayeem *et al*. (2016), this substance has shown the potential to combat oxidative damage, cancer manifestations, microbial infestations, neurodegenerative disorders, and aging. Caffeic acid has high free radical scavenging activity and inhibits lipid peroxidation as well as protecting against LDL oxidation (KHAN; MAALIK; MURTAZA, 2016). Ferulic acid had the lowest bioaccessible fraction among the cultivars analyzed, as it conjugates to the cell wall with other polysaccharides, and is fairly resistant to gastric digestion. This acid has a wide variety of biological activities as an antioxidant in addition to its anticancer, hypocholesterolemic, and anti-inflammatory activities (FALLER; FIALHO; LIU, 2012).

The lesser bioaccessibility of phenolic acids after *in vitro* digestion obtained in the present study can be attributed either to the interactions existing in the food matrix or to the way in which they were converted, which can make them poorly soluble in gastrointestinal fluids (ALMINGER *et al*., 2014). In addition, the enzymatic digestion process may release more phenolic compounds than those investigated in this study.

The influence of *in vitro* gastrointestinal digestion on raw and cooked grains in the ABTS radical capture activity, as well as in the reduction of ferric (Fe^{3+}) to ferrous (Fe^{2+}) by the FRAP assay of the BRS Aracê and Tumucumaque cultivars are shown in Tables 5 and 6.

Before digestion, similar to that obtained for the content of bioactive compounds, thermal processing significantly reduced ($p < 0.05$) the antioxidant activity of both cultivars. This result was expected, considering that there was a loss in the content of bioactive compounds. The reduction in antioxidant activity was also consistent with other research on cowpea grains, as in the study by Barros *et al*. (2017), Cavalcante *et al.* (2017), and Yadav *et al*. (2018). The main differences between these studies in the present research are the cultivars analyzed, as Cavalcante *et al*. (2017), evaluated the Brazilian cowpea cultivars BRS Marataoã, BR 17-Gurguéia, BRS Itaim, BRS Cauamé, and BRS Guariba, while Yadav *et al*. (2018) studied four Indian

Table 4 - Phenolic compounds identified before and after gastrointestinal digestion *in vitro* in the duodenal phase, in raw and cooked grains of the cultivar BRS Tumucumaque

Grains	Compounds	mg.100g^{-1}		BI %
		Before digestion in vitro	After digestion in vitro	
Uncooked	Gallic	55.02 ± 0.17 ^{a}A	34.00 ± 1.33 bA	61.8
	Chlorogenic	1.28 ± 0.01 B	-	-
	Caffeic	25.34 ± 0.35 ^{a}C	18.40 ± 0.40 bB	72.6
	p-coumaric	1.86 ± 0.04 ^{a}BD	2.45 ± 0.07 bC	131.7
	Ferulic	18.70 ± 0.71 ^{a}E	4.35 ± 0.47 bD	23.3
Cooked	Gallic	45.10 ± 0.42 ^{a}A	22.83 ± 0.61 bA	50.6
	Chlorogenic	-	-	-
	Caffeic	16.28 ± 0.27 ^{a}B	9.31 ± 0.29 bB	57.2
	p-coumaric	-	-	-
	Ferulic	10.90 ± 0.03 ^{a}C	2.35 ± 0.23 bC	21.6

Legend: Results are expressed as mean ± standard deviation. BI: Refers to the Bioaccessibility Index, calculated from the equation BI (%) = 100. B/C (see Materials and Methods). The same lowercase superscripted letters between the bio-accessibility before and after signifies no significant difference between the averages according to Student's t-test ($p<0.05$, 95% CI). The same uppercase letters between the compounds before and after imply no significant difference between the averages according to the one-way Test ANOVA (Post Hoc Multiple comparisons, the Tukey test, $p<0.05$, 95% CI)

cultivars: EC4216, BL2, Kohinoor, and Gomati. In addition to the genetic factors, there were differences related to the soil, region, and temperature of the crops.

Table 6 shows the results of antioxidant activity by the two methods evaluated for the cultivar BRS Tumucumaque.

Considering Tables 5 and 6, it was observed that gastrointestinal digestion *in vitro* promoted an increase in the antioxidant activity evaluated by the two methods, with emphasis on greater accessibility of the compounds in the duodenal phase. Hachibamba *et al.* (2013), suggested that the simulated digestion

Table 5 - Antioxidant activity by the ABTS and FRAP method before and after gastrointestinal digestion *in vitro* considering each stage, in raw and cooked grains of the biofortified cowpea cultivar BRS Aracê

Steps of digestion	ABTS (μmol.Trolox.100g^{-1})		FRAP (μmol.Trolox.100g^{-1})	
	Uncooked grains	Cooked grains	Uncooked grains	Cooked grains
Before digestion	799.21 ± 15.71 ^{a}A	428.88 ± 6.74 bA	356.53 ± 7.85 ^{a}A	162.30 ± 7.85 bA
Oral	437.67 ± 1.87 ^{a}C	398.84 ± 0.00 bC	232.86 ± 2.21 ^{a}C	147.64 ± 2.21 bC
Gastric	794.51 ± 0.00 aAB	517.15 ± 0.00 bB	317.06 ± 2.62 ^{a}B	217.11 ± 0.00 bB
Duodenal	1579.22 ± 0.00 aD	1331.8 ± 5.02 cD	658.89 ± 0.00 aD	433.78 ± 0.00 bD
Colonic	227.98 ± 0.00 aE	203.96 ± 0.02bE	188.98 ± 2.19 ^{a}E	88.63 ± 0.00 bE
BI (%)				
Oral	54.8	93.0	65.3	91.0
Gastric	99.4	120.6	88.1	133.8
Duodenal	197.6	310.5	184.8	267.3
Colonic	28.5	47.6	53.0	54.6

Legend: Results are expressed as mean ± standard deviation. BI: Refers to the Bioaccessibility Index, calculated from the equation BI (%) = 100. B/C (see Materials and Methods). The same lowercase superscripted letters between the types of bean raw and cooked imply no significant difference between the averages according to Student's t-test ($p<0.05$, 95% CI). The same uppercase letters between the phases imply no significant difference between the averages according to the one-way test ANOVA (post hoc multiple comparisons, Tukey test) ($p<0.05$, 95% CI)

Table 6 - Antioxidant activity by the ABTS and FRAP method before and after gastrointestinal digestion *in vitro* considering each stage, in raw and cooked grains of the cultivar BRS Tumucumaque

Steps of digestion	ABTS (μmol.Trolox.100g^{-1})		FRAP (μmol.Trolox.100g^{-1})	
	Uncooked grains	Cooked grains	Uncooked grains	Cooked grains
Before digestion	837.73 ± 15.40 ^{a}A	528.92 ± 17.84 bA	453.11 ± 6.79 ^{a}A	274.86 ± 2.61 bA
Oral	436.30 ± 0.00 ^{a}B	300.18 ± 0.00 bB	346.79 ± 3.57 aB	169.41 ± 0.00 bB
Gastric	1164.53 ± 26.16 aC	584.70 ± 0.00 bC	553.54 ± 0.00 ^{a}C	308.28 ± 0.54 bC
Duodenal	1654.28 ± 26.54 ^{a}D	703.30 ± 2.65 bD	701.78 ± 2.22 ^{a}D	515.10 ± 2.76 bD
Colonic	227.98 ± 0.00 aE	200.70 ± 0.00 bE	160.19 ± 2.13 ^{a}E	114.68 ± 0.00 bE
BI (%)				
Oral	52.1	56.8	76.5	61.6
Gastric	139.0	110.5	122.2	112.2
Duodenal	197.5	133.0	154.9	187.4
Colonic	27.2	37.9	35.4	41.7

Legend: Results are expressed as mean ± standard deviation. BI: Refers to the Bioaccessibility Index, calculated from the equation BI (%) = 100. B/C (see Materials and Methods). The same lowercase superscripted letters between the types of bean raw and cooked imply no significant difference between the averages according to Student's t-test ($p<0.05$, 95% CI). The same uppercase letters between the phases imply no significant difference between the averages according to the one-way test ANOVA (post hoc multiple comparisons, Tukey test) ($p<0.05$, 95% CI)

promoted the release of phenolic compounds from the glycosidic forms to aglycone due to hydrolysis during the digestion process and surmised that the antioxidant activity of the aglycone may be greater than that of the glycosides.

It is important to highlight that the FRAP test presented lower levels than the ABTS test in all the evaluated phases. Changes in pH and enzymatic action may produce new chemical compounds with greater or lesser antioxidant activity compared to the original compounds before digestion. Although the digested extracts have demonstrated a relevant neutralization capacity against ABTS radicals, the presence of other non-phenolic compounds, such as peptides derived from protein hydrolysis and polyamines, can contribute to high antioxidant activity (BARROS *et al*., 2107).

The phenolic compound content and antioxidant activity were significantly reduced ($p < 0.05$) in the colonic phase, indicating low extraction of the food matrix or reduced metabolism. Chen *et al*. (2015), showed that phenolic compounds that are bound to the cell wall and are not released after chemical solvent extraction, require chemical hydrolysis to completely dissociate. Many colonic bacteria have enzymes that promote carbohydrate hydrolysis (pectinases, hemicellulases, and cellulases), releasing phenolic compounds that may play a role during digestion and fermentation in the small and large intestine.

Thus, the bioactive compounds of cowpea seeds can have direct protective effects *in situ* in the capture of reactive oxygen species, since the gastrointestinal tract is constantly exposed to these species, both from the diet and from the activation of phagocytes in the intestine, as well as systemic beneficial effects, as reported by Sancho, Pavan and Pastore (2015).

Cooking promoted a reduction in the content of total phenolic compounds and antioxidant activity, and *in vitro* gastrointestinal digestion increased the bioaccessibility of these compounds, making them potentially available for absorption. It was also possible to observe different behaviors for the contents of bioactive compounds according to the stage of the digestive process, which were verified by the characteristics of the food matrix and possible interactions between phenolic compounds and amino acids, peptides, proteins, enzymes, and other food constituents. Although phenolic acids are degraded under gastrointestinal conditions, the grains of the cowpea cultivar BRS Tumucumaque maintained compounds with relevant bioactivity and antioxidant activity, which can potentially protect against chronic non-communicable diseases.

CONCLUSIONS

1. Cooking promoted a reduction in the content of total phenolic compounds, total flavonoids, and antioxidant activity in the grains of the cowpea cultivars evaluated;
2. Simulated *in vitro* digestion made some phenolic acids less extractable, such as chlorogenic and ferulic acids (due to their connection with other food components) or more extractable, as observed in raw grains with gallic, caffeic, and p-coumaric acids, and cooked grains showing gallic and caffeic acids (by releasing linked forms);
3. Simulated *in vitro* digestion of the grains promoted an increase in the content of phenolic compounds, total flavonoids, and antioxidant activity, demonstrating that in the organism *in vivo*, these antioxidant compounds may be bioaccessible to the cells of the gastrointestinal tract and able to exert their beneficial health effects, aiding in protection against chronic non-communicable diseases.

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