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Cellular Antioxidant Activity of *Feijoada* Whole Meal Coupled with an in Vitro Digestion

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ABSTRACT: Consumption of plant food rich meals, such as *feijoada*, a traditional meal in Brazil, is associated with the reduction of chronic disease. The objectives of this study were to determine phytochemical content and antioxidant activity by chemical and cellular antioxidant assays (CAA) of *feijoada* with or without in vitro digestion. *Feijoada* showed no difference in phenolics and flavonoids after digestion. Bound and residue contributions to total phenolics were 20.9% and 32.2%, respectively, suggesting that phenolics reach the colon after intake. Flavonoids in residue and bound fractions represented 50% of total flavonoids. Antioxidant activity of *feijoada* without digestion was higher than that with digestion; however, it showed lower antiproliferative activity and CAA. *Feijoada* with in vitro digestion also yielded phenolics with higher CAA. Analyses of whole meals should be used to evaluate phytochemicals present in food mixtures consumed, especially with digestion models coupled with CAA resulting in information similar to those in physiological conditions.

KEYWORDS: *in vitro digestion, phenolics, flavonoids, ORAC, cellular antioxidant activity, feijoada*

INTRODUCTION

Plant foods provide a rich mixture of phytochemicals to which the health benefits observed by the regular intake of these foods are associated.[1] Fruits and vegetables are well-known as the primary sources of phytochemicals in the diet. Whole grains were not considered as a good source for antioxidants and phytochemicals previously because their phytochemical content was underestimated.[2,3] Actually, whole grains are rich in phytochemicals, which are complementary to the phytochemicals from fruits and vegetables.[3] Phenolics in grains are mainly in bound form, associated with fiber or the cell wall, and can pass through the stomach and small intestine to the colon, being released by bacterial fermentation.[2,4] In addition, consumption of legumes has been associated with reduced risk of chronic diseases such as cardiovascular (CDV) disease[5] and cancer.[6]

In South American countries, legumes are an important energy and protein source.[7] The combination of grains and legumes, such as rice and beans, not only is the main staple food but also improves the nutritional quality of protein and phytochemicals. *Feijoada* is one of the common meals in Brazil and is composed of rice, black beans, toasted cassava flour, kale, and slices of orange.[8] This meal contains a wide variety of phytochemicals and bioactive compounds from grains, legumes, fruits, and vegetables. Phytochemicals have been suggested to be responsible for the health benefits of plant-based foods, especially due to the additive and synergistic effects of these compounds in the whole meals instead of individual and isolated pure compounds.[1] Previous studies in antioxidant research mainly focused on individual fruits, vegetables, and whole grains,[2,9,10] and a few studies investigated the phytochemical profiles and antioxidant activity of whole meals.[11]

Cellular antioxidant activity (CAA) assay is a new approach to quantify antioxidant activity at the physiological conditions when compared to the chemical antioxidant activity assays.[12−14] The CAA assay has been widely used for fruits and vegetables recently but not yet for the whole meals. In addition, previous research using the CAA assay measured the cellular antioxidant activity of samples without the incorporated in vitro digestion model. We believe that in vitro digestion will affect cellular antioxidant activity of tested samples. Measurement of cellular antioxidant activity of food samples coupled with in vitro digestion will provide further information close to the real physiological conditions. The objectives of this study were to determine the phytochemical profiles including total phenolics and flavonoids, and antioxidant activity, and investigate antiproliferative activity and cellular antioxidant activity using the CAA assay with or without in vitro digestion of *feijoada* whole meal.

MATERIALS AND METHODS

Chemicals. Methanol (MeOH), hydrochloric acid (HCl), acetic acid, acetone, phosphate-buffered saline (PBS), sodium carbonate, and potassium phosphate were purchased from Mallinckrodt Chemicals (Phillipsburg, NJ). Ethanol (EtOH, anhydrous, 100%), sodium borohydride ($NaBH_4$, reagent grade), chloranil (analytical grade), vanillin (analytical grade), butylated hydroxytoluene (BHT), bile extract (from porcine), pepsin (from porcine stomach mucosa), pancreatin (from porcine pancreas), DCFH-DA, fluorescein disodium salt, 6-hydroxy-2,5,7,8-tetramethylchoman-2-carboxylic acid (Trolox), Folin−Ciocalteu reagent, and quercetin dehydrate were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). Tetrahydrofuran (THF, analytical grade), aluminum chloride ($AlCl_3 \cdot 6H2O$, analytical grade), and dimethyl sulfoxide were purchased from Fisher Scientific (Pittsburgh, PA). 2,2-Azobis(2-amidinopropane) (ABAP) was pur-

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chased from Wako Chemicals USA, Inc. (Richmond, VA). Gallic acid was purchased from ICN Biomedical Inc. (Costa Mesa, CA). HepG2 human liver cancer cells were obtained from the American Type Culture Collection (ATCC) (Rockville, MD). Williams' Medium E (WME) and Hanks' Balanced Salt Solution (HBSS) were purchased from Gibco Life Technologies (Grand Island, NY). Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Lawrenceville, GA).

Sample Preparation. *Feijoada* whole meal is a traditional Brazilian dish consisting of black beans (*Phaseolus vulgaris* L.), rice (*Oryza sativa* L.), Brazilian kale (*Brassica oleracea* L. *acephala* DC.), cassava (*Manihot esculenta*) flour, and orange (*Citrus sinensis* L. Osbeck) as the main food ingredients. All food ingredients were purchased from the main markets of Rio de Janeiro, Brazil, and were chosen based on the common varieties consumed by the population. All ingredients were taken to the Dietetic Laboratory of the Nutrition Institute of the Federal University of Rio de Janeiro for the preparation of meals. All ingredients, except orange, were cooked individually based on a laboratory standardized technique using standard technical cards.[15] After cooking, each food ingredient was weighed and mixed as the serving (Table 1). The final dish was thoroughly mixed in a blender, lyophilized, and stored at −40 °C until analyses. Each individual food component of the meal was also mixed in a blender, lyophilized, and stored at −40 °C until analyses.

Table 1. Composition of Cooked *Feijoada* Whole Meal

	weight (g)	proportion (%)
black beans	463	63.6
rice	138	18.9
kale	47	6.5
cassava flour	35	4.8
orange	45	6.1
feijoada meal (1 serving)	728	100

Gastrointestinal in Vitro Digestion. In vitro digestion was conducted by the method described by Miller et al.[16] and modified by our laboratory.[17] Briefly, the sample (4.5 g) was mixed with saline (140 mM NaCl, 5 mM KCl, and 150 μM BHT) in 1:4 w/w ratio (meal/saline) to create a final volume of 18 mL. The mixture was placed in an orbital shaker at room temperature for 10 min. After that, it was acidified to pH 2.0 with 0.1 M/1 M HCl. Then the sample was mixed with 0.5 mL of pepsin solution (0.2 g of pepsin in 5 mL of 0.1 M HCl) and incubated in a shaking water bath at 37 °C for 1 h. After gastric digestion, the pH of the digestate was increased to 6.9 with 0.1 M/1 M $NaHCO_3$. Further intestinal digestion was performed with the addition of 2.5 mL of pancreatin−bile solution (0.45 g of bile extract and 0.075 g of pancreatin in 37.5 mL of 0.1 M $NaHCO_3$) and incubated in a shaking water bath at 37 °C for 2 h. The total digestate weight was adjusted to 28 g with saline, centrifuged at 2700*g* for 10 min and separated in two fractions: supernatant, as the bioaccessible fraction, and the residue fraction. A scheme of the digestion procedure is shown in Figure 1. All fractions were stored at −70 °C until analyses.

Phytochemical Extraction. Since the sample consisted mainly of grains and legumes, which have higher lipid content than that of fruits and vegetables, lipid removal was added prior to the extraction according to method developed in our laboratory.[2] In 50 mL conical tubes, 2 g of the lyophilized sample was weighed. To that, 10 mL of deionized water was added before the addition of 10 mL of hexane. The mixture was thoroughly shaken for 10 min at room temperature and centrifuged for 5 min at 2700*g*. The hexane layer was removed and the procedure repeated twice. To the residue, 25 mL of pure acetone was added and mixed for 10 min using a high-speed homogenizer. Then, the sample was centrifuged at 2700*g* for 5 min and the supernatant removed. Another 15 mL of pure chilled acetone was added and the procedure repeated. After centrifuging, the supernatants were pooled together, evaporated to dryness at 45 °C with a rotary evaporator, and resuspended in 10 mL of 70% methanol. This fraction was used for the analysis of free phenolics.

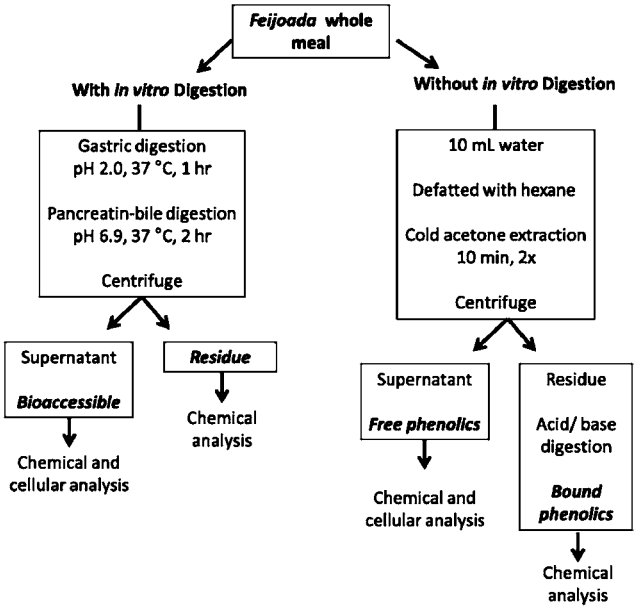

Figure 1. Experimental design scheme.

The bound phenolics were extracted using the method reported by Adom and Liu.[2] To the residue of the free phenolic extraction, 20 mL of 2 M NaOH was added to each tube, flushed with nitrogen (2 min), and digested for 1 h at room temperature with shaking. After that, 4 mL of concentrated HCl was added and left to stand for 2 min. Then, 20 mL of hexanes was added and extracted for 10 min before centrifuging at 2700*g* for 5 min. The supernatant was removed and the procedure with hexane repeated once. Finally, 20 mL of ethyl acetate was added and the mixture extracted for 10 min at room temperature with shaking. The tubes were centrifuged for 5 min at 2700*g* and the supernatant collected. Addition and extraction with ethyl acetate were repeated five times. The supernatants were pooled together, evaporated to dryness at 45 °C with rotary evaporator, and resuspended with 10 mL of 70% methanol. This fraction was used for bound phenolic analysis. All samples were stored at −40 °C until analyses.

Determination of Total Phenolic Content. The total phenolic content of each sample treatment was determined using the colorimetric method described by Singleton et al.[18] and modified in our laboratory.[12,14] Briefly, extracts were reacted with Folin−Ciocalteu reagent and then neutralized with sodium carbonate. After 90 min, the absorbance of the resulting solution was measured at 760 nm. Gallic acid was used as the standard, and total phenolic content was expressed as milligrams of gallic acid equivalents (GAE) per 100 g of dry weight (DW) of sample.

Determination of Total Flavonoid Content. The total flavonoid content of each sample was determined using the sodium borohydride/chloranil-based assay developed by our laboratory.[19] Briefly, 4 mL extracts of tested samples were added into test tubes (15 × 150 mm), solvent evaporated under nitrogen gas, and reconstituted in 1 mL of tetrahydrofuran/ethanol (THF/EtOH, 1:1, v/v). Catechin standards (0.1−10.0 mM) were prepared fresh before use in 1 mL of THF/EtOH (1:1, v/v). Then, 1 mL of 50 mM $NaBH_4$ solution and 0.5 mL of 74.6 mM $AlCl_3$ solution were added into each test tube with 1 mL of sample solution or 1 mL of catechin standard solution. Then, the test tubes were shaken in an orbital shaker at room temperature for 30 min. An additional 0.5 mL of 50.0 mM $NaBH_4$ solution was added into each test tube with continued shaking for another 30 min at room temperature. Then, 2.0 mL of cold 0.8 M acetic acid solution was added into each test tube, and the solutions were kept in the dark for 15 min after a thorough mix. Then, 1 mL of 20.0 mM chloranil was added into each tube, which was heated at 95 °C with shaking for 60 min. The reaction solutions were cooled using tap water, and the final volume was brought to 4 mL using methanol. Then, 1 mL of 1052

mM vanillin was added into each tube and mixed. Then 2 mL of 12 M HCl was added to each tube, and the reaction solutions were kept in the dark for 15 min after a thorough mix. Aliquots of final reaction solutions (200 μL) were added into each well of a 96-well plate, and the absorbances were measured at 490 nm using an MRX Microplate Reader with a Revelation workstation (Dybex Technologies, Inc., Chantilly, VA). Total flavonoid content was expressed as milligrams of catechin equivalents per 100 g of DW of sample. Data were reported as the mean ± SD for at least three replicates.

Phenolic Acids Analysis. Free phenolic acids were quantified by HPLC according to Queiroz et al.[20] Briefly, 500 mg of lyophilized samples were extracted with 5 mL of methanol, water, acetic acid, and BHT (85:15:0.5:0.2, v/v/v/w) for 4 h at room temperature. Samples were then brought to dryness in a rotary evaporator at 40 °C under vacuum. The residue was redissolved in 1 mL of 25% methanol and filtered with a 0.2 μm PTFE syringe before free phenolics analysis. The analyses were done using a Shimadzu Chromatography workstation and C18 Waters Symmetry column (250 mm × 4.6 mm, 5 μm). The flow rate was 1.0 mL/min. Mobile phase A was water/0.02% TFA, and phase B was methanol/0.02% TFA. The gradient conditions were as follows: 0−5 min, 25% B; 5−10 min, 25−30% B; 11−16 min 30−45% B; 17−18 min, 45% B; 19−40 min, 45−85% B; and 41−50 min, 80−25% B. The detection weight length was set to 270 nm. Authentic standards of acids included gallic, protocatechuic, chlorogenic, caffeic, ferulic, and cinnamic acids at concentrations 0.25, 0.5, 1, 2.5, 5, 10, 25, 50, and 100 μg/mL. Phenolic acid recovery rates were >94%. Phenolic acid contents were expressed as mg per 100 g dry weight.

Total Antioxidant Activity. The total antioxidant activity was determined using the oxygen radical absorbance capacity (ORAC) assay described by Huang et al.[21] and modified in our laboratory.[12,14] Briefly, sample extract dilutions were prepared with 75 mM phosphate buffer (pH 7.4). The assay was performed in black-walled 96-well plates (Corning Scientific, Corning, NY). The outside wells of the plate were not used as there was much more variation from them than from the inner wells. Each well contained 20 μL of extracts or 20 μL of Trolox standard (range = 6.25−50 μM) and 200 μL of fluoroscein (final concentration = 0.96 μM), which were incubated at 37 °C for 20 min. After incubation, 20 μL of 119 mM ABAP was added to each well. Fluorescence intensity was measured using a Fluoroskan Ascent FL plate-reader (Thermo Labsystems, Franklin, MA) at an excitation of 485 nm and an emission of 520 nm for 35 cycles every 5 min. ORAC values were expressed as micromoles of Trolox equivalents per 100 g of DW.

Cell Culture. HepG2 cells were grown in Complete Medium (WME supplemented with 5% FBS, 10 mM Hepes, 2 mM L-glutamine, 5 μg/mL insulin, 0.05 μg/mL hydrocortisone, 50 units/mL penicillin, 50 μg/mL streptomycin, and 100 μg/mL gentamycin) and were maintained at 37 °C and 5% CO_2 as described previously.[22] Cells used in this study were between passages 12 and 32.

Cytotoxicity and Inhibition of Proliferation Assays. Cytotoxicity toward HepG2 cells was measured using the method developed in our laboratory.[22] HepG2 cells in growth media were placed in each well of a 96-well flat-bottom plate at a density of 4.0×10^4 cells/well. After 24 h of incubation at 37 °C with 5% CO_2, the growth medium was removed, each well washed with 100 μL of PBS, and replaced by media containing different concentrations of sample tested. Control cultures received the extraction solution minus the extracts, and blank wells contained 100 μL of growth medium with no cells. After another 24 h of incubation, cytotoxicity was determined by the methylene blue assay.[23] Cytotoxicity was determined by a 10% reduction of absorbance at 570 nm reading for each concentration compared to the control. A minimum of three replications for each sample was used to determine the cytotoxicity.

Antiproliferative activity against human HepG2 liver cancer cells was determined using the method reported previously.[24] Briefly, HepG2 cells in growth media were placed in each well of a 96-well flat-bottom plate at a density of 2.5×10^4 cells/well. After 4 h of incubation at 37 °C with 5% CO_2, the growth medium was replaced by media containing different concentrations of extracts or digested extracts. Control cultures received the extraction solution minus the sample extracts, and blank wells contained 100 μL of growth medium with no cells. After 72 h of incubation, cell proliferation was determined by the methylene blue assay.[23] Cell proliferation (percent) was determined from the absorbance at the 570 nm reading for each concentration compared to the control. A minimum of three replications for each sample was used to determine the antiproliferative activity.

Cellular Antioxidant Activity. *Preparation of Solutions.* A 200 mM stock solution of DCFH-DA in methanol was prepared, aliquoted, and stored at −20 °C until use as described previously by our laboratory.[12−14] A 200 mM ABAP stock solution in water was prepared, aliquoted, and stored at −40 °C until use. Quercetin solutions were prepared in dimethyl sulfoxide before further dilution in treatment medium (WME with 2 mM L-glutamine and 10 mM Hepes).

CAA of Samples. The CAA was determined using the protocol described previously by our laboratory.[12−14] Briefly, HepG2 cells were seeded at a density of 6×10^4/well on a 96-well microplate in 100 μL of complete medium/well. Twenty-four hours after seeding, the growth medium was removed, and the wells were washed with 100 μL of PBS. Wells were then treated with 100 μL of treatment medium containing solvent control, control extracts, or tested extracts plus 25 μM DCFH-DA for 1 h. After 1 h, the media were removed, the cells washed with 100 μL of PBS, and 600 μM ABAP was applied to the cells in 100 μL of oxidant treatment medium (HBSS with 10 mM Hepes). The 96-well microplate was placed into a Fluoroskan Ascent FL plate reader at 37 °C. Emission at 538 nm was measured after excitation at 485 nm every 5 min for 1 h.

Quantification of CAA. After blank subtraction and subtraction of the initial fluorescence values, the area under the curve for fluorescence versus time was integrated to calculate the CAA value at each concentration of extract as follows:

$$\text{CAA unit} = 100 - \left(\int \text{SA} / \int \text{CA}\right) \times 100$$

where $\int$SA is the integrated area under the sample fluorescence versus time curve, and $\int$CA is the integrated area from the control curve. The median effective dose (EC_{50}) was determined for the sample extracts from the median effect plot of log(*fa*/*fu*) versus log(dose), where *fa* is the fraction affected (CAA unit), and *fu* is the fraction unaffected (1-CAA unit) by the treatment. The EC_{50} values were stated as the mean ± SD for triplicate sets of data obtained from the same experiment. EC_{50} values were converted to CAA values, which are expressed as micromoles of quercetin equivalents (QE) per 100 g of DW, using the mean EC_{50} value for quercetin from at least four separate experiments.

Statistical Analysis. Statistical analysis were conducted using SigmaPlot Version 2000 (Aspire Software International, Ashburn, VA). Results were subjected to ANOVA and differences between means were located using Tukey's multiple comparison test. Significance was determined at $p < 0.05$. All data were reported as the mean ± SD for three replications

RESULTS AND DISCUSSION

Total Phenolic Content. Free and bound phenolics, as for the phenolic contents in the bioaccessible and residue fractions, and percentage contribution to the total are shown in Table 2, expressed as milligrams of gallic acid equivalents per 100 g of sample on a dry weight basis.

Samples without digestion had 102.6 ± 13.4 mg of gallic acid equiv/100 g of DW for total phenolics, from which the free and bound phenolics were 81.2 ± 13.0 and 21.4 ± 1.4 mg of gallic acid equiv/100 g of DW, respectively. The contributions of free and bound fractions to the total phenolics were 79.1% and 20.9%, respectively, indicating the major phenolics of *feijoada* whole meal were from the free fraction.

Total phenolic content of the sample with digestion was 93.5 ± 1.6 mg of gallic acid equiv/100 g of DW. The phenolic contents of the bioaccessible fraction and residue fraction in the

Table 2. Total Phenolics, Total Flavonoids, and Antioxidant Activity of *Feijoada* Whole Meal with or without in Vitro Digestion[a]

	total phenolics (mg GAE/100 g DW)	total flavonoids (mg catechin/100 g DW)	ORAC (μmol Trolox/100 g DW)
	Without in Vitro Digestion		
free	81.2 ± 13.0 (79.1) ab	420.4 ± 27.9 (52.4) b	4187.1 ± 767.8 (71.3) ae
bound	21.4 ± 1.4 (20.9) c	376.9 ± 10.0 (47.6) b	1681.8 ± 92.5 (28.7) b
total	102.6 ± 13.4 a	797.3 ± 35.7 a	5869.0 ± 856.5 a
	With in Vitro Digestion		
bioaccessible	64.3 ± 1.3 (67.8) b	356.9 ± 7.1 (47.1) c	2392.1 ± 231.8 (76.8) c
residue	29.2 ± 2.9 (32.2) d	398.5 ± 31.9 (52.9) cb	722.3 ± 143.5 (23.2) d
total	93.5 ± 1.6 a	755.4 ± 25.7 a	3114 ± 369.8 e

[a]Values with no letters in common in each column are significantly different (p <0.05). Values in parentheses indicate percentage contribution to the total.

digested sample were 64.3 ± 1.3 and of the residue 29.2 ± 2.9 mg of gallic acid equiv/100 g of DW, respectively. The contributions to the total phenolics from the bioaccessible fraction and residue fraction were 67.8% and 32.2%, respectively. This was similar to the phenolic profiles in the sample without in vitro digestion in which the major phenolics were from the free fraction.

There was no difference between total phenolic contents from the samples without in vitro digestion and those with in vitro digestion (Table 2). The differences of phenolic contents between the bound fraction and residue fraction were probably due to the incomplete digestion with the in vitro digestion model, as suggested by Adom and Liu previously.[2] Bound phenolics or phenolics from the residue fraction are mainly in β-conjugation and can pass through the stomach and small intestine digestions to reach the colon[2] where those bioactive compounds can be released after the fermentation by colon bacteria.[3,4]

Phytochemicals of whole grains have been reported mainly in bound form 75% in wheat and 62% in rice.[2] Without analyzing the phytochemicals in bound fraction, the whole grain phytochemical were commonly underestimated previously.[2−4] For the *feijoada* whole meal, bound phenolics or phenolics from the residue fraction were 20.9% and 32.2%, respectively (Table 2). Therefore, bound phenolics and free phenolics of the *feijoada* whole meal should be included in the evaluation of its nutritional quality.

The *feijoada* whole meal is a ready-to-eat cooked meal, commonly consumed in Brazil. Food processing, including cooking, will facilitate the release of bound phytochemicals from the cell wall or fibers and other larger molecules.[24,25] This may partially explain why the *feijoada* whole meal has relatively higher amounts of free phenolics. *Feijoada* is also mainly composed of black beans which are rich in anthocyanins, commonly found in nonbound forms.[26]

Total Flavonoid Content. Flavonoid content in samples with or without in vitro digestion and the percentage contribution to the total flavonoids are shown in Table 2, expressed as milligrams of catechin equivalents per 100 g of sample on a dry weight basis. Samples without digestion had a total flavonoid content of 797.3 ± 35.7 mg of catechin equiv/100 g of DW, from which 420.4 ± 27.9 mg of flavonoids were for the free fraction and 376.9 ± 10.0 mg of flavonoids for the bound fraction. The total flavonoid content of the sample with digestion was 755.4 ± 25.7 mg of catechin equiv/100 g of DW. The flavonoid contents of the bioaccessible fraction and residue fraction in the digested sample were 356.9 ± 7.1 and 398.5 ± 31.9 mg of catechin equiv/100 g of DW, corresponding to 47.1% and 52.0% of total flavonoids, respectively.

Previous reports on total flavonoid content in food[27,28] used the aluminum chloride spectrophotometer assay described by Zhishen et al.[29] Actually, this assay cannot quantify total flavonoids because not all the subgroups of flavonoids reacted with aluminum chloride to yield color reaction at a visible range.[19] He and Liu developed a specific assay for total flavonoids which can detect all types of flavonoids including flavones, flavonols, flavonones, flavononols, isoflavonoids, flavanols, and anthocyanins.[19]

Phenolic Acids Analysis. Free phenolics were analyzed by HPLC, and the results are shown in Table 3. The main phenolic acids present in the ingredients which compose *feijoada* whole meal were caffeic and ferulic acids. Ferulic acid was present in all ingredients and was the main phenolic acid present in the *feijoada* whole meal. Cooked kale was the ingredient with the highest amount of ferulic acid (11.71 mg/100 g DW), cassava flour (0.504 mg/100 g DW) the second, and cooked rice the third (0.430 mg/100 g DW). The presence of ferulic acid in all ingredients of *feijoada* whole meal is expected since this phenolic acid is abundant in plant foods, being found in citrus, vegetables, and cereals. In addition, the presence of ferulic acid could represent beneficial consequences from the consumption of *feijoada* whole meal since it has been associated not only with a high antioxidant activity but with anticancer effect, cholesterol-lowering, and anti-inflammatory activity among others.[30] Caffeic acid was detected in all samples except for cooked rice and roasted cassava flour. For this particular phenolic acid, orange was the ingredient with the highest concentration 0.479 mg/100 g DW. However, since orange corresponds to only 6.1% of the whole meal, *feijoada* caffeic acid content was much lower: 0.018 mg/100 g DW.

Table 3. Phenolic Acids in *Feijoada* Cooked Ingredients and Whole Meal[a]

	gallic acid	protocatechuic acid	chlorogenic acid	caffeic acid	ferulic acid	cinnamic acid
feijoada	nd	nd	nd	0.018	0.225	nd
cooked bean	nd	nd	nd	0.018	0.207	nd
cooked rice	nd	nd	nd	nd	0.430	nd
toasted cassava flour	nd	nd	nd	nd	0.504	nd
cooked kale	nd	nd	nd	0.264	11.710	nd
orange	nd	nd	nd	0.479	0.373	nd

[a]Values expressed as mg/100 g dry weight. nd: values were not detected.

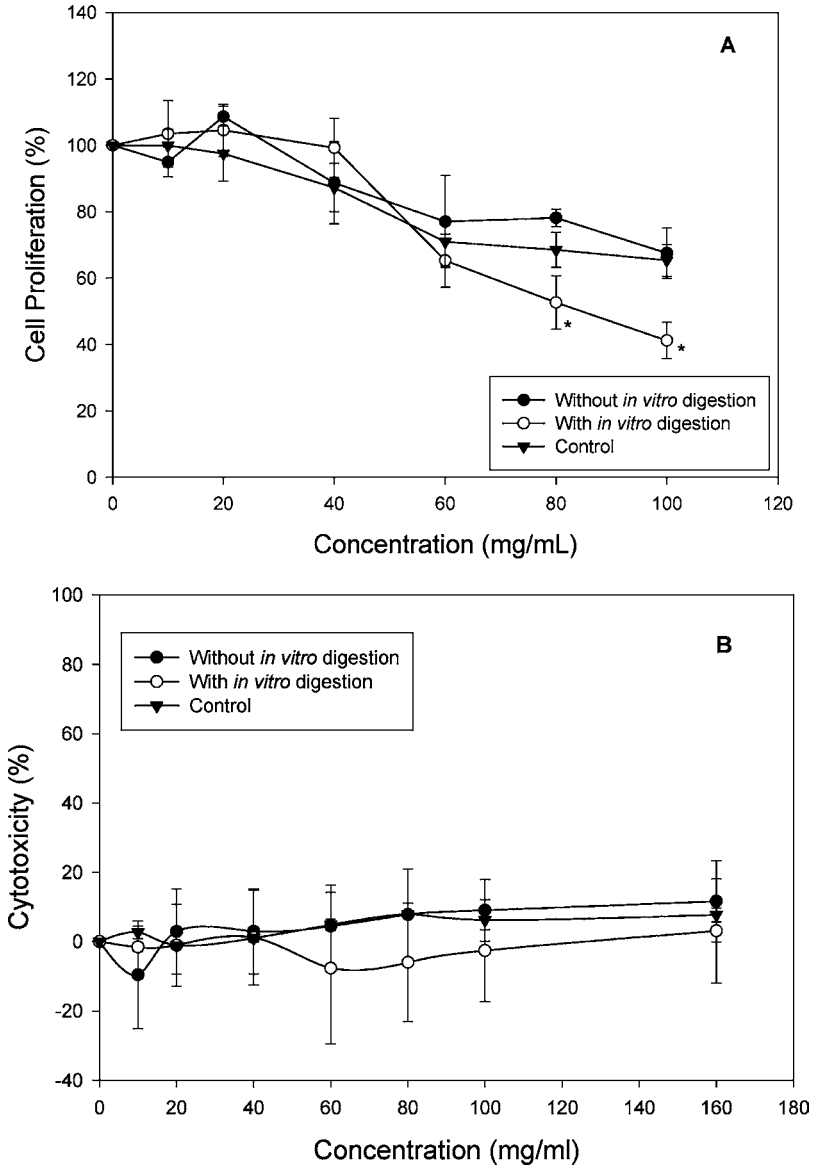

Figure 2. (A) Antiproliferative activity and (B) cytotoxicity against human $HepG_2$ liver cancer cells by *feijoada* whole meal extracts with or without digestion (mean ± SD, $n = 3$). Values marked with * are significantly different compared to the control ($p < 0.05$).

Total Antioxidant Activity. The total antioxidant activities, measured by ORAC, of *feijoada* whole meal extracts with and without digestion and percentage contribution to the total are shown in Table 2, expressed as μmol Trolox equiv/100 g dry weight basis. Total antioxidant capacity of *feijoada* whole meal without digestion, 5869.0 ± 856.5 μmol Trolox/100 g DW, was significantly higher ($p < 0.05$) than that of *feijoada* whole meal with digestion, 3114 ± 369.8 μmol Trolox/100 g DW. For *feijoada* whole meal without digestion, free phenolics fraction, 4187.1 ± 767.8, contributed to 71.3% of total antioxidant capacity, and bound phenolics fraction, 1681.8 ± 92.5, contributed to 28.7% of total antioxidant capacity (Table 2).

Despite the lower total antioxidant capacity for *feijoada* whole meal with digestion, compared to the sample without digestion, the percentage contributions of bioaccessible and residue fractions were similar to those of free and bound fractions. Bioaccessible, 2392.1 ± 231.8 μmol Trolox equiv/100 g DW, and residue fraction, 722.3 ± 143.5 μmol Trolox equiv/100 g DW, contributions to total antioxidant capacity of *feijoada* whole meal with digestion were 76.8% and 23.2%, respectively (Table 2). Contributions of approximately 20% to 30% of total antioxidant activity of both bound and residue fractions are physiologically important, as those fractions can pass through stomach and small intestines reaching the colon, where they can have beneficial antioxidant activity in situ.

Total antioxidant activity of *feijoada* whole meal with and without digestion were similar to those described previously for vegetables, such as spinach, with 2605 μmol Trolox equiv/100 g.[13] It is interesting to point out that the total antioxidant activity values of *feijoada* whole meal, with and without digestion, were similar to those of raw vegetables, even though it is a processed, ready-to-eat, meal. Previously, Dewanto et al.[24,25] showed that processing can increase the total antioxidant capacity of food.

Antiproliferative Activity. The antiproliferative and cytotoxic activities human $HepG_2$ liver cancer cells by *feijoada* whole meal extracts with or without digestion are shown in Figure 2. The extract of *feijoada* whole meal with in vitro digestion significantly inhibited the proliferation of human

Table 4. Cellular Antioxidant Activity and Cellular Antioxidant Quality of *Feijoada* Whole Meal with and without Digestion[a]

	EC_{50} (mg/mL)	CAA (μmol QE/100 g DW)	CAA (μmol QE/100 μmol phenolics)	cytotoxicity (mg/mL)
without digestion	173.1 ± 36.2	2.1 ± 1.0	0.2 ± 0.1	>160
with digestion	104.7 ± 5.4*	4.4 ± 1.2*	0.6 ± 0.2*	>160

[a]Values marked with * in each column are significantly different ($p < 0.05$).

$HepG_2$ liver cancer cells at concentrations above 80 mg/mL when compared to the control ($p < 0.05$) (Figure 2A). However, there were no differences observed using the extract without digestion when compared to the control ($p < 0.05$) (Figure 2A). This is indicates that the extract with the in vitro digestion yielded more bioactive compounds than the extract without digestion. There was no significant cytotoxicity for both *feijoada* whole meal extract with or without digestion at concentrations up to 160 mg/mL (Figure 2B). This suggested that the antiproliferative activity was not caused by the cytotoxicity.[22]

The EC_{50} of *feijoada* whole meal with digestion against human $HepG_2$ liver cell growth was 87.3 mg/mL, which was higher than that of fruits like cranberry (14.5), apple (49.3), and red grapes (71.0) but still lower than banana (110.1), grapefruit (130.0), and peach (156.2).[9] The higher the EC_{50}, the lower is the antiproliferative activity.

Cellular Antioxidant Activity. The cellular antioxidant activities of the *feijoada* whole meal extract with and without in vitro digestion were measured using the CAA assay. The EC_{50} and CAA values are listed in Table 4. The EC_{50} values of *feijoada* whole meal extract without digestion and with digestion were 173.1 ± 36.2 mg/mL and 104.7 ± 5.4 mg/mL, respectively. The CAA values of *feijoada* whole meal extract with digestion, 4.4 ± 1.2 μmol QE/100 g DW, was significantly higher ($p < 0.05$) than that of the extract without digestion, 2.1 ± 1.0 μmol QE/100 g DW (Table 4).

To compare the quality of the phenolic compounds, CAA values were expressed as μmol QE/100 μmol phenolics. The quality of *feijoada* whole meal extract with *in vitro* digestion was much higher than that of extract without digestion ($p < 0.05$) (Table 4). There was no cytotoxicity observed at the concentrations tested for CAA values (Table 4). It is interesting to point out that despite similar values for phenolic and flavonoid content, and a higher antioxidant activity for the nondigested sample, measured as ORAC, the cellular antioxidant activity and the quality of the cellular antioxidant activity were significantly higher in the sample extract with in vitro digestion when compared to the extract without digestion (Table 4).

Cellular antioxidant activity assay has been considered as a more physiologically relevant assay in the measurement of antioxidant activity of food when compared to the common chemistry antioxidant activity assays.[12,31] Until today, there have been no reports on the use of the CAA assay on whole meal. Although the meal tested consisted mainly of black beans and rice, 63.6% and 18.9%, respectively, CAA values obtained here were compared to those of vegetables. The digested *feijoada* whole meal resulted in a CAA value of 4.4 μmol QE/100 g DW, which was similar to those reported for aspargus (4.35), red pepper (4.64), and eggplant (4.35).[13] For the *feijoada* whole meal without digestion, the CAA value of 2.1 μmol QE/100 g DW was similar to those reported for chilli pepper (1.81), sweet corn (1.82), and Brussel sprout (1.82).[13] Since the *feijoada* whole meal mainly contains cereals and vegetables, the CAA values of the sample were lower than those reported for fruits, which vary from 3.68 to 163 μmol QE/100 g fruit for lemons and pomegranate, respectively.[14]

However, the CAA values of the tested meal being similar to those observed for some raw vegetable is important since we are considering the meal cooked and in its ready-to-eat form. In addition, the CAA value of the sample with the in vitro simulated digestion was increased two times higher than that of the samples without digestion.

Consumption of a diet rich in plant foods is known to reduce the risk of chronic diseases.[1] *Feijoada* whole meal is a traditional Brazilian meal composed of a mixture of grains and vegetables. *Feijoada* whole meal with or without digestion showed no significant difference for total phenolics and total flavonoids. For both *feijoada* whole meal extracts, with and without digestion, flavonoid contribution to total flavonoids were approximately 50% for free and bound fractions for bioaccessible and residue fractions.

Total antioxidant activity, measured by ORAC, was higher for *feijoada* whole meal without digestion compared to extracts with digestion. However, *feijoada* whole meal with digestion showed higher antiproliferative activity and higher CAA values. The application of in vitro digestion seems to yield higher phenolics quality resulting in higher bioactivity in the cellular antioxidant assay. *Feijoada* whole meal is an already cooked and ready-to-eat meal; however, its ORAC and CAA values were similar to that of raw vegetables, suggesting that processing can result in beneficial modification in both bioactive compound contents and antioxidant activity values.[24,25]

Ready-to-eat meals composed of a variety of plant foods, such as *feijoada* whole meal, can provide a variety of bioactive compounds such as phenolic compounds and flavonoids, and exhibit high antioxidant activity. More studies using whole meals instead of individual foods are needed in order to identify the actual amount of bioactive compounds consumed during a meal and in a population dietary pattern. In addition, the use of in vitro digestion coupled with cellular antioxidant activity can be used to obtain more physiological information on the health effects of bioactive compounds found in food, being a quick screening technique for following bioavailability studies.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

ABAP, 2,2-azobis(2-amidinopropane) dihydrochloride; $AlCl_3$, alunminiun chloride; BHT, butylated hydroxytoluene; CAA, cellular antioxidant activity; CE, catechin equivalents; DCFH, 20,70-dichlorofluorescin; DCFH-DA, 20,70-dichlorofluorescin diacetate; DW, dry weight; EtOH, ethanol; FW, fresh weight; GAE, gallic acid equivalents; MeOH, methanol; $NaBH_4$, sodium borohydride; ORAC, oxygen radical absorbance

capacity; QE, quercetin equivalents; TE, Trolox equivalents; THF, tetrahydrofuran.

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