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Extraction of protein concentrate from red bean (*Phaseolus vulgaris* L.): antioxidant activity and inhibition of lipid peroxidation

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ABSTRACT

Red Bean Protein Concentrate (RBPC) and their hydrolysates were used to evaluate the antioxidant capacity. The RBPC protein content was in the range of 57.38%–72.68% of the total sample content. RBPC protein profile showed a range of 15–100 kDa. Phaseolin protein was identified with bands of 45 and 50 kDa. Phaseolin protein was found in all the RBPC samples at the different pHs assayed. In the gastric digestion phase, bands from 60 to 100 kDa were totally hydrolyzed with pepsin. Phaseolin protein (45 and 50 kDa) presented resistance to gastric hydrolysis. All the RBPCs and gastrointestinal digest presented antioxidant activity using ferric-reducing antioxidant power (FRAP), 2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS), oxygen radical absorbance capacity (ORAC), and thiobarbituric acid reactive substances using the *in vitro* and *in vivo* methods. RBPC at pH 7.0 presented a value of 95.80 µmoL TE/g of RBPC (FRAP); 257.12 µmoL TE/g of RBPC (ABTS), and 1960 µmoL TE/g of RBPC (ORAC). Duodenal digest of RBPC presented high antioxidant activity with 225.77 µmoL TE/g of digest (FRAP); 345.21 µmoL TE/g of digest (ABTS); and 3256 µmoL TE/g of digest (ORAC). Gastric and duodenal digest of RBPC were used to inhibit lipid peroxidation using the *in vitro* method presenting a value of 87.95% and 93.0%, respectively. When the *in vivo* method in zebrafish larvae was used, values were 79.03% and 86.76%, respectively. RBPCs showed no reactive oxygen species (ROS) inhibition. However, RBPCs with gastric and gastrointestinal digests, presented ROS inhibition, 75.30% for gastric digests and 66.40% for gastrointestinal digests.

INTRODUCTION

Legumes are important in the human nutrition for their bio components, such as proteins, carbohydrates, fiber, minerals, and lipids. Legumes proteins have a high percentage of lysin amino acids and can complement the proteins from cereals which are deficient in lysin amino acids. In human nutrition, different beans are used for their high nutritional and biological properties. We can mention the beans, such as *Phaseolus vulgaris, Cajanus cajan*, *Lens culinaris*, *Pisum sativum*, and *Cicer arietinum*, which are the important source of protein in the under-developed countries (Achouri and Boye, 2013; Achouri *et al*., 2012; Boye *et al*., 2010; Carbonaro *et al*., 2015; Foschia, 2016; Tosh and Yada, 2010). Legumes proteins can be used in the production of Protein Concentrates (PC) and Protein Isolates (PI). Proteins used in the food industry can be of animal or vegetal origin (e.g., milk and eggs proteins, soybean, lupines, quinoa, amaranth, and bean proteins) (Rodríguez Saint Jean *et al*., 2013; Boye *et al*., 2016; Toapanta *et al*., 2016; Acosta *et al*., 2016; Vilcacundo *et al*., 2018a; Carrillo *et al*., 2017b).

The oxidative stress is defined as a disequilibrium in the production of harmful substances in the organism and the production of antioxidant substances. The oxidative stress is recognized as an important cause of a variety of degenerative diseases, such as Parkinson disease and arthritis. Common free

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radicals produced in the body are oxygen based and termed as reactive oxygen substances. They include different radicals, such as superoxide, hydroxyl, and peroxyl. Many exogenous molecules, that the body requires, help to keep the redox balance and include ascorbic acid, tocopherols, tocotrienols, polyphenols, carotenoids, proteins, and peptides. These components are abundantly found in fruits, vegetables, and legumes. In the past years, food proteins and peptides have been a research subject for their antioxidant activity and the evaluation of ROS inhibition (Carrillo *et al*., 2017a; Galadari *et al*., 2017; Vilcacundo *et al*., 2017).

Phaseolus vulgaris L. belongs to the Fabaceae family. It is an important crop in legume grains with a high protein content, with an important consumption in different places, such as South America, Central America, and Africa. In 2011, the world production of these beans was more than 20 million tons, the area dedicated to this crop was around 30 million hectares (Luna-Vital *et al*., 2015). *Phaseolus vulgaris* seeds have an important nutritional and biological value in the human diet. *Phaseolus vulgaris* has a protein content ranging from 16% to 33%, a big fraction represented by the storage protein phaseolin (30% to 50%) and lectins (10% to 12%) (Boschin *et al*., 2014; García-Mora *et al*., 2015, Torres *et al*., 2016). Phaseolin contains trimeric proteins that belong to the 7S vicilin class. Different authors have described antihypertensive, antitumoral, antifungal, and antioxidant activities of hydrolysates obtained from *P. vulgaris* L. (Akıllıoğlu and Karakaya, 2009; Lin and Lai, 2006; Mamilla and Mushra, 2017; Pazmiño *et al*., 2018).

Lipid oxidation is an important issue for the food industry because many processed products contain fats of animal and vegetable origin in their formulations. Fats produce food spoilage. It is known that linoleic acid is prone at process of oxidation during the storage of processed food (Barden and Decker, 2016). Thiobarbituric acid reactive substances (TBARS) *in vitro* method can serve as screening to select antioxidant samples to be evaluated in an *in vivo* model that allows to understand the mechanism of action and the implication of the reduction of reactive substances (ROS).

Zebrafish (*Danio rerio*) is an emerging animal model with many uses in medicine, pharmacy, molecular biology, and biotechnology and recently in food science as its genomic expression for certain diseases is similar to humans. It is an easy-to-use, low-cost, and fast-growing animal model with few ethical restrictions for laboratory management (Sprague *et al*., 2006). Zebrafish is a model that allows to evaluate the inhibition of ROS substances and the inhibition of TBARS lipid peroxidation using zebrafish embryos and larvae. At the same time, it allows to evaluate the toxicity of the molecules studied. Rat and mouse animal models are used to evaluate TBARS and ROS inhibition but present the disadvantages of the ethical restrictions, high cost, and sacrifice of the animals used. In the zebrafish model, five-day post-fertilization larvae are used.

The aim of this research was to produce Red Bean Protein Concentrate, RBPC, from *P. vulgaris* and evaluate the digestibility using standardized *in vitro* digestion methods (Minekus *et al*., 2014). The antioxidant capacity and inhibition of lipid peroxidation *in vitro* and *in vivo* (zebrafish) were also evaluated.

MATERIAL AND METHODS

Isolation of red bean protein concentrate (RBPC)

Phaseolus vulgaris L. seeds were obtained from a germplasm bank at the State Bolivar University, campus Alpacha (Guaranda-Ecuador). RBPC was prepared according to Poveda *et al*. (2016). The defatted flour was suspended in water (1:10, *w:v*) at pH 8.0. The suspension was centrifuged at 4500 × g during 30 minutes. The precipitate was removed, and the pH of the solution was adjusted at pH (3.0, 4.0, 5.0, 6.0, and 7.0). Finally, the pH of the precipitate was neutralized and lyophilized. It was kept frozen until its use. The RBPC protein content was determined using the Dumas method (Serrano *et al*., 2013). Moisture, lipids, total fiber, soluble solids, and ash of RBPCs were determined according to AOAC 2012 using the methods: 950.10, 930.09, 985.29, 923.09, and 942.05. The carbohydrate content was also determined using the method described by AOAC (2012).

RBPC *in vitro* gastrointestinal digestion

The *in vitro* gastrointestinal simulation was made according to Minekus *et al*. (2014) with minor changes for this study. The oral phase was not considered in this study. RBPC (5.0 mg/ml) was subject to a gastric phase digestion at pH 3.0 using pepsin enzyme which was added to 2,000 U/ml at 37°C for 2 hours. Then, the pH was adjusted at pH 7.0 for the intestinal phase and the pancreatin enzyme was used. The percentage of hydrolysis degree (%DH) of RBPC hydrolyzed protein was determined according to Adler-Nissen (1979). Gastric and duodenal digests were fractionated using the ultrafiltration method with a hydrophilic cutoff membrane using Vivaspin 500 (GE Healthcare, Little Chalfont, UK). Fractions with molecular weight lower than 3 and 10 kDa were lyophilized and stored at −20°C. The protein content of samples was determined using the Lowry protocol.

RBPC characterization and RBPC digests by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE electrophoresis of all the samples was made in a concentration of 12% polyacrylamide solution in a Mini-Protean electrophoresis system (Bio-Rad, Hercules, CA, USA). The standard proteins (10–250 kDa) were used. Gels were stained for 12 hours with a Coomassie Blue G-250 solution (Cardenas *et al*., 2018).

RBPC 2-DE electrophoresis analysis

Nearly, 50 µg of sample were dissolved in lysis and rehydration buffers. Samples were loaded on Ready Strip IPG Strips with a pH (3.0−10.0), using 0.6% dithiothreitol and 1% IPG buffer (Bio-Rad). The first-dimensional isoelectric focusing (IEF) was made in a PROTEAN IEF cell (Bio-Rad, Hercules, CA). Strips were submerged in the equilibration solution with 1% dithiothreitol for 15 minutes at 25°C and then was added 2.5% iodoacetamide to the solution. 2DE (SDS-PAGE), equilibrated strips were loaded on 10% (w:v) gels, and were run vertically in a PROTEAN system (Bio-Rad) (Quinteros *et al*., 2016).

RBPCs RP-UHPLC analysis and RBPC digests

RBPCs and their digest were analyzed using the RP-UHPLC methods, (Agilent 1200 infinity, Agilent Technologies, Waldbron, Germany). The detector uses a wavelength of 214 nm.

The separation was made using the column (Zorbax EC C18, Agilent Poroshell 120,). The samples were eluted using the lineal gradient method. Samples were eluted at 1.0 ml/minute using a lineal gradient of 0% to 70% of organic solvent (Lara *et al*., 2017). Trifluoroacetic acid was added to the solution to improve the segregation of proteins.

Fourier transform infrared spectroscopy (FTIR)

RBPCs and gastric and gastrointestinal samples were analyzed using the FTIR spectrometer method (PerkinElmer, FT-IR spectrometer Frontier, UK). All the spectrums were obtained by comparison between 32 scans at 4 cm^{-1} from 4,000 to 650 cm^{-1} (Zhao *et al*., 2008). The spectrum data were analyzed using the PerkinElmer Spectrum software (Version 10.4, UK). All the assays were made twice.

Extraction of polyphenols from RBPC

The extraction of polyphenols from RBPC was carried out according to Hue *et al*. (2014). Nearly, 0.3 g of RBPC lyophilized was added to 5 ml of solution with 70% of methanol, 30% H_2O, and 0.1% of formic acid *v/v*. This step of the extraction was made four times. Samples were shaken (Mist10ral Multi-Mixer; Melrose Park, USA) for 5 minutes, followed by an ultrasound treatment (Cole-Parmer model 8892; Chicago, USA) for 10 minutes. Then, the samples were centrifuged at 5,700 rpm (Damon EC DIVISION; USA) for 10 minutes. The extract of each cycle was collected in 25 ml flasks with a methanol solution.

Total polyphenols content (TPC) from RBPC

RBPC samples TPC were calculated according to the Singleton and Rossi (1965) method using small adjustments. One milliliter of Follin solution was added.to 1 ml of sample work. At minute three, 1 ml of Na_2CO_3 solution was added. The reaction was made in dark conditions for 90 minutes. Then, the absorbance was measured at 725 nm (Thermo Scientific Evolution 200). Gallic acid was used to make the standard curve at concentrations of 0–0.075 mg/ml. The results were represented as mg of gallic acid equivalents (GAE)/100g sample.

ABTS analysis

ABTS method was used according to Arnao *et al*. (2001) using a 7.4 mM ABTS solution and a 2.6 mM K_2SO_8 solution. Ten milliliter of ABTS solution was mixed with 10 ml of K_2SO_8 solution for 12 hours at 25°C in dark conditions. This solution was diluted by mixing 1 ml with 50 ml of methanol to obtain an absorbance of 1.1 at 734 nm (Thermo Fisher Scientific Evolution 200 UV/Vis, Waltham, MA USA). Nearly, 150 µl of sample were mixed with 2,850 µl of ABTS solution and the mixture was kept at 25°C for 2 hours in dark conditions. The blank was prepared in the same form, but methanol was replaced with ABTS. Trolox standard was made with a standard curve. The antioxidant activity was expressed as µmoL Trolox equivalents (TE)/g sample.

Oxygen radical absorbance capacity-fluorescein (ORAC-FL) assay

The worked solution was of 200 ml and had fluorescein (FL) (70 nM), 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) 14 mM, and antioxidant Trolox (0.2-1.6 nmoL). The fluorescence emitted was read for 137 minutes using a FLUOstar OPTIMA plate reader (BMG Labtech, Offenburg, Germany). The assay was controlled using the FLUOstar Control software (version 1.32 R2). Black 96-well microplates (Nunc, Denmark) were used. All the experiments were prepared in three times. ORAC-FL values were represented as µmoL of Trolox equivalent (TE)/g sample (Vilcacundo *et al*., 2018b).

Ferric-reducing antioxidant power assay (FRAP)

One milliliter of samples was diluted (1:2 and 1:4) and then mixed with 2.5 ml of buffer phosphate (pH 6.6) and 2.5 ml of the potassium ferrocyanide solution 1% (w:v). The solution was heated at 50°C for 20 minutes. Then, 2.5 ml of trichloroacetic acid at 10% (w:v), 2.5 ml distilled water and 0.5 ml of ferric chloride at 1% (w:v), were added. The solution was kept for 30 minutes at 25°C (Benzie and Strain, 1996).

The absorbance was measured at 700 nm by UV-VIS spectrophotometry (Shimadzu Spectrophotometer model 2600, Kyoto, Japan) and Trolox standard was used to standard the curve. The data obtained were expressed as µmoL Trolox Equivalents (TE)/g sample.

DPPH assay

RBPCs, hydrolysates and fractions were used to evaluate their antioxidant activity with the DPPH method. The ability to capture free radicals by antioxidants was analyzed using the radical species DPPH according to Brand-Williams *et al*. (1995), measuring the decrease of absorbance at 517 nm spectrophotometrically (SP-2100UV/SP spectrophotometer, China). Each assay was made in triplicate with the value of activity represented as mg of Trolox equivalents (TE)/100 g sample.

***In vitro* thiobarbituric acid reactive substances (TBARS)**

RBPCs and digest were used to calculate % TBARS. Nearly, 0.5 g of sacha inchi oil was oxidized by heating. Samples (2.0 mg/ml) were added in the oil and were heated at 30°C for 48 hours. Butylhydroxytoluene (BHT) was used as a positive control. One milliliter of sample was mixed with 1 ml of the 1% thiobarbituric acid (TBA) solution. The solution was heated at 95°C for 1 hour. The absorbance was measured at 532 nm (Thermo Scientific Evolution 200). % TBARS was represented as % TBARS = $A_s/A_b \times 100$, where A_b is the absorbance of blank and A_s is the absorbance of the sample (Carrillo *et al*., 2016b).

***In vivo* TBARS evaluation in zebrafish**

Thirty larvae of zebrafish were incubated in 24-well plates with the samples. Lipid peroxidation was started with 1 ml of 1.5% ethanol for 8 hours at 28°C. Then, 500 µl of Tween 0.1% was added. Larvae were homogenized (T25 basic Ultra Turrax IKA, Thermo Fisher Scientific, Germany). One milliliter of 1% TBA was added and the solution was heated at 95°C for 1 hour. Absorbance of the solution was measured at 532 nm (Thermo Scientific Evolution 200, Germany). % TBARS were expressed as % TBARS= $[1 - (A_b - A_s)/A_b \times 100]$, where A_b is the absorbance of blank and A_s is the absorbance of the sample (Carrillo *et al*., 2016a).

Incubation of zebrafish embryos with AAPH reactive

Then, 7–9 hours post-fertilization (7–9 hpf), embryos (group = six embryos) were transferred to a 12-well plate and submerged in an osmotic embryo medium E2 1X (15 mM NaCl, 0.5 mM KCl, 1.0 mM $CaCl_2$ $2H_2O$, 50 µM Na_2HPO_4, 150 µM KH_2PO_4, 10 mM $MgSO_4$ $7H_2O$, 0.7 mM $NaHCO_3$, and 0.5 mg/l of methylene blue dissolved in distillated water) containing 1 ml of vehicle (0.1% DMSO) with samples for 2 hours. After embryos were treated with 25 mM AAPH or treated with AAPH plus samples for up to 24-hour post-fertilization (24 hpf) (Cunliffe, 2003).

Evaluation of ROS formation in zebrafish embryos

Formation of ROS in zebrafish embryos was analyzed using an fluorescent assay 2,7-dichlorofluorescein diacetate (DCFH-DA). DCFH-DA is changed intracellularly in high fluorescent compound dichlorflouorescein (DCF) (Rosenkranz *et al*., 1992). The embryos were treated with 0.1% dimethyl sulfoxide (DMSO) to permeabilize the chorion of zebrafish eggs. At 3–4 hpf, the embryos were incubated with the samples. Two hours later, 25 mM AAPH was added and was incubated for 24 hours. The embryos were transferred into 96-well plates and treated with a DCFH-DA solution (2.0 µl/ml). The plates were incubated for 2 hours in the dark at 28.5°C. Then, the chorion was removed with the help of tweezers. The image of stained embryos was registered using a fluorescent microscope (Leica DM1000 LED, Wetzlar, Germany), equipped with a camera Moticam 2000 (Taiwan, China).

Statistical analysis

Results are expressed as means ± standard deviation of five replicates for assay. The differences were analyzed using ANOVA one-way followed by the Tukey test. All the results were considered statistically significant at $p < 0.05$ using the software GraphPad Prism 4.

RESULTS AND DISCUSSION

% of RBPC yield and % of RBPC protein content

In this study, RBPC from *P. vulgaris* L. cultivated in Ecuador was obtained. The protein concentrates from red bean were obtained by the alkaline method (pH 8.0) and isoelectric precipitation (pH 3.0–pH 7.0). At a pH 4.0 of precipitation in the RBPC isolation, a 14.91% yield was obtained, this was the highest value, followed by the RBPC obtained at pH 5.0 with a yield value of 13.69%. These pH values are near the isoelectric point of the proteins (pI 4.5). This is the reason that explains high yields.

RBPC protein content was determined using the Dumas method (Table 1). Red bean flour presents a 23.71% protein content. All the protein concentrates presented higher protein contents than the red bean flour. RBCP at pH 7.0 present a higher value with 72.68% and the lower value was for the RBCP at pH 5.0 with a value of 57.38% of protein content. These samples present statistical differences ($p < 0.05$). The presence of other components in RBPCs was also determined. For example, RBPC at pH 7.0 present 72.68% of protein, 0.84% of lipids, 0.86% of fiber, 5.5% of ash, 3.20% of moisture, 1.23% of soluble solids, and 15.69% of carbohydrates.

Characterization of RBPC by RP-UHPLC, SDS-PAGE, and 2DE electrophoresis analysis

RBPCs were analyzed using a RP-UHPLC (Fig. 1). All the RBCPs present similar profiles of peaks observed in the chromatogram. This indicates that proteins obtained at the pHs used were the same proteins. These peaks were named P1–P3. P1 and P3 peaks were the main fractions present in the protein concentrates. P3 from RBPC present a higher intensity of absorbance, this indicates that this fraction is very soluble at a neutral pH (pH 7.0).

RBPCs were also analyzed using the SDS-PAGE electrophoresis (Fig. 2). In the gel, it can be observed that the proteins profile obtained is the same at all the pHs assayed in this study. Bands were observed from 15 to 100 kDa molecular weights. Bands with 45 and 50 kDa were the ones with the highest intensity in all the pHs assayed. The intensity increases when pH increases, at pH 7.0 bands of 45 and 50 kDa are very intense, showing a correlation with the high percentage of protein content (72.68%) determined with the Dumas method. The bands (45 and 50 kDa) were identified as vicilin (globulin fraction). Also, three bands were identified with high molecular weights of 60, 80, and 100 kDa approximately. Other three bands were identified with mass of 15, 20, 25, and 30 kDa, these bands can be lectin proteins from *P. vulgaris*.

RBPC at pH 7.0 was analyzed using the 2-DE electrophoresis method. Figure 3 shows a RBPC proteins profile at pH 7.0. This profile shows two bands with high intensity and molecular weights between 50 and 60 kDa. Two bands show 26 spots in the profile, these bands can be phaseolin polypeptides. Lopez-Pedrouso *et al*. (2014) reported a protein profile of common beans from MesoAmerican and the Andean regions using the 2-DE electrophoresis, with two bands with mass corresponding to 40 to 60 kDa, these bands were identified as phaseolin subunits. They reported variations in the number of spots present in the varieties (Lopez-Pedrouso *et al*., 2014).

Montoya *et al*. (2008) and (2010) reported the protein profile of different variety of Phaseolus. They reported high content of globulins and reported 2–6 bands between 40 kDa and 54 kDa, they identified these bands as Vicilin (7S globulin). Carrasco-Castilla *et al*. (2012) reported protein profiles of *P. vulgaris*. They identified 10 protein bands with mass ranging from 15 to 200 kDa in the sample. The 41 and 46 kDa bands correspond to the phaseolin subunits and the most abundant proteins. The bands of 15 kDa, 18 kDa, 25 kDa and 32 kDa were identified as lectin familiy proteins.

Table 1. Proteins content of flour and concentrates from red bean. % Yield of RBPC.

Sample	(%) Protein ± SD	% Yield RBPC
RB Flour	23.71 ± 0.13^a	
RBPC pH 3.0	64.47 ± 0.01^b	12.91^b
RBPC pH 4.0	65.53 ± 1.90^c	14.43^d
RBPC pH 5.0	57.38 ± 0.00^d	13.69^c
RBPC pH 6.0	65.00 ± 0.20^c	8.66^a
RBPC pH 7.0	72.68 ± 0.10^c	12.70^b

Data analyzed by one-way ANOVA and followed by Tuckey´s test. Different letter represents significant differences between sample as $p < 0.05$ ($n = 5$).

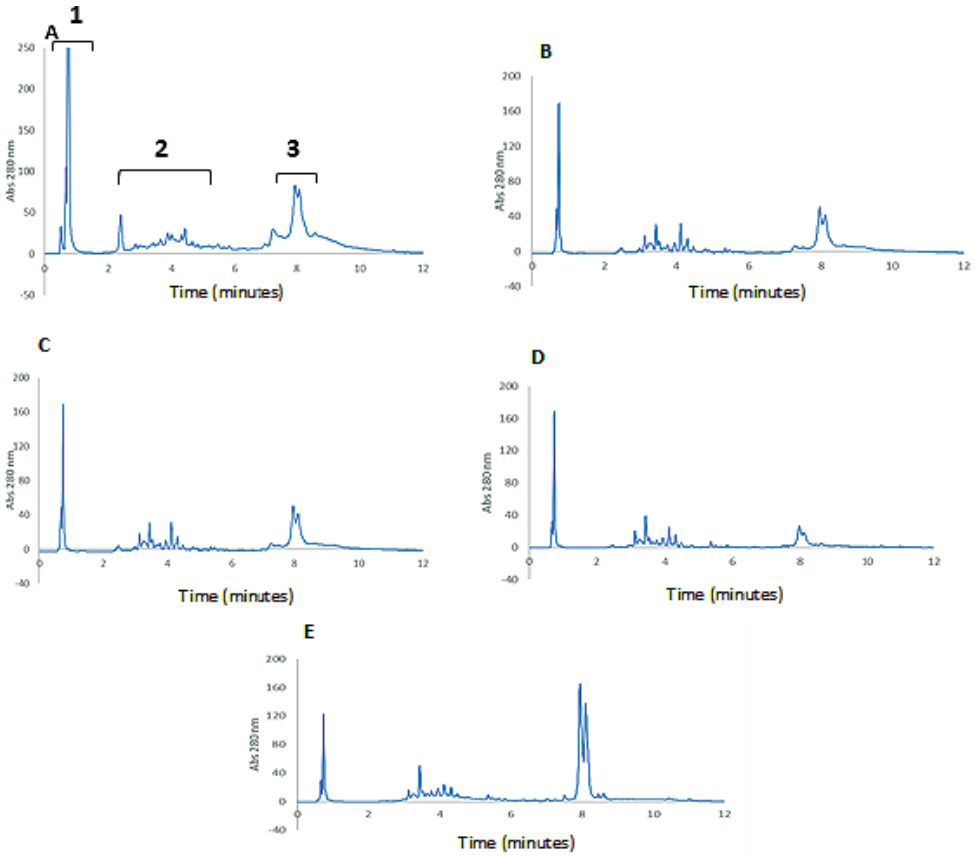

Figure 1. RP-UHPLC analysis of red bean concentrate (RBPC). (A) RBPC at pH 3.0, (B) RBPC at pH 4.0, (C) RBPC at pH 5.0, (D) RBPC at pH 6.0, and (E) RBPC at pH 7.0.

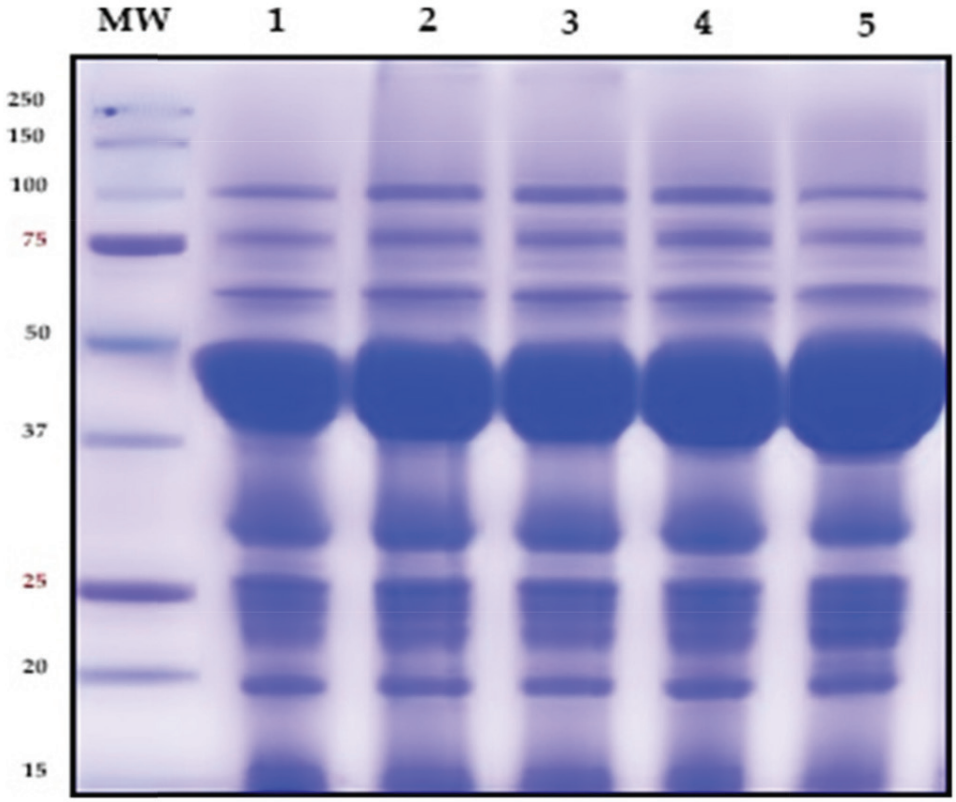

Figure 2. SDS-PAGE electrophoresis analysis of RBPC. Lane 1: RBPC at pH 3.0, lane 2: RBPC at pH 4.0, lane 3: RBPC at pH 5.0, lane 4: RBPC at pH 6.0, and lane 5: RBPC at pH 7.0. MW (molecular weight standard).

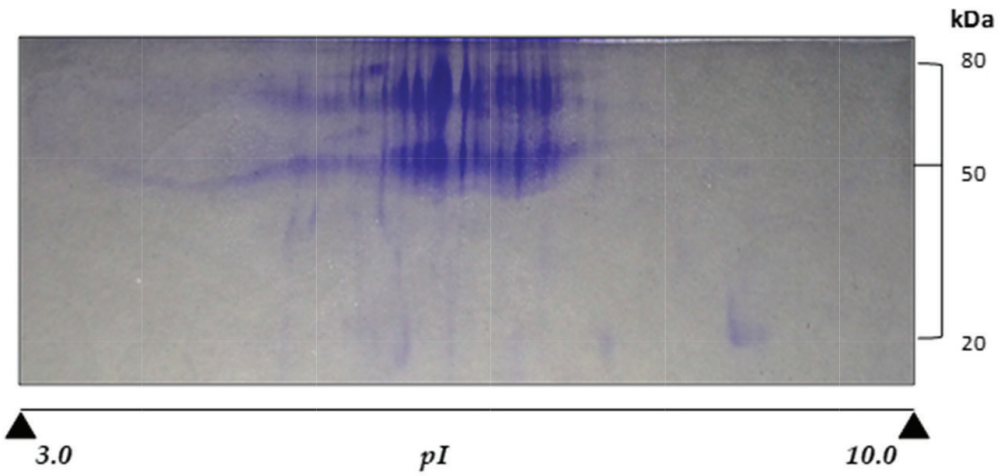

Figure 3. 2-DE electrophoresis analysis of RBPC at pH 5.0. Positions of spots according at their isoelectric point (pI) and molecular mass (Mr) are shown.

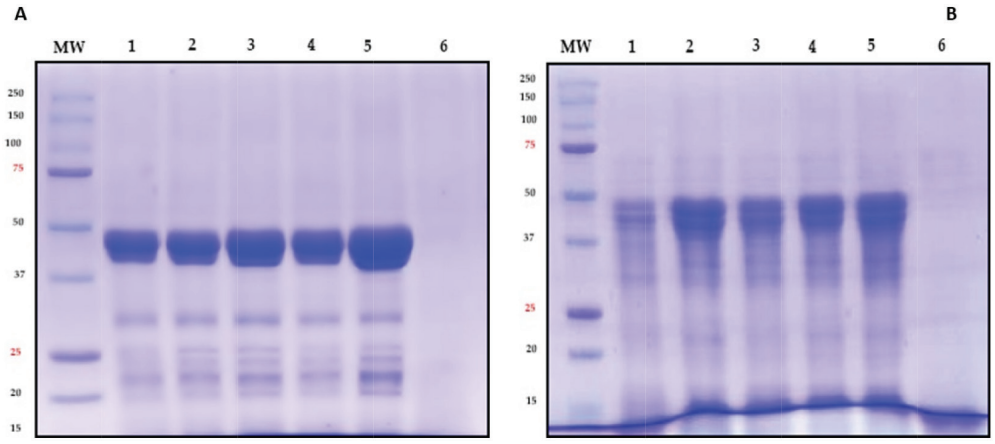

Figure 4. SDS-PAGE electrophoresis analysis of RBPC under simulated gastrointestinal digestion. A) gastric digest and B) duodenal digest. Lane 1: RBPC at pH 3.0, lane 2: RBPC at pH 4.0, lane 3: RBPC at pH 5.0, lane 4: RBPC at pH 6.0, and lane 5: RBPC at pH 7.0. MW (molecular weight standard).

García-Mora *et al*. (2015) reported a protein profile from *P. vulgaris* L. var. pinto, with bands between 10 and 97 kDa. Bands with molecular weights of 25 kDa, 45 kDa, and 50 kDa were identified as phaseolin. Phytohemagglutinins (32 kDa), α-amylase inhibitor (18 kDa), and α-amylase β subunit (15 kDa) were identified in the pinto bean protein concentrate. Our RBPCs protein profiles, from to 100 kDa, are similar to the ones reported by these authors, phaseolin was identified with the same molecular weight 45 and 50 KDa.

Hall *et al*. (1999) reported three isolated bands of common beans identified as phaseolin subunits with molecular weights of 43, 47, and 53 kDa. Felsted *et al*. (1981) reported lectin subunit from *P. vulgaris* with a molecular weight of 32 kDa. Also, protease inhibitors have been reported with molecular weights of 10 kDa and α-Amilase inhibitor with a mass of 12.4, 15.2, 33.6, and 45 kDa in *P. vulgaris* (Carrasco-Castilla *et al*., 2012). The mass of phaseolin proteins described in this study are similar to the masses described by different works on these proteins. The small differences reported in the molecular weights of proteins from *P. vulgaris* L. are due to the high genetic variety existing in these plants.

RBPC digests under simulated gastrointestinal digestion

In this study, RBPCs at different pHs (pH 3.0–pH 7.0) were subjected to a simulated gastrointestinal digestion using an *in vitro* method to determine the *in vitro* digestibility following the harmonized protocol according to Minekus *et al*. (2014). RBPC gastrointestinal digests were analyzed using the SDS-PAGE electrophoresis method.

Gastric phase digestion

Figure 4a shows the RBPC protein profile with bands of molecular weights from 15 to 50 kDa. RBPCs were hydrolyzed with pepsin at pH 3.0 for 2 hours. In the gastric phase, all the RBPCs present similar profile of hydrolysis with pepsin. Bands of 60, 80, and 100 kDa were totally hydrolyzed with pepsin. Pahseolin fraction (50 kDa) presents partial hydrolysis in all the RBPC assayed, this band contains two bands overlapping in the

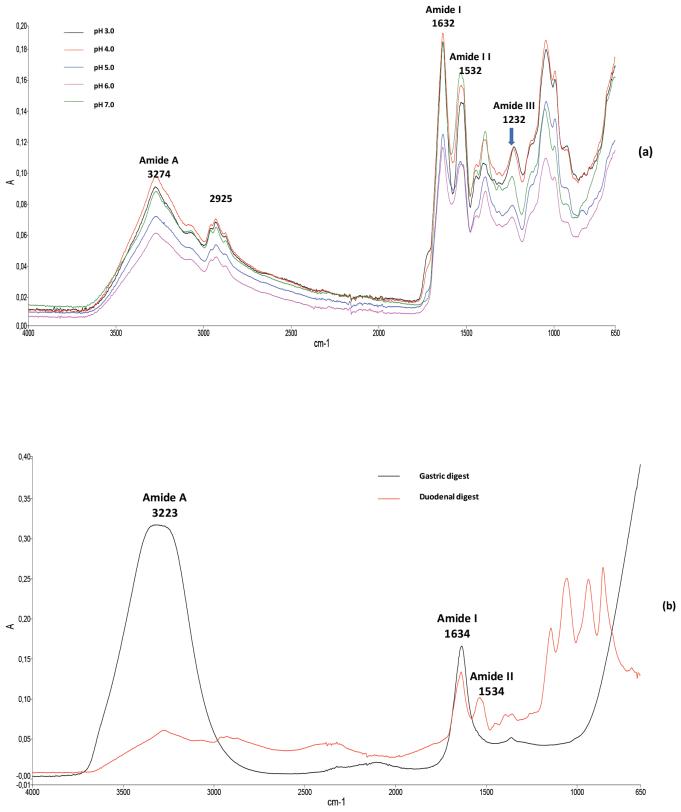

Figure 5. FTIR analysis of RBPC and gastrointestinal digests. (a) RBPC at pH 3.0 to pH 7.0 and (b) gastric and duodenal digests of RBPC.

gel. The lectins bands of low molecular weight (15, 20, 25, and 30 kDa) also present resistance to gastric hydrolysis with the pepsin enzyme. % DH of gastric digests of RBPCs was determined, showing values from 10% to 12% DH. RBPC at pH 3.0 and pH 6.0 presents higher values with 11.85% and 12% DH, respectively.

Duodenal phase digestion

RBPCs were hydrolyzed with pepsin and pancreatin at pH 7.0 for 2 hours in the presence of bile salt. In the duodenal phase, the phaseolin (50-kDa band) present resistance to hydrolysis with pepsin and pepsin/pancreatin preparation (Fig. 4b). Other bands of high and low molecular weights were hydrolyzed in this phase. Phaseolin protein from *P. vulgaris* presents resistance to gastric and gastrointestinal hydrolysis under the simulated gastrointestinal *in vitro* method. RBPC obtained at pH 3.0 present higher hydrolysis with pepsin and pepsin/pancreatin. RBPCs % DH in duodenal digest was determined with a value from 74.58% to 75.60% DH. RBPC at pH 3.0 and pH 5.0 present a higher value with 75.60% and 74.93% DH, respectively. All the RBPCs gastrointestinal digest were higher than the RBPCs gastric digest.

Different *in vitro* hydrolysis methods were used to evaluate hydrolysis of common bean seeds. There are differences in the % DH reported in the studies. These differences must be due to the type and variety of seeds, geographic position of the cultivar and the differences in the method of hydrolysis and enzymes used, time of incubation, pHs of simulation, temperature, proportion of enzymes, and combination of enzymes. For example, Montoya *et al.* (2008) and (2010) reported hydrolysis of phaseolin isolated treated thermally and not treated thermally of 43 varieties hydrolyzed with pepsin dissolved in HCl at pH 2.0 incubated for 0, 30, and 120 minutes and hydrolyzed with pepsin and pancreatin dissolved in a phosphate buffer at pH 7.5. In the gastric phase at 120 minutes of incubation with pepsin, the % DH was 5.2% in the unheated sample and 7.5% in the heated sample. In the duodenal phase, at 360 minutes of incubation with pepsin and pancreatin, the % DH was 11% to 27% for the unheated sample, and 57% to 96% for the heated sample. The gastrointestinal digest presents a high % DH but the results were different depending on the variety of *P. vulgaris* used.

Torruco-Uco *et al.* (2009) reported hydrolysates of *P. vulgaris* from Mexico obtained with Alcalase and Favourzyme for 30 minutes. They found a % DH of 49.48 and 26.05, respectively. Valdez-Ortiz *et al.* (2012) reported % DH of three varieties of sulfur yellow bean (*P. vulgaris*) Azufrado Higuera, Azufrado Noroeste, and Azufrado Regional hydrolyzed with three different enzymes. They reported that Azufrado Regional present the lowest value for the three enzymes with 38% (alcalase), 33% (thermolysine), and 18% (pancreatin)%DH.

Characterization of RBPC and RBPC digest by FTIR analysis

RBPCs, gastric digest, and gastrointestinal digest were analyzed using the FTIR technique with wavelengths from 4,000 to 650 cm^{-1}. Figure 5A showed relevant peaks at 1,632, 1,532, and 1,232 cm^{-1}, characteristic of amide I region (C=O), amide II region (N-H bending), and amide III region (C-N and N-H stretching) typical when proteins are identified. The region between 3,000 and 3,500 cm^{-1} corresponds to Amide A region. Peaks range from 1,460 to 1,380 cm^{-1} was attributed to the symmetric and asymmetric bending vibrations of the methyl group. The amide I band (1,632 cm^{-1}) presents a high absorption band of proteins, thus is used as a model of protein secondary structure. This band presents stretching vibration of C=O bonds (70%–85%) and is strongly related to the conformation of the polypeptide backbone (Liu *et al.*, 2014; Luján-Facundo *et al.*, 2015). RBPC at pH 3.0, pH 4.0, and pH 7.0 shows strong intensity in the band 1,632 and 15,321 cm^{-1}, this band was identified as amide I and amide II regions. Different authors have reported the characterization of proteins using these bands (Amide I, II, and III). Navarro-Lisboa *et al.* (2017) reported the identification of proteins from quinoa (*Chenopodium quinoa* Willd) using the FTIR analysis with the presence of bands (1,632, 1,532, and 1,232 cm^{-1}). These bands were identified as amide I, II, and III, respectively. De la Caba *et al.* (2012) described the identification of proteins in soybean protein concentrate with the presence of relevant peaks 1,632, 1,532, and 1,230 cm^{-1} identified as amide I, II, and III. Das *et al.* (2017) reported the identification of gelatin using the FTIR analysis. They found the relevant peaks 1,630, 1,565, and 1,240 cm^{-1} identified as amide I, II, and III, respectively.

Figure 5B showed relevant peaks of RBPC gastric digest and RBPC gastrointestinal digests of RBPC at pH 7.0. The spectrum profiles of FTIR analysis were different. RBPC gastrointestinal digest shows peaks with higher intensity. Peaks were identified at 1,634 and 1,534 cm^{-1} corresponding to amide I and amide II, respectively. Gastric digest presented no typical peaks of amide II and amide III. The only peak identified was the one at 1,634 cm^{-1}, corresponding to the amide I region. However, this peak presented

a lower intensity than the gastric digest. This fact suggests that the gastric digest presented a low hydrolysis with a correlation with the result of the SDS-PAGE electrophoresis and the % DH. The region from 3,500 to 3,000 cm^{-1} shows the highest intensity in gastric digest. FTIR analysis can be used to characterize the gastric and gastrointestinal hydrolysates.

These hydrolysates allow to identify the amide regions used as typical regions used to identify intact proteins.

RBPC and digests antioxidant activity

Antioxidant activity in fresh fruits, vegetables, legumes and their products, and foods has been described for *in vitro* and *in vivo* studies, including using the ABTS, FRAP, TBARS, and ORAC-FL methods. ORAC-FL assay is considered as the most relevant antioxidant method, using a biologically relevant radical source. These antioxidant methods present different results depending of crop species and laboratories. Ou *et al*. (2002) reported no correlation of antioxidant activity between the FRAP and ORAC methods in a high number of vegetable samples.

The RBPC antioxidant activity (pH 3.0–pH 7.0) and their gastric and gastrointestinal digests antioxidant activity were evaluated using the FRAP, ABTS, and ORAC methods.

RBPCs presented values from 45.13 ± 0.55 to 95.80 ± 0.55 µmoL of TE/ g per sample using the FRAP method. The highest value corresponds to RBPC at pH 7.0. Gastric and duodenal digest were more active than RBPCs. For example, duodenal digests presented the highest value with 225.77 ± 0.03 µmoL of TE/g sample (Table 2).

In the ABTS method, RBPC at pH 5.0 and pH 7.0 presented the highest antioxidant activity with values of 273.66 ± 0.55 and 257.12 ± 0.55 µmoL of TE/g per sample respectively (Table 2). Similar results for gastric and duodenal digests were obtained. These digests presented higher antioxidant activity than RBPCs. Duodenal digests presented the highest value with 345.21 ± 0.23 µmoL of TE/g per sample.

The RBPCs antioxidant activity was also evaluated using the ORAC method. Table 2 shows the ORAC method results. RBPC at pH 7.0 presented an ORAC value of 1960 ± 0.10 µmoL of TE/g sample, this value was the highest ORAC value in RBPCs. Gastric and gastrointestinal digests obtained with pepsin and pepsin/pancreatin presented the highest antioxidant activity. Gastric digest presented a value of 2423 ± 0.17 µmoL of TE/g sample and duodenal digest presented a higher value with 3256 ± 0.20 µmoL of TE/g sample. When using FRAP, ABTS, and ORAC methods, RBPC gastric and duodenal digest were the samples with the highest antioxidant activity. RBPCs presenting value between 68.23 ± 0.24 and 71.82 ± 0.36 and their hydrolysates present value of 85.42 ± 0.11 to gastric digest and 102.33 ± 0.09 to duodenal digest. Duodenal digest present higher value of DPPH than other samples (Table 2). Gastric and duodenal digest from RBPCs were fractionated using ultrafiltration membrane of 3 and 10 kDa to determine the effect of mass of the peptides in the antioxidant capacity using the DPPH method. Gastric fractions present the same antioxidant activity (Fig. 6).

García-Mora *et al*. (2015) described pinto bean protein concentrate and hydrolysates obtained with alcalase and savinase enzymes. Their antioxidant activity was evaluated using the FRAP, ORAC, and ABTS methods. The hydrolysates obtained with savinase also were more active than the pinto bean protein concentrate. The RBPCs values using the ORAC and ABTS and the RBPCs digest were higher than the ones reported by García-Mora *et al*. (2015). RBPCs have a high content of protein and polyphenols compounds, we suggest that the relation of these molecules in RBPCs can be liable of the antioxidant capacity described in this study.

***In vitro* and *in vivo* TBARS in zebrafish larvae**

RBPCs and gastric and gastrointestinal digest of RBPC were used to determinate their capacity to inhibit TBARS using the *in vitro* and *in vivo* zebrafish larvae models. The activity was expressed as % of inhibition TBARS. The MDA content was calculated using an MDA standard curve. *In vitro* TBARS: Figure 7 shows the results of % of inhibition TBARS using the *in vitro* model. BHT was used as a control for its strong antioxidant activity and for the wide use in Ecuador in the food industry for conservation of processed food (oils, snacks, and bakery processed foods). BHT was used as a positive control (0.25 mg/ml) presenting a value of 80.28% of inhibition *in vitro* TBARS. RBPCs and gastric and gastrointestinal digests were evaluated at 2.0 mg/ml concentrations. RBPC from pH 3.0 to pH 7.0 present a higher percentage than the BHT positive control at all the concentrations assayed. For example, RBPC at pH 7.0 presented at value of 84.76% of inhibition *in vitro* TBARS. RBPC gastric digests and duodenal digests presented a higher value of % of inhibition TBARS than BHT and RBPCs without hydrolysis. These samples presented values of 87.95% and 93.0%, respectively (Fig. 7).

In vivo TBARS

The cytotoxicity of RBPCs and their hydrolysates was evaluated using *in vivo* zebrafish embryos and the larvae model. In

Table 2. Total Polyphenol Content (TPC) and antioxidant activity of RBPC using FRAP, ABTS, ORAC and DPPH methods.

Sample	TPC (mg GAE/100 g sample) ± SD	FRAP (µmoL TE/g sample) ± SD	ABTS (µmoL TE/g sample) ± SD	ORAC (µmoL TE/g sample) ± SD	DPPH (µmoL TE/g ample) ± SD
RBPC pH 3.0	246.15 ± 0.55	65.70 ± 0.25	116.19 ± 0.16	1250 ± 0.15	68.23±0.24
RBPC pH 4.0	135.57 ± 0.55	45.13 ± 0.42	81.55 ± 0.14	870 ± 0.07	70.45±0.17
RBPC pH 5.0	204.82 ± 0.00	87.00 ± 0.28	273.66 ± 0.02	1320 ± 0.04	69.66±0.24
RBPC pH 6.0	161.63 ± 1.07	81.26 ± 0.16	142.29 ± 0.39	1030 ± 0.12	68.98±0.22
RBPC pH 7.0	521.66 ± 0.85	95.80 ± 0.37	257.12 ± 0.78	1960 ± 0.10	71.82±0.36
GD	N/D	146.89 ± 0.02	297.63 ± 0.44	2423 ± 0.17	85.42±0.11
DD	N/D	225.77 ± 0.03	345.21 ± 0.23	3256 ± 0.20	102.33±0.09

Data analyzed by one-way ANOVA and followed by Tuckey´s test. Different letter represents significant differences between sample as $p < 0.05$ ($n = 5$).
GD (gastric digest) and DD (duodenal digest)

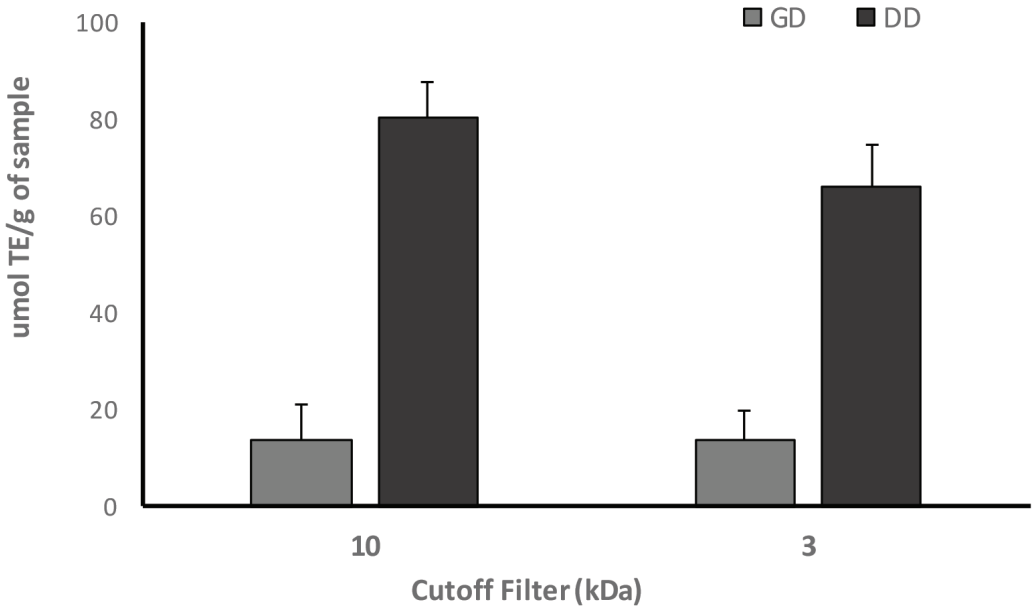

Figure 6. DPPH analysis of fractions of gastric and duodenal digest from RBPC. GD (gastric digest of RBPC at pH 7.0 and DD (duodenal digest of RBPC at pH 7.0).

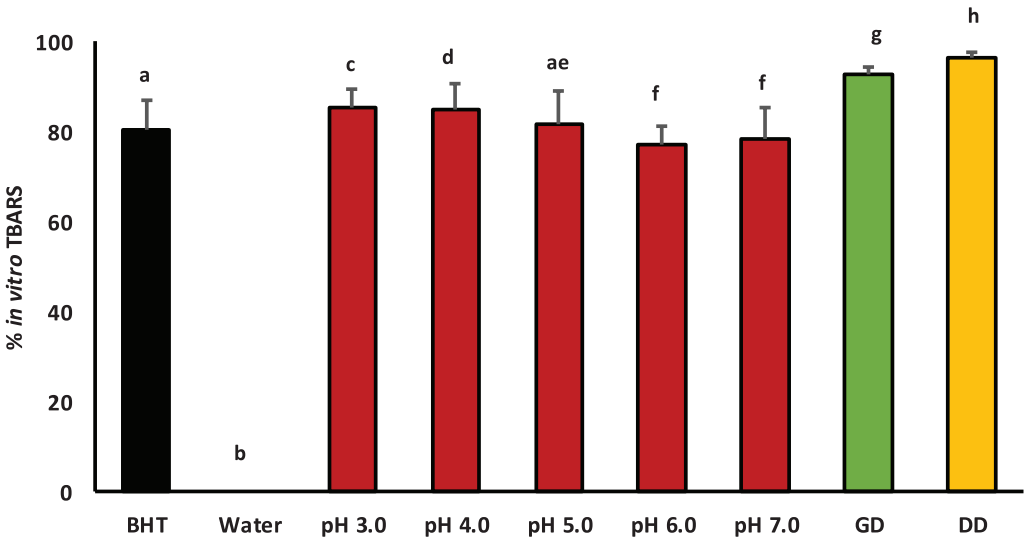

Figure 7. *In vitro* lipid peroxidation inhibitory activity of RBPC and gastrointestinal digests. BHT (positive control), water (negative control), RBPC (RBPC at pH 3.0–7.0), GD (gastric digest) and DD (duodenal digest). Data were analyzed using one-way ANOVA and followed by Tukey's test. Different letters over bars represent statistical differences between group samples with $p < 0.05$ ($n = 5$).

all the samples, pH was adjusted to 7.0 as low pHs or high pHs can kill zebrafish embryos. RBPCs and digest presented inhibition of lipid peroxidation in zebrafish embryos. RBPC at pH 3.0 presented a value of 53.91%, RBPC at pH 4.0 showed a value of 57.21%, RBPC at pH 5.0 had a value of 55.62%, RBPC at pH 6.0 showed a value of 60.31%, and RBPC at pH 7.0 presented a value of 63.06%. Gastric digest presented a value of 79.03% and duodenal digest presented a value of 86.76% (Figure 8). After 48 hours of exposure to different concentrations of sample, zebrafish larvae presented an absence of coagulation and the survival percentage was >95% of survived larvae. Zebrafish larvae were observed in different times with the help of a stereoscopic microscopy and zebrafish larvae exhibit the same morphology than zebrafish embryos of the control group without the sample. These results indicate that RBPCs and gastric and gastrointestinal digest showed an absence of cytotoxic effect for the development of larvae. Then, after 96 hours of incubation at different concentrations of samples, zebrafish larvae presented no coagulation and the percentage of survival was 100% of survived larvae. RBPCs and gastric and gastrointestinal digest presented no morphologic damage to the zebrafish larvae. All the larvae were observed in their development stages for 3 months without presenting problems of fertility. Normal eggs, embryos, and larvae were observed.

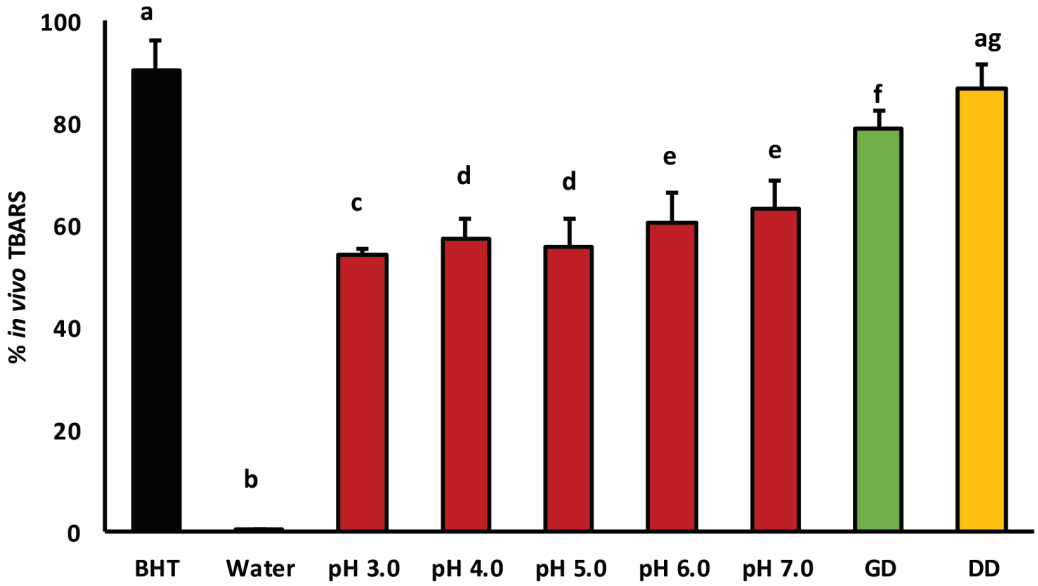

Figure 8. *In vivo* lipid peroxidation inhibitory activity of RBPC and gastrointestinal digests in zebrafish larvae. BHT (positive control), water (negative control), RBPC (RBPC at pH 3.0 to pH 7.0), GD (gastric digest) and DD (duodenal digest). Data were analyzed using one-way ANOVA and followed by Tukey's test. Different letters over bars represent statistical differences between group samples with $p < 0.05$ ($n = 5$).

Protection of RBPC digest in the *in vivo* model produced for AAPH reactive

The DCFH-DA fluorescence experiment is the fastest method used to determine and quantify ROS production using *in vivo* animal models. In the last years, this method has been used in the zebrafish embryos model. It has been reported recently the use of an *in vivo* antioxidant method in Caco-2 cells and zebrafish embryos using DCFH-DA (2,7-dichlorofluorescein diacetate). This compound is a nonionic substrate and a nonpolar substrate, the cell membrane is hydrolyzed by enzymes with cellular esterase to the non-fluorescent DCFH. DCFH molecule is oxidized to the DFC molecule in the presence of ROS. For this reason, DFC fluorescence represents a measure of ROS in the cells. Kang *et al*. (2014) reported evaluation of polysaccharide purified from aloe Vera (*Aloe barbadensis*) to inhibit the production of ROS in zebrafish embryos using DCFH-DA assay (Kang *et al*., 2014). Lee *et al*. (2013) described the *Ecklonia cava* extract with capacity to inhibit the production of ROS in zebrafish embryos using DCFH-DA assay. Other authors have described the use of DCFH-DA assay to inhibit ROS in cells model. Goh *et al*. (2016), described ROS reduction in human cell lines, HaCaT, and human monocytic cell lines, THP-1 from *Aronia melanocarpa*. Jensen *et al*. (2015) have described ROS reduction in cells using isolate algae extract. Carrasco-Castilla *et al*. (2012) reported high antioxidant capacity of the protein hydrolysates determined in Caco-2 cell lines. ROS reduction was quantified using the DCFH-DA assay. Gastric digest and gastrointestinal digest of RBPC at pH 7.0 were used to evaluate their protective effect against oxidative stress in the *in vivo* zebrafish embryos produced for AAPH reactive.

ROS is used as an important relevant indicator in the determination of damage and oxidative cellular stress. The survival rate of embryos in the experiment was calculated. The non-treated group (basal control) presented a 100% survival rate of zebrafish embryos. AAPH group presented 75% live embryos. Gastric and duodenal digests presented 100% live embryos used in the assay. RBPC, gastric, and gastrointestinal digests antioxidant effects on ROS intensity (DCFH-DA) can be seen in Figure 9. AAPH group shows strong intensity of fluorescence of embryos. RBPC gastric and gastrointestinal digests could inhibit the formation of ROS in zebrafish embryos. Hydrolysates are usually more active than parental proteins due to the formation of bioactive peptides. The antioxidant peptides are usually small fragments with molar weights between 1,500 and 6,000 Da and with an amino acid sequence composed of 5–16 amino acids. The type of amino acid is also determinant in antioxidant activity as the different amino acid tend to be positively charged peptides. Future work can be used to identify the sequences of peptides responsible for antioxidant activity and with the capacity to inhibit ROS in embryos of zebrafish. Other studies are also necessary to determine the mechanism of action of these molecules. Zhao *et al*. (2004) developed synthetic peptides with aromatic amino acids and positive charge in their sequences: SS-02 (Dmt-DArg-Phe-Lys-NH2; Dmt 2,6-dimethyltyrosine), SS-20 (Phe-D-ArgPhe-Lys-NH2), SS-31 (D-Arg-Dmt-Lys-Phe-NH2), and [3H] SS-02. These peptides were able of inhibiting lipid peroxidation *in vitro* of linoleic acid and were able of reducing the formation of ROS in the mitochondria of Caco- 2 cells. Carrillo *et al*. (2016b) described five antioxidant peptides from Hen Egg White Lysozyme (HEWL) with the sequences f(109-119) VAWRNRCKGTD, f(111-119) WRNRCKGTD, f(122-129) AWIRGCRL, f(123-129) WIRGCRL, and f(124-129) IRGCRL. These peptides inhibited the production of TBARS in the zebrafish model. These peptides have strong positive charge and have aromatic amino acids, such as tryptophan. Carrillo *et al*. (2016a) reported hydrolysates from native and heat-HEWL with ability to reduce the production of TBARS in the zebrafish model. Vilcacundo *et al*. (2018a) described

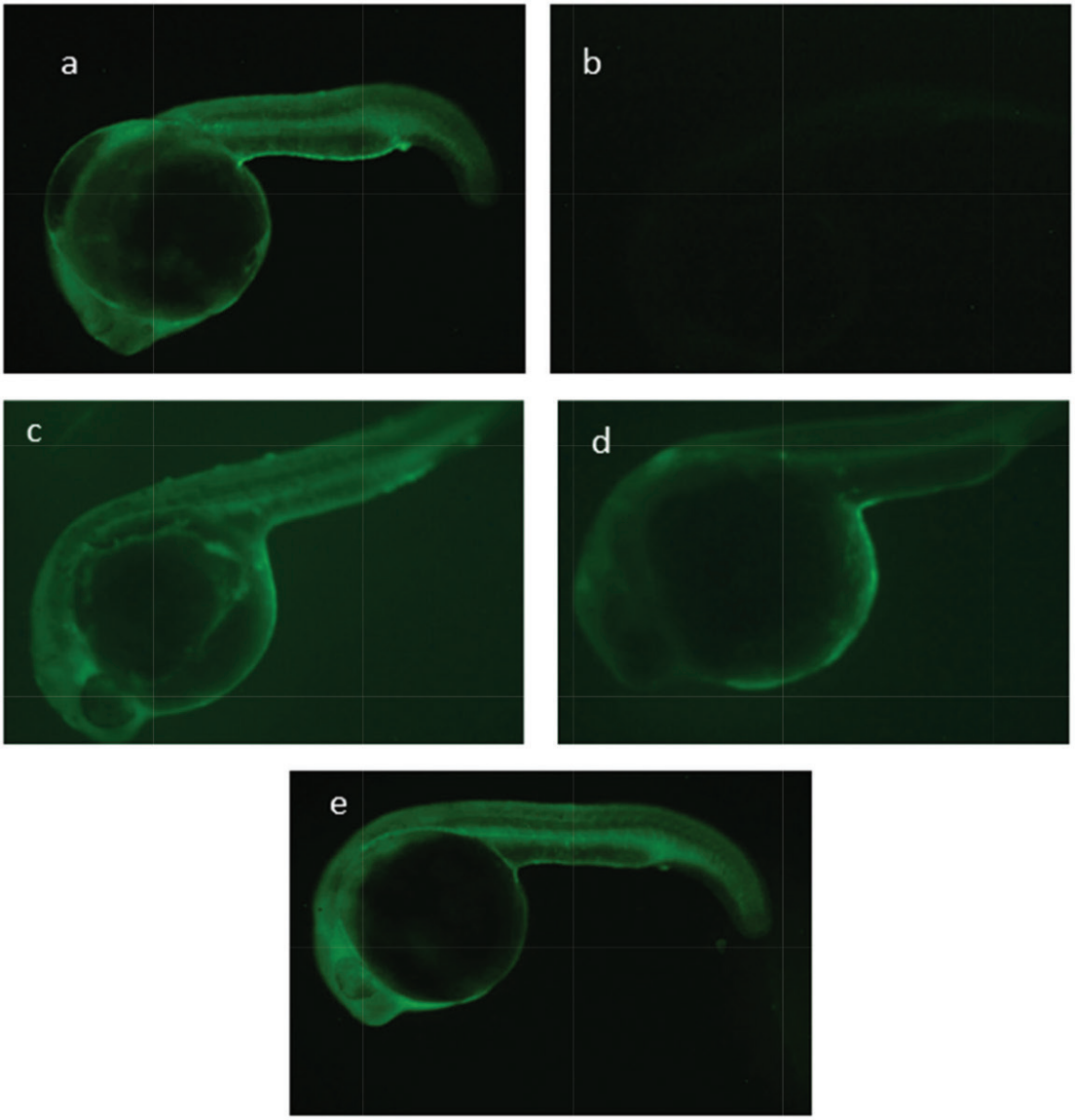

Figure 9. Micrographs of reduction of ROS in zebrafish embryos of RBPC and their gastrointestinal digest. (a) AAPH (positive control), (b) water (control without AAPH), (c) gastric digest of RBPC + AAPH), and (d) duodenal digest of RBPC + AAPH (n= 5).

amaranth protein concentrate (APC) and their gastrointestinal digest with the capacity to reduce TBARS in the *in vitro* and *in vivo* models (zebrafish larvae and embryos). Gastric digest and duodenal digest present the capacity to reduce ROS in zebrafish embryos using DCFH-DA as a fluorescent agent. These studies indicate that hydrolysates and peptides from food protein can have the capacity to inhibit TBARS *in vitro* and *in vivo* with a possible use in the food industry for the conservation of processed oils and food.

It was observed that the intensity of the fluorescence of gastric digest and duodenal digest was lower when compared to the intensity of the group of embryos treated with AAPH. It was observed a higher intensity fluorescence emitted for the group embryos treated with RBPC This fact indicates that RBPC sample produces no protection in the production of ROS. The non-treated group basal control presented the lowest intensity of fluorescence in all the groups. It was difficult to obtain the microphotograph of the basal control group being the intensity extremely low.

The quantification of intensity of the fluorescence of zebrafish embryos was made using software to analyze digital images (ImageJ). The percentage of intensity of fluorescence was compared to the percentage obtained for the positive control (AAPH reactive). 100% of intensity of fluorescence obtained was assigned to the group of embryos treated with AAPH reactive. Figure 10 shows the % of intensity of fluorescence of the group of embryos treated with RBPC and gastric digest and gastrointestinal digest of RBPC. RBPC gastric digest at pH 3.0 present a value of intensity of fluorescence of 75.30% and RBPC gastrointestinal digests present a value of 66.40% of intensity of fluorescence when compared to the AAPH signal. RBPC without hydrolysis present 100% of intensity of fluorescence. This sample presents no protection against oxidation induced for AAPH. The

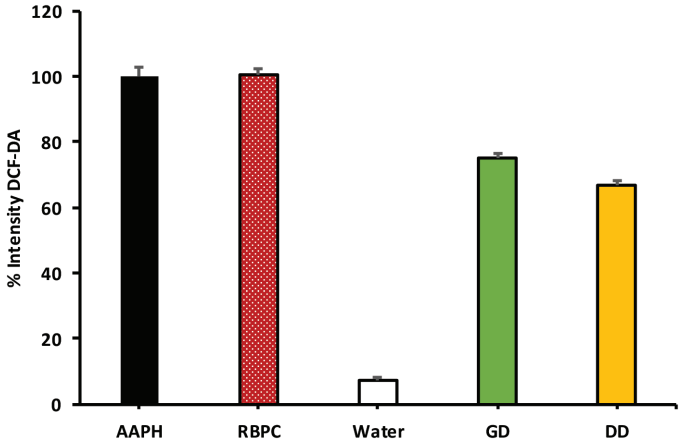

Figure 10. % of intensity by DFC-DA fluorescence in presence of AAPH. AAPH (positive control), water (basal control without AAPH), DG (gastric digest incubated with AAPH), DD (duodenal digest incubated with AAPH) and RBPC (red bean protein concentrate incubated with AAPH). Results were analyzed using one-way ANOVA and followed by Tuckey´s test. Different letter represents significant differences between sample as $p < 0.05$ ($n = 5$).result.

group of embryos treated with water present 7.0% of intensity of fluorescence when compared to the group of embryos treated with AAPH reactive.

CONCLUSION

Results obtained in this study demonstrate that RBPCs and RBPCs hydrolysates produced with *in vitro* gastrointestinal digestion model present antioxidant activity and inhibition of TBARs using the *in vitro* and *in vivo* zebrafish larvae model. RBPCs and their hydrolysates were able of inhibiting ROS formation in zebrafish embryos. RBPCs and their hydrolysates, due to their antioxidant capacity and their protein content, can be used in the food industry as functional ingredients. Other studies can be used to identify the sequences of antioxidant peptides and study their mechanisms of action. Moreover, a study is needed to determine their technological properties, such as protein solubility, water absorption capacity, oil absorption capacity, the emulsifying activity index, and finally determine their possible use in the food industry.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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