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Peptides in common bean fractions inhibit human colorectal cancer cells

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ABSTRACT

The aim of this study was to characterize peptides present in common bean non-digestible fractions (NDF) produced after enzymatic digestion and determine their antiproliferative action on human colorectal cancer cells. Five NDF peptides represented 70% of total protein (GLTSK, LSGNK, GEGSGA, MPACGSS and MTEEY) with antiproliferative activity on human colon cancer cells. Based on the antiproliferative effect, HCT116 cell line was most sensitive to bean Azufrado Higuera (IC_{50} = 0.53 mg/ml) and RKO to Bayo Madero (IC_{50} = 0.51 mg/ml) peptide extracts. Both cultivars increased significantly ($p < 0.05$) the expression of p53 in HCT116 by 76% and 68%, respectively. Azufrado Higuera modified the expression of cell cycle regulation proteins p21 and cyclin B1. Bayo Madero modified the expression of mitochondrial activated apoptotic proteins BAD, cytC, c-casp3, Survivin, BIRC7. Results suggest that peptides present in common bean NDF contributed to the antiproliferative effect on human colorectal cancer cells by modifying molecules involved in either cell cycle arrest or apoptosis.

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1. Introduction

Colorectal cancer (CRC) is the fourth most common cancer in men and third most common cancer in women in Latin America (Goss et al., 2013). The relationship between increasing the risk of colorectal cancer and eating habits, physical activity and obesity is well established (Wolin, Carson, & Colditz, 2010). In a study with participants which had histologically confirmed colorectal adenomas, identified by complete colonoscopy, high consumption of legumes was related to low risk of CRC development (Lanza et al., 2006).

Common bean (*Phaseolus vulgaris* L.) consumption is part of the traditional diet in many countries around the world, including Mexico, whose consumption is higher than other countries. In 2009, yearly *per* capita consumption of common bean in Mexico oscillated around 10.3 kg, compared to some countries of the European Union with 2.5 kg and the United States with 3.5 kg (FAO, 2014). At present, common bean consumption confronts important modifications in face of a changing society, including eating habits due to urbanism and lifestyle. The Mexican Department of Economy (Dirección General de Industrias Básicas, 2012), reported that the production and consumption of common bean have been decreasing in the past 5 years. Additionally, common beans are an excellent source of complex carbohydrates (50–60%) (Paredes-López, Guevara-Lara, & Bello-Pérez, 2006) and proteins (20–25%) constituted mainly of globulins, albumins and glutelins (Montoya, Lalles, Beebe, & Leterme, 2010).

Resistant starch, proteins, oligosaccharides and some polyphenols also present in common bean are fermented by colonic microbiota and constitute the non-digestible fraction (NDF) (Saura-Calixto, García-Alonso, Goñí, & Bravo, 2000) recognized as an important component in protecting colon health. Common bean NDF, has the ability to be fermented producing, among other compounds, short chain fatty acids (SCFA), mainly acetate, propionate and butyrate; this SCFA are recognized to be preventive agents due to their capability to inhibit tumor cell proliferation and induce apoptosis (Campos-Vega, Oomah, Loarca-Piña, & Vergara-Castañeda, 2013; Sengupta, Muir, & Gibson, 2006). *In vitro* fermentation products of common bean NDF have shown to regulate gene expression in HT-29 cells related with p53-mediated signal transduction response genes involved in cell cycle and apoptosis (Campos-Vega, Loarca-Piña, & Oomah, 2010). Furthermore, the effect of common bean NDF has also been reported in rat models azoxymethane-induced colon cancer, through cell-cycle arrest in G1/S and G2/M phases and cell death by apoptotic induction (Feregrino-Perez et al., 2008; Vergara-Castañeda et al., 2010; Vergara-Castañeda, Guevara-González, Guevara-Olvera, Oomah, & Loarca-Piña, 2012). Moreover, the proximate composition of the NDF indicates not only the high content of carbohydrates (∼77%)

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but also significant amounts of protein (~17%) (Vergara-Castañeda et al., 2010), for which the role on chemoprevention is still unknown.

The transcription factor p53 is an important molecular target in CRC and it is involved in cell cycle regulation and apoptosis. The role of tumor suppressor p53 is to maintain cell homeostasis and provide specific and adapted responses to stress, for instance by mediating antiproliferative responses to DNA damage signals, including cell cycle arrest, cell senescence and apoptosis (Millau, Bastien, & Drouin, 2009). In the HCT-116 colorectal cancer cell line, p53 has been shown to regulate both cell cycle (Petersen, Hasvold, Lukas, Bartek, & Syljuåsen, 2010) and apoptotic (Lan et al., 2012) pathways depending on the cellular events surrounding and DNA damage stimuli. The objective of this study was to characterize peptides of common beans NDF produced after *in vitro* simulated gastrointestinal digestion and to evaluate their antiproliferative and protein expression effect in an *in vitro* model using HCT116, RKO and KM12L4 human colorectal cancer cells. We found that both Azufrado Higuera and Bayo Madero NDF bean cultivars increased the expression of p53 in HCT116 leading to modifications of markers associated with cell cycle arrest or apoptosis.

2. Materials and methods

2.1. Materials

Human colorectal cancer cells HCT116 (positive for transforming growth factor beta 1 (TGF-β1) and beta 2 (TGF-β2) expression), RKO (lack endogenous human thyroid receptor nuclear receptor (h-TRbeta1)), and KM12L4 (high producer of interleukin 18 (IL18)), were obtained from Dr. Lee M. Ellis (MD Anderson Cancer Center, University of Texas). These cells were selected due to their clinical relevance since they are well-known metastatic human colorectal cancer cells. Eagle's Minimum Essential Medium and 0.25% (w/v) Trypsin – 0.53 mM EDTA were purchased from American Type Culture Collection (Manasas, VA). Primary antibodies nucleolin (C23, 271-520), GSK-3β (345-420), casp3 (full-length protein), cytC (full-length protein), casp8 (217-350), clu (120-449), p65NFκB (526-539), TNFR1 (30-301), cyclin B1 (400-433), radio immunoprecipitation assay (RIPA) buffer were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Primary antibodies VEGF (206–317), VEGF-R2 (full-length protein), GAPDH (full-length protein), Alexa-Fluor 488, ITsignal FX, prolong gold antifade reagent DAPI stain were purchased from Invitrogen (Carlsbad, CA). Secondary antibodies anti-mouse IgG and anti-rabbit IgG horseradish peroxidase conjugated were purchased from GE Healthcare (Buckinghamshire, UK). All other reagents were purchased from Sigma–Aldrich (St. Louis, MO) unless otherwise specified.

2.2. Samples

Common bean (*P. vulgaris* L.) cultivars Azufrado Higuera (AH), Bayo Madero (BM), Negro (N) 8025, and Pinto Durango (PD) harvested in 2011 in Central Mexico were provided by the Instituto Nacional de Investigaciones Forestales Agrícolas y Pecuarias (INIFAP, Mexico). Dry common bean seeds were cooked according to the method of Aparicio-Fernandez, Manzo-Bonilla, and Loarca-Piña (2005). Briefly, beans were placed in a beaker with HPLC-grade water (1:5 w/v) and heated at 90 ± 3 °C for 2.5 h or until seeds were soft enough for consumption. Cooked seeds and broth were ground in a domestic grinder (Osterizer, Mexico), lyophilized, and the samples were passed through a 80-US mesh (0.180 mm) screen, and stored in 50 ml centrifuge tubes at 4 °C until use.

2.3. Extraction and quantification of the non-digestible fraction

NDF was determined as the sum of soluble and insoluble material, following the procedure of Saura-Calixto et al. (2000). 300 mg of the samples treated, as described above, were incubated with pepsin (300 mg/ml, Sigma–Aldrich P7000) and porcine pancreatic α-amylase (120 mg/ml Sigma–Aldrich A3176). The samples were centrifuged (3000×*g*, 15 min) and the supernatants were taken. The residue (insoluble NDF) were dried and quantified gravimetrically. Supernatants were dialyzed against water (25 °C, 48 h, water flow 7 l/h) (cellulose dialysis membranes, 12,000–14,000 MWCO; Sigma–Aldrich). Dialysates with the soluble NDF were separated for further analysis, and a portion was treated with 1 M sulfuric acid (100 °C for 90 min) for measuring insoluble NDF after reaction with 3,5-dinitrosalicylic acid. The soluble and insoluble NDF were combined and lyophilized before protein extraction. Nitrogen, lipid, and ash contents of cooked bean NDF samples were determined by procedures of the Association of Official Analytical Chemists (AOAC) (methods 920.87, 920.39, and 923.03, respectively). Protein content was calculated as nitrogen × 5.85.

2.4. Preparation of common bean NDF peptide extract

Common bean protein extracts were produced according to Pedroche et al. (2002) with minor modifications. Briefly, 10 g of lyophilized NDF was suspended in 100 ml of 0.2% NaOH solution pH 12, and stirred for 1 h. The solution was centrifuged at 8000×*g* for 20 min; two additional extractions were carried out with half of the volume of NaOH solution. The pH of the supernatant was adjusted to 4.4 and the precipitate formed was recovered by centrifugation at 8000×*g* for 20 min. The precipitate was washed with distilled water, cold saturated ammonium acetate in methanol, methanol, acetone and water again, and then it was lyophilized. *In vitro* simulated digestion of protein isolate was performed according to Megías et al. (2009) with minor modifications. Protein isolate was suspended in water (1:20 w/v); a sequential enzyme digestion was performed with pepsin (1:20 w/w, EC 3.4.23.1, Sigma–Aldrich P7000) and pancreatin (1:20 w/w, a mixture of several digestive enzymes produced by the exocrine cells of the porcine pancreas, EC 232-468-9, Sigma–Aldrich P7545) at 37 °C for 1.5 h for each enzyme. The hydrolysis was stopped by incubating at 75 °C for 20 min, the suspension was centrifuged at 20,000×*g* for 15 min at 4 °C, and the supernatant was desalted through electro-dialysis using the ElectroPrep System (Harvard Apparatus, Hill Road MA) with 500 Da dialysis membrane. After desalting, the samples were lyophilized and kept at −20 °C until further use. Lyophilized material was called NDF peptide extract of Azufrado Higuera (AH-PE), Bayo Madero (BM-PE), Negro 8025 (N8-PE) and Pinto Durango (PD-PE) common bean cultivars.

2.5. Peptide extracts characterization

For sample preparation, 1 mg of AH-PE, BM-PE, N8-PE and PD-PE were dissolved in 1 ml of deionized water and analyzed by using an UltrafleXtreme MALDI TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a frequency tripled Nd:YAG solid state laser using the FlexControl 1.4 software package (Bruker Daltonics). MS/MS analysis of each ion of interest was performed at 500 Hz in LIFT mode using a randomized raster, summed, and saved for analysis. Data processing was performed using the FlexAnalysis 3.4 software package (Bruker Daltonics, Bremen, Germany) and Biotools 3.2 (Bruker Daltonics, Bremen, Germany). The percentage of each peptide was calculated based on the area under the curve method. The total area for each sample was calculated by taking the sum of all the areas under the curve. Then the area under the curve for each peptide identified was

divided by the total area and the percentage was calculated. Potential biological activities of the peptides were obtained using BioPep tool (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep, accessed 05/16/2013). Presence of peptides in common bean (*P. vulgaris* L.) protein sequences were confirmed using BLAST® tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed 06/08/2013). Physicochemical properties were predicted using PepDraw tool (http://www.tulane.edu/~biochem/WW/PepDraw/index.html, accessed 07/26/2013).

2.6. Cell culture and cell proliferation assay

Human colon cancer cells HCT-116, RKO and KM12L4 were cultured in Eagle's Minimum Essential Medium Containing 10% FBS, 1% penicillin/streptomycin and 1% sodium pyruvate at 37 °C in 5% CO_2/95% air. The cell proliferation assay was performed using the CellTiter® 96 Aqueous One Solution Proliferation assay containing 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenil)-2-(4-sulphophenil)-2H-tetrazolium, inert salt (MTS), and an electron coupling reagent, phenazine ethosulfate (PES) (Promega Corporation, Madison, WI). Ten thousand cells per well were seeded in a 96-well plate and total volume was adjusted to 200 µl with growth medium. The cells were allowed to grow for 24 h at 37 °C in 5% CO_2/95% air and treated with different concentrations of AH-PE, BM-PE, N8-PE and PD-PE ranging from 0.125 to 1 mg/ml for 24 h. After treatment, the culture medium was replaced by 100 µl of fresh medium and 20 µl of MTS/PES reagent were added to each well. The plate was incubated for 2 h at 37 °C in 5% CO_2/95% air and the absorbance was read at 515 nm. The percentage of viable cells was calculated with respect to the cells treated with water as a vehicle. Inhibitory concentrations of 20%, 30% and 50% of the cells (IC_{20}, IC_{30} and IC_{50}) were calculated using Prism 6 software.

2.7. Immunoblotting

HCT-116 cells were seeded at a density of 2×10^5 cells per well in a 6-well plate for 24 h at 37 °C in 5% CO_2/95% air. After 24 h, cells were treated with either molecular biological grade distilled water as control, or 0.5 mg/ml of AH-PE, or 0.5 mg/ml BM-PE. For measurement of nuclear markers p65, NF-κB, GSK-3β, and Clu, then the cells were washed with PBS twice, trypsinized, washed again with PBS and nuclear and cytoplasmic fractions were separated using NE-PER nuclear extraction kit (ThermoScientific, Pierce, IL) following manufacturer's protocol. To measure caspases 3 and 8, cytochrome C, vascular endothelial growth factor VEGF, and cyclin B1, after treatment, cells were washed with PBS twice, trypsinized and suspended in RIPA buffer, and the cell suspension was sonicated and boiled for 5 min before use. Equal amounts of protein were loaded in 4–20% gradient polyacrylamide gels (BioRad Laboratories, Inc.). Separated proteins were transferred to PVDF membranes and these were blocked with 5% non-fat dry milk in 0.1% Tris-buffered saline with Tween 20 (TBST) for 1 h at 4 °C. After blocking, membranes were washed 5 times 5 min each with TBST, and primary antibody was added at the manufacturer recommended dilution and incubated at 4 °C overnight. After primary antibody incubation, membranes were washed with TBST and secondary antibody was added at the manufacturer recommended dilution and incubated for 1 h at room temperature, membranes were washed five times, 5 min each with TBST and protein expression was detected using 1:1 chemiluminescent reagents A and B of ECL Prime Western Blotting kit (GE Healthcare, Buckinghamshire, UK) and visualized using a GelLogic 4000 Pro Imaging System (Carestream Health, Inc., Rochester, NY).

2.8. Protein apoptosis array

The profile of apoptosis-related proteins expression was performed and analyzed using a Human Apoptosis Array (Ary009, R&D Systems, Minneapolis, MN) according to the manufacturer instructions. Briefly, HCT-116 cells were seeded at a density of 1×10^6 cells in a 25 cm^2 canted neck flask for 24 h at 37 °C in 5% CO_2/95% air. After 24 h, cells were treated with 0.5 mg/ml of AH-PE and BM-PE separately; with molecular biological grade distilled water used as control and harvested using provided lysis buffer reagent. A provided membrane containing immobilized antibodies was blocked with blocking solution reagent for 1 h at room temperature, and treated with equal amounts of cell lysates overnight at 4 °C. The membrane was washed with the provided washing buffer and incubated subsequently with detection antibody cocktail and streptavidin horseradish peroxidase-conjugated for 1 h at room temperature each. Protein expression was detected using 1:1 provided chemiluminescent reagents A and B and visualized using a GelLogic 4000 Pro Imaging System (Carestream Health, Inc., Rochester, NY).

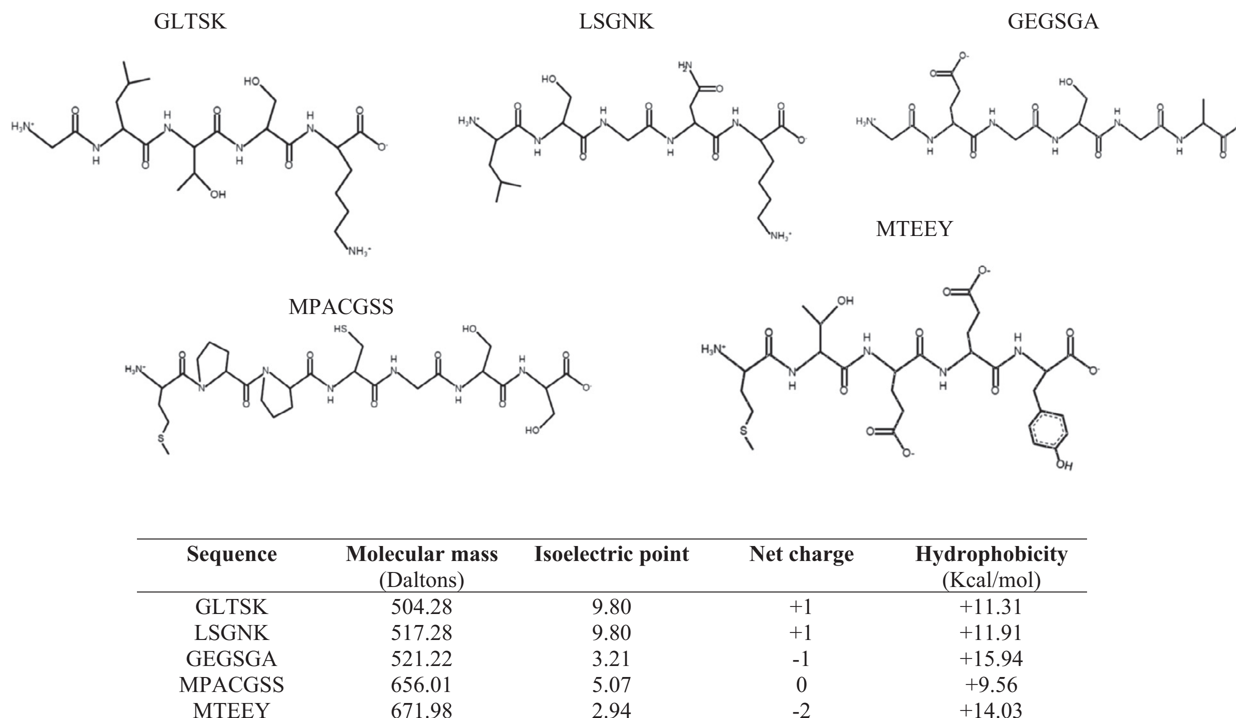
2.9. Immunofluorescence confocal microscopy

Briefly, 1×10^4 HCT116 cells were seeded in 300 µl phenol red-free Opti-MEM® medium (Life Technologies, Carlsbad, CA) in ibiTreat microscopy chambers (ibidi, Verona, WI) for 48 h at 37 °C in 5% CO_2/95% air. After incubation, cells were treated with 0.5 mg/ml BM-PE for 24 h. Cells were washed three times with PBS and fixed with 4% paraformaldehyde aqueous solution (Electron Microscopy Sciences, Hatfield, PA) for 30 min at room temperature, washed three times 5 min each with PBS, and permeabilized with 0.1% Triton X-100 in PBS for 15 min at room temperature. Cells were washed once with PBS and incubated with ultracold HPLC-grade methanol for 15 min at −20 °C. Methanol

Table 1
Characterization of NDF major peptides.

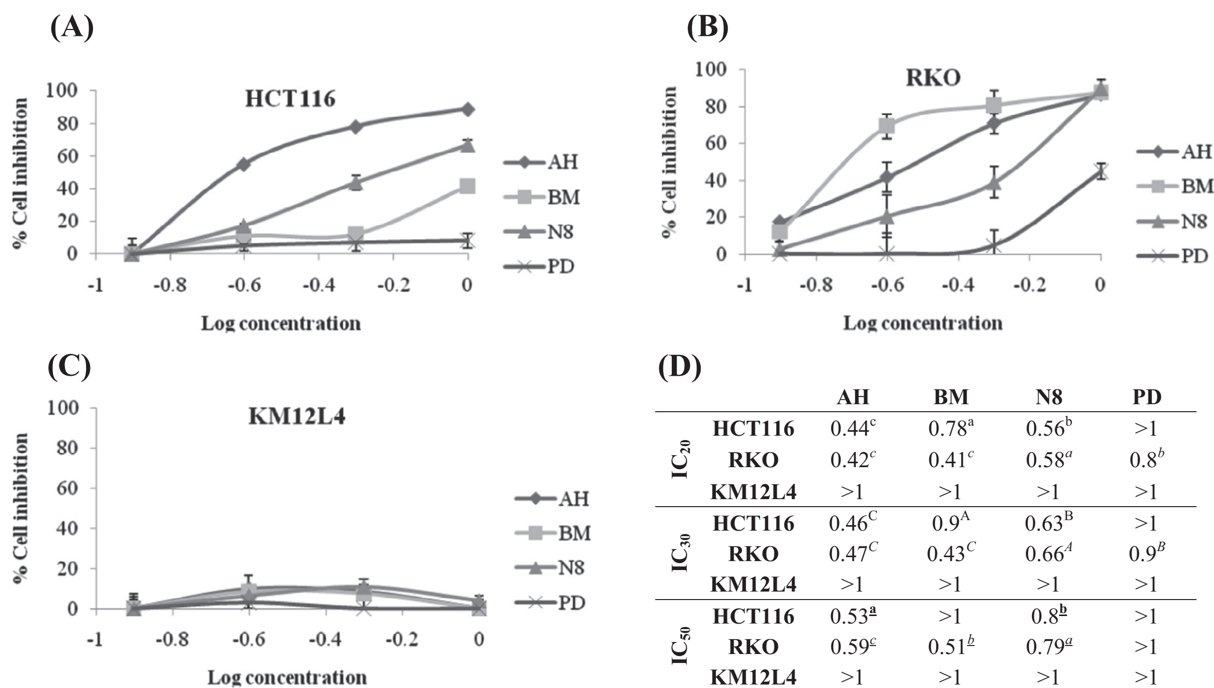
Molecular mass (Da)[a]	% Production (area)				Sequence	Biological activity[b]	*Phaseolus vulgaris* protein source
	Azufrado Higuera	Bayo Madero	Negro 8025	Pinto Durango			
505.48	13.73	12.05	12.76	14.59	GLTSK	ACE inhibitor	Ribosomal protein S4
518.29	9.56	7.6	4.18	5.73	LSGNK	ACE inhibitor	NBS-LRR type putative disease resistance protein CNL-B18
521.22	6.28	5.31	7.8	5.81	GEGSGA	ACE inhibitor	Glycine-rich cell wall structural protein
656.01	30.59	29.94	32.44	35.23	MPACGSS	ACE inhibitor, dipeptidyl peptidase IV inhibitor	Albumin 1-A
671.98	11.12	14.01	10.85	13.57	MTEEY	ACE inhibitor , stimulating vasoactive substance release	NADH-plastoquinone oxidoreductase subunit I

Da, daltons.
[a] Percentage of production of peptide of total protein detected using MALDI-TOF; these peptides represented ~70% of total area.
[b] Determined using BioPep.



Sequence	Molecular mass (Daltons)	Isoelectric point	Net charge	Hydrophobicity (Kcal/mol)
GLTSK	504.28	9.80	+1	+11.31
LSGNK	517.28	9.80	+1	+11.91
GEGSGA	521.22	3.21	-1	+15.94
MPACGSS	656.01	5.07	0	+9.56
MTEEY	671.98	2.94	-2	+14.03

Fig. 1. Physicochemical properties of the main peptides found on four cultivars of common bean NDF using PepDraw tool, including amino acid sequence and structure, molecular mass, net charge, isoelectric point and hydrophobicity.



		AH	BM	N8	PD
IC_{20}	HCT116	0.44^{c}	0.78^{a}	0.56^{b}	>1
	RKO	0.42^{c}	0.41^{c}	0.58^{a}	0.8^{b}
	KM12L4	>1	>1	>1	>1
IC_{30}	HCT116	0.46^{C}	0.9^{A}	0.63^{B}	>1
	RKO	0.47^{C}	0.43^{C}	0.66^{A}	0.9^{B}
	KM12L4	>1	>1	>1	>1
IC_{50}	HCT116	0.53^{a}	>1	0.8^{b}	>1
	RKO	0.59^{c}	0.51^{b}	0.79^{a}	>1
	KM12L4	>1	>1	>1	>1

Fig. 2. Effect of peptide extracts treatments on cell survival on (A) HCT116, (B) RKO and (C) KM12L4 human colorectal cancer cells treated with 0.125, 0.25, 0.5 and 1 mg/ml of common bean cultivars Azufrado Higuera (AH), Bayo Madero (BM), Negro 8025 (N8) and Pinto Durango (PD) peptide extracts for 24 h at 37 °C. Data are expressed as the mean ± standard error of three independent experiments ($n = 6$). (D) IC_{20}, IC_{30} and IC_{50} values (the concentration indicated as mg/ml needed to inhibit cell proliferation by 20%, 30%, and 50% respectively) determined using Prisma 6 software, the smaller the value, the higher the potency. Different letters in rows mean significantly different ($p < 0.05$). IC values > 1 show that the highest concentration (1 mg/ml) was not effective to inhibit 20%, 30% or 50% of cell survival.

was removed and replaced with PBS and incubated for 30 min at room temperature. Cells were blocked with Image-iT FX Signal Enhancer (Life Technologies) for 30 min at room temperature, washed once with PBS and incubated with TNFR1 monoclonal antibody diluted in PBS (1:50) for 5 h at 37 °C. After incubation, cells were washed three times 5 min each with PBS and incubated with Alexa Fluor 488 Goat Anti-mouse (Life Technologies) secondary antibody (1:200) for 3 h at 37 °C. Cells were washed three times with PBS and cured with ProLong gold antifade reagent with DAPI (Life Technologies) for 24 h at 25 °C in dark. The chamber was stored at 4 °C until further use. The cells were visualized using a Carl Zeiss LSM 700 Laser Scanning Microscope (Carl Zeiss AG,

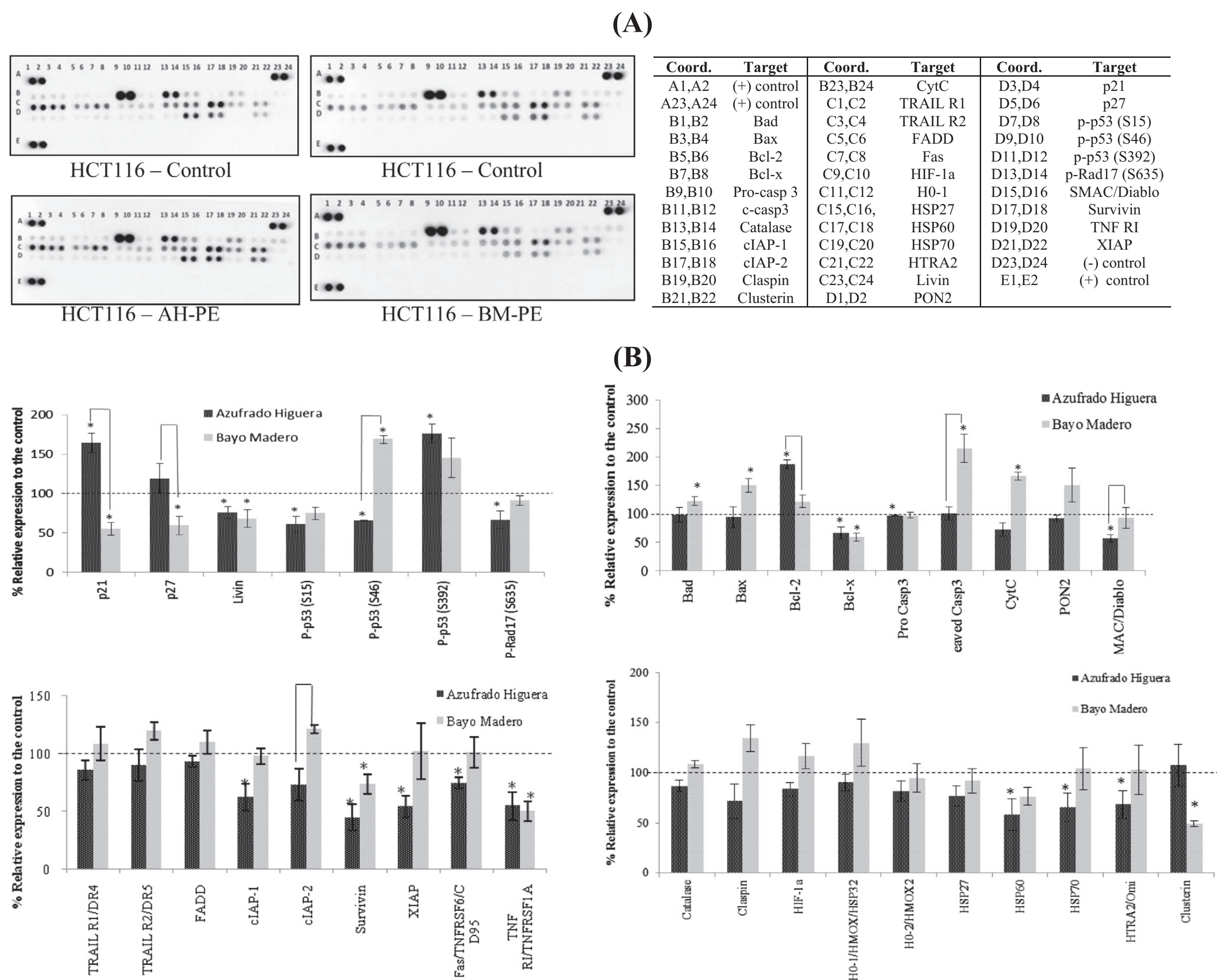


Fig. 3. Effect of AH-PE and BM-PE on HCT116 colorectal cancer cells protein expression using an apoptosis array analysis. HCT116 cells were treated with 0.5 mg/ml of common bean cultivars Azufrado Higuera and Bayo Madero peptide extracts for 24 h at 37 °C. (A) A representative image is shown containing an individual control for each treatment; the table shows the coordinates and the protein target within the array membrane. (B) Compiled bar graphs show the relative expression of the proteins of interest, means with star are significantly different from its control ($p < 0.05$) and lines binding bars means significantly different between cultivars ($p < 0.05$). Data are expressed as the mean ± standard error of three independent experiments ($n = 6$).

Germany) with 63× oil immersion objective. Total intensities and area sums were quantified with Zen 10 software (Carl Zeiss).

2.10. Statistical analysis

The results were expressed as the mean ± standard error of at least two independent experiments and analyzed by ANOVA. Statistical significance was determined using Student's t test ($\alpha = 0.05$) with software JMP version 7.0.

3. Results and discussion

3.1. Bioactive peptides were found in common bean non-digestible fraction

Azufrado Higuera yielded the lowest (41.3%) content of NDF and Bayo Madero the highest (46.6%). NDF protein, ash and total carbohydrate content were not significantly different among cultivars but ranged between 16.3–20.16%; 2.2–3.2%, and 73.5–78.8%, respectively. Lipids content showed significant differences, ranging between 0.9% and 2.1% (Supplementary Table 1). In this study beans were cultivated and processed under the same conditions, nevertheless there were differences in the yield and composition of NDF among cultivars. This could be the result of different genetics, among other environmental factors (Fenner, 1992). Pepsin-pancreatin hydrolysis increased the concentration of molecules below 10 kDa (Supplementary Fig. 1), confirming the conversion of proteins into peptides. Table 1 summarizes the characteristics of most abundant peptides which comprised roughly 70% of total detected protein in the peptides extract. According to MALDI-TOF-MS/MS analysis (Supplementary Fig. 2), similar molecular mass profiles were observed among peptides; there were some differences in the percentage of the least abundant peptides among cultivars (Supplementary Table 2). The resulting peptides in the NDF peptide extracts had small molecular masses suggesting that they can cross cell membranes and get internalized into the cells (Lindgren, Hällbrink, Prochiantz, & Langel, 2000). The variability of the responses on proliferation of human colon cancer cells to different cultivar treatments could be influenced by the PE composition. Even though the molecular masses of the peptides were similar, and the sequenced peptides represented around

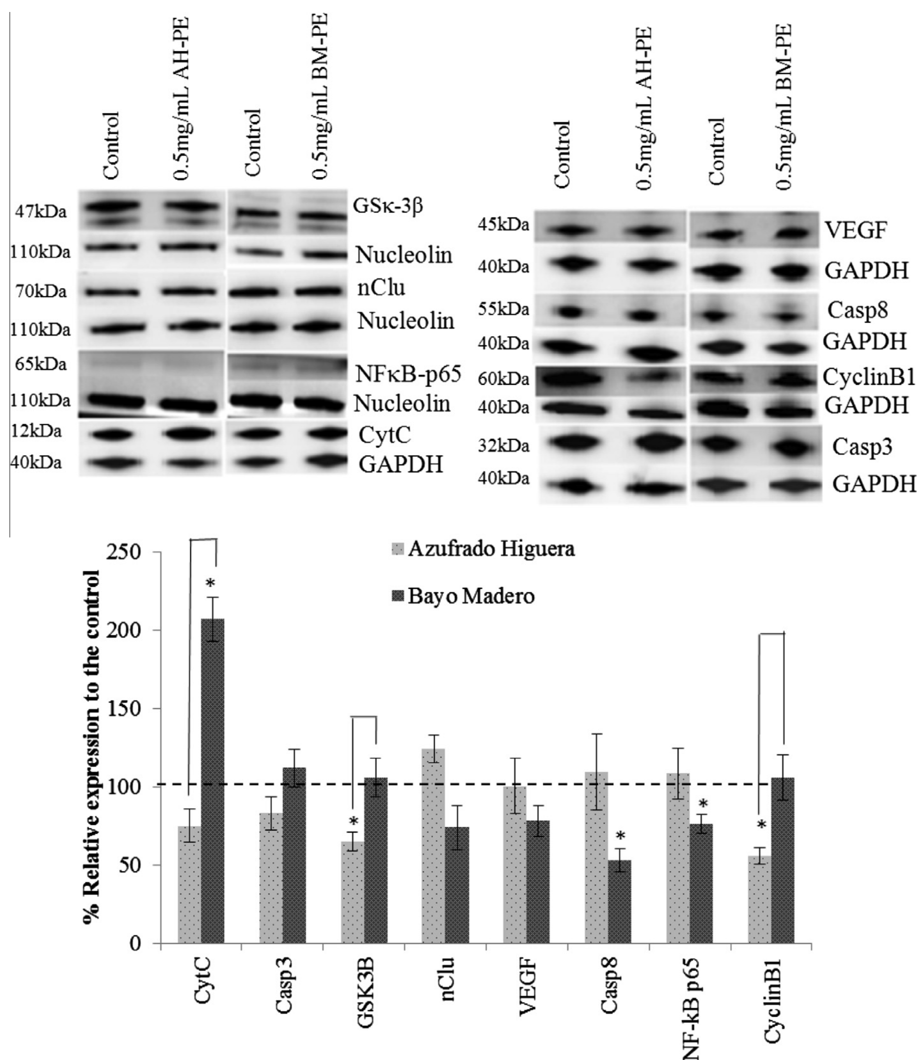


Fig. 4. Effect of AH-PE and BM-PE on HCT116 colorectal cancer cells protein expression. HCT116 cells were treated with 0.5 mg/ml of common bean cultivars Azufrado Higuera and Bayo Madero peptide extracts for 24 h at 37 °C. Nuclear and cytoplasmic cell lysates were extracted and protein expressions were analyzed by Western blot. Means with star are significantly different from its control ($p < 0.05$) and lines binding bars means significantly different between cultivars ($p < 0.05$). Data are expressed as the mean ± standard error of three independent experiments ($n = 3$). A representative image is shown containing an individual control for each treatment.

70% of the protein, small differences in their characteristics and abundance among cultivars were observed. There were seventeen peptides with a molecular mass ranging from 505 to 1019 Da, some of which were not present in all of the cultivars.

Fig. 1 depicts the physicochemical properties determined for the main peptides found. The reported biological activity for these peptides included angiotensin converting enzyme (ACE) inhibition, dipeptidyl peptidase IV (DPP-IV) inhibition, and stimulating vasoactive substance release (Table 1). This finding is in agreement with previous studies with common bean hydrolysates where ACE inhibition was reported (Hernández-Álvarez et al., 2012; Rui, Boye, Simpson, & Prasher, 2012).

Peptides present in common bean NDF could be providing indirect protection for colon health through their biological activities, since sequenced peptides had mainly angiotensin converting enzyme (ACE) inhibition potential.

3.2. Cultivar affected the capability of common bean NDF peptide extract to reduce colon cancer cell proliferation

Fig. 2 is showing proliferation reduction, in a dose–response manner, by the common bean peptide extracts AH-PE, BM-PE, N8-PE and PD-PE. The most potent were for HCT116 (AH-PE-IC_{50} = 0.53 mg/ml; N8-PE-IC_{50} = 0.80 mg/ml) and for RKO (BM-PE-IC_{50} = 0.51 mg/ml, AH-PE-IC_{50} = 0.59 mg/ml, and N8-PE-IC_{50} = 0.79 mg/ml). The effectiveness to reduce cell proliferation varied depending on the cultivar. KM12L4 cell line showed all IC_{50} values >1 mg/ml among the four cultivars. PD-PE showed the highest IC_{50} values, therefore less potency, for all three cell lines. Proliferation of HCT116, RKO and KM12L4 has been reported to be regulated by peptides from food sources such as lunasin, a 43 aminoacid peptide isolated from soybean, which presented an antiproliferative effect on the cell lines mentioned above in a dose-dependent manner (Dia & González de Mejia, 2011).

The difference in the concentration of the peptides per cultivar could be inducing different responses. Nonetheless, an interaction between dietary compounds could also be taking place, including the polyphenol–protein bindings, which may irreversibly be enhanced by heating treatments, for instance while the seeds were cooked (Odzal, Capanoglu, & Altay, 2013).

3.3. Azufrado Higuera and Bayo Madero NDF peptide extract altered protein expression in HCT116 cells

Regarding cultivar Azufrado Higuera, the inhibition of proliferation caused by AH-PE in HCT116 cells stimulated the activation

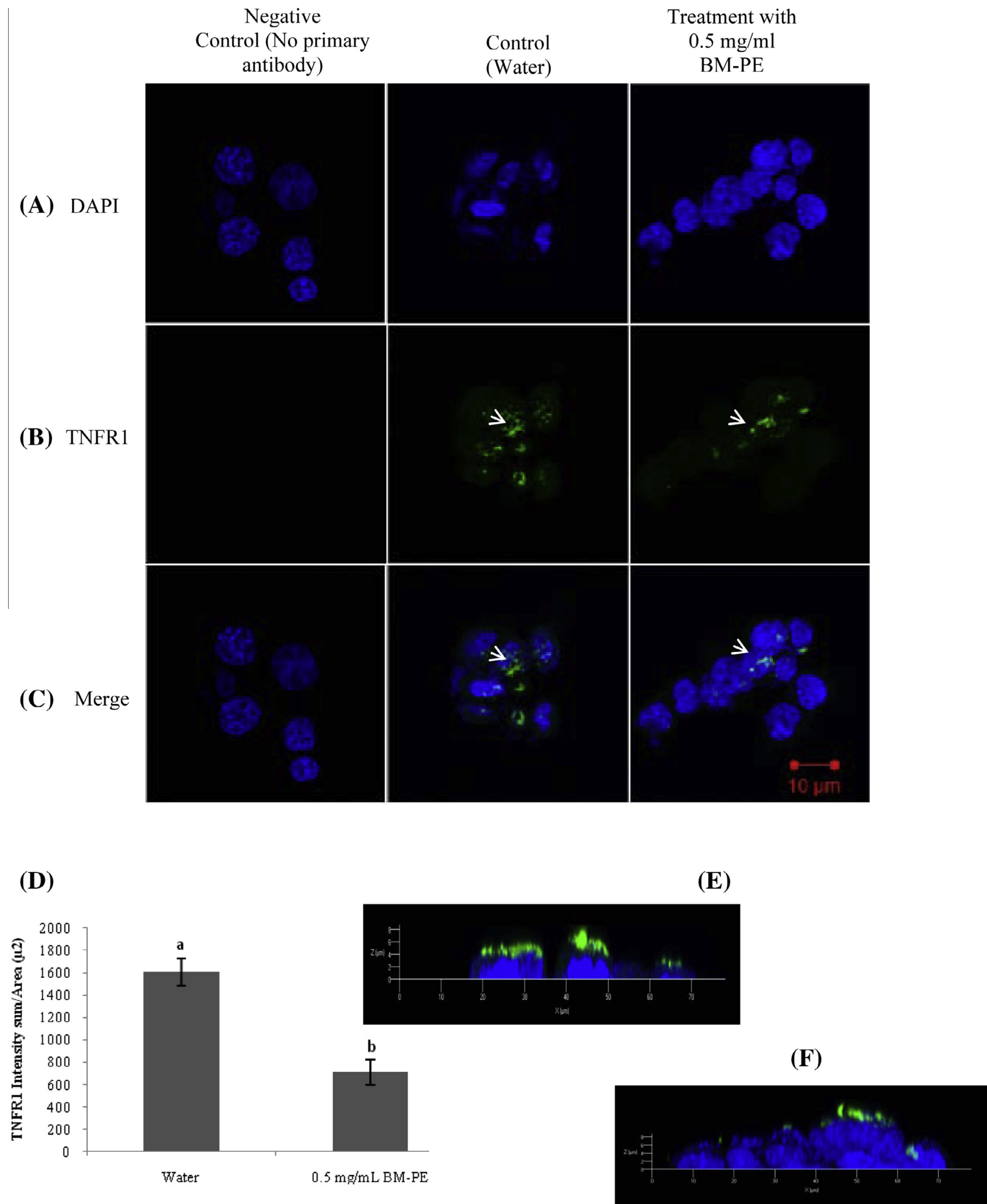


Fig. 5. TNFR1 expression in HCT116 colorectal cancer cells treated with 0.5 mg/ml of common bean cultivar Bayo Madero peptide extract for 24 h at 37 °C. After the treatment, cells were fixed and treated with antibodies for TNFR1 and Alexa Fluor 488 for confocal immunofluorescence microscopy. TNFR1 expression decreased after the treatment, marked by arrows showing high intensity of green fluorescence in the HCT116 membrane. (A) DAPI-stained cells for nucleus. (B) TNFR1 mouse monoclonal antibody and Alexa-Fluor 488-conjugated secondary antibody-stained cells. (C) Merged images from A and B. (D) Bar graph of treated cells and control (molecular biology grade distilled water) Data are expressed as the net intensity/area (μm^2) of two independent experiments mean ± standard error ($n = 6$). Transversal view of (E) untreated and (F) BM-PE treated cells indicating the location of the receptor on the membrane. Image analyses were performed using LSM 700 confocal microscope (Zeiss, Germany).

of tumor suppressor p-p53 Ser^{392} (76% respect to the control). Cyclins are key cell cycle control proteins with specific association with cell cycle progression. Other cell cycle control molecules include cyclin dependent kinase (CDK) inhibitors, such as p21,

which tightly regulate the activities of cyclin/CDK enzyme complex genes (Tenderenda, 2005). The expression of p21 was higher, in HCT116 cells after treatment with AH-PE, with respect to the control (64%), whereas cyclin-B1 expression was lower (−45%); this could regulate cyclin-CDK complex formation (Figs. 3 and 4). Cyclin-B1 is a regulatory protein expressed predominantly during G2/M transition phase (Lindqvist, Rodríguez-Bravo, & Medema, 2009); therefore, the inhibition of cyclin-B1 and overexpression of p21 could be a potential mechanism of action in the antiproliferative effect of AH-PE. This effect was observed previously in KM12L4 cell line treated with lunasin, which promoted an increase in expression of p21 and caused cell cycle arrest in G2/M phase (Dia & González de Mejia, 2011), as well as in HT-29 cell line (Dia & González de Mejia, 2010).

Moreover, for cultivar Bayo Madero, in Figs. 3 and 4 it can be observed that BM-PE induced a different type of antiproliferative mechanism in HCT116 cells, due to p-p53 Ser^{46} being overexpressed (68%) and potentially triggered the activation of mitochondrial apoptosis pathway, which is a mechanism by which cells are able to induce themselves to cell death in an ordered manner. Thus, p-p53 Ser^{46} led to the activation of proapoptotic proteins such as cyt C (66% in the apoptosis array and 106% by western blot), Bcl-2-associated with death promoter BAD (22%), Bax (50%), and cleaved-caspase 3 (115%), an effector caspase which plays an important role in the execution-phase of apoptosis. A concomitant effect contributing with apoptosis process was the decrease in expression of TNFR1 (−51% in the apoptosis array and −56% with immunofluorescence confocal microscopy, Fig. 5), a transmembrane receptor that in some cases induce the activation of antiapoptotic proteins through subunit NF-κB p65 signaling (Baud & Karin, 2001). The decrease of this receptor could be carried to downstream down-regulation of NF-κB p65 (−24%) and, subsequently, of survivin (−27%) and BIRC7 (−32%) which are potential caspases inhibitors.

Depending on the stimuli and stress conditions, p53 can be modified at some amino acid residue; for example, phosphorylation, acetylation among others, thus, depending on the post-translational modification, p53 is able to induce either cell cycle arrest or apoptosis (Kang, Kim, Jang, & Bae, 2009; Smeenk et al., 2011; Thomas et al., 2013). This could partially explain the increased expression of p53 in HCT116 cells exposed to peptides of common bean NDF of both cultivars but phosphorylated in different residues leading to modifications of markers associated with cell cycle arrest or apoptosis. Mitochondrial pathway of apoptosis activation by legume-derived peptides has been reported previously with lunasin in KM12L4 (Dia & González de Mejia, 2011) and HT-29 (Dia & González de Mejía, 2010) cell lines, modifying some markers, such as cytC, Bax and casp3.

4. Conclusion

Peptides GLTSK, LSGNK, GEGSGA, MPACGSS and MTEEY represented 70% of total protein in NDF hydrolysates with ACE inhibition properties. We demonstrated the antiproliferative effect of Azufrado Higuera, Bayo Madero and Negro 8025 cultivars of common bean NDF peptide extract against HCT116 and RKO colorectal cancer cells. Moreover, Azufrado Higuera cultivar induced modification in the expression of markers associated with cell cycle arrest by increasing the expression of p-p53 Ser^{392} and p21, and decreasing the expression of cyclin-B1. Bayo Madero cultivar induced modification in the expression of markers related to mitochondrial activated apoptosis and on the transmembrane receptor TNFR1. Further research is needed regarding the action of the most abundant pure peptides and their potential beneficial effects in protecting colon health.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2014.02.050.

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