

Antioxidant Capacity of Flaxseed Products: The Effect of *In vitro* Digestion

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Abstract This study evaluated the effect of *in vitro* digestion of flaxseed products on Folin-Ciocalteu reagent reducing substances (FCRRS), its antioxidant capacity and prevention of oxidative DNA damage in human monocyte cell line U937. Flaxseed protein isolate was obtained from defatted flaxseed meal and the protein hydrolysate with high antioxidant capacity was obtained from hydrolysis of the protein isolate with Alcalase in a two factor central composite rotatable design (pH 8.5 and enzyme: substrate 1:90, w/w). The FCRRS content and antioxidant capacity measured by FRAP and ORAC in aqueous and 70 % methanol extracts were the highest in protein hydrolysate, followed by protein isolate, while the defatted meal showed the lowest values. After *in vitro* gastrointestinal digestion, the FCRRS content of protein isolate and hydrolysate reached similar values, however the hydrolysate had the highest antioxidant capacity, measured by FRAP while the isolate had the highest ORAC values. The defatted meal showed the lowest capacity in all assays ($p < 0.05$). The hydrolysate did not protect against DNA damage induced by H_2O_2 in U937 cells under the conditions of the present study. The results suggest that flaxseed protein isolate and hydrolysate are potential functional food ingredients with antioxidant capacity.

Keywords Hydrolysate · Phenolic compounds · Comet assay · Methanolic extract · Aqueous extract

Abbreviations

CCRD	Central composite rotatable design
DFM	Defatted flaxseed flour
FCRRS	Folin-Ciocalteu reagent reducing substances
FPI	Flaxseed protein isolate
FPH	Flaxseed protein hydrolysate
FRAP	Ferric reducing antioxidant power
GI	Gastrointestinal
ORAC	Oxygen radical absorbance capacity
PDFM	Partially defatted brown flaxseed meal
ROS	Reactive oxygen species

Introduction

Antioxidant compounds limit deterioration and preserve the nutritional and sensory qualities of foods. In biological systems, antioxidants such as vitamins A, C, E and phenolic compounds are involved in promoting health and prevent chronic diseases and aging [1]. In recent years, peptides with antioxidant capacity have gained significant interest. These peptides can be released from the parent protein by *in vitro* hydrolysis using enzymes isolated from microorganisms, plants and animals or *in vivo* by the gastrointestinal (GI) enzymes and/or intestinal microbiota [2].

Simulated GI digestion has been used to evaluate the effect of digestion on the antioxidant capacity of compounds and their role in the prevention of GI diseases associated to oxidative damage from reactive oxygen species (ROS) [3]. Antioxidant compounds may also protect macromolecules such as lipids and DNA against damage caused by free radicals through scavenging activity [4]. Thus, there is an increasing interest in studying natural substances that can exhibit protective activities against genotoxicity caused by

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oxidative stress in DNA and thus prevent pathological conditions such as inflammation, diabetes, carcinogenesis, and aging [4].

Flaxseed (*Linum usitatissimum* L.) is known as a rich source of α -linolenic acid, dietary fiber and lignans [5]. More recently, it has also received attention as a source of bioactive peptides [5]. *In vitro* studies showed that flaxseed protein hydrolysates have biological activities such as antioxidant, anti-inflammatory and cholesterol-lowering ability [5–7]. However, the stability of the antioxidant compounds from flaxseed meal, its protein isolate and hydrolysate to *in vitro* digestion was not studied. The aims of the present study were to investigate the effects of the protein isolation and enzymatic treatment on the antioxidant capacity of flaxseed, to evaluate the stability of its antioxidant compounds to *in vitro* digestion and to investigate the ability of its isolate and hydrolysate, before and after *in vitro* digestion, to prevent oxidative changes to the DNA of U937 human lymphocyte cells.

Materials and Methods

Materials

Partially defatted brown flaxseed meal (PDFM) was obtained from Cisbra Ltd. (Panambi, RS, Brazil). The enzymes Alcalase 2.4 L, pepsin and pancreatin, bile salts, Folin-Ciocalteu reagent, gallic acid, Trolox, 2,4,6-tri (2-pyridyl)-s-triazine (TPTZ), 2,2'-azobis (2-methylpropionamide) dihydrochloride (AAPH), sodium dodecyl sulfate (SDS) and tricine were purchased from Sigma (St. Louis, MO, USA). The sodium fluorescein was purchased from Synth (São Paulo, Brazil). Acrylamide, bis-acrylamide and Tris were purchased from Bio-Rad (Irvine, CA, USA). Methanol, trifluoroacetic acid (TFA), acetonitrile, coomassie brilliant blue G-250 and sodium hydroxide were purchased from Merck (Hohenbrunn, Germany).

Protein Isolate and Hydrolysate from Defatted Flaxseed Meal

PDFM was defatted with hexane (1:3, w/v) at ambient temperature (23 ± 2 °C) for 24 h with renewal of hexane every 6 h, yielding defatted flaxseed meal (DFM). To obtain the protein isolate (FPI), DFM was dispersed in deionized water (1:10), the pH was adjusted to 9 with 1 N NaOH and the mixture was stirred in a homogenizer (Fisaton, São Paulo, Brazil) at ambient temperature for 60 min, followed by centrifugation ($16,274 \times g$ for 30 min). The extraction process was repeated and supernatants of all steps were pooled. The pH was adjusted to 4.2 with 1 N HCl and the precipitated protein was separated by centrifugation, washed

three times with acidified water (pH 4.2) and suspended in deionized water and pH was adjusted to 6 with 1 N NaOH. The protein isolate was freeze-dried and stored at -20 °C until analysis.

In order to obtain a flaxseed protein hydrolysate with high antioxidant capacity, a central composite rotatable design (CCRD) with 2 factors, 2 levels, 3 center points and 4 axial points with a total of 11 trials was employed. The independent variables were enzyme:substrate ratio (E/S) (1:150 to 1:30, w/w) and pH (7.5 to 9.5). The dependent variables were the antioxidant capacity and degree of hydrolysis (DH). To obtain the hydrolysates, FPI (5 %w/v protein in distilled water) was hydrolyzed with Alcalase at 60 °C for 180 min, under the conditions established for each CCRD assay. The pH was maintained by adding 0.25 N NaOH using an automatic titrator (DL50 Graphix Mettler Toledo, Schwerzenbach, Switzerland). The reaction was stopped by heating the reaction mixture at 90 °C for 10 min to inactivate the enzyme [8]. The hydrolysates were cooled (7 °C), frozen (-20 °C) and freeze-dried. A control test was performed with no Alcalase addition.

Characterization of PDFM, DFM, FPI and FPH

The ash, moisture, protein, fat, carbohydrate and dietary fiber contents of the PDFM, DFM and FPI was determined according to AOAC methods [9]. Fat and protein contents were determined by Bligh and Dyer [10] and Kjeldahl ($N\% \times 6.25$) [11] methods, respectively.

The Folin-Ciocalteu reagent reducing substances (FCRRS) content was determined in aqueous and methanolic (70 %) extracts [12]. The result was defined as the content of FCRRS, since the reagent is not specific for phenolic compounds and may react with other reducing substances, such as aromatic amino acids residues and ascorbic acid [12]. The measurements were carried out in triplicate, and the results were expressed as mg of gallic acid equivalents (GAE)/g sample.

The electrophoretic profiles of DFM and FPI were determined by SDS-PAGE in a Mini Protean II apparatus (Bio-Rad, Hercules, CA, USA) using 4–15 % polyacrylamide gradient gel under reducing conditions [13]. The samples (5 mg ml^{-1}) were dispersed in buffer (2 % SDS and 5 % β -mercaptoethanol) and were boiled at 96 °C for 10 min. A 10 μl aliquot was applied to each well. After the run, the gels were stained with 0.1 % coomassie blue and destained in an acetic acid/methanol/distilled water solution (1:4:5). A 14.4–94.7 kDa marker kit (Bio-Rad) was used as molecular weight standard. The hydrolysate and digested samples were evaluated by Tricine-SDS PAGE [14]. The gels consisted of a resolving gel (14.6 %T, 4 %C), spacing gel (10 %T, 3 %C) and stacking gel (4 %T, 3 %C). The samples were dissolved

(1 % protein w/v) in reducing buffer (0.5 M Tris-HCl, pH 6.8, 10 % SDS, 10 % glycerol, 5 % β -mercaptoethanol and 0.1 % bromophenol blue) and heated at 40 °C for 30 min. Aliquot of 10 μ l was loaded in each well. After the run, the gels were fixed for 1 h in a methanol/acetic acid/water solution (5:1:4), stained with coomassie brilliant blue G-250 (0.04 % in 10 % acetic acid) and destained with 10 % acetic acid.

Antioxidant Capacity

The antioxidant capacity was measured in aqueous and methanolic (70 %) extracts. The ferric reducing antioxidant power (FRAP) was performed according to Thaipong et al. [15] with adaptations. FRAP values of the extracts were determined from a standard curve of ferrous sulfate solution and the results were expressed in mg FeSO₄ (FS)/g sample. The oxygen radical absorbance capacity (ORAC) method was performed according to the methodology described by Dávalos et al. [16]. Fluorescein reacted with free radicals generated by AAPH, yielding a non-fluorescent product. The fluorescence measurements were made using a Synergy™ HT Multi-Mode Microplate Reader with an excitation wavelength of 485 nm and an emission wavelength of 520 nm. The antioxidant activity was expressed as μ mol of Trolox equivalent (TE)/g sample. The assays were carried out in triplicate.

Degree of Hydrolysis (DH)

The DH of the hydrolysates was determined using the pH-stat procedure [8]. The OPA method [17] was used to determine the DH of the samples before and after *in vitro* digestion. All measurements were performed in duplicate.

Simulated GI Digestion

The simulated GI digestion of the products was carried out as described by Martos et al. [18] with adaptations. To simulate gastric digestion, the samples were dispersed in a simulated gastric fluid (35 mM NaCl), the pH was adjusted to 2 by adding 1 N HCl and the volume was adjusted to reach a final concentration of 5.9 mg protein/ml. The mixture was incubated in a shaking water bath at 37 °C, 60 rpm. After 15 min, pepsin was added (E/S 1:20, w/w), the pH was adjusted to 2 and the mixture was incubated at 37 °C for 60 min under continuous stirring. The intestinal digestion was simulated by using an aliquot of 2.9 ml of gastric digest adjusted to pH 6.5 by adding 1 M NaHCO₃, 1 M CaCl₂, and 240 μ l of bile salts at a concentration 9 mg/ml. The mixture was incubated at 37 °C for 15 min under stirring. Pancreatin (E/S 1:25, w/w) was added and after stirring at 60 rpm for

60 min at 37 °C the reaction was stopped by heating to 90 °C for 10 min. At the end of the simulated GI digestion, the digest presented the following final concentrations: 3.9 mg/ml protein, 7.6 mM CaCl₂, 0.07 mg/ml bile salts, 0.16 mg/ml pancreatin and 0.2 mg/ml pepsin. The digest was centrifuged (11,000 \times g for 15 min) and the supernatant containing the digested product was stored at -20 °C. The procedure was performed in triplicate.

Cell Culture and DNA Damage (Comet Assay)

Human monocytic U937 cells were obtained from the European Collection of Animal Cell Cultures (ECACC) and maintained in RPMI-1640 medium enriched with 10 % FBS (v/v). Cells were incubated at 37 °C in a humidified atmosphere of CO₂ in air in the absence of antibiotics. U937 cells (1 \times 10⁵ cells/ml) were supplemented with the test compounds for 24 h in 6-well plates to a final volume of 2 ml. After incubation, the cells were exposed to 40 μ M H₂O₂ for 30 min at 37 °C. Cells were harvested, embedded in a low melting point agarose (1 %) and placed on microscope slides coated with a layer of normal gelling agarose. Slides were placed in lysis solution [2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10, 1 % (w/v) sodium sarcosinate, 1 % (v/v) Triton X-100 and 10 % (v/v) DMSO; 4 °C] for 2 h. Slides were then placed in a horizontal gel electrophoresis tank (Horizon 20 25, GIBCO BRL, Life Technologies, Scotland) containing fresh electrophoresis solution (300 mM NaOH, 1 mM EDTA) for 30 min. Electrophoresis was performed at 25 V and 300 mA for 25 min at 4 °C. After electrophoresis, the slides were washed with neutralizing buffer (0.4 M Tris, pH 7.5). Slides were stained with ethidium bromide (20 μ g/ml) and then covered with coverslips. Cells were visualized at a magnification of 200X (Nikon Optiphot-2) and the Komet 5.5 image analysis software program (Andor Technology, Northern Ireland) was used to determine the level of DNA damage, which was expressed as percentage of tail DNA. The results represent mean \pm standard error (SE) of three independent experiments.

Table 1 Chemical composition of partially defatted flaxseed meal (PDFM), defatted flaxseed meal (DFM) and flaxseed protein isolate (FPI)

Composition (%)	PDFM	DFM	FPI
Proteins ^a	29.3 \pm 0.38	39.0 \pm 0.25	71.8 \pm 0.94
Lipids	14.9 \pm 0.87	5.4 \pm 1.74	4.1 \pm 0.13
Ash	4.2 \pm 0.00	4.0 \pm 0.45	1.5 \pm 0.05
Moisture	8.4 \pm 0.00	9.3 \pm 0.00	9.1 \pm 0.00
Dietary fiber	35.1 \pm 0.31	38.8 \pm 0.32	13.0 \pm 0.29

^a N% \times 6.25; mean \pm standard deviation of three replicates

Table 2 Degree of hydrolysis and antioxidant capacity as determined by the ORAC and FRAP methods from aqueous and methanol extracts of hydrolysates produced under the specified CCRD conditions

Trial	Uncoded values		DH%	FRAP (mg FS/g FPH)		ORAC ($\mu\text{mol TE/g FPH}$)	
	pH	E/S		Aqueous extract	Methanol extract	Aqueous extract	Methanol extract
1	7.8	1:133	14.4	35.6 \pm 2.0	33.0 \pm 1.7	335.9 \pm 27.1	335.8 \pm 32.1
2	7.8	1:48	19.3	33.7 \pm 2.2	29.0 \pm 0.9	331.7 \pm 26.6	326.7 \pm 32.8
3	9.2	1:133	13.6	41.7 \pm 2.5	35.6 \pm 0.3	330.0 \pm 28.6	291.8 \pm 6.1
4	9.2	1:48	17.5	39.2 \pm 4.6	34.7 \pm 0.8	319.3 \pm 16.3	314.3 \pm 42.0
5	7.5	1:90	17.3	32.4 \pm 1.3	27.7 \pm 1.0	251.4 \pm 8.8	281.8 \pm 39.6
6	9.5	1:90	12.7	41.1 \pm 3.7	36.6 \pm 2.6	271.8 \pm 27.4	266.8 \pm 10.8
7	8.5	1:150	13.8	37.5 \pm 1.0	38.6 \pm 1.7	338.0 \pm 42.1	320.4 \pm 24.6
8	8.5	1:30	18.9	35.1 \pm 3.4	36.2 \pm 1.9	324.2 \pm 20.1	291.2 \pm 31.9
9	8.5	1:90	14.8	37.6 \pm 3.1	37.7 \pm 3.6	291.4 \pm 19.8	331.9 \pm 15.7
10	8.5	1:90	14.7	37.8 \pm 2.4	38.5 \pm 0.6	284.6 \pm 25.3	353.5 \pm 12.7
11	8.5	1:90	15.3	37.2 \pm 1.1	38.1 \pm 0.3	260.6 \pm 23.9	347.1 \pm 24.0
Control	8.5	–	2.5	23.4 \pm 0.6	21.6 \pm 1.3	153.6 \pm 14.8	176.42 \pm 6.5

Mean \pm standard deviation of three repetitions with three replicates; DH% degree of hydrolysis; TE trolox equivalents; FS ferrous sulfate; FPH flaxseed protein hydrolysate. Hydrolysis conditions: Alcalase; 60 °C; 180 min. Control: trial without Alcalase, 60 °C, 180 min

Statistical Analysis

A paired *t*-test was performed to determine the difference between the contents of the FCRRS and the antioxidant capacity before and after *in vitro* digestion. The FCRRS content and antioxidant capacity were analyzed by ANOVA followed by the Tukey's test. The software STATISTICA version 7.0 (Stat Soft, Tulsa, USA) was used to analyze the CCRD.

Results and Discussion

The composition of PDFM, DFM and FPI is shown in Table 1. The protein content of FPI (71.8 %) was lower than that reported in literature [11, 19]. The difference in the contents may be either due to the removal of the seed coat prior to obtaining the flaxseed meal or the extraction method [11]. The dietary fiber content of DFM and FPI samples were 38.8 % and 13 %, respectively. Due to its high dietary fiber content, particularly mucilage, flaxseed products can play an important role in preventing diseases such as diabetes, obesity and cancer [11].

Flaxseed Hydrolysates

The antioxidant capacity and the DH of the hydrolysates produced under the CCRD conditions are shown in Table 2. The highest FRAP values in aqueous and methanolic extracts were 41.7 mg FS/g FPH (E/S: 1:133, pH 9.2) and 38.6 mg FS/g FPH (E/S: 1:150, pH 8.5), respectively. The

highest ORAC values shown by the FPHs were 338.0 $\mu\text{mol TE/g FPH}$ (E/S: 1:150, pH 8.5) in the aqueous extract and 353.5 $\mu\text{mol TE/g FPH}$ (E/S: 1:90, pH 8.5) in the methanol extract. Based on mathematical models derived from the CCRD (Online Resource 1), the hydrolysis condition for obtaining hydrolysate with the highest antioxidant capacity in aqueous and methanolic extracts, measured by ORAC and FRAP, was E/S 1:90 and pH 8.5 (Online Resource 2). The hydrolysate obtained under this condition was designated as FPH 0.

The DH of the hydrolysates ranged from 12.7 to 19.3 %. The hydrolysates with the highest antioxidant capacity measured by FRAP and ORAC methods showed DH values of

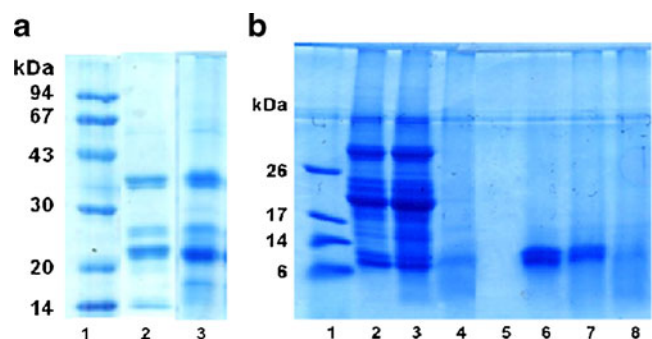


Fig. 1 Electrophoresis of samples before and after *in vitro* digestion. **a** SDS PAGE: 1 - molecular weight standards, 2 - defatted flaxseed meal (DFM), 3 - flaxseed protein isolate (FPI). **b** tricine SDS-PAGE: 1 - molecular weight standards, 2 - defatted flaxseed meal (DFM), 3 - flaxseed protein isolate (FPI), 4 - flaxseed protein hydrolysate (FPH 0), 5 - control digestion, 6 - DFM, 7 - FPI, 8 - FPH 0, after *in vitro* digestion

Table 3 Degree of hydrolysis, content of the Folin-Ciocalteu reagent reducing substances and antioxidant capacity as determined by the FRAP and ORAC methods from aqueous and methanol extracts of DFM, FPI and FPH 0, before and after *in vitro* digestion

Samples	DH%	Aqueous extract			Methanol extract		
		FCRRS (mg GAE/g sample)	FRAP (FS mg/g sample)	ORAC (μ mol TE/g sample)	FCRRS (mg GAE/g sample)	FRAP (FS mg/g sample)	ORAC (μ mol TE/g sample)
Before <i>in vitro</i> digestion							
DFM	0.00 (0.03)	1.4 ^{a,A} (0.14)	5.9 ^{a,A} (0.15)	40.9 ^{a,A} (1.30)	0.7 ^{a,A} (0.05)	3.9 ^{a,A} (0.30)	27.0 ^{a,A} (3.57)
FPI	0.00 (0.02)	2.7 ^{a,B} (0.28)	13.0 ^{a,B} (0.34)	64.6 ^{a,A} (9.93)	2.2 ^{a,B} (0.10)	10.5 ^{a,B} (0.35)	60.6 ^{a,B} (14.27)
FPH 0	25.15 (0.62)	24.1 ^{a,C} (0.26)	37.5 ^{a,C} (0.30)	300.1 ^{a,B} (21.18)	19.5 ^{a,C} (0.57)	38.1 ^{a,C} (0.39)	325.5 ^{a,C} (31.67)
After <i>in vitro</i> digestion							
DFM	18.41 (0.32)	10.4 ^{b,A} (0.58)	18.1 ^{b,A} (0.79)	188.4 ^{b,A} (23.43)	12.2 ^{b,A} (0.72)	30.6 ^{b,A} (2.27)	279.4 ^{b,A} (6.72)
FPI	28.28 (0.45)	24.7 ^{b,B} (1.87)	31.1 ^{b,B} (1.94)	421.2 ^{b,B} (49.20)	24.7 ^{b,B} (2.08)	34.8 ^{b,B} (1.65)	436.8 ^{b,B} (7.98)
FPH 0	25.21 (0.35)	24.1 ^{a,B} (0.32)	47.1 ^{b,C} (1.06)	485.1 ^{b,B} (38.07)	21.5 ^{a,B} (0.76)	48.3 ^{b,C} (1.63)	391.1 ^{b,C} (17.11)

Values in parentheses represent standard deviation values; different lowercase letters, on the same column, for the same sample and extract, before and after digestion, are statistically significant ($p < 0.05$); different capital letters in the same column for the same test are statistically significant ($p < 0.05$); *GAE* gallic acid equivalents; *TE* trolox equivalents; *FS* ferrous sulfate; *DFM* defatted flaxseed flour; *FPI* flaxseed protein isolate; *FPH* flaxseed protein hydrolysate

13.6 and 14.7 %, respectively, which are in the middle of this range. Marambe et al. [6] reported that increasing the DH value led to decreased hydroxyl radical scavenging activity of flaxseed protein hydrolysate with Flavourzyme. These results are in accordance with other studies, which suggested that there is an optimal range of DH values to produce peptides with antioxidant capacity [20]. As the peptide size increases, the access of reactive species to nucleophilic centers becomes more difficult while low molecular weight peptides and free amino acids exhibit low antioxidant capacity, since they have reduced solubility in lipophilic medium [2]. The antioxidant activity is a synergistic effect of the combination of amino acid with antioxidant potential and their position in the peptide sequence [2].

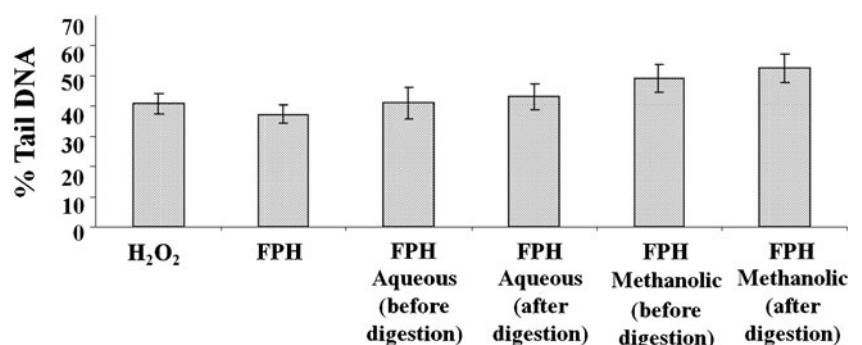
Effect of *In Vitro* Digestion on the Content of Reducing Substances and Antioxidant Capacity

Electrophoretic profile of DFM and FPI showed bands between 6–67 kDa (Fig. 1a) and of FPH 0 consisted of a diffused band at approximately 6 kDa (Fig. 1b), which indicates that

none of the original fractions found in FPI (Fig. 1a, b) was resistant to the Alcalase action. Following the digestion, the bands >14 kDa were no longer visible, and two bands between 14 and 6 kDa remained visible. The existence of these bands in the FPH 0 after digestion suggests that these fractions are at least partially resistant to hydrolysis.

Prior to the *in vitro* digestion, the FCRRS content of the DFM in the methanol extract (0.7 mg GAE/g) was lower than that reported for whole flaxseed meal (2.7 to 3.25 mg GAE/g) [12]. These differences may be due to the loss of phenolic compounds and lignans during the defatting process [21]. The FCRRS content of FPI was two to three times higher than that of DFM in the aqueous and methanol extracts, and up to 9 times lower than the hydrolysates (Table 3). The increase in the FCRRS content in FPI compared to the meal, which has also been observed for amaranth [22] and buckwheat [20], may be due to the solubilization of phenolic compounds during the alkaline extraction step [11]. The highest FCRRS content was shown by FPH (24.2 mg GAE/g FPH). The hydrolysis of FPI catalyzed by Alcalase was expected to release small peptides and phenolic compounds associated

Fig. 2 DNA damage in U937 cells treated with flaxseed protein hydrolysate (FPH) extracts before and after simulated GI digestion. DNA damage was expressed as percentage of tail DNA. Data represent the mean \pm standard deviation of three independent experiments



with flaxseed protein by breaking down the protein-polyphenol complexes [3], leading to increased FCRRS content. In addition, the alkaline pH and temperature used for Alcalase activity may have caused a partial hydrolysis of the lignans, releasing phenolic compounds [23, 24], as was shown by the control assay, which exhibit higher antioxidant capacity than the FPI ($p < 0.05$).

The releasing of FCRRS during the protein concentration and hydrolysis processes affected the ability of neutralizing free radicals. FPI showed FRAP value two times higher in the aqueous extract and three times higher in the methanol extract and up to two times higher ORAC values than the DFM. The antioxidant capacity of FPI measured by FRAP and ORAC was up to four to six times lower than of FPH ($p < 0.05$).

After *in vitro* digestion, the FCRRS content of DFM increased 9 times in the aqueous extract and 17 times in the methanol extract ($p < 0.05$), while that of FPI increased 20 times in both extracts, reaching values similar to the digested hydrolysates ($p < 0.05$). DFM also showed an increase in antioxidant capacity, being four times higher in the aqueous extract and 10 times in the methanol extract. In addition, FPI exhibited an increase in both extracts, being three times higher as determined by FRAP and eight times by ORAC. During *in vitro* hydrolysis, DFM and FPI generated peptides with molecular weight lower than 6 kDa (Fig. 1), which have higher antioxidant capacity than the parent proteins [25]. These results suggest that flaxseed antioxidant compounds remained stable under the simulated GI conditions, possibly due to the protection exerted by the food matrix. Siracusa et al. [26] observed that the behavior of phenolic compounds toward simulated digestion strongly depends on the matrix which exerts a sort of “self-protection” effect on its components when submitted to the digestion process. The actions of the digestive enzymes and bile salts can also promote the release of phenolic compounds stored in plant cells or bound to other compounds [27].

Overall, the FPH exhibited less dramatic changes after *in vitro* digestion, showing small but significant, increases in FCRRS content in both extracts and a 1.4-fold increase ($p < 0.05$) in antioxidant capacity (FRAP and ORAC). These results suggest that compounds with antioxidant capacity present in the hydrolysates were already accessible and stable under the digestion conditions. The low effect of digestive enzymes on antioxidant capacity of FPH should be due to the broad specificity of Alcalase, which may have already cleaved the specific sites required for pepsin and pancreatin action [28] and released phenolic compounds during hydrolysis. Furthermore, after *in vitro* digestion, the antioxidant capacity of FPI as determined by ORAC was 30 % higher than of the FPH but 10 % lower when determined by FRAP ($p < 0.05$). The ORAC method mimics both oxidative deterioration and the mechanism of action of antioxidants that occur *in vivo* [29]. Thus, the results suggest that FPI may have the potential to maintaining the redox equilibrium in the GI tract. These results are

relevant since FPI may contribute to prevent diseases caused by ROS production by immune cells during the digestive process [3, 30] or by a pro-oxidation environment induced by a diet rich in saturated fat and meat [31].

In order to investigate the antioxidant potential of the extracts at cellular level, the human macrophage cell line U937 was employed. The conditions of oxidative stress were simulated in the cells by the addition of H_2O_2 , which produces hydroxyl radicals in the presence of iron, by means of the Fenton reaction [32]. The addition of 40 μM H_2O_2 to U937 cells increased the level of DNA damage in the cell from a baseline level of approximately 5 to 40 % tail DNA (Fig. 2) as measured by the comet assay. The preincubation of the cells with aqueous and methanolic FPH extracts, before and after digestion, did not significantly protect ($p > 0.05$) the cells against H_2O_2 -induced DNA single strand breaks (Fig. 2). A similar effect was obtained at concentrations of 0.125, 0.25 or 1.0 $\mu g/ml$ (data not shown). Despite good radical scavenging activity shown by the extracts and digests (Table 3), they failed to demonstrate any radical scavenging in U937 cells. Plant extracts have demonstrated an ability to scavenge ROS and prevent hydroxyl radical induced damage to macromolecules including DNA in human cells [4]. Thus, neither the main polyphenols found in flaxseed, the lignans, which require hydrolysis by colonic microflora to exert biological effects *in vivo* [33] nor the peptides released by Alcalase and digestive enzymes showed protective activity against DNA damage.

These results suggest that the use of flaxseed protein isolate and hydrolysate as ingredients to formulate functional foods and nutraceuticals may be a promising alternative. However, studies that characterize the antioxidant compounds in the aqueous and methanol extracts are necessary to better understand the contribution of peptides and phenolic compounds to the antioxidant activity of flaxseed protein products.

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