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Article

Utilization of Hardened *Phaseolus Lunatus* Beans as a Source of Naturally Occurring Bioactive Peptides

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RESUMO

Feijões endurecidos são rejeitados pelos consumidores e frequentemente utilizados como ração animal, apesar de seu conteúdo nutricional permanecer inalterado após o endurecimento. Em particular, os feijões endurecidos ainda representam uma rica fonte de proteína e carboidrato, em um mundo onde a fome é uma realidade. Existem diversos estudos que descrevem peptídeos bioativos encapsulados; no entanto, pouco se sabe sobre peptídeos naturais e suas propriedades. Neste estudo, peptídeos naturais de *Phaseolus lunatus* endurecido foram extraídos, parcialmente purificados e examinados quanto ao seu potencial biológico. Soluções extratoras foram testadas para obter o máximo rendimento e atividade antioxidante. A solução menos polar foi a mais eficaz para a obtenção de peptídeos antioxidantes (DPPH – 962 ± 29 e FRAP – 2567 ± 83 µmol Trolox/mg de proteína). O tratamento por 30 minutos a 90 °C aumentou em 2,6 vezes (2497 µmol de Trolox/mg de proteína) a atividade antioxidante pelo ensaio DPPH e em 1,2 vezes (3149 µmol de Trolox/mg de proteína) pelo ensaio FRAP. Os peptídeos antioxidantes na fração <3 kDa resistiram à digestão gástrica/intestinal e apresentaram atividade antiperoxidativa próxima à do BHT. Finalmente, os peptídeos apresentaram atividade vasodilatadora na faixa de 30% (F<3 kDa) a 17% (F<3 kDa hidrolisada) e atividade inibitória da ECA na faixa de 15% (F<3 kDa) a 85% (F<3 kDa hidrolisada).
Palavras-chave: pesca INN; afundamento; poluição marinha.

ABSTRACT

Hardened beans are rejected by consumers and frequently used as animal feed despite their unchanged nutritional content after hardening. Particularly, hardened beans remain a rich source of protein and carbohydrates in a hungry world. There are several studies describing encrypted bioactive peptides; however, little is known about naturally occurring peptides and their functionalities.

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In this study, naturally occurring peptides from hardened *Phaseolus lunatus* were extracted, partially purified and examined regarding their biological potential. Extractor solutions were tested to obtain the maximum yield and antioxidant activity. The most nonpolar was the more effective for obtaining antioxidant peptides (DPPH - 962±29 and FRAP - 2567±83 μmol Trolox/mg protein). Treatment for 30 minutes/90 ºC increased 2.6-fold (2497 μmol Trolox/mg protein) the antioxidant activity by DPPH and 1.2-fold (3149 μmol Trolox/mg protein) the FRAP assay. Antioxidant peptides in the <3kDa-fraction resisted gastric/intestinal digestion and presented anti-peroxidative activity close to the BHT. Finally, peptides presented vasorelaxant activity in the range of 30% (F<3 kDa) and 17% (hydrolyzed F<3 kDa) and ACE-inhibitory activity in the range of 15% (F<3 kDa) and 85% (hydrolyzed F<3 kDa).
Keywords: antioxidant, antiperoxidative, thermal-resistant, vasoactive, ACE-inhibitor.

Introduction

Beans are a staple food that contains nutrients capable of partially replacing protein products of animal origin, such as meat and cheese, assuming significant importance in the diet of low-income populations in rural and urban areas. *Phaseolus lunatus*, also known as lima or butter bean, is the second most important species of the Phaseolus genus, behind only the common bean (*Phaseolus vulgaris*) in terms of cultivated area. It is found in South America and Asia, where it is used as food and for medicinal purposes (Li et al., 2024).

Like other beans, *P. lunatus* beans undergo a natural hardening process after harvest, which leads consumers to reject them. Hardened beans are often used as animal feed, even though their nutritional content remains the same, which constitutes a significant waste of food in a hungry world. Therefore, studies that allow the inclusion of hard beans in food formulations are aligned with the ONU SDGs to minimize waste and generate sustainable options for the food sector.

Beans have been reported as a source of bioactive peptides since they have high protein content that can be relatively well digested. However, the knowledge about the presence of naturally occurring antioxidant peptides in beans is scarce, as well as their properties.

Antioxidant defense systems co-evolved along with aerobic metabolism to counteract oxidative damage produced by oxygen-free radicals and other reactive oxygen species (ROS). The imbalance between the antioxidant defense systems and ROS production has been associated with many chronic diseases, such as atherosclerosis, cancer, diabetes, and rheumatoid arthritis (Miao et al., 2018; Quiao et al., 2021). Moreover, redox imbalance and dysregulated ROS production are hallmarks of hypertension in humans, and consequently heart disease and chronic kidney disease (Touyz et al., 2020; Camargo et al., 2025).

Another key action of antioxidants is the protection against neurovegetative disorders. The brain has a high metabolic rate, depending on oxidative phosphorylation for its energy source. Additionally, brain tissue has high levels of lipids, which are significantly susceptible to oxidative attack. Thus, oxidative stress can affect the function of neurons and trigger a broad spectrum of neurodegenerative disorders (Houldsworth, 2024).

Additionally, the free radical theory of aging emphasizes that the generation of oxygen free radicals, an unavoidable consequence of life in an aerobic environment, results in cumulative damage to critical cellular components, leading to functional disorders or tissue injuries related to the natural aging process (Wang et al., 2011; Urrutia & Bórquez, 2023; Wang et al., 2024; Houldsworth 2024).

Substantial evidence indicates that dietary antioxidants play an important role in the redox equilibrium because they can reduce the initiation and propagation of free radical cascades *in vivo* and, therefore, minimize the free radical-induced damage, preventing and delaying the aging process (Qiao et al., 2021; Houldsworth, 2024). Synthetic antioxidants are cost-effective and efficient, but since they display some toxic and hazardous effects, there is a trend in the food and pharmaceutical industries to replace them with natural products (Parcheta et al., 2021; Wang et al., 2024).

In this sense, this work aimed to investigate the presence of naturally occurring antioxidant peptides in hardened *Phaseolus lunatus* beans, to evaluate the stability of these peptides under thermal treatment, simulated gastric digestion, and test their antioxidant activity, angiotensin I-converting enzyme (ACE) inhibition and *ex vivo* vasorelaxant effect.

Materials and Methods

Materials

Hardened seeds of *Phaseolus lunatus* were identified in the catalog from National Agro-Research Institute (INIA) and were collected in the farm Russia, Iguatu, Ceara State, Brazil. The hull was manually removed, and the whole beans were ground to produce the flour with a particle size equivalent to 32 mesh. The common bean flour was stored at -20ºC until processing.

Qubit® protein assay reagent was purchased from Invitrogen. 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,4,6-tris (2-pyridyl)-*s*-triazine (TPTZ) and 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), phenylephrine, acetyl choline, thio barbituric acid (TBA), malonaldehyde (MDA), 2,6-bis (1,1-dimethylethyl)-4-methylphenol beta (BHT), Pepsin (porcine) and Pancreatin (porcine), N_{α}-Benzoyl-L-arginine 4-nitroanilide hydrochloride (BApNA), dimethyl-sulphoxide (DMSO), were purchase from Sigma-Aldrich. All other reagents were analytical grades.

Extraction method

To obtain the maximum yield of antioxidant peptides, four extraction solutions were tested to determine the best extraction condition. Methods 1- 3 were based on the methodology described by Mahatmanto et al. (2014), and method 4 was based on the methodology of Oseguera-Toledo et al. (2011).

Method 1: extraction was conducted by mixing 1 g of bean flour with 5 mL of a solution containing dichloromethane and methanol (1:1 v/v). The mixture was left stirring for 1h, at room temperature, for 15 minutes at 4ºC.

The supernatant was separated, and after evaporation of the solvent, the pellet was suspended in 2.5 mL of 0.3 mol L^{-1} sodium acetate buffer, pH 3.4. This protein solution was stored for further analysis.

Method 2: extraction was conducted by mixing 1 g of flour and 5 mL of a solution containing acetonitrile, water, and formic acid in the proportion of 25:24:1. The mixture was stirred for 1 h at 25ºC. After extraction, the supernatant was separated, and the organic solvents were evaporated. The protein concentrates were frozen at -80ºC overnight and lyophilized for further analysis.

Method 3: extraction was made by mixing 1 g of bean flour with 5 mL of 20 mmol L^{-1} sodium acetate solution, pH 5.0. The mixture was maintained under orbital stirring for 1 h, at 4ºC. The mixture was centrifuged, and 5 mL of cold acetone was added to the supernatant. After 15 minutes of incubation at 4ºC, the suspension was centrifuged, and the supernatant was stored for further analysis.

Method 4: extraction was made by mixing 1 g of bean flour with 5 mL of distilled water, pH 8.0 adjusted with NaOH 100 mmol L^{-1}. The mixture was maintained under stirring for 1 h, at 35 ºC. The mixture was centrifuged, the supernatant was stored, and the precipitate was submitted to a new extraction procedure. The supernatant was pooled and stored for further analysis

The protein content of all samples was determined using the Qubit® Protein Assay Kit, following the manufacturer's instructions. Alternatively, the protein content was determined by Bradford (1976) methodology, using standard protein bovine serum albumin (BSA).

Peptide Purification - Ultrafiltration

The ultrafiltration runs were performed in a 400 mL stirred dead-end cell using 3 kDa ultrafiltration membranes (regenerated cellulose Millipore reference PLBC07610 and PLGC07610, respectively). The system was operated at 1.5 kgf cm^{-2} and 4ºC with a suitable pH (Lemes et al., 2014). Fractionation of the extract components was conducted through sequential filtration using membranes with decreasing pore sizes (cut-off).

Trypsin Inhibitory Activity

The activity of trypsin was measured by the method of Dantzger et al. (2015). Briefly: 50 μL of trypsin solution (0.1 mg mL^{-1}) prepared in 0.1 mol L^{-1} Tris-HCl buffer, pH 8.0, containing 20 mmol L^{-1} $CaCl_2$ were added to 500 μL of 0.1 mol L^{-1} Tris-HCl buffer, pH 8,0, and then, 150 μL of 4 mmol L^{-1} BApNA prepared in DMSO were added to the mixture. The assay was incubated at 37°C for 30 minutes. The reaction was subsequently stopped by the addition of 300 μL of 30% (v/v) acetic acid. After centrifugation at 2000 × g, the absorbance of the supernatant was measured at 405 nm on a Pharmacia (GEHealthcare, Germany) Ultrospec 2000 spectrophotometer. One enzyme unit (U) was defined as the amount of enzyme that produced an increase of 0.1 in absorbance under the assay conditions.

The inhibitory activity was measured by adding to 50μL of the trypsin solution, 450 μL of 0.1 mol L^{-1} Tris-HCl buffer, pH 8.0, and 50 μL of the respective protein fraction. The assay was incubated at 37 ºC for 30 minutes before the addition of 150 μL of 4 mmol L^{-1} BApNA prepared in DMSO. The reaction proceeded for 30 minutes and was subsequently stopped by the addition of 300 μL of 30% (v/v) acetic acid. After centrifugation at 2000 × g, the absorbance of the supernatant was measured at 405 nm on a Pharmacia (GEHealthcare, Germany) Ultrospec 2000 spectrophotometer. One unit of inhibitor (UI) was defined as the amount of inhibitor that reduces 0.1 unit of the absorbance compared to the reading of the assay free of protein fractions.

Simulated Gastric/Intestinal Digestion

Simulated protein digestibility was determined by a multi-enzymatic method described by Megías et al. (2004), using pepsin and pancreatin. Pepsin (1 mg mL^{-1}) was prepared in HCl 0.2 mol $L^{-1,}$ and pancreatin (1 mg mL^{-1}) was prepared in 50 mmol L^{-1} sodium phosphate buffer, pH 7.5. Samples of protein fraction were first hydrolyzed with pepsin (enzyme to substrate ratio 1:20) at pH 1.5, 37 °C for 2 h. Then, the samples were neutralized with 0.2 mol L^{-1} NaOH solution to pH 7.5 and, after the addition of pancreatin (1:20), the digestion was carried out for 2 h at 37 ºC. The digestion was interrupted by heating the samples to 75 ºC for 20 minutes.

Bioactivities

Antioxidant assays

DPPH radical scavenging activity

The determination of DPPH scavenging activity was carried out based on the method described by Brand-Williams et al. (1995), with modifications. An aliquot of 50 μL sample (50 μg protein) was mixed with 200 μL of DPPH radical (0.15 mol L^{-1}). The mixture was homogenized and incubated at room temperature for 15 minutes, and then the absorbance against blank was measured at 520 nm, using an Epoch microplate spectrophotometer (Biotek Instruments, VT, USA). A Trolox calibration curve was prepared for a concentration range of 0 to 300 mM, and the inhibition obtained for the sample was interpolated to calculate the concentration in Trolox equivalents (μmol of Trolox mg^{-1} protein).

Ferric reducing antioxidant power (FRAP)

The FRAP assay was performed according to the method described by Rufino et al. (2006). The FRAP reagent was prepared as a mixture of 2.5 mL of 0.01 mol L^{-1} TPTZ prepared in 0.04 mol L^{-1} HCl and 2.5 mL of 0.02 mol L^{-1} $FeCl_3$ in 25 mL of 0.3 mol L^{-1} acetate buffer pH 3.4. 1.3 mL of freshly prepared FRAP reagent was mixed with 50 μL sample (50 μg protein) and 150 μL deionized water, and incubated at 37ºC for 30 minutes. The absorbance was determined at 595 nm. In the FRAP assay, the antioxidant potential of the sample was determined from a standard curve plotted using Trolox. FRAP values were expressed as μmol of Trolox mg^{-1} protein.

Lipid Peroxidation

Thio barbituric acid reactive substances (TBARS) were assayed by the method of Ohkawa et al. (1979) using brain tissue homogenates (samples of cortex and hippocampus) from Wistar rats. Homogenates were prepared in 0.2 mol L^{-1} sodium phosphate buffer, pH 7.4, in a ratio of 1:5 (w/v). The test consisted of incubating 200 μL of brain tissue homogenate with 10 μL of H_2O_2 0.3 mol L^{-1}, in an ice bath, for 30 minutes. Then, the mixture was centrifuged at 4000 rpm for 15 minutes, at 2 ºC. Next, 25 μL of the supernatant was mixed with 25 μL of distilled water, 75 μL of TCA, and 50 μL of TBA. The solution was vortex-mixed in tightly capped tubes and then placed in a 100 ºC water bath for 15 minutes. The samples were then cooled to room temperature, and then 2 mL of butanol was added to extract the TBA-MDA complex, which was subsequently separated into phases by centrifugation. The determination of the TBARs complex in the n-butanol extract was done at 535 nm. Results were expressed as nmol of MDA mg^{-1} of protein, according to the equation:

$$\mathbf{C = \Delta A \times 38.43 / C_0}$$

Where:
ΔA = change in absorbance
Conversion factor = 38.43 of the calibration curves
C = Malondialdehyde concentration (MDA) in nmol mg^{-1} protein.
Co = Protein concentration (mg sample)

Tests of anti-peroxidative activity were done by pre-incubating 200 μL of brain homogenate with 2 μL of 0.2 mol L^{-1} sodium phosphate buffer (negative control), or 2 μL peptide solution (F<3kDa or hydrolyzed F<3kDa) containing 200, 400, or 600 μg of protein, for 30 minutes, in an ice bath. Following, 10 μL of 0.3 mol L^{-1} H_2O_2 was added and the mixture incubated for 30 minutes. The assay proceeded, and the amount of MDA produced was determined at 535 nm. A control test was run with brain homogenate and H_2O_2. As a positive antioxidant control was used 2 μL of BHT at a concentration of 400 μg.

Study of vascular reactivity

Male Wistar rats, 10–12 weeks of age (from the Federal University of Goiás, Goiás, Brazil), were used. After euthanasia, the thoracic aorta was quickly dissected and cleaned in a physiological solution. Four millimeter rings of aorta were mounted on two stainless steel hooks and suspended in a 10 mL organ bath containing a modified Krebs-Henseleit buffer at 37°C and bubbled with a gas mixture (95% O_2 and 5% CO_2). Isometric force generation was recorded with an isometric force transducer. A resting tension of 1.5 g was imposed on each ring, and the rings were allowed to equilibrate for 1 h. Endothelium integrity was assessed by measuring the dilatory response to a solution of 10 μM acetylcholine (Ach) in 1 μM phenylephrine (Phe) contracted vessels. For studies of endothelium-intact vessels, a ring was discarded if relaxation with a 10 μM solution of ACh was not 90% or greater. After some washout and tension stabilization, the arterial rings were

again pre-contracted with Phe and randomly exposed to cumulative concentrations (10^{-7}–10^{-3} g mL^{-1} – final dilutions in organ bath) of one of the tested samples or vehicles (distilled water) (n = 5–7 per sample) [22]. All experimental protocols were approved by the Institutional Ethics in Research Committee at the Federal University of Goiás, Goiás, Brazil (Protocol CEP/UFG 20/2013).

In vitro ACE-inhibitory activity

Preparation of ACE was done according to Hayakari et al. (1978). Rabbit lungs were used as starting material, and the resulting aliquots were stored at -20 ºC. This method is based on the colorimetric reaction of hippuric acid with 2,4,6-trichlore-s-triazine (TT). A 3 g L^{-1} solution of hippuryl-l-histidyl-l-leucine (HHL) prepared in 0.1 mol L^{-1} phosphate-based buffers (pH 8.3) and a 5 mol L^{-1} NaCl was used as substrate. Once assay time was complete, the reaction was stopped with TT in dioxane (30 mg mL^{-1}), and 5 minutes later the samples were centrifuged at 15,250 x g for 10 minutes at 4 ºC. Absorbance was read at 382 nm. All runs were done in triplicate. ACE inhibitory activity (%) was determined as the difference between the initial ACE activity and the remaining activity after incubation with peptides. The IC50 value (the peptide concentration required to produce 50% ACE inhibition) was quantified by a regression analysis of ACE inhibitory activity (%) versus peptide concentration (µg protein mL^{-1}).

Results and Discussion

Extraction of the target bioactivity

DPPH radical scavenging activity and Ferric reducing antioxidant power (FRAP)

The beans are already recognized as an important nutraceutical food due to their content of bioactive compounds, especially bioactive peptides (Pereira & Tavano 2014; Tacias-Pascacio, 2020; Qiao et al., 2021). However, bean flour is a complex mixture of components and therefore, the efficient extraction of bioactive peptides constitutes a key step for obtaining these active ingredients to the maximum extent. The extraction efficiency of any substance depends on several factors, including the method employed, the time spent, the temperature, the type of solvent, and the solvent-to-material ratio (Dorta et al., 2011). Especially in the case of antioxidant peptides, the extraction solvent is always the most important factor. Many water-miscible organic solvents are commonly used for peptide extraction, and the selection of the best extractor should consider the targeted peptide and its ratio of hydrophobic/hydrophilic amino acids (Recharla et al., 2017; Nwachukwu & Aluko, 2019). In this work, four different solvents were tested as peptide extractors, and the results are shown in Table 1.

Table 1: Amount of protein from *Phaseolus lunatus* extracted using different methods.

Method / Solvent	Protein content (mg g^{-1} flour)	DPPH (µmol Trolox mg^{-1} protein)	FRAP (µmol Trolox mg^{-1} protein)
M1	1.5^{d} ± 0.1	962^{a} ± 29	2567^{a} ± 83
M2	12^{c} ± 0.04	118^{b} ± 14	1936^{b} ± 86
M3	56^{b} ± 0.01	_	_
M4	133^{a} ± 0,02	_	_

Results expressed as means ± standard deviation (p> 0.05). Values with different letters in the column are statistically different. 50 µg of protein were used in the antioxidant tests. Source: Authors

The importance of using an adequate extraction method for the target activity was confirmed by the DPPH radical scavenging activity and Ferric reducing antioxidant power (FRAP) tests. As can be seen in Table 1, although the extracts obtained by M3 and M4 presented the highest efficiency in protein extraction, they were not effective for the extraction of antioxidant peptides, as denoted by the DPPH and FRAP assays.

The highest values of antioxidant activity measured by DPPH and FRAP was obtained in the M1 extract, which was 8.15-fold and 1.33-fold higher than those obtained for the protein extracted using M2. These results suggest that naturally occurring antioxidant peptides from *Phaseolus lunatus* have a hydrophobic nature and can be better extracted using a low dielectric constant medium.

The differences observed in the antioxidant activity of the extracts M1 and M2 are related to a combination of peptide composition and the mechanism involved in the antioxidant assay. FRAP assay has been used to evaluate the antioxidant activity by assessing the electron-transferring potential of a compound under acidic pH (redox potential ~0.70 V), and DPPH radical scavenging assay can evaluate the antioxidant activity by the electron-transferring followed by proton acceptance, in an intermediary mechanism between SET (single electron transference) and HAT (hydrogen atom transference) redox reaction (Recharla et al., 2005; Munteanu & Apetrei, 2021). Therefore, as seen in Table 1, different sets of peptides were extracted in M1 and M2, with specific antioxidant capacity as function of the solvent employed in the extraction method.

Trypsin Inhibitor and thermal treatment

Two main families of trypsin inhibitors are present in beans – Kunitz family, which has a molecular mass of around 20 kDa, and Bowman-Birk family, with molecular mass around 9 kDa. Despite the new trends to consider these inhibitors as health beneficial as cancer suppressor compounds (Batista et al., 2010), their ingestion must be carefully controlled due to their antinutritional main effects and the abundance of them in bean seeds. According to Laurena et al. (1994) and Egbe & Akinyele (1990), the content of trypsin inhibitors in *Phaseolus lunatus* beans is 2000 - 3400 IU g^{-1} of the grain. As can be seen in Table 2, only a small fraction of trypsin inhibitors was present in the M1 and M2 extracts, representing respectively 1.3 and 2.3% of the total trypsin inhibitor present in *Phaseolus lunatus* beans.

Table 2: Amounts of trypsin Inhibitor and their thermal stability.

Methods/Solvents	**Inhibition (IU g^{-1} flour)**		
	T_0	$T_{10\ min}$	$T_{30\ min}$
M1- Dichloromethane / Methanol	41.5^{a}±0.2	0.5^{b}±0.02	-
M2- Acetonitrile / water / formic acid	49^{a}±0.05	35^{b}±0.04	32.5^{c}±0.03

Results expressed as averages of three determinations ± standard deviation. In the same line, data followed by different letters differ significantly (p> 0.05).

Additionally, the trypsin inhibitors present in M1 showed thermolability, being destroyed after 30 min of heat treatment at 90 ºC, whereas the inhibitor fraction present in M2 was thermoresistant (Table 2), confirming the presence of a different pool of peptides in the extracts.

Interestingly, the thermal treatment applied to reduce trypsin inhibitor activity (90 ºC for 30 min) did not affect the antioxidant power. On the contrary, results showed an increase in the antioxidant activity in both extracts, which was more pronounced in M1. After heating for 30 min at 90 °C a 2.6-fold increase was observed

in the antioxidant activity, which changed from 962 at $t_{o,}$ to 2497 μmol of Trolox mg^{-1} protein at t_{30}). Although less pronounced, the heating of M2 resulted in a 1.2-fold increase in the antioxidant activity (from 2567 at $t_{o,}$ to 3149 μmol of Trolox mg^{-1} protein at t_{30}).

The explanation for this apparent increase in antioxidant activity is related to the extraction medium. The extraction in both methods 1 and 2 is conducted in a low dielectric constant medium, which favors the dissolution of peptides with relatively low hydrophilicity. Conversely, the antioxidant assay is conducted in a highly hydrophilic environment, which energetically favors aggregation of hydrophobic compounds present in the M1 and M2 extracts (Nwachukwu & Aluko, 2019). Heating promotes disaggregation, exposing the molecules to exert their antioxidant activity. Therefore, the higher the hydrophobicity of the extraction medium, the higher the increase observed in the antioxidant activity after heating.

Since the goal of this study was the extraction of a higher amount of antioxidant peptides from *P. lunatus* grains, the extract obtained in method 1 (M1) was chosen for further fractioning and analysis.

Peptides Fractioning and Simulated Gastric/Intestinal Digestion

Proteins present in M1 were further fractionated and concentrated in an ultrafiltration device using a 3kDa membrane. After ultrafiltration, the protein (F>3kDa) and peptide fractions (F<3kDa) were tested for antioxidant activity. Following, the fractions were submitted to a simulated gastric digestion with pepsin and pancreatin, to evaluate if the antioxidant activity could persist through gastric/intestinal transit (Table 3).

Table 3: Antioxidant activity measured by DPPH and FRAP for extract obtained in method 1, protein fraction (F>3kDa), and peptide fraction (F<3kDa) before and after simulated digestion.

Sample	DPPH	FRAP
	μmol of trolox mg^{-1} protein	
M1 extract	962^{c} ± 29	2567^{B} ± 83
F > 3 kDa	161^{e} ± 42	_
Hydrolyzed F > 3 kDa	538^{d} ± 41	_
F < 3 kDa	$2011^{b,B}$ ± 204	3297^{A} ± 37
Hydrolyzed F < 3 kDa	$3002^{a,A}$ ± 126	3215^{A} ± 87

Results expressed as averages of three determinations ± standard deviation. (p> 0.05). In the column, data followed by different lowercase letters differ significantly (p> 0.05). In the lines, data followed by the same uppercase letter are not statistically different (p<0.05).

As shown in Table 3, antioxidant activity is predominant in the peptide fraction compared to the corresponding protein fraction, intact or hydrolyzed. The antioxidant activity in the F<3kDa increased after fractioning and separation from the total protein extract, being more evident in the DPPH activity.

After simulated *in vitro* gastric/intestinal digestion, the values of antioxidant activity of the F<3kDa increased 22% for the FRAP assay and 33% in the DPPH scavenging potential, probably due to the exposure of new fragments of active peptides that were still aggregated and were released by enzymatic hydrolysis. The resistance to simulated gastric/intestinal digestion is a characteristic of naturally occurring peptides, which frequently present in their composition uncommon amino acids and other types of linkage in addition to the common peptide bonds in their backbone (Ehinger & Hertweck, 2024).

Anti-peroxidative activity

Neuronal components are, in general, very susceptible to ROS attack since the double bonds of the unsaturated lipids present in those structures are labile to peroxidation and oxidative modification, initiating a

chain reaction that damages neighboring unsaturated fatty acids (Houldsworth 2024). The major reactive aldehyde resulting from the peroxidation of biological membranes is malondialdehyde (MDA), which is classically used as an indicator of neuronal tissue damage.

Tests with brain homogenate showed that naturally occurring peptides present in the F<3kDa were able to prevent the oxidative action of hydrogen peroxide, similarly to BHT, a classical antioxidant compound (Figure 1A). The same preventive effect was obtained with the hydrolyzed F<3kDa, meaning that anti-peroxidative activity is resistant to gastric/intestinal digestion (Figure 1B).

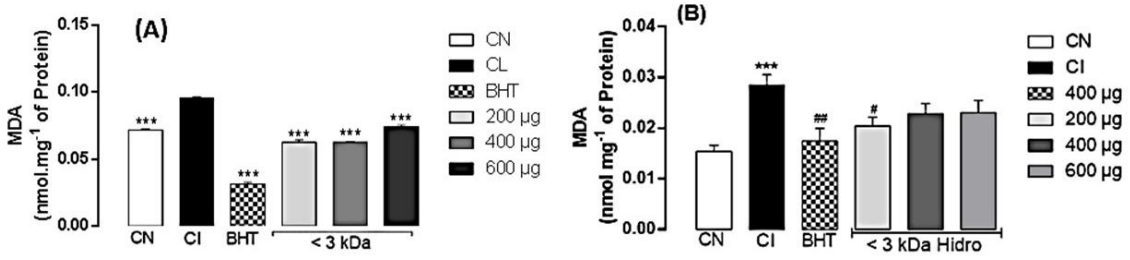

Figure 1: Malonaldehyde levels obtained by the TBARS methodology. (A) F<3kDa; (B) hydrolyzed F<3kDa. CN – negative control; CL – control; BHT – positive control. The columns and vertical bars represent the mean ± SEM (*** p <0.001 vs CN, #p <0.05 vs. CL, ##p <0.01 vs. CL).

The brain produces large amounts of ROS, as it is a highly metabolically active organ. In addition, generation of reactive oxygen species (ROS) and depletion of intracellular antioxidants following cerebral ischemia/reperfusion are hallmarks of oxidative stress and lead to tissue injury. Brain tissue is protected by an elaborate network of antioxidants that maintain a delicate redox equilibrium, including a family of small cell-permeable antioxidant peptides (Cho et al., 2007). Fails in this protective network or depletion of antioxidants are associated with neurodegenerative pathogenesis (Houldsworth, 2024). The presence of antioxidant peptides as part of the daily diet may favor the redox balance, preventing ROS accumulation, thus providing a healthier cellular environment.

In vitro ACE-inhibitory activity and vasorelaxant activity

Oxidative stress has been implicated in numerous vascular diseases. Several studies have reported the use of dietary antioxidants as an important alternative therapeutic in the prevention and treatment of cardiovascular diseases (Touyz et al., 2020; Griendling et al., 2021; Camargo et al., 2024). The endothelium plays an important role in maintaining vascular homeostasis, and the increase of oxidative stress alters several physiological functions, such as leukocyte adhesion, platelet aggregation, and blood flow in the endothelium.

The F<3kDa and the hydrolyzed F<3kDa were tested as a vasorelaxant agent in aortic rings segments (Figure 2). As can be observed, a 30% vasorelaxant rate was obtained after incubation of the aortic ring segments with 10^{-6} g mL^{-1} with F<3kDa (Figure 2A). Moreover, the naturally occurring peptides present in the F<3kDa exhibited 15% (±2.4) of ACE-inhibitory activity, which implies these naturally occurring peptides may be exploited as nutraceutical ingredients.

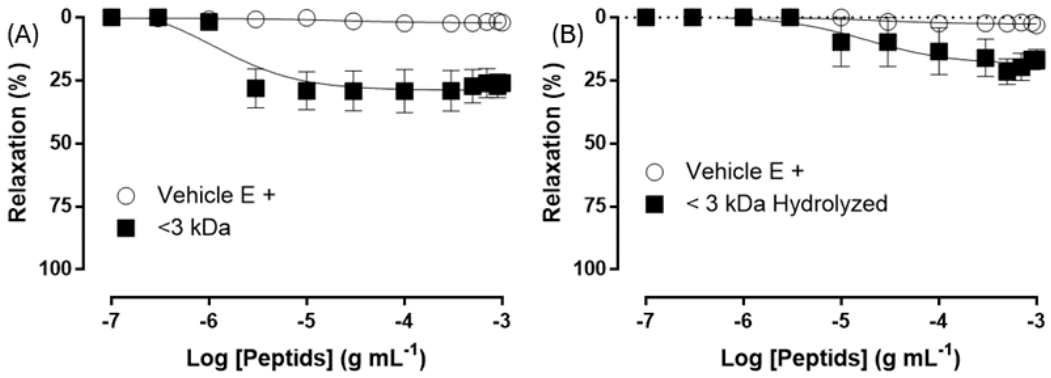

Figure 2: Relaxant action of peptides from (A) F<3kDa and (B) hydrolyzed F<3kDa on thoracic aorta rings of Wistar rats with preserved endothelium

On the other hand, the hydrolyzed F<3kDa presented a 17% vasorelaxant rate, when incubation occurred with 10^{-5} g mL^{-1} of the sample. This indicates that gastric/intestinal simulated digestion affected the structural integrity of some peptides, reducing the action on specific vascular receptors. However, this fraction presented 85% (±3.3) of ACE-inhibitory activity, which is close to the percentages reported for pepsin-based hydrolysates from legumes such as chickpea (86%), *Jamapa bean* (77%), lentil (79%), lupine (*L. albus*) (80%), lupine (*L. angustifolius*) (89%), pea (71%), and soy (88%) (Boschin et al, 2014).

Although the simulated gastric/intestinal digestion reduced the amount of active ligand molecules for vasorelaxant activity, antioxidant activity was preserved since this activity depends more on the type and sequence of amino acids than the steric arrangement (Kaur et al., 2021). On the other hand, the simulated digestion increased the ACE inhibitory activity (IC_{50} = 1.4 mg mL^{-1}), probably due to the exposition of amino acids such as tyrosine, phenylalanine, tryptophan, proline, lysine, leucine, isoleucine, and arginine, which have been proved to present a strong influence on the anchoring peptides to ACE active site (Kaur et al., 2021).

It is important to notice that the number of vasoactive peptides present in 1g of the hydrolyzed F<3kDa is very high, considering the recommendation of beans in the range of 187 g per week or 25 g per meal, according to the Dietary Guidelines Advisory Committee (2020). Moreover, the decrease in vasorelaxant activity may be counterbalanced by the amount of ACE-inhibitory active compounds generated in the process.

Gathering the findings regarding the anti-peroxidative, vasorelaxant, and ACE inhibitory activities, it is possible to consider the F<3kDa form hardened *P. lunatus* beans as an important source of naturally occurring peptides with potent antioxidant activity, which additionally could aid in the prevention of the initial events of cardiovascular and neurodegenerative diseases.

Conclusion

In this work, hardened *Phaseolus lunatus* beans, an agro-industrial residue, have been shown to be a very promising source of naturally occurring bioactive peptides. These peptides presented consistent antioxidant, anti-peroxidative, vasorelaxant, and ACE-inhibitory activities. Additionally, their inherent bioactivity resisted thermal treatment and simulated gastric/intestinal digestion, an advantage compared to other antioxidant compounds sensitive to thermal and pH changes. Their antioxidant activity has been associated with the presence of amino acids with some degree of hydrophobicity, since aromatic amino acids can act as good electron/proton acceptors in a redox reaction. In this case, the choice of the proper extraction medium for these compounds was crucial to reach better extraction yields, considering the hydrophobic characteristics.

It has been indicated that a portion of bioactive peptides can pass the intestine barrier and present biological effects at the tissue level. Intact absorption of peptides is regarded as a normal physiological process,

and molecular size and structural properties, such as hydrophobicity, seem to be determinant factors involved in this transport route for peptides.

In this sense and considering the advances in the understanding of peptides' bioactivities and their physiological absorption, the results obtained in this work are very promising, indicating that *Phaseolus lunatus* beans as a source of bioactive molecules, adding health beneficial properties to this functional food.

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