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ORIGINAL PAPER

Neuroprotective Effect Of Peptide Fractions from Chia (*Salvia hispanica*) on H_2O_2-Induced Oxidative Stress-Mediated Neuronal Damage on N1E-115 Cell Line

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Abstract
Neurodegenerative diseases (ND) affect around a billion people worldwide. Oxidative stress plays a critical role in the activation of neuronal death mechanisms, implicated in the ND etiology. In the present research, the neuroprotective effect of the *S. hispanica* protein derivatives is evaluated, on neuronal cells N1E-115, after the damage induction with H_2O_2. From the protein-rich fraction of *S. hispanica,* three peptide fractions were obtained (3–5, 1–3 y < 1 kDa) and its neuroprotective effect on neuronal cells N1E-115 was evaluated, through the antioxidant pathway. In the toxicity assay, the peptide fractions showed viability greater than 90%. When N1E-115 cells were incubated with 100 µM H_2O_2, fractions 1–3 and < 1 kDa, presented cell viability of 66.64% ± 3.2 and 67.32% ± 2.8, respectively. Fractions 1–3 and < 1 kDa reduced by 41.73% ± 3.2 and 40.87% ± 2.8, respectively, the ROS production compared to the control, without significant statistical difference between both fractions ($p < 0.05$), while F3–5 kDa, only reduced the ROS production by 21.95% ± 2.4. The protective effect observed in the < 3 kDa fractions could be associated with its antioxidant activity, which represents an important study target.

Keywords Parkinson's disease · Alzheimer's · Peptides · Neuroprotection · Antioxidant

Introduction

According to the report of the World Health Organization (WHO), neurodegenerative diseases (ND) affect around a billion people worldwide. In the report Neurological Disorders: Public Health Challenges, it's clear that 50 million people worldwide suffer from epilepsy and 24 million Alzheimer's and other dementias [1, 2]. Although the etiology of this group of diseases is unclear, it is known that oxidative stress (OS) and neuroinflammation play an important role in its genesis [3, 4].

The brain is susceptible to OS lesions because it is an organ with high energy use and metabolic demand, therefore minimal imbalances of the redox state, as occurs in mitochondrial dysfunction, favor tissue injury, damage, and activation of neuroinflammatory mechanisms [5, 6]. The chronicity and perpetuation of OS and neuroinflammation play an important role in neurodegeneration [7]. Although initially, neuroinflammation is a mechanism of immune protection against damage to central nervous system (CNS) structures, the inflammation chronicity, characterized by the prolonged microglial activation and the proinflammatory mediator's perpetuation, increases OS and nitrosative cell. Therefore, a response chain is generated between OS-inflammation-OS, which increases necroptosis and favors cognitive decline [8].

Proteins and peptides with biological activity represent an opportunity area for functional food development aimed at the prevention and treatment of chronic diseases [9]. Recently, interest in the development of molecules with a neuroprotective effect, specifically peptides, has increased [10]. The ability of a peptide to show neuroprotective activity is closely related to its amino acid sequence, structure, and degree of hydrophobicity. The hydrophobicity of the peptide increases the antioxidant activity since it allows the peptide to reach hydrophobic targets such as cell membranes [11]. Also, the peptide must include amino acids polarized at one or both ends to increase its solubility and the peptide must be between 3 and 15 amino acids long with a molecular

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weight below 1 kDa [12]. With their small molecular weight, neuroprotective peptides may have the potential to cross the blood–brain barrier (BBB) without compromising the CNS [13].

Salvia plants have been used historically in traditional medicine for the treatment of various diseases, including cognitive and neurological conditions [14]. Research findings confirm that many Salvia species and their bioactive compounds influence several biological processes that may have an impact on neurological and cognitive function [15]. Particularly, the peptides research and protein derivatives from *S. hispanica*, it has focused on its potential antioxidant effect. Such is the case of peptides obtained from the albumin fractions and *S. hispanica* globulin, which exhibit antioxidant activity against 2,2′-azino-bis (3-ethylbenzthiazolin-6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH); likewise, peptides from the prolamin and globulin fractions showed the ability to chelate the ferrous ion [16].

S. hispanica (Chia) protein hydrolyzate refers to an antioxidant activity associated with the neutralization of free radicals [17]. Fractions < 1 kDa of *S. hispanica* also report antihypertensive activity (69.31%) attributed to a high content of hydrophobic amino acid residues, which contribute to the inhibitory force of the peptide on ACE, for blocking the angiotensin II production [18]. A review by Grancieri et al. [19], summarizes amino acid sequences of peptides obtained from *S. hispanica*, with potential antioxidant, hypotensive, hypoglycemic, and anti-cholesterolemic activities. New research development that focuses on *S. hispanica* proteins and their bioactive peptides are needed to specifically demonstrate the mechanisms of action that contribute to the observed health benefits. In the present research, the neuroprotective effect of the *S. hispanica* protein derivatives is evaluated, on neuronal cells N1E-115, after the damage induction with H_2O_2, expanding the other applications reference of chia proteins on the antioxidant pathway.

Materials and Methods

Materials

S. hispanica (Chia) seeds were obtained in the Jalisco State from Mexico. All chemicals used were analytic grade reagents from Sigma Chemical Co. (St. Louis, MO, USA), Merck (Darmstadt, Germany), and Bio-Rad (Bio-Rad Laboratories, Inc. Hercules, CA, USA).

Obtaining of Protein-Rich Fraction from *S. hispanica*

Using the method reported by Segura-Campos et al. [18]. Chia seeds were submitted to gum extraction with water at a 1:40 ratio (w/v) for 90 min at room temperature. After that, the suspension was left to dry in a Fisher Scientific® brand stove at 50 °C for 24 h. Defatted seeds were obtained by pressure with a TRUPER® brand 8-ton hydraulic jack and, to extract the residual oil, the Soxhlet system was applied using hexane (High Purity®) for 4 h at 70 °C. The defatted seeds were allowed to dry in an extraction hood for 12 h to evaporate the solvent residues. Finally, the seeds were ground and sifted with a mesh of 0.5 mm and 140 µm for 30 min in a Ro-Tap® model E. As a result of the above, protein-rich fraction (flour) was obtained.

Chemical Characterization of the Protein-Rich Fraction (Flour)

Standard AOAC [20] procedures were used to determine nitrogen (method 954.01) and moisture (method 925.09) contents in the protein-rich fraction.

Enzymatic Hydrolysis

Enzymatic hydrolysis was done according to Martínez-Leo et al. [21]. A sequential enzymatic system was used with pepsin (Sigma®, P7000-100G) and pancreatin (Sigma®, P3292-100G) (45/45 min). The enzymatic conditions were: 1:10 (w/v) enzyme-to-substrate ratio, pH 2 for pepsin, and pH 7.5 for pancreatin, at 37 °C for 90 min. The samples were centrifuged at 3350 × *g* for 20 min to remove the insoluble portion in Hermle® Z300K.

Hydrolyzate Fractionation by Ultrafiltration

The hydrolyzate was fractionated by ultrafiltration using a high-performance ultrafiltration cell (Model 2000, Millipore, Inc., Marlborough, MA). Three fractions were prepared using three molecular weight cut-off (MWCO) membranes: 1, 3 and 5 kDa. Peptide fractions were prepared by ultrafilter the hydrolyzate through the MWCO membranes beginning with the lower weight (1 kDa) and ending with the largest cartridge (5 kDa). Thus, three peptides fractions were obtained: F < 1, F1–3, F3–5 kDa. Protein content in ultrafiltered peptide fraction (PF) was quantified using the method of Lowry et al. [22]

Cell Cultures

N1E-115 cell lines (ATCC® CRL-2263) were cultured in sterile Costar® 75 cm^2 flasks, with Dulbecco's Modified Eagle's Medium (DMEM) (ATCC® 30-2002) containing 4 mM L-glutamine, 4500 mg/L glucose, 1 mM sodium pyruvate, and 1500 mg/L sodium bicarbonate. Cells were cultured routinely in DMEM, with 10% Fetal Bovine Serum (FBS) and Penicillin Streptomycin (Gibco®), in an atmosphere of 95% relative humidity and 5% CO_2, at 37 °C.

Cell Toxicity Assay

To determine the viability cell from peptide fractions from *S. hispanica*, 96-well plates were used a cell density 1×10^4 cells per well, for 48 h at 37 °C, under conditions described in "Cell cultures" section. When the cells reached 80% confluence, the medium was changed without FBS and was treated with the *S. hispanica* protein derivatives, at a concentration of 25 and 50 µg/mL. It was incubated for 48 h at 37 °C in an atmosphere of 5% CO_2 and 95% humidity. After 48 h of treatment, the culture medium was extracted and the cells were fixed with 10% trichloroacetic acid (TCA, Aldrich®). Cells were incubated at 4 °C for 1 h, TCA was discarded and 0.4% sulforhodamine B (SRB) in acetic acid was added, it was incubated for 20 min at 25 °C. Finally, plates were washed with 1% acetic acid, rinsed four times until it was possible to observe the dye adhered to the cells and allowed to stand at 25 °C for 24 h. The plates were read at 540 nm in a microplate reader (GloMax-Multi + Detection System with Instinct Software).

Dimethyl-sulfoxide 20% (DMSO) (Sigma®) was used as a positive control. To determine the DMSO concentration to be used, a toxicity curve was performed with different DMSO concentrations (5–100%) in the N1E-115 cell line (Fig. 1a). Non-treated cells are negative controls.

Determination of Protective Effect After Damage H_2O_2-Induced

To determine the protective effect from peptide fractions from *S. hispanica*, 96-well plates were used a cell density 1×10^4 cells per well, for 48 h at 37 °C, under conditions described in "Cell cultures" section. When the cells reached 80% confluence, a 48 h treatment from *S. hispanica* peptide fractions was performed at a final concentration per well of 25 and 50 µg/mL in an atmosphere of 5% CO_2 and 95% humidity. After 48 h of treatment, two washes with PBS were performed, a medium change was performed and the damage was induced with H_2O_2. At the end of 24 h, cell viability was measured using the SRB assay, described previously. The plates were read at 540 nm in a microplate reader (GloMax-Multi + Detection System with Instinct Software).

Cells treated with H_2O_2 was used as a positive control. A toxicity curve of 50–300 µM was made (Fig. 2a), to determine the concentration required to damage more than 50% of N1E-115 cells. Non-treated cells are negative controls.

Measurement of ROS Generation

To determine the ROS generation, the N1E-115 cells were pre-treated with *S. hispanica* peptide fractions for 48 h at 25 µg/mL. After 48 h of treatment, the oxidation-sensitive dye DCFH-DA (1 mM) was added to the cells and incubated for 30 min. Was damage induce with hydrogen peroxide at 30% for 2 h. The cells were then collected after washing twice with PBS the intracellular ROS formation

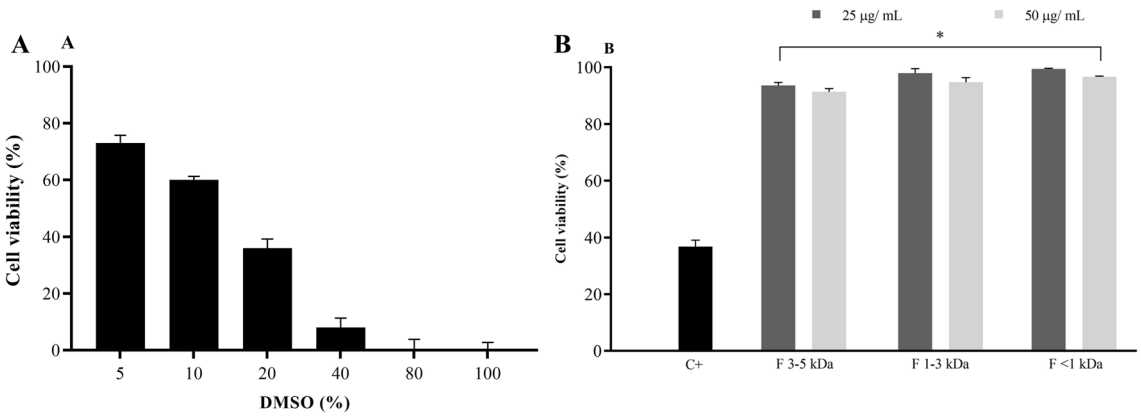

Fig. 1 **a** Cell viability (%) with respect to DMSO on N1E-115. **b** Cell viability (%) with respect to control, after 48 h of protein hydrolyzate (PH) treatment and peptide fractions from *S. hispanica* in N1E-115 cells. Data expressed as mean ± SD (n = 3), normalized with respect to the negative control (cells without treatment). Cells treated with 20% DMSO represent the positive control (C+). *$p < 0.05$ vs. positive control (Statistical significance assessed by one-way ANOVA, for comparison between the control was used Dunnett post hoc test)

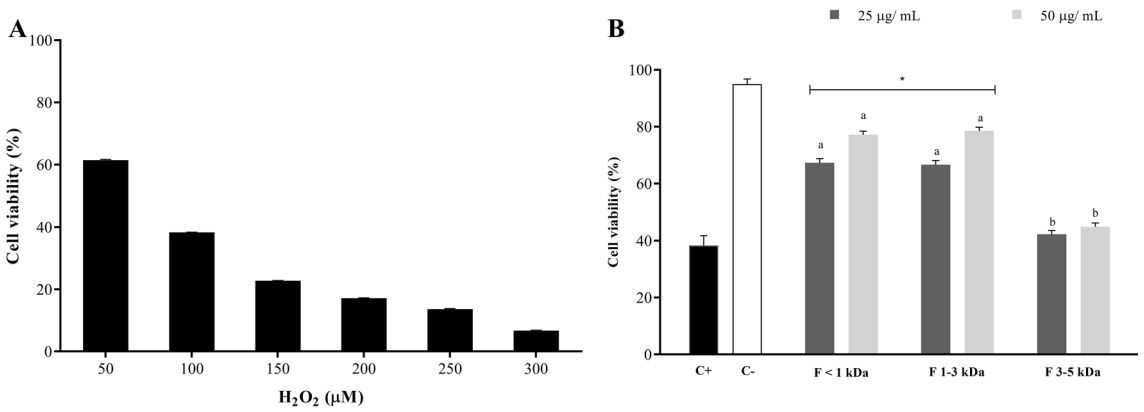

Fig. 2 **a** Cell viability with respect to concentrations different of H_2O_2 (μM), **b** Cell viability concerning 48 h pretreatment of proteins derivatives from *S. hispanica*, with induction of damage by 24 h 100 μM H_2O_2. Data expressed as means ± SD (n = 3). [a, b]Different letters in protein derivatives at the same concentration indicate statistical difference assessed by one-way ANOVA, Tukey post hoc test. *$p < 0.05$ versus positive control (C +) (Dunnett post hoctest)

was detected at an excitation wavelength of 485 nm and an emission wavelength of 535 nm in a multidetector reader (GloMax-Multi + Detection System with Instinct Software). 1 mM H_2O_2 and Non-treated cells, were used as damaged and negative controls, respectively.

2-Diphenyl-1-Picrylhydrazyl Radical (DPPH) Scavenging Assay

The technique proposed by Sharma & Bhat [23] was used, with some modifications, to determine the DPPH radical scavenging activity of the peptide fractions. For this, the DPPH solution (300 μM) was mixed with 100 μL of the peptide fractions at various concentrations (25, 50, 75, 100 y 125 μg/mL). Then, the mixture was left to stand for 30 min in the dark at room temperature. The absorbance was measured at 517 nm. IC_{50} (i.e., the concentration of peptide required to reduce 50% of the DPPH radicals) was calculated from the percentage of radical scavenging activity, using the following equation: Scavenging effect (%) $= 1 - [1 - [A_{\text{sample}} - A_{\text{sample blank}}] / A_{\text{control}}] \times 100\%$. Where A_{control} is the absorbance of the control (DPPH solution without sample), A_{sample} is the absorbance of the test sample (DPPH solution + test sample), and $A_{\text{sample blank}}$ is the absorbance of the sample only (sample without DPPH solution).

Statistical Methods

All results were processed using descriptive statistics, to measure the central tendency (mean) and dispersion (standard deviation). The data obtained from the biological activities were evaluated by one-way analysis of variance (ANOVA) ($p < 0.05$) a comparison of the means (Tukey's post hoc test) and control comparison (Dunnett post hoc test), to establish the statistical differences between treatments. All these analyses were performed using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA) software package.

Results and Discussion

S. hispanica Flour Characteristics

A protein-rich flour with a moisture content of 8.4% ± 0.04 and dry-based protein content of 75.28% ± 1.08 was obtained. Unlike other studies, such as Segura-Campos et al. [18] and Sosa et al. [24] who reported protein content of 44.62 and 49.51%, respectively in degummed and degreased chia flour, in the present study, the protein content was higher.

In the protein concentrates of *S. hispanica*, protein contents have been observed (Chim et al. [17] 77.26% and Segura-Campos et al. [18] 83.59%) like those reported in the present study, which implies the potential use of the flour itself, rich in protein, for biological studies and the development of functional foods. The biological use of plant-based foods as a source of protein is increasing, more widely studied, in favor of a reduction in the consumption of food of animal origin [25]. To respect, obtaining protein-rich flour, from plant-based foods, is becoming an ideal and key ingredient for the development of protein-based foods and products [18, 26].

Enzymatic Hydrolysis of the Protein-Rich Fraction

After enzymatic hydrolysis and ultrafiltration of the protein hydrolyzate, 3 peptide fractions of different molecular cuts were obtained (F3–5, F1–3 y F‹1 kDa) with a protein content between 0.069 ± 0.01 and 0.074 ± 0.02 mg/mL. Since the antioxidant activity at the CNS level is related to low molecular weight peptides, the ultrafiltration process is a factor that favors its obtaining.

Assessment of N1E-115 Cell Toxicity

In this study, the cytotoxic effect of the *S. hispanica* peptide fractions on the cell viability of N1E-115 was evaluated. To present the results, the values obtained were normalized concerning negative control. Incubation for 48 h with 20% DMSO (Positive control) produced cytotoxicity of 57.15% ± 3.2. As shown in Fig. 1b, the peptide fractions showed significant statistical difference concerning the positive control ($p < 0.05$), exhibiting viability greater than 90% for both concentrations studied (25 and 50 µg/mL). As reported by Chan et al. [27], who studied the cytotoxicity of *S. hispanica* peptide fractions in mouse macrophages of the BALBc strain, no cytotoxic effect was found in the protein derivatives of the present study. Similar data have been reported in Salvia species by Gong et al. [28] and Shaerzadeh et al. [29].

Neuroprotective Effects of *S. hispanica* Protein Derivatives on H_2O_2-Induced Cytotoxicity

When N1E-115 cells were incubated with 100 µM H_2O_2 there was a mortality of 61% ± 4.2, compared to the control group ($p < 0.05$). In contrast, cell mortality was significantly reduced in pre-incubated cells for 48 h with the *S. hispanica* peptide fractions before exposure to H_2O_2 ($p < 0.05$). At a concentration of 25 µg/mL, fractions 1–3 and < 1 kDa, presented a cell viability of 66.64% ± 3.2 and 67.32% ± 2.8, respectively, with no statistically significant difference between them ($p < 0.05$). The 3–5 kDa fraction showed the lowest cell viability percentage (42.29% ± 3.8). The same trend was observed at a concentration of 50 µg/mL (Fig. 2b).

Previous research exhibits a lower neuroprotective effect than reported in the present study. Such is the case of the *Benthosema pterotum* protein hydrolyzate that exhibited cell viability close to 70% at a concentration of 160 µg/mL. On the other hand, Wang et al. [30] report neuroprotection on SH-5YSY cells of 82%, in < 3 kDa fractions of porcine hide gelatin, an effect attributed to the presence of the amino acids Gly, Pro and Tyr. Lee et al. [31] refer to a 48.5% reduction in neuronal damage of PC12 cells, from *Hericium erinaceum* protein hydrolysate, also neuroprotective effect of hydrolysates obtained by different proteases was compared, being the hydrolyzate with pepsin who exhibited the greatest neuroprotective effect against OS, the enzyme used in the present study, and that could also represent a reason why the neuroprotective effect from *S. hispanica* peptide fractions is attributed.

In comparison with other plant sources, Li et al. [32], reported in peptide fractions of 1–3 kDa of soy, nut, and peanut, a behavior similar to that obtained in the present study, observing a concentration-dependent effect, reached 50 µg/mL, cell viability greater than 90%. According to Lee & Hur [33], the peptides exhibit several mechanisms of neuroprotective action, such as (1) Inhibition of calcium influx by blocking the DAPK1/NR2B combination; (2) Inhibition of cytochrome c release and Bax expression, an increase of Bcl-2 expression; (3) Inhibition of caspase pathways related to cell death, (4) Prevention of DNA fragmentation and (5) Inhibition of ROS and inflammatory cytokine generation through antioxidant activities. Specifically, the neuroprotective mechanism associated with antioxidant activity brings greater survival benefits, by reducing oxidative stress, neuroinflammation, and providing survival to neurons. Since peptide fractions of low molecular weight, usually present greater antioxidant activity than their counterparts of greater weight [21], this could be associated with the neuroprotective activity found in the < 3 kDa fractions.

Antioxidant and Free Radical Scavenging Activity

In the present study, the CM-H_2DCFDA staining method was applied to assess whether *S. hispanica* protein derivatives can inhibit the ROS production on N1E-115 cells treated with H_2O_2. When N1E-115 cells were incubated with 1 mM H_2O_2 at 30%, the ROS increased significantly 3 times, compared to the control group ($p < 0.05$) (Fig. 3a). The ROS were significantly reduced in preincubated cells for 48 h with 25 µg/mL of *S. hispanica* peptide fractions, before exposure to H_2O_2 ($p < 0.05$). Fractions 1–3 and < 1 kDa reduced by 41.73% ± 3.2 and 40.87% ± 2.8, respectively, the ROS production compared to the control, without significant statistical difference between both fractions ($p < 0.05$), while F3–5 kDa, only reduced the ROS production by 21.95% ± 2.4.

H_2O_2 has been widely used as an oxidative stress inducer in biological studies [34]. Cells exposed to H_2O_2 generally produce an accumulation and ROS overproduction that causes DNA damage and leads to neuronal cell apoptosis, a typical phenomenon and highly related to neurodegenerative diseases. Since the fractions with the lowest molecular weight had the greatest antioxidant effect, they are an important target for neuroprotective study.

According to Dave et al. [35] peptide fractions < 3 kDa hydrolyzed from enzymes such as pepsin-pancreatin, include TRYP, LYS, and GPV sequences, to which their potential

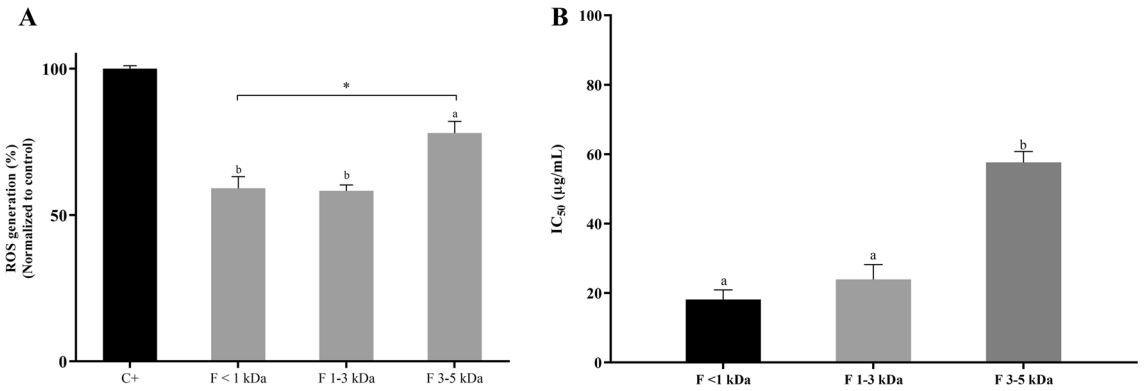

Fig. 3 **a** ROS generation with respect to the control after 48 h pretreatment of peptides fraction from *S. hispanica* on N1E-115 cells. ROS generation was monitored by fluorescent probe CM-H_2DCFDA. Data expressed as means ± SD (n = 3), normalized with the negative control. Cells treated only with H_2O_2 were the damage control group (C+). [a, b]Different letters in protein derivatives indicate statistical difference assessed by one-way ANOVA, Dunnett's post hoc test. *$p < 0.05$ vs. H_2O_2 (damage control) (Tukey post hoc test). **b** IC_{50} from fractions peptides from *S. hispanica*. Data expressed as means ± SD (n = 3). [a, b]Different letters in protein derivatives indicate statistical difference assessed by one-way ANOVA, Tukey post hoc test

Table 1 DPPH radical scavenging activity (%) of peptide fractions from *S. hispanica* L

Concentration mg/mL	DPPH radical scavenging activity (%)		
	F 3–5 kDa	F 1–3 kDa	F < 1 kDa
25	$35.71 \pm 1.8^{B,d}$	$47.27 \pm 1.0^{A,d}$	$48.52 \pm 2.4^{A,d}$
50	$48.26 \pm 2.4^{B,c}$	$60.96 \pm 1.8^{A,c}$	$63.74 \pm 1.3^{A,c}$
75	$59.32 \pm 2.2^{B,b}$	$70.17 \pm 1.0^{A,b}$	$72.19 \pm 2.1^{A,b}$
100	$67.78 \pm 1.3^{C,a}$	$78.68 \pm 0.6^{B,a}$	$82.58 \pm 1.9^{A,a}$
125	$68.48 \pm 0.8^{B,a}$	$82.06 \pm 2.1^{A,a}$	$83.37 \pm 1.2^{A,a}$

Data expressed in means ± DS (n = 3)

[a–d] Different lowercase letters indicate significant statistical difference between the study concentrations of peptide fractions from *S. hispanica* L

[A–C] Different capital letters indicate significant statistical difference between the peptide fractions from *S. hispanica* L, at the same concentration. Both evaluated by one-way ANOVA, Tukey test's (p<0.05)

antioxidant activity is attributed. To know if the antioxidant effect present in the peptide fractions of lower molecular weight, could be due to the interaction of their amino acids with free radicals, the DPPH scavenging activity was performed. Table 1 shows the percentages of DPPH scavenging by the *S. hispanica* peptide fractions at three study concentrations (25, 50 and 100 µg/mL). The F< 1 kDa had the highest antioxidant activity (82.58% ± 1.9 at 100 µg/mL), with a concentration-dependent ratio. On the contrary, a significant statistical difference is observed concerning the F3-5 kDa ($p < 0.05$), which presented the lowest antioxidant activity (67.78% ± 1.3 at 100 µg/mL).

The mean inhibitory concentration (IC_{50}) of the *S. hispanica* peptide fractions resulted in a favored antioxidant activity for F ≤ 1 kDa and F1–3 kDa (18.13 ± 2.9 µg/mL and 23.93 ± 3.1 µg/mL, respectively), no statistical difference between them ($p < 0.05$) (Fig. 3b). Previous research in hydrolysates and peptide fractions with a neuroprotective effect use the DPPH free radical scavenging assay as a benchmark for antioxidant activity. Such is the case of Lee et al. [31] who obtained a *Hericium erinaceum* protein hydrolysate from hydrolysis with pepsin and exhibited an IC^{50} of 205 ± 0.015 µg/mL and Venuprasad et al. [36] who reported an IC_{50} of 395 µg/mL, from *Ocimum sanctum* protein hydrolyzate. Values like those of the present research are reported by Orona et al. [16], in F < 10 kDa from *S. hispanica*, obtained from hydrolysis with pepsin-pancreatin.

It is known that the enzyme system used to obtain protein derivatives plays an important role in the antioxidant effect. Particularly, the antioxidant activity of the peptides is related to their high content of amino acids, such as His, Met, Val, Asp, and Glu, which are associated with strong antioxidant properties and, which in turn contributes to a greater ability to DPPH scavenging [37]. Although certain aspects of the structure–function relationship of antioxidant peptides are still poorly understood [38], it is believed that the type and sequence of amino acids are important determinants of the antioxidant property in peptides [39, 40], which could be linked to a neuroprotective activity.

In the present research, the neuroprotective effect observed in the N1E-115 cell line could be related to the antioxidant activity of the *S. hispanica* peptide fractions, since the enzyme system used, and the cut of the fraction are aspects related to the antioxidant property. Although the amino acid content is unknown, the study showed the relationship between amino acid interaction and neutralization of the DPPH radical, which leads to researches on the amino acid sequence and identification of the peptides involved in the observed response.

Conclusion

The < 3 kDa peptide fractions had a neuroprotective effect on N1E-115 cells, related to a mechanism of protection against oxidative stress, by reducing the ROS production, unlike their higher molecular weight counterparts. The 1–3 kDa fraction is a potential target for the development of scientific researches on neuroprotection and antioxidant activity.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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