# CAPÍTULO 15

# ANTIOXIDANT CAPACITY AND CYTOPROTECTIVE/CYTOTOXIC EFFECT OF BOVINE PLASMA HYDROLYSATES ON INTESTINAL-LIKE CACO-2 CELLS

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**ABSTRACT**: Enzymatic hydrolysates of food by-products have gained significant importance in recent years as a viable strategy for the use and valorization of these. This study evaluates the antioxidant capacity of bovine plasma hydrolysates (BPH) and their ultrafiltrate fractions using chemical assays ($ABTS^+$, ORAC, FRAP, CBA) and their protective effect in differentiated Caco-2 cells stressed with $H_2O_2$ (5 mM/2h). The highest antioxidant capacity was obtained with a degree of hydrolysis of 19.1% (BPH-19) and its 1-3 kDa fraction (FBPH-3). Pre-treatment 24 h with BPH-19 and FBPH-3 at 0.01-0.25 mg/mL alone without oxidative stress did not exert a cytotoxic effect and prevented a decrease in cell viability versus pre-treated with $H_2O_2$. Pre-treatment with BPH-19 after adding $H_2O_2$, showed a decreased intracellular ROS accumulation (15%), increase in the glutathione levels (19.5%), and a distribution in the cell cycle close to that of control levels. These results suggest that BPH-19 have antioxidant properties and a cytoprotective effect with potential action against ROS-mediated intestinal injuries.

**KEYWORDS:** Enzymatic hydrolysis, Bioactive peptides, Reactive oxygen species, Apoptosis, Necrosis.

## CAPACIDAD ANTIOXIDANTE Y EFECTO CITOPROTECTOR/CITOTOXICO DE HIDROLIZADOS DE PLASMA DE BOVINO SOBRE CÉLULAS INTESTINALES TIPO CACO-2

**RESUMEN:** La hidrólisis enzimática de subproductos alimentarios ha ganado importancia en los últimos años, como estategia viable para la revaloración de estos sustratos. Este estudio evalúa la capacidad antioxidante de hidrolizados de plasma de bovino (HPB) y de sus fracciones usando ensayos químicos (ABTS$^{+}$, ORAC, FRAP, CBA) y su efecto protector contra la producción de especies reactivas de oxígeno en células diferenciadas Caco-2 estresadas peroxide de hidrógeno ($H_2O_2$, 5 mM/2h). La mayor capacidad antioxidante se obtuvo con grado de hidrólisis de 19.1% (HPB-19) y su fracción de 1-3 kDa (FBPH-3). Un pretratamiento por 24 h con HPB-19 y FBPH-3 a 0.01-0.25 mg/mL solo sin estres oxidative nopresentó efecto citotóxico y previno el descenso en la viabilidad cellular con respect a las células tratadas con $H_2O_2$. El pretratamiento con HPB-19 despúes de la adición de $H_2O_2$, mostró un descenso intracellular de la acumulación de especies reactivas de oxígeno (15%), incremento en los niveles de glutation (19.5%) yuna distribución del ciclo celular similar a ensayo control. Estos resultados sugieren que HPB-19 tiene propiedades antioxidantes y efecto citoprotector con potencial de prevenir daño intestinal mediado por especies reactivas de oxígeno.

**PALÁBRAS CLAVE:** Hidr**ó**lisis enzimática, Péptidos bioactivos, Especies reactivas de oxígeno, Apóptosis, Necrósis.

## 1 | INTRODUCTION

Reactive oxygen species (ROS) are the product of cellular metabolic processes, they play a vital role in the body's biological signal transduction and homeostasis. An accumulation in ROS production, produce in cell damage or other possible pathologies such as cancer, leukemia, uremia, neurological or autoimmune diseases, sepsis, and rheumatoid arthritis, among others (Carocho and Ferreira 2013). ROS are normally neutralized by antioxidants, and intracellular glutathione (GSH) is one of the most effective defense systems that the body has against ROS (Gómez at al. 2019a). The relationship between reduced and oxidized glutathione (GSH:GSSG) is used both clinically and biologically as an emblematic marker of a cell's antioxidant capacity (AC) (Enns and Cowan 2017). An imbalance of the pro-oxidant/antioxidant relationship can cause oxidative stress, which is a clinical condition that is associated with a decrease in GSH and, therefore, a reduced cellular redox potential (Mischley et al. 2016). The production of ROS in high concentrations in the redox potential of the cells can be reflected in the deregulation of the cell cycle (Foyer et al. 2018), alterations in cellular metabolism and cell death by necrotic or apoptosis pathways (Gomez et al. 2019). This process can be off-set by the effects of antioxidants, which act through electron or hydrogen atom transfer to the free radicals, or by counteracting initiators, activators, or intermediate radicals in the reaction medium. This occurs because free radicals are singlet oxygen species and can be neutralized by means of the activation of metallic catalysts (Exner et al. 2000). In this vein, the intake of antioxidants could help

restore intracellular GSH levels, reduce oxidative damage and the subsequent appearance of diseases associated with this type of oxidative process (Devi et al. 2017).

Numerous studies have obtained protein hydrolysates with AC, which are considered nutritionally healthy, safe, and inexpensive (Chang et al. 2015). Both vegetable and animal-based biological protein sources have been used to obtain these types of hydrolysates. Proteins from by-products of the food industry have been considered feasible and sustainable sources to this end, due to the lower environmental impact they entail (Samaranayaka and Li-Chan 2011). No previous studies have evaluated the efficacy of antioxidant peptides from by-products of the bovine slaughter industry using cell cultures, which provide biologically more relevant results than chemical tests because they integrate processes such as the uptake, absorption, distribution, and cellular metabolism of the antioxidant compounds (Gómez-Grimaldos et al. 2020). However, there are studies on the AC of protein hydrolysates, such as the in vitro evaluation of the protective effects of Nile tilapia scales hydrolysates on DNA damage and reduction in the ROS level on RAW264.7 cells (Ngo et al. 2010). Likewise, other studies have observed that antioxidant peptides from hazelnut by-products can exhibit a cytoprotective effect in HUVECs cells through the prevention of ROS production (Liu et al. 2018). Finally, bone and skin of bream fish hydrolysates show AC and cytoprotective effects on the cytotoxicity produced by tert-butyl hydroperoxide (TBPH) on Caco-2 cells (Wiriyaphan et al. 2015).

The applications of antioxidant substances are broad, from the chemical industry (Liu et al. 2020; Rani et al. 2017), the pharmaceutical and food industries, as well as in human health (Zhu et al. 2017). The hydrolysates are from a biological source, their main applications are preferably as preservatives at products for health and pharmaceuticals (medicines), in the cosmetic industry (preservatives) and in the food industry (functional compounds) (Gómez at al. 2019b; Wiriyaphan et al. 2015).

Today it is necessary to take advantage of and revalue the waste from the food and agricultural industry, in order to generate new products with added value and potential applications in the pharmaceutical and food industry (Ramos et al. 2019). Proving that the products generated from biological industrial waste are not toxic to consumer health is also very important, and this can be achieved, for instance, with analysis carried out through in vitro tests on differentiated Caco-2 cells (Juan-García et al. 2021; Simitzis 2018). Bovine plasma is an industrial waste that is a high-quality and cost-effective source of proteins. This by-product accounts for 3% of the waste derived from the industry but is generally thrown away (FAO 2021), which makes it an ideal raw material for obtaining value-added compounds. This commitment has been framed in the Sustainable Development Goals (SDG), specifically in target 12.3, which aims to reduce losses and waste in food production chains by 2030 (Leal et al. 2018).

Enzymatic hydrolysates of food by-products have gained significant importance in recent years as a viable strategy for the use and valorization of these since it can generate

added value products considered nutritionally healthy, low cost, and safe (Gómez and Zapata 2016). This strategy allows us to revalue bovine plasma hydrolysates (BPH) and their fractions. This study aims to obtain bovine plasma hydrolysates with cytoprotective activity against $H_2O_2$ oxidant injury induced in differentiated Caco-2 cells.

## 2 | MATERIALS AND METHODS

### 2.1 Samples and Reagents

Bovine plasma was acquired from a commercial supplier Colombia and was stored at -20 °C until hydrolysis. Propidium iodide (PI), 2',7'-dicholofluorescein diacetate (DCFDA), fluorescein sodium salt, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid(Trolox), 2,2'-Azino-bis 3-ethylbenzenothiazoline-6-sulfonic acid (ABTS), 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), 2,2'-azobis2-metilpropionamidine dichlorhydrate (AAPH), Crocin and 3-4,5-dimethylthiazol-2-yl-2,5-diphenylthiazolium bromide (MTT) were obteined from Sigma-Aldrich (Ontario, Canada). Annexin V apoptosis detection kit was acquired from eBioscience (San Diego, CA, USA). 5-chloromethylfluorescein diacetate (CMFDA) was purchased from Abcam (Massachusetts, USA). Dulbecco's Modified Eagle Medium (DMEM+GlutaMAXTM), antibiotic solution, HEPES, fetal bovine serum (FBS) and trypsin-EDTA solution were purchased from Gibco (Scotland, UK), 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6$) was from Molecular Probes (Eugene, OR, USA). Alcalase 2.4 L was purchased from Novo Nordisk Co. (Bagsvaerd, Denmark).

### 2.2 Enzymatic hydrolysis

A stirred bioreactor of 7.5 L New Brunswick Scientific BIOFLO® & CEL-LIGEN 310 (Edinson, NJ, USA, G628-011) was used to perform the enzymatic hydrolysis with Alcalase 2.4L (enzyme/substrate ratio of 0.89 AU/g protein), using a protein concentration of 42 g/L. For the analysis, six aliquots with different DH (0; 3.8; 6.7; 10.8; 15.3 and 19.1%) were taken during the reaction to determine the sample antioxidant capacity. The enzyme in each aliquot was inactivated by heating at 85 °C for 600 s.

### 2.3 Fractionation of BPH by ultrafiltration

The hydrolyzate with a DH of 19.1% (BPH-19) which presented highest antioxidant activity than other samples were diluted, fractionated and filtered through ultrafiltration membranes with molecular weight cut-off 100, 10, 3 and 1 kDa, according to Gomez-Grimaldos et al. (2020). Fractions were labeled as follows: FBPH>100, the 100 kDa membrane retentate; FBPH-100, the 10 kDa membrane retentate of the 100 kDa permeate; FBPH-10, the 3 kDa membrane retentate of the 10 kDa permeate; FBPH-3, the 1 kDa membrane retentate of the 3 kDa permeate; and FBPH<1, the 1 kDa membrane permeate. The fractions obtained were lyophilized.

## 2.4 Antioxidant parameters

### *2.4.1 ABTS⁺*

Following the methodology described by Gómez and Zapata (2016), $ABTS^{+}$ radical was mixed with sample or standard. After 60 min, the absorbance was measured at 730 nm in a spectrophotometer (GENESYS 10S, Thermo Scientific™). The Trolox was used as standard (0–250 µM) to report the results as micromoles of Trolox equivalents per gram of protein (µmol TE/g of protein).

### *2.4.2 Oxygen Radical Absorbance Capacity (ORAC)*

The assay was evaluated by monitoring changes in fluorescent signal from a probe (FL) (Gómez and Zapata 2016). AAPH solution was added to FL – samples/Trolox, which had been incubated at 37 °C for 30 min. Fluorescence intensity was measured for 120 min (λexc=485 nm and λem=520 nm). The results were registered as µmol TE/g of protein using a Trolox calibration curve (0–200 µM).

### *2.4.3 Crocin bleaching assay (CBA) assay*

Solutions of AAPH (18.5 mM) and Crocin (100 µM) were prepared in PBS (0.01 M, 0.12 M NaCl, pH 7.4). The reaction began with the addition of 400 µL AAPH to a mixture of the 200 µL sample (1 mg/mL) plus Crocin (400 µL), according to the methodology described by Ordoudi and Tsimidou (2006). The absorbance of the solution was measured at 442 nm after 30 s and 20 min. The crocin bleaching inhibition percentage (%Inh) was calculated as:

$$\%\boldsymbol{Inh} = [(\Delta \boldsymbol{A_b} - \Delta \boldsymbol{A})/\Delta \boldsymbol{A_b}] \times \mathbf{100} \qquad (\mathbf{1})$$

Where, $\Delta A$ and $\Delta A_b$ represent the variation in absorbance values after 20 min of reaction, with and without the sample, respectively.

### *2.4.4 FRAP assay*

The FRAP assay was evaluated according to Bedoya-Ramírez et al. (2017) with FRAP reagent warmed at 37 °C. 30 µL of a sample (or Trolox standard), 90 µL of water, and 900 $\mu$L of FRAP were mixed, and the solution was left for 60 min at 37 °C. Subsequently, the absorbance was measured at 595 nm. The results were registered as µmol TE/g of protein using a Trolox calibration curve (0–500 µM).

## 2.5 Cytotoxic/cytoprotective assays

### *2.5.1 Culture and treatment of Caco-2 cell*

Human colon adenocarcinoma cells (Caco-2) from the American Type Culture

Collection (HTB-37, Rockville, MD, USA) were cultured in DMEM+ GlutaMAX™ supplemented glucose, no-essential amino acids, HEPES, antibiotic solution and FBS. The cells were incubated at 37 °C with 5% of $CO_2$ and the medium was replaced every two days, as previously described by López-García, et al (2017). Caco-2 cells were seeded onto 24-well plates (Costar Corp., USA) at a cell density of 5 x $10^4$ cells/$cm^2$ with 1 mL of DMEM. Caco-2 cells were used between passages 6–24 and cell differentiation was reached 7 days post-seeding and remained constant until 12 days confirmed by mea suring the transepithelial electrical resistance (722 ± 88.7 Ω $cm^{-2}$). After 7 days post-seeding, the culture medium was aspirated, and cell cultures were preincubated for 24 h with samples or DMEM according to the analysis.

### *2.5.2 Cell viability*

MTT assay was used to determine Cell viability (Cilla et al. 2015). Cells were incubated for 24 h with BPH-19 and FBPH-3 at different concentrations (0.01–0.5 mg/mL for BPH-19 and 0.01–0.25 mg/mL for FBPH-3). The reduction of the tetrazolium ring of MTT by viable cells was measured at 570 nm with a background subtraction of 690 nm in a thermostated UV-VIS spectrophotometer (Perkin Elmer). The concentration with the highest cytoprotective effect in the MTT assay was used for the other cytoprotection assays (0.25 mg/mL for BPH-19 and 0.1 mg/mL for FBPH-3).

### *2.5.3 Induction of oxidative stress*

Oxidative stress was induced according to previous studies carried out in the Bionutest group of the University of Valencia (Cilla et al. 2018; García-Nebot et al. 2017). After pre-incubation for 24 hours with the samples or with DMEM (stress control), the cells were treated for 2 h with 5 mM $H_2O_2$ solution in the culture medium to induce oxidative stress. The cells only treated with DMEM and not exposed to stress were used as a control.

### *2.5.4 ROS*

Changes in fluorescence caused by intracellular oxidation of DCFDA were used to monitor the ROS level, according to López-García et al. (2017). Cells were incubated for 24 h with BPH-19 and FBPH-3 (at the concentrations with the highest cytoprotection in the MTT test), then the culture medium was removed, and cells were incubated with DCFDA solution for 30 min at 37 °C. Later, the cells with and without oxidative stress treatment were analyzed via a flow cytometer at λexc=495 nm and λem=529 nm (FACSCanto, BD Biosciences). At least 1×104 cells were analyzed for each sample.

### *2.5.5 Intracellular glutathione determination*

Intracellular levels of glutathione (GSH) were measured by CMFDA According to

the methodology described by Álvarez-Sala et al. (2018). Cells were incubated with 1 µM CMFDA for 40 min at 37 °C, and the fluorescent intensity was determined via flow cytometry at λexc=492 nm and λem=516 nm. Fluorescent intensities were analyzed in at least $1\times10^4$ events for each sample.

### *2.5.6 Cell cycle analysis*

The cell cycle was analyzed by DNA content in the cells, as described by Cilla et al. (2018). The DNA was quantified by flow cytometry using PI staining. Fluorescent intensities were measured at λexc=351 nm and λem=617 nm in at least 1×104 events for each sample.

### *2.5.7 Determination of apoptosis/necrosis*

Apoptosis or necrosis in cells was determined using staining with Annexin V and PI according to the protocol described by López-García et al. (2017). Cells were incubated with 5 µL PI solution and 5 µL Annexin V for 15 min. Later, fluorescent intensities were measured by flow cytometry at λexc=494 nm and λem=519 nm for Annexin V, and λexc=351 nm and λem=617 nm for PI. The two-dimensional gating method, an analysis of $1\times10^4$ events for each sample, was used to determine the percentage of viable cells (Annexin-/PI-), cells in the state of early apoptosis (Annexin+/PI-), late apoptosis (Annexin+/PI+), and necrosis (Annexin-/PI +).

### *2.5.8 Membrane potential evaluation*

Changes in mitochondrial membrane potential were evaluated by flow cytometry staining, using the cationic lipophilic dye DiOC6 (10$\mu$M) (λexc=485 nm and λem=499 nm) that accumulates in the mitochondrial matrix, according to the protocol described by Cilla et al. (2015). The cell suspension (100 µL) was added to a new tube and incubated with 5 µL $DiOC_6$ at room temperature in the dark for 15 min. Then, 400 µL of PBS was added and cells were studied using flow cytometry and an analysis of $1\times10^4$ events for each sample were carried out.

## 2.6 Statistical analysis

All values were expressed in terms of mean ± standard deviation of at least four separate experiments (n=4). Statistical differences among the means were identified by One-way analysis of variance (ANOVA), followed by Tukey's HSD test ($p<0.05$). Correlations of the antioxidant activity values obtained with the different in-vitro methods were obtained with Pearson's correlation coefficient ($p<0.05$). Statistical software Statgraphics® Centurion XV, Virginia, USA was used.

## 3 | RESULTS AND DISCUSSION

### 3.1 Effect of DH on BPH antioxidant activity

The AC of the BPH samples was evaluated by means of four well-known in vitro methods: ORAC, FRAP, $ABTS^+$ and CBA. In general, the higher the DH, the greater biological AC (Table 1), with a statistically significant relationship between them ($R^2$: 0.87 and 0.95 with $p<0.05$). The DH of 19.1% (BPH-19) show the highest antioxidant activity, with an increase in AC of between 4.8-4.9 times the biological activity, in the ORAC, FRAP, and ABTS+ tests. Moreover, with regard to the ability to inhibit crocin bleaching, the results showed that non hydrolyzed BP proteins show very low activity (9.92 ± 0.20%) but when raw material is hydrolyzed reaches an inhibition value of 78.75 ± 0.09% with a DH of 19.1%.

Previous studies have shown that AC is directly proportional to DH (De Castro and Sato 2015; Hamzeh et al. 2016). This behavior may be due to size and amino acid sequence changes compared to the native protein. On the other hand, side chains and terminal amino groups exposed by hydrolysis can more easily react with reactive species such as oxidant molecules (Phanturat et al. 2010; Tovar-Pérez et al. 2017). The chemical properties conferred by the peptide sequence with the ability to inhibit oxidative reactions also explain this phenomenon.

| DH(%) | CBA (%) | FRAP (μmol ET/g) | ORAC (μmol ET/g) | $ABTS^+$ (μmol ET/g) |
|---|---|---|---|---|
| 0.0 | $9.92 \pm 0.20^a$ | $85.6 \pm 0.3^a$ | $196.47 \pm 0.28^a$ | $293.31 \pm 0.42^a$ |
| 3.8 | $61.15 \pm 0.03^b$ | $229.7 \pm 0.1^b$ | $791.67 \pm 0.52^b$ | $1181.87 \pm 0.78^b$ |
| 6.7 | $72.27 \pm 0.11^c$ | $150.5 \pm 0.1^c$ | $892.38 \pm 0.31^c$ | $1332.22 \pm 0.46^c$ |
| 10.8 | $70.87 \pm 0.17^d$ | $282.8 \pm 0.3^d$ | $915.49 \pm 0.28^d$ | $1366.73 \pm 0.41^d$ |
| 15.3 | $69,46 \pm 0.21^e$ | $268.5 \pm 0.1^e$ | $955.12 \pm 0.46^e$ | $1425.88 \pm 0.69^e$ |
| 19.1 | $78.75 \pm 0.09^f$ | $411.3 \pm 0.2^f$ | $969.15 \pm 3.91^f$ | $1446.83 \pm 0.12^f$ |

Mean ± standard deviation (n=4). $^{a\text{-}f}$ Different letters in the same column show statistically significant differences ($p<0.05$).

Table 1. Antioxidant activity of bovine plasma hydrolysates with different degree of hydrolysis (DH)

The ORAC assay is based on transferring hydrogen atoms from the antioxidant to peroxyl radicals. It is one of the most used for estimating the scavenging capacity of a biologically relevant radical (Jiménez-Morales et al. 2022). BPH showed ORAC capacity similar to other studies with plant-based substances (Giorgi et al. 2015; Intiquilla et al. 2016) and other animal-based hydrolysates (Shanura et al. 2020; Wachirattanapongmetee et al. 2019; Zhang et al. 2020). The highest ORAC value achieved was 969.15 ± 3.91 μmol TE/g protein; this value is within the range of the ORAC capacity for foods reported in United States Agriculture Department (USDA) databases (Daneshzad et al. 2020). In the

CBA analysis, results showed that the AC of the BP is not significant enough to prevent the crocin bleaching, but that when the protein is hydrolyzed and reaches a DH of 19.1%, higher values are obtained than those reported in the literature for oil palm kernel protein hydrolysates (Chang et al. 2015), abalone viscera hydrolysate (Je et al. 2015), and tuna protein hydrolysates (Bougatef et al. 2012).

The results obtained for ORAC and CBA assays suggest that BPH can inhibit peroxy radical oxidation by transferring hydrogen atoms, which is of great relevance due to the importance of this type of radical on the lipid oxidative degradation mechanism (Jiménez-Morales et al. 2022).

### 3.2 Separation of antioxidant peptides from BPH

The result showed that a decrease in the molecular weight leads to an increase in the AC, and FBPH-3 was the fraction that showed the greatest AC (see Table 2). It is possible that the AC of peptides from BP are more active and stable when they have peptide bonds in their structure, rather than having single amino acid residues or dipeptides (Gómez-Grimaldos et al. 2020). These results are consistent with some studies which have obtained low molecular weight peptides with elevated AC (Gómez and Zapata 2016; Wiriyaphan et al. 2015).

The FBPH-3 fraction has 10.8%, 67.8% and 29.3% more AC than BPH-19 at the CBA, FRAP and $ABTS^{+}$ assays respectively, suggesting that this fraction can more easily donate hydrogen atoms or electrons to free radicals (Gómez and Zapata 2016). FBPH-3 shows a greater ability to reduce $Fe^{3+}$-TPTZ ferrous compounds than hydrolysates obtained from other by-products (Villamil et al. 2017).

| Fraction (kDa) | CBA (%) | FRAP (μmol ET/g) | $ABTS^{+}$ (μmol ET/g) |
|---|---|---|---|
| FBPH < 1 | 65,24 ± 0.09[a] | 1189.78 ± 0.46[a] | 2040.70 ± 0.13[a] |
| FBPH-3 | 88.32 ± 0.03[b] | 1267,6 ± 0.34[b] | 2045, 56 ± 0.44[b] |
| FBPH-10 | 67.77 ± 0.13[c] | 597.14 ± 0.21[c] | 1816.24 ± 0.09[c] |
| FBPH-100 | 65.09 ± 0.15[a] | 784.05 ± 0.28[d] | 2034,63 ± 0.12[d] |
| FBPH>100 | 68.89 ± 0.23[d] | 605.48 ± 0.22[e] | 1835,66 ± 0.15[e] |

Mean ± standard deviation (n=4). [a-e] Different letters in the same column show statistically significant differences ($p<0.05$).

Table 2. Antioxidant capacity of bovine plasma hydrolysates ultrafiltrates with (DH): 19.1 %

According to the results, FBPH-3 showed a higher AC than the molecular fractions with a higher weight, which suggests that small peptides are more potent antioxidants than larger peptides. This could be due to the side chains and their amino ends, which are more available to donate electrons or hydrogen atoms and react with free radicals or reduce complexes such as $Fe^{3+}$-TPTZ (Gómez-Grimaldos et al. 2020; Phanturat et al. 2010).

Peptides with AC have been reported in the literature by means of gel chromatography with values of 318 µmol ET/g of protein (Hamzeh et al. 2016). Likewise, peptides separated by ultrafiltration with considerable AC and with low molecular sizes between 1 and 5 kDa have also been observed in the literature (Wiriyaphan et al. 2015). When hydrolyzed, protein origin substances release amino acids, which are known for their ability to donate electrons such as tryptophan (Try), tyrosine (Tyr), and histidine (His), which increase AC when exposed by hydrolysis processes and are contained in BPH in concentrations between 3.3 ± 0.02 to 28.7 ± 0.3 mg/g of protein (Gómez-Grimaldos et al. 2020).

### 3.3 Cytotoxicity Tests

The BPH-19 and FBPH-3 fractions (samples with the highest AC in the in vitro tests) were chosen to analyze their cytotoxic and cytoprotective effect on intestinal-like Caco-2 cells. According to the results shown in figure 1, treatment with BPH-19 does not affect cell viability, although at concentrations of 0.01-0.05 mg/mL a slight increase is observed, possibly due to the fact that BPH-19 contains amino acids that can favour cell development (Gómez-Grimaldos et al. 2020). Based on the results, a concentration of 0.25 mg/mL was chosen for subsequent bioactivity studies, since it does not show differences with respect the control.

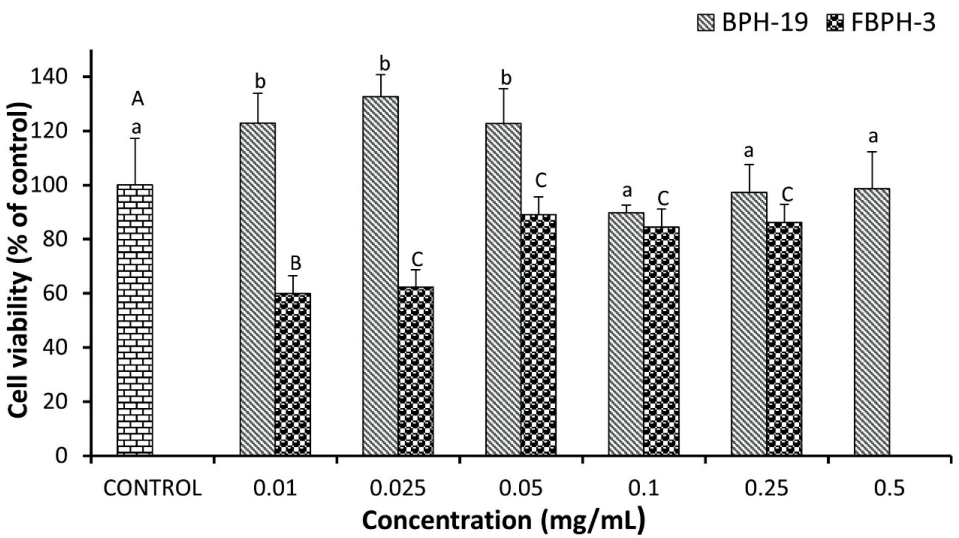


Figure 1. The effect of pretreatment with BPH-19 and FBPH-3 on cellular viability (MTT assay) on the differentiated Caco-2 cells after treatment of 24 h. Values are expressed as mean ± standard deviation (n=4). The different superscript lowercase letters (a-b) for BPH-19 and uppercase letters (A-C) for FBPH-3 show statistically significant differences between the treatments ($p<0.05$).

FBPH-3 at concentrations between 0.05 to 0.25 does not show great significant changes in the viability of the culture with values higher than 92.6 %. However, treatment at low concentrations (0.01 and 0.025 mg/mL), decreases the viability of Caco-2 cells up to

59.98% and 62.31%, respectively. The decrease in cell viability may occur because amino acid residues at low concentrations can be easily transported through the cell membrane, which can affect the intracellular electrolyte concentration and change the internal osmotic pressure, causing cells to become hyper hydrated and strongly decrease their viability (Tang et al. 2017). Similarly, a decrease in the viability of cell cultures has been observed when treated with abalone viscera hydrolysate at concentrations between 0.5 and 1 mg/mL (Je et al. 2015).

Likewise, other studies which have analyzed concentrations under 0.2 mg/mL of hydrolyzed red tilapia viscera and threadfin bream surimi by-products and their respective ultrafiltered fractions, have been reported to be non-cytotoxic on Caco-2 cells (Gómez et al. 2019a; Wiriyaphan et al. 2015).

## 3.4 Cytoprotective effect of BPH-19 and FBPH-3 against $H_2O_2$-induced stress

### *3.4.1 Cell Viability*

Figure 2 shows the results of MTT assay in stressed differentiated Caco-2 cells pre-incubated with BPH-19 and FBPH-3 for 24 h. The results show that cell viability suffers a significant decrease ($p<0.05$) of 31.6% when cells are subjected to stress conditions. After 24 h of pre-treatment with BPH-19, a preventive effect was observed on the decrease in cell viability at BPH-19 concentrations of 0.25 and 0.5 mg/mL. At lower concentrations of BPH-19 the compound fails to counteract the effect of the oxidative stress, perhaps because the concentration of BPH-19 is not sufficient to neutralize the radicals formed by the reaction with $H_2O_2$ 5 mM. In pre-treatments with FBPH-3 at concentrations between 0.05-0.1 mg/mL, there is partial prevention of oxidative stress induced by $H_2O_2$, increasing cell viability by 19.1% vs. the control with stress. Therefore, this concentration was chosen for subsequent bioactivity studies. Although FBPH-3 showed higher AC in vitro than BPH-19 (Tables 1 and 2), it also exhibited some cytotoxicity in Caco-2 cells as mentioned in the previous section, which leads to an expected lower cytoprotective effect as can be seen in figure 2. Cell model systems allow the evaluation of bioavailability, reactivity, and bioactivity of antioxidant compounds in cell metabolism (Zhang et al. 2019). These results are similar to those obtained by Zhong et al. (2011), who showed that the protein hydrolysate isolated from marine by-products has a greater capacity to neutralize radical DPPH and exert a cytoprotective effect in Caco-2 cells exposed to 0.15 µM of $H_2O_2$ for 12 h.

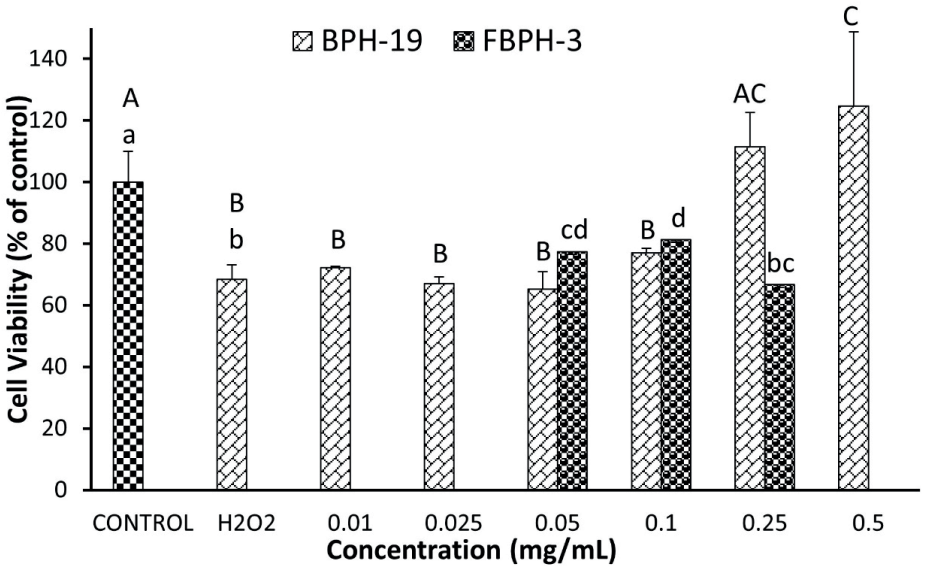


Figure 2. The effect of pretreatment with BPH-19 and FBPH-3 on cellular viability (MTT assay) on the differentiated Caco-2 cells with oxidative stress induced by $H_2O_2$ 5 mM. Values are expressed as mean ± standard deviation (n=4). The different superscript lowercase letters (a-d) for BPH-19 and uppercase letters (A-C) for FBPH-3 show statistically significant differences between the treatments ($p<0.05$).

### *3.4.2 ROS accumulation*

Figure 3 shows ROS intracellular level in Caco-2 cells pre-incubated with BPH-19 and FBPH-3, with and without the effect of oxidative stress. No significant differences ($p>0.05$) in the ROS intracellular accumulation were detected in the tests with the absence of induced oxidative stress. The cells treated with 5 mM $H_2O_2$ showed an increase in intracellular ROS levels of 15.6 times more than those of the control without stress. Cells pre-treated with FBPH-3 under stress conditions had an increase of 18.7 times the ROS levels compared to the control without induced stress. In addition, an increase of 16.5% in ROS levels was also observed with this sample compared to the control with stress-induced. By contrast, in cells with induced stress pre-treated with BPH-19, ROS levels decreased by 15.2% with respect to the $H_2O_2$ control. The decrease in intracellular ROS levels observed with BPH-19, together with its high capacity to transfer hydrogen atoms and/or electrons (Table 1), suggests that the mechanism of action of the antioxidant peptides contained in BPH-19 it is the sequestration or neutralization of the charges of free radicals, expressed in situations such as stress induced by $H_2O_2$ (Carocho et al. 2018). Other hydrolysates with significant AC at the cellular level from marine and chickpea by-products which have the ability to neutralize the increase in intracellular ROS can be found in the literature (Ngo et al. 2010; Wang et al. 2016).

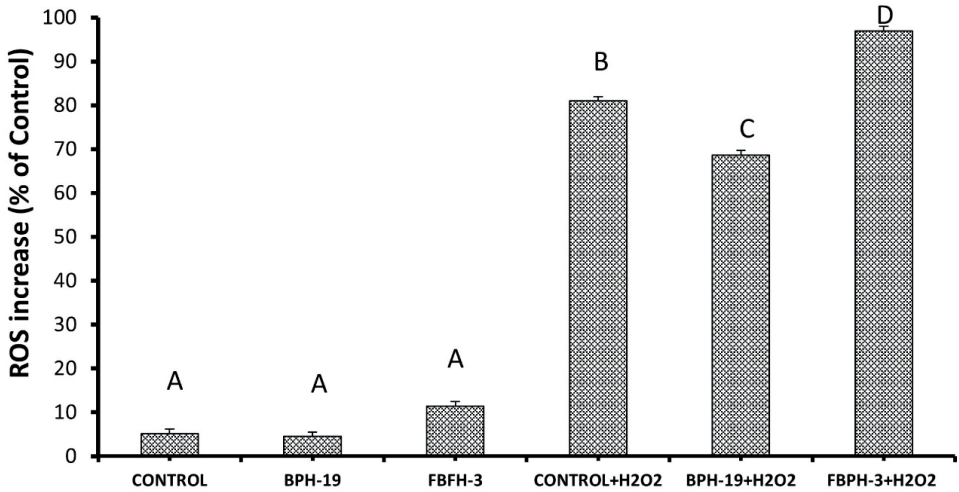


Figure 3. The effect of pretreatment with BPH-19 (0.25 mg/mL) and FBPH-3 (0.1 mg/mL), with and without oxidative stress induced by $H_2O$ 5mM on the oxygen reactive species production. Values are expressed as mean ± standard deviation (n = 4). The different superscript letters (A-D) show statistically significant differences between the treatments ($p<0.05$).

### *3.4.3 Intracellular GSH Levels*

Cellular redox state was established as a measure of the effect of BPH-19 and FBPH-3 on GSH levels in Caco-2 cells with and without conditions of oxidative stress. Figure 4 shows that the levels of intracellular GSH in the control and cells pre-treated with BPH-19 and FBPH-3, without the induction of oxidative stress, do not show significant changes ($p>0.05$). After exposing the cells to $H_2O_2$, the levels of intracellular GSH decrease significantly ($p<0.05$) by 26.2% in the control and FBPH-3, compared to the control without induced stress conditions. In contrast, in the sample pre-treated with BPH-19, GSH levels only decreased by 10.1% ($p<0.05$) versus control but increase 19.5% compared with control of stress. This indicates that FBPH-3 does not exert any protective effect on the cell under stress conditions. These results are consistent with the results regarding toxicity, cell viability and accumulation of intracellular ROS, where the greatest protective effect is offered by BPH-19. Some researchers have obtained similar results and report that the fractions smaller than 3 kDa from gluten do not change intracellular GSH in HepG2 cells. However, in the presence of $H_2O_2$ 0.5 mM, GSH levels decrease (Gabrielsson et al. 2014). These results suggest that BPH-19 does not present a pro-oxidant effect, considering its capacity to inhibit ROS increase and GSH decrease in the context of cellular oxidative stress (figure 3 and 4), unlike other antioxidants for which a pro-oxidant effect has been reported (Carocho and Ferreira 2013). The mechanism by which BPH-19 provides certain recover in the GSH levels under $H_2O_2$-induced stress conditions may be due to the modulation of

GSH-related antioxidant enzymes such as glutathione peroxidase (GPx) (Brigelius-Flohé and Maiorino 2013).

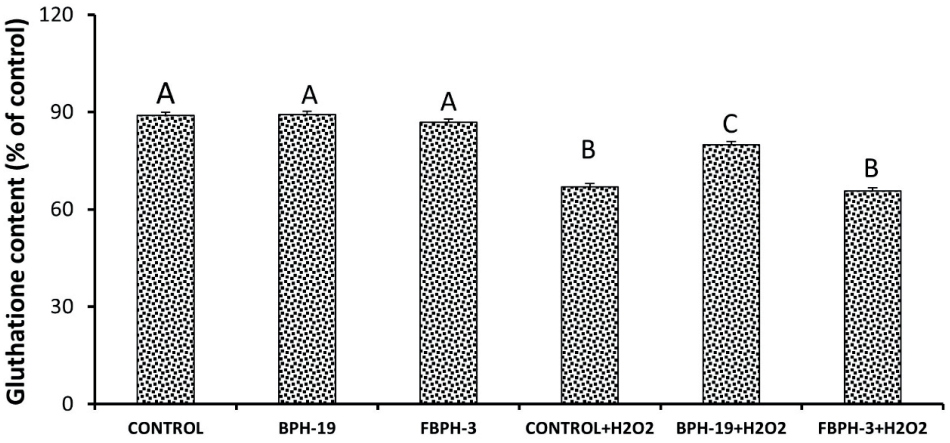


Figure 4. The effect of pretreatment with BPH-19 (0.25 mg/mL) and FBPH-3 (0.1 mg/mL), with and without oxidative stress induced by H2O2 5mM on the intracellular content of glutathione in differentiated Caco-2 cells. Values are expressed as mean ± standard deviation (n = 4). The different superscript letters (A-C) show statistically significant differences between the treatments ($p < 0.05$).

### *3.4.4 Cell Cycle*

Figure 5 shows the distribution of the cell cycle of the differentiated Caco-2 cells, which were pre-incubated with 0.25 mg/mL of BPH-19 and 0.1 mg/mL of FBPH-3, under conditions of oxidative stress or the absence thereof. The stress condition induced by $H_2O_2$ was seen to decrease the percentage of cells in the G1 phase by 11.0%, in the S phase by 21.7% and in the G2/M phase by 37.8%, which causes an accumulation of the cell population in the subG1 phase (89.2%) with respect to the control ($p<0.05$). Moreover, pre-treatment with BPH-19 and FBPH-3 was seen to prevent the alteration of the cell cycle in the G1, S and G2/M phases, maintaining the cell population in each phase at the basal control level. Additionally, when cell culture is pre-treated with BPH-19 or FBPH-3, cell accumulation in the subG1 phase is less than stress control a 57% or 71%, respectively.

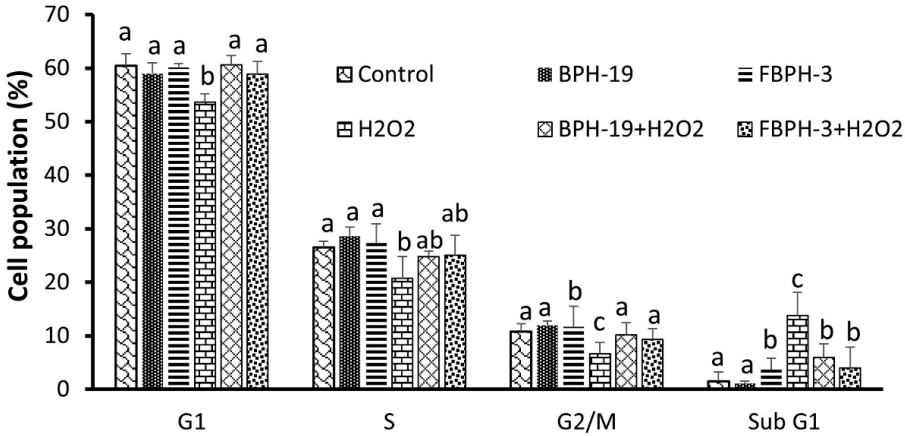


Figure 5. The effect of pretreatment with BPH-19 (0.25 mg/mL) and FBPH-3 (0.1 mg/mL), with and without oxidative stress induced by $H_2O_2$ 5 mM on the distribution of cellular cycle phases of culture differentiated Caco-2 cells. Values are expressed as mean ± standard deviation (n=4). The different superscript letters (a-c) show statistically significant differences between the treatments in each cell cycle phase ($p<0.05$).

Oxidative stress processes can alter some important control points in cell cycle regulation, as peroxides are known to alter the G1 checkpoint in Caco-2 cell cycle progression. In this regard, both BPH-19 and FBPH-3 manage to re-establish the cell cycle at the control level (figure 5 In line with these results, other authors have reported that pre-treatment with peptides from krill protein hydrolysates can prevent subG1 phase accumulation in H2O2-stimulated cells (Fernando et al. 2020).

### *3.4.5 Determination of apoptosis and cell necrosis*

Oxidative stress can cause cell death by different pathways, necrosis (non-physiological) or regulated apoptosis (Ryter et al. 2007). The results of figure 6 show that stress by $H_2O_2$ 5 mM causes a decrease in viable cells by 73.6%, increasing the number of cells in apoptosis by 44.1% and cells in a necrotic state by 343.3% versus control. Pre-treatment of Caco-2 cells with BPH-19 and FBPH-3 with/without oxidative stress does not affect basal cell viability significantly ($p>0.05$) and does not have a significant protective effect on cells in a necrotic state, but it does show a decrease in apoptotic cells both without stress (80.6%) as with stress (27.5%) ($p<0.05$). This finding suggests that both samples help preventing the cells from entering apoptosis without affecting the cell viability. However, this protective effect under stress conditions is more pronounced with BPH-19 vs. FBPH-3 (27.5% and 18.4%, respectively). These results are consistent with the previous parameters of ROS, GSH and the subG1 phase of the cell cycle. Some studies have obtained similar results showing a significant increase in Caco-2 necrotic cells due to treatment with 20 mM

of $H_2O_2$ (Devi et al. 2017).

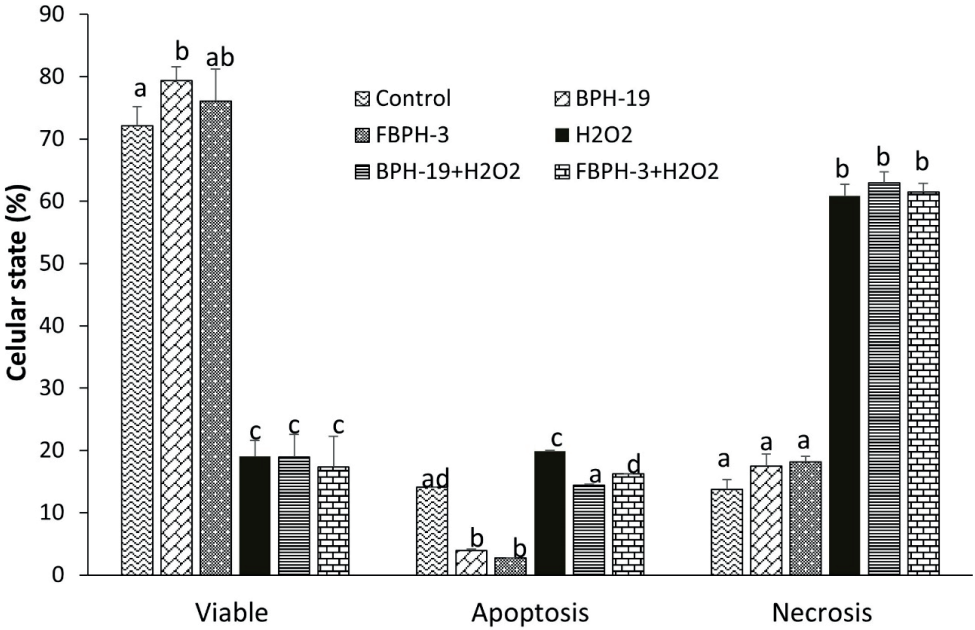


Figure 6. The effect of pretreatment with BPH-19 (0.25 mg/mL) and FBPH-3 (0.1 mg/mL), with and without oxidative stress induced by $H_2O_2$ 5 mM on the state cellular state of differentiated Caco-2 cells. Values are expressed as mean ± standard deviation (n=4). The different superscript letters (a-d) show statistically significant differences between the treatments in each cellular state condition ($p<0.05$).

There are few studies that analyze the ability of hydrolysates with cytoprotective effect to prevent cell death by different pathways. Devi et al. (2017), have carried out pre-treatments for 24 h with buffalo milk casein peptides (0.1 µg/mL) in fibroblast cultures, which managed to reverse necrosis produced by 20 mM of $H_2O_2$ for 6 h. However, these studies found that at higher concentrations of the peptides (0.5 µg/mL), the protective effect is lost, producing a state of necrosis at a similar level to cells treated exclusively with $H_2O_2$. The clear deduction is that the cytoprotective effect exerted by BPH-19 to prevent or reverse the state of apoptosis is due to its AC (figure 4) caused by $H_2O_2$ treatment and a sustained adequate cytoprotective cell environment.

### *3.4.6 Evaluation of mitochondrial membrane potential*

Changes in mitochondrial membrane potential are indicated by a reduction in the $DiOC_6$-induced fluorescence intensity. Thus, the $DiOC_6$ fluorescence decrease is an early indicator of cellular damage such as early and late apoptosis; processes where the integrity of the membrane is lost (Cilla et al. 2015). The results show that BPH-19 and FBPH-3 do not significantly reduce the fluorescence of $DiOC_6$ in Caco-2 cells. In contrast, $H_2O_2$ at 5 mM

produces a decrease in fluorescence of 54.1% versus control, which indicates a dissipation of the intracellular mitochondrial membrane potential. Furthermore, neither BPH-19 and FBPH-3 offer protection against induced oxidative stress, showing a similar decrease in fluorescence of 61.8% and 53.7%, respectively, versus control (figure 7).

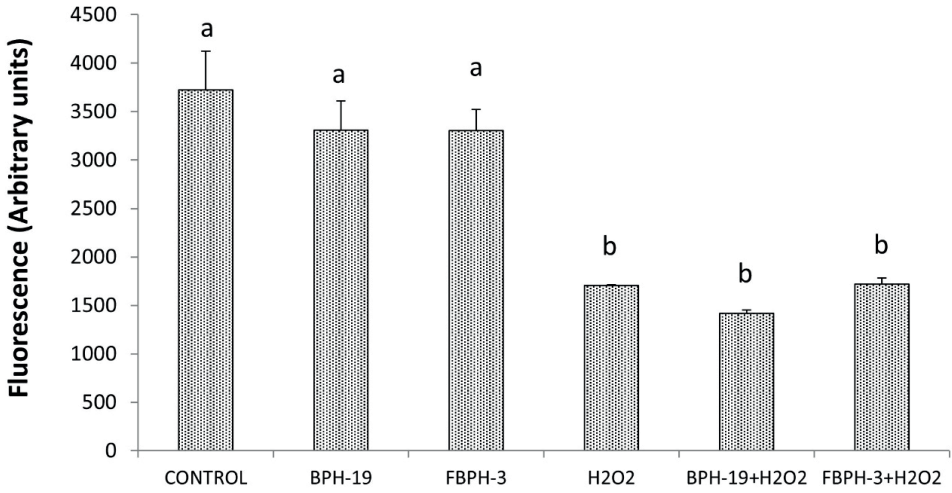


Figure 7. The effect of pretreatment with BPH-19 (0.25 mg/mL) and FBPH-3 (0.1 mg/mL), with and without oxidative stress induced by $H_2O_2$ 5 mM on the level of alteration of the mitochondrial membrane of differentiated Caco-2 cells. Values are expressed as mean ± standard deviation (n = 4). The different superscript letters (a-b) show statistically significant differences between the treatments ($p < 0.05$).

In the literature there are not many studies evaluating the cytoprotective effect of hydrolysates or other antioxidant substances on the integrity of the mitochondrial membrane potential. Some studies have obtained similar results, such as García-Nebot et al. (2011), who observed that casein phosphopeptides, despite having a cytoprotective effect, do not protect against the loss of membrane potential induced by oxidative stress with $H_2O_2$ in Caco-2 cells.

## 4 | CONCLUSION

The results indicate that hydrolysates from bovine plasma have potent antioxidant properties and that the highest activity is achieved with a degree of hydrolysis of 19.1%. BPH fractionation shows that peptides with a molecular weight of less than 3 kDa possess a higher AC. However, it does not correlate with a greater cytoprotective effect in Caco-2 cells, where the complete hydrolysate presented better results than the fraction. This shows the importance of performing screening studies with biological models such as cell

cultures, to establish the real cytoprotective effect of the samples. The results suggest that the by-products of bovine slaughterhouses, such as plasma, can be used as a valuable raw material with antioxidant capacity. This product has a potential cellular biological capacity with cytoprotective effects against ROS-mediated intestinal lesions. This can provide a cost-effective commercial process, as well as reduce the environmental impact associated with the disposal of the industry's waste.

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