




***In vitro* anticancer and antioxidant potential of *Amaranthus cruentus* protein and its hydrolysates**

Shanece RAMKISSON¹, Depika DWARKA¹, Sonja VENTER², John Jason MELLEME^{1*} 

Abstract

New insights on the use of peptides as therapeutic agents in the treatment of cancer have emerged with reports showing anti-tumour activity of peptides, predominantly derived from animals or microorganisms. Amaranth is a pseudocereal traditionally acknowledged to possess pharmacotherapeutic properties. Thus, the aim of this study was to compare the *in vitro* anti-cancer effect of amaranth protein hydrolysates (alcalase, trypsin, and pepsin). Protein hydrolysates were tested for their antioxidant activity together with the anticancer and apoptotic potential. Antioxidants results revealed hydrolysates to have a greater antioxidant effect than un-hydrolysed protein, with results exceeding that of controls. The MTT cytotoxicity assay conducted on MCF-7, A549 and HEK 293 cell lines showed the trypsin hydrolysate to exhibit a preeminent anti-cancer effect. Annexin V-FITC flow cytometry showed an increased number of early apoptotic and late necrotic cells compared to untreated cells, further validated by caspase 3/7 activity. These assays confirmed the induction of apoptosis of the trypsin hydrolysate thereby demonstrating that the hydrolysate can be used as a potential therapeutic against selected cancers.

Keywords: *Amaranthus cruentus*; antioxidant; cytotoxicity; apoptosis.

Practical Application: Control of cancer cells using hydrolysates.

1 Introduction

Dixit & Ali (2010) reported the contribution of previous studies in the widely accepted figure that 80-90% of human cancer is caused by environmental factors. Cancer has become a severe health concern across the world with the World Health Organization (WHO) estimating 13.1 million cancer-related deaths in 2030 (Senthilkumar et al., 2014). Current epidemiological studies have shown consistent consumption of selected nutrients to be associated with a reduced prevalence of cancer and chronic diseases. This has resulted in functional foods and nutraceuticals becoming popular alternatives or adjuvants to chemotherapy and radiotherapy.

Proteins are believed to be an imperative source of therapeutic peptides and have been scientifically validated as promoters in preventing certain diseases including cancer. When food proteins are hydrolysed peptides are produced, which are complex mixtures of inactive molecules used for the isolation of bioactive peptides (BAPs) through post-hydrolysis (Kannan, 2009). In most cases, greater bioactivity has been demonstrated by protein hydrolysates and peptides in comparison to their parent protein (Udenigwe & Aluko, 2012). Some food derived proteins and peptides are currently under preclinical or clinical studies as a possible alternative therapy to commonly used treatments for cancer (Quiroga et al., 2015). Initiation, promotion and progression are stages of cancer development which peptides and proteins exert their action using various mechanisms (Ortiz-Martinez et al., 2014).

Recognised examples of plant derived peptides and proteins with known antitumor activity include lunasin and Bowman

Birk inhibitor from soybean as well as some plant lectins (Hernandez-Ledesma et al., 2013). Amaranth a well-known pseudocereal belonging to the *Amaranthaceae* family are known to be rich in protein and pose an excellent amino acid balance and has previously been reported as a vital source of bioactive components (Fritz et al., 2011, Montoya-Rodríguez et al., 2015, Quiroga et al., 2015). Recently, amaranth derived protein peptides have exhibited different biological activities including antioxidant, antimicrobial and antihypertensive activity (Montoya-Rodríguez et al., 2015).

Amaranth proteins have been associated with potential hypocholesterolemic effect with lunasin-like peptides found in *Amaranthus hypochondriacus* glutelin fractions with potential antitumor properties (Quiroga et al., 2015). Barrio & Añón (2010), reported that *Amaranthus mantegazzianus* protein isolate antiproliferative activity was present and revealed its putative mechanism of action in various non-tumor and tumor cell lines. Thus, the aim of this study was to compare the *in vitro* anti-cancer and antioxidant effect of amaranth protein, as well as hydrolysates prepared using alcalase, trypsin and pepsin.

2 Materials and methods

2.1 Flour preparation

Amaranthus cruentus grains were obtained from the Agricultural Research Council-VOPI, cleaned, washed, ground into flour and passed through a 180 micron mesh. The flour was then

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¹Department of Biotechnology and Food Technology, Durban University of Technology, Durban, South Africa

²Agricultural Research Council-Vegetable and Ornamental Plant Institute, Pretoria, South Africa

*Corresponding author: johnm@dut.ac.za

defatted with hexane (1:10 w/v), centrifuged (20 min, 13000xg), dried at room temperature and stored at 4 °C until further use (Silva-Sánchez et al., 2008).

2.2 Protein isolate/hydrolysate preparation

The extraction of protein was conducted according to Silva-Sánchez et al. (2008). Briefly, PBS (pH 7.4) was used for the extraction of total protein in a ratio of 1:10 (w/v) flour/buffer. The suspension was placed in an ultrasonic bath (60 min), and thereafter centrifuged (20 min, 13000 xg). Supernatants were collected and dialyzed at 4°C against distilled water, freeze-dried. The protein concentration of *A. cruentus* protein isolates were evaluated using the standard Bio-Rad protocol. Hydrolysis of protein was conducted using a method by Silva-Sanchez et al., 2008. Briefly, *A. cruentus* protein (5% w/v) was dispensed in deionized water. The proteolytic enzyme was added (enzyme to substrate ratio (E/S) of 1:100), digested (4 h) and heated (10 min). The pH was adjusted to pH 4.0 and the suspension centrifuged (60 min, 8000 xg), freeze dried and kept at a temperature of -20 °C until further use.

2.3 SDS PAGE

The SDS-PAGE of amaranth protein isolate and hydrolysate was done according to Arise (2016) (reducing conditions). Polyacrylamide Tris-HCl gels (10-15%) were used in conjunction with the Bio-Rad Criterion cell under a constant voltage (200 V).

2.4 Antioxidant activity

DPPH radical scavenging assay

Antioxidant activity of protein and hydrolysates were evaluated using α , α -diphenyl- β -picrylhydrazyl (DPPH) radical scavenging assay according to Karamać et al. (2014). Briefly, Protein isolate and hydrolysates (100 μ L) and dilutions of the sample mixed with DPPH (100 μ L). Samples were incubated (30 min) in the dark before reading the absorbance read at 517 nm. Scavenging activity was calculated using the following formula:

$$\text{Scavenging effect} = \frac{\text{Absorbance}(\text{blank}) - \text{Absorbance}(\text{sample})}{\text{Absorbance}(\text{blank})} \times 100$$

ABTS assay

The 2,2 - azinobis - 3-ethylbenzothiazoline-6-sulfonic acid (ABTS) was performed according to Re et al. (1999) and Karamać et al. (2014). The ABTS and potassium persulfate were mixed in a ratio of 1:0.5. The ABTS stock solution was dissolved in water to an absorbance of 0.70 at 734 nm. Then 10 μ L of sample at various concentrations (100-2000 μ g/mL) and ABTS solution (200 μ L) were added to the 96-well plate. The absorbance was read after 6 min at 734 nm and results were expressed in mmol of glutathione per g of hydrolysate.

FRAP assay

The ferric reducing antioxidant power (FRAP) was evaluated using the method by Karamać et al. (2014). Hydrolysates (40 μ L, 100-1000 μ g/mL) were dissolved in deionized water.

Two hundred microlitres of reagent containing 0.3 mol acetate buffer (pH 3.6), 10 mM 2,4,6-Tris (2-pyridyl)-s-triazine in 40 mmol HCl and 20 mmol $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in a ratio of 5:1:1 (v/v/v) was heated to 37°C and added to the samples. Samples were incubated (37 °C, 30 min) and absorbance read at 593 nm. The results were calculated using a standard curve for $\text{FeSO}_4 \cdot x\text{H}_2\text{O}$

2.5 Anticancer activity

Cells (HEK-293 human embryonic kidney, MCF-7 breast cancer and A-549 human lung cancer) were grown at 37°C, 5% CO_2 in DMEM. The 3-(4,5 dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide (MTT) assay was conducted according to Dwarka et al. (2017). Briefly, cells (1×10^2 cells/mL) as well as 50 μ L media were seeded into 96-well flat bottom plate and incubated (24 h) then treated with 50 μ L of isolates prepared (1000-7.8 μ g/mL) and incubated for 24 h. MTT reagent (20 μ L, 5 mg/mL) was added to the cells and incubated (4 h at 37 °C), after which 100 μ L of DMSO was added and the absorbance read at 570 nm on a microplate spectrophotometer (Multiscan Go, Thermo Scientific). The percentage viability was determined using the following formula:

$$\% \text{Cell viability} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of untreated cells}} \times 100 \quad (1)$$

Quantification of apoptosis

The Annexin V-PE Apoptosis detection kit (BD Biosciences) was used as per manufacturer's protocol. Briefly, cells were seeded in plates and left to adhere overnight. They were then treated with *A. cruentus* isolate, trypsin hydrolysate and camptothecin.

After 24 h, cells were trypsinized, resuspended in binding buffer. FITC Annexin V (5 μ L) and propidium iodide (PI) (5 μ L) were added. Thereafter, results analysed by flow cytometry (BD FACS Aria).

Caspase-Glo 3/7

Caspase-3/7 levels were detected according to Caspase-Glo 3/7 assay kit (Promega Corporation (2019) Cat No. G8090). Seeded cells were incubated for 24 h. *A. cruentus* isolate, trypsin hydrolysate and camptothecin were added. Caspase-Glo 3/7 reagent (100 μ L) was added to each well and incubated for an hour. The luminescence of samples was measured using a GlowMax luminescence .

2.6 Statistical analysis

All data was subjected to analysis of variance (ANOVA) for determination of significant differences ($p < 0.05$) using Graph Pad Prism software, San Diego, CA, USA.

3 Results and discussion

Amaranthus cruentus protein isolate had a protein concentration of 61.52%. The SDS profile showed well-defined bands with molecular weights between 57-67 kDa (Figure 1). Silva-Sánchez et al. (2008), Tiengo et al. (2009), Barrio & Añón (2010), Sabbione et al. (2015) and Sabbione et al., (2016) revealed similar results for amaranth isolates of different cultivars.

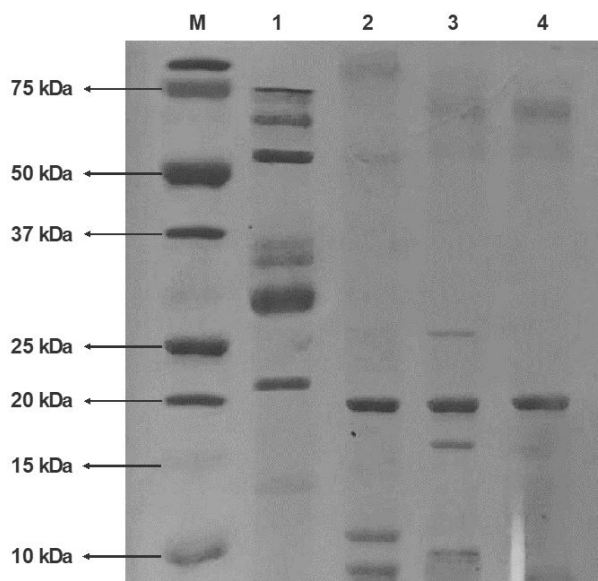


Figure 1. SDS - Page of *A. cruentus* isolate and hydrolysates under reducing conditions. Lane M - molecular weight marker, 1 - isolate, 2 - pepsin hydrolysate, 3 - alcalase hydrolysate and 4 - trypsin hydrolysate.

As seen in Figure 2a before hydrolysis, the unhydrolyzed protein isolate showed low DPPH radical scavenging percentage (14-18.3%) and after hydrolysis increased (12-26.8%). Pepsin hydrolysate had the lowest IC_{50} values of 23.06 $\mu\text{g/mL}$ while trypsin hydrolysate had the highest of 34.41 $\mu\text{g/mL}$. *Amaranthus cruentus* isolate, alcalase hydrolysate and glutathione had IC_{50} values of 29.48, 26.21 and 26.06 $\mu\text{g/mL}$ respectively. During pepsin digestion, the peptide bonds between hydrophobic and aromatic amino acids, including phenylalanine, tryptophan, and tyrosine, were cleaved, which resulted in increased hydrophobicity, allowing the pepsin hydrolysate to react with DPPH radicals in the methanol system (Phongthai et al., 2018). This study is in line with results found by Arise (2016) and Phongthai et al. (2018) who revealed higher scavenging activity for pepsin and trypsin hydrolysate from Bambara protein. Phongthai et al. (2018) found that the digestion by pepsin and pepsin-trypsin increased the DPPH radical scavenging activity of rice bran protein concentrate by 3.1-4.9 fold.

The reducing power of *A. cruentus* protein isolate and its hydrolysates are shown in Figure 2b. Glutathione had the highest FeSO_4 concentration amongst all samples (0.8-1.2 $\mu\text{mol/g FeSO}_4$). Before hydrolysis, *A. cruentus* isolate showed low FeSO_4 concentration (0.6-0.8 $\mu\text{mol/g}$), however after hydrolysis the FeSO_4 concentration increased (0.6-0.92 $\mu\text{mol/g}$). Trypsin hydrolysate was found to have the highest FeSO_4 concentration. *Amaranthus cruentus* isolate had the lowest IC_{50} (17.57 $\mu\text{g/mL}$), while glutathione had the highest (79.81 $\mu\text{g/mL}$). Pepsin, alcalase and trypsin hydrolysates had IC_{50} values of 28.28, 23.92 and 25.29 $\mu\text{g/mL}$ respectively. Similar results were found by Karamać et al. (2014) who showed that fractionation of hydrolysates exhibited greater reducing

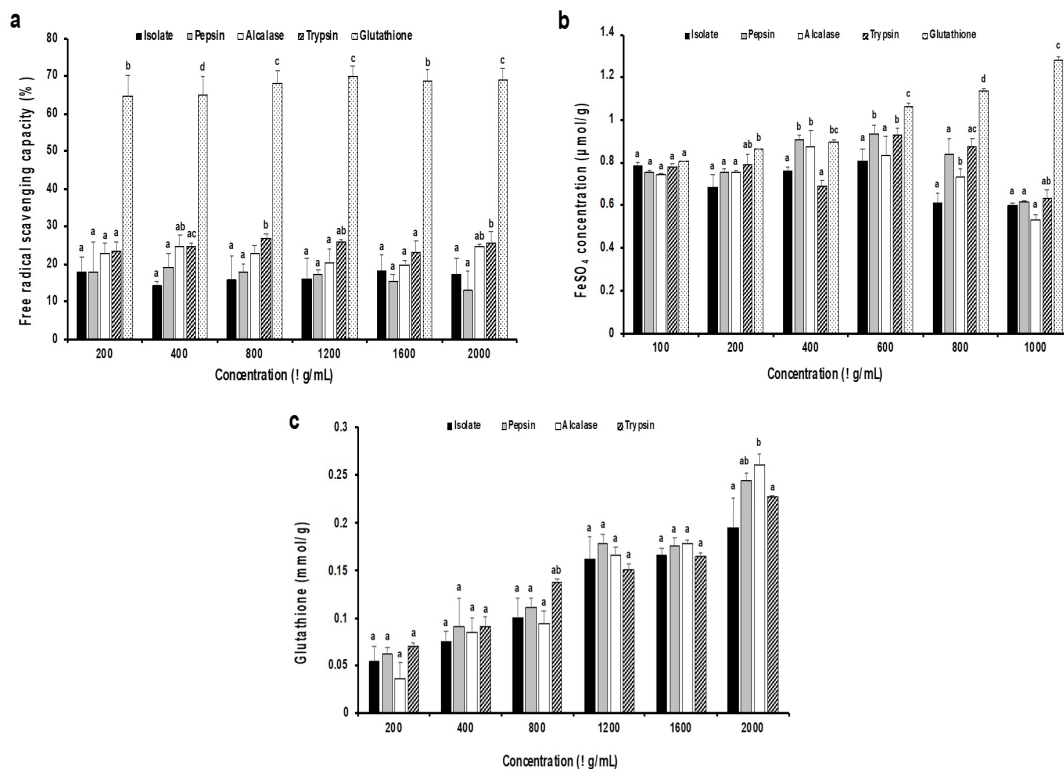


Figure 2. Antioxidant activity (a-DPPH, b-FRAP and c-ABTS) of *Amaranthus cruentus* protein isolate and hydrolysates. Mean values with different letters are significantly different at $p \leq 0.05$.

ability than high molecular weight fractions. Oyedeji (2018) demonstrated that in African Yam bean, alcalase hydrolysate had an increase in peptide size caused which caused a decrease in reducing activity.

The ABTS assay is expressed in equivalents of reduced glutathione (Figure 2c). The protein isolate had the lowest glutathione equivalent before hydrolysis (0.05-0.19 mmol/g). After hydrolysis there was a notable increase with all hydrolysates (0.03-0.25 mmol/g).

Generally, among the hydrolysates, trypsin hydrolysate showed a greater glutathione content for most concentrations. The IC₅₀ revealed that the trypsin hydrolysate had the lowest (IC₅₀ 114 µg/mL), while the alcalase hydrolysate had the highest (206.6 µg/mL). The results obtained in this study is similar to studies by Arise (2016) who reported Bambara protein hydrolysates had superior ABTS^{•+} scavenging ability when compared to the unhydrolysed protein isolate, with trypsin hydrolysate found to be a greater scavenger (EC₅₀ 22 µg/mL) compared to alcalase and pepsin hydrolysate. Karamać et al. (2014) showed similar results for flaxseed protein hydrolysates. Lower antioxidant activity can be attributed peptide mixtures of higher chain lengths being not as effective in scavenging the ABTS^{•+} (Dryáková et al., 2010).

The antioxidant capacity of peptides is dependent on size as well as on amino-acid composition, sequence, and structural characteristics; these considerations would explain the notable differences between hydrolysates obtained from different

operational conditions (Delgado et al., 2015). The results obtained in this study for all antioxidant activities show that hydrolysis of the protein isolate improved the reducing ability of *A. cruentus* hydrolysates.

MCF-7 cells treated with isolates and hydrolysates are shown in Figure 3a. *Amaranthus cruentus* isolate had the lowest IC₅₀ of 3.55 µg/mL while alcalase hydrolysate had the highest of 965.50 µg/mL. Trypsin hydrolysate, pepsin hydrolysate and camptothecin had an IC₅₀ of 3.87 µg/mL, 173 µg/mL and 9.35 µg/mL respectively. A549 cells treated with isolates and hydrolysates are shown in Figure 3b. Before hydrolysis, *A. cruentus* isolate had the highest cell viability for most concentrations (21-91%). After hydrolysis there was a decrease in cell viability (18-68.6%). Trypsin hydrolysate had the lowest IC₅₀ of 14.10 µg/mL, while camptothecin had the highest of 304.9 µg/mL. HEK-293 cells shown in Figure 3c. The cell viability for camptothecin was 53-80.8%.

For the protein samples, pepsin hydrolysate had the highest cell viability for most concentrations (66-84.2%). *Amaranthus cruentus* isolate had the lowest IC₅₀ of 8.57 µg/mL, while alcalase hydrolysate had the highest of 11.07 µg/mL. Camptothecin had an IC₅₀ of 9.10 µg/mL.

Figure 4 shows control (cells only) profiles showed that most of the cells remained alive (Q4), whereas it was observed that after the treatments of *A. cruentus* isolate and trypsin hydrolysate there was an increase of the number of early apoptotic (Q3) and late apoptotic (Q2) compared to the control. Trypsin hydrolysate

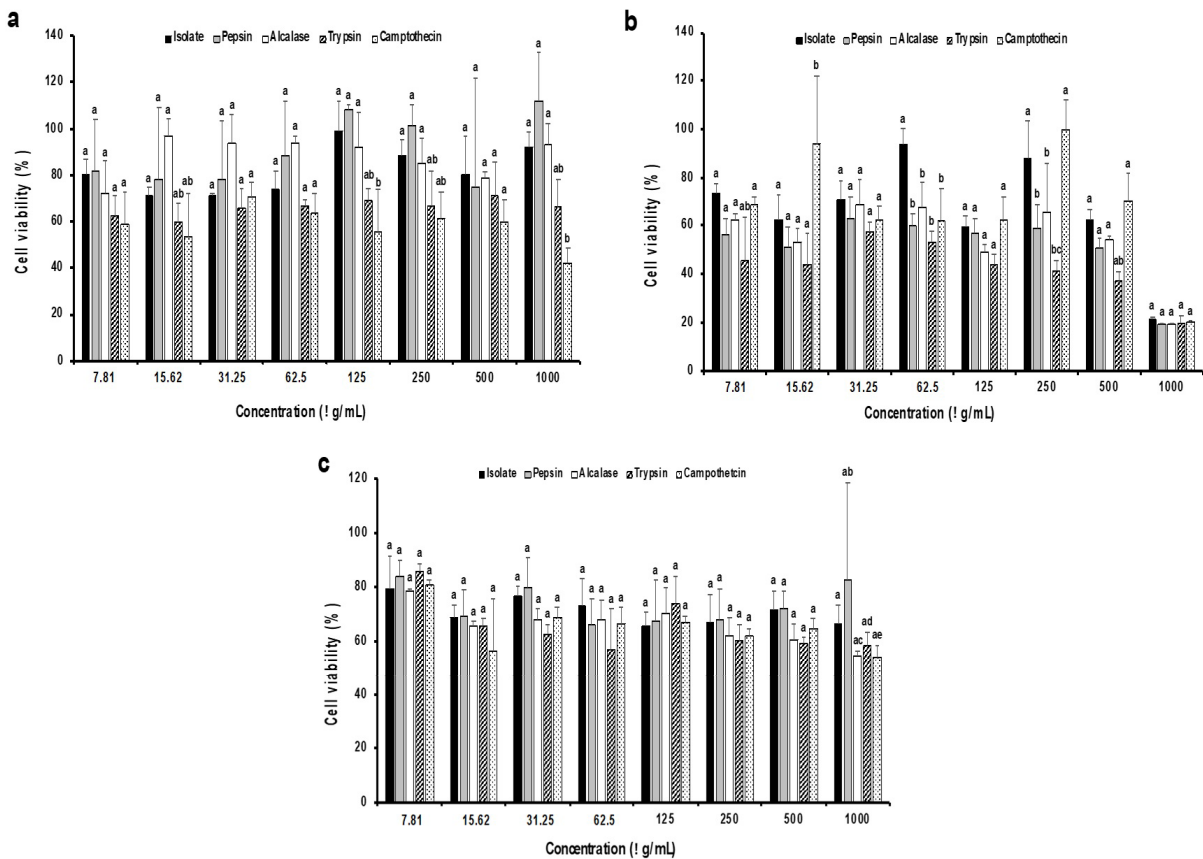


Figure 3. Cell viability (%) of MCF-7(a), A549 (b) and HEK 293 (c) cell lines treated with *Amaranthus cruentus* isolate and hydrolysate in comparison to camptothecin. Mean values with different letters are significantly different at p≤0.05.

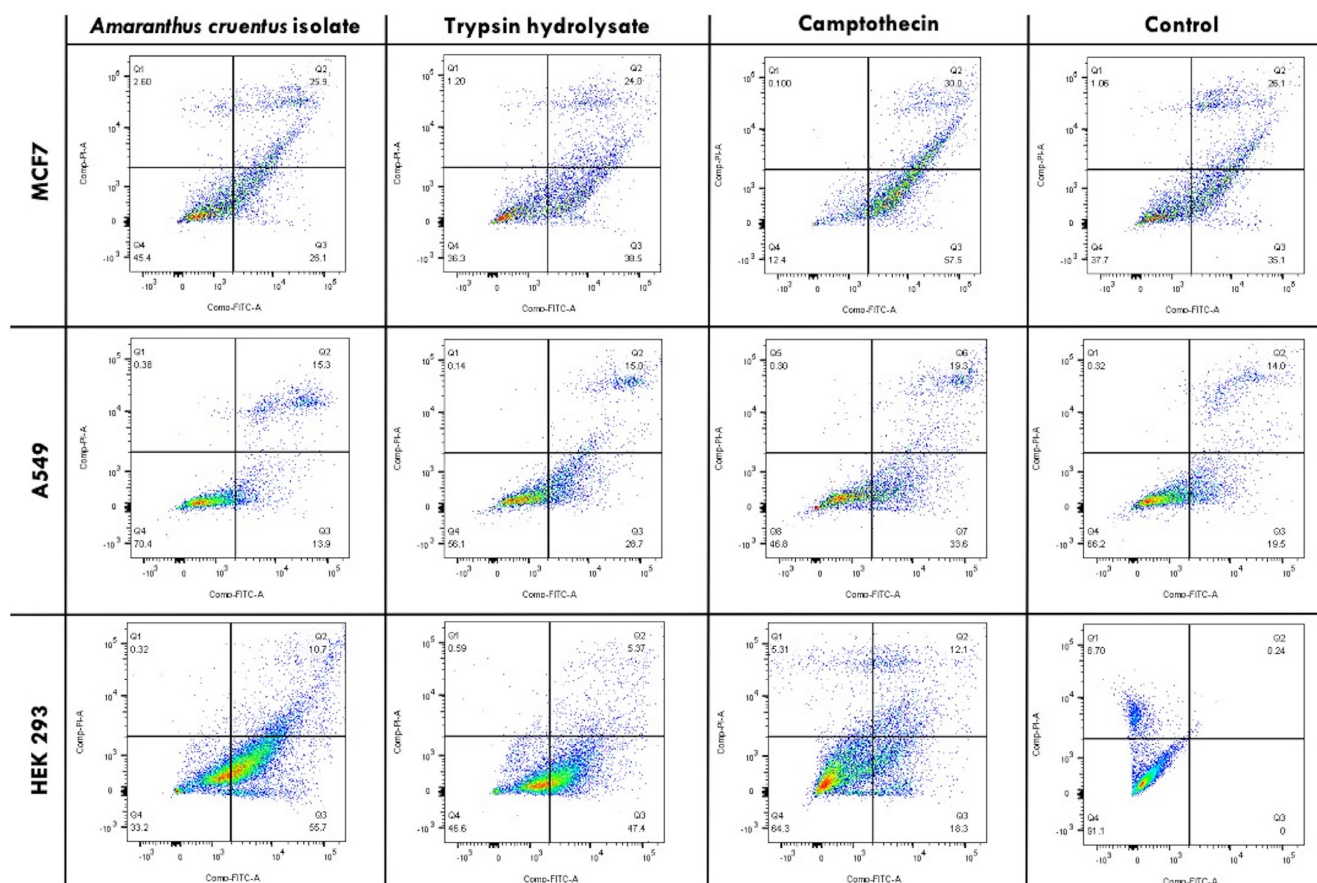


Figure 4. Flow cytometry analysis of MCF-7, A549 and HEK 293 cells treated with isolate, trypsin and camptothecin.

was shown to be the most effective, with 38.5 and 28.7% of early apoptotic cells for MCF-7 and A549 respectively.

Silva-Sánchez et al. (2008) found a peptide able to induce apoptosis against HeLa cells after trypsin proteolysis contained in amaranth seeds that matched more than 60% with lunasin sequence. Hsieh et al. (2010) reported that a combination of 10 µM lunasin with 2mM aspirin significantly increased early stage apoptosis (7%) and late stage (12%) of MDA-MB-231 cells. Recently, Sabbione et al. (2019) revealed that amaranth protein isolate produced 46.23 ± 8.08 and $4.59 \pm 1.19\%$ of late apoptosis and early apoptosis.

As seen in Figure 5 luminescence is proportional to the amount of caspase activity present. All treatments for all cell lines expressed higher caspase-3/7 compared with the control. HEK treated cells had the highest fluorescence units. The cells had to be transported in order for readings to be capture and the high caspase reading is probably due to stress of the cells, since HEK cells are extremely sensitive. Castro et al. (2009) revealed that all whey protein isolates and fractions showed higher caspase 3 activity in comparison to the control. Li et al. (2013) observed that corn protein induced apoptosis of HepG2 cells by increasing expressions of the Cleaved-caspase-3. Sabbione et al. (2019) observed that amaranth proteins from simulated gastrointestinal digestion at 1 and 2 mg/mL showed significantly higher values of caspase-3 activity compared to control.

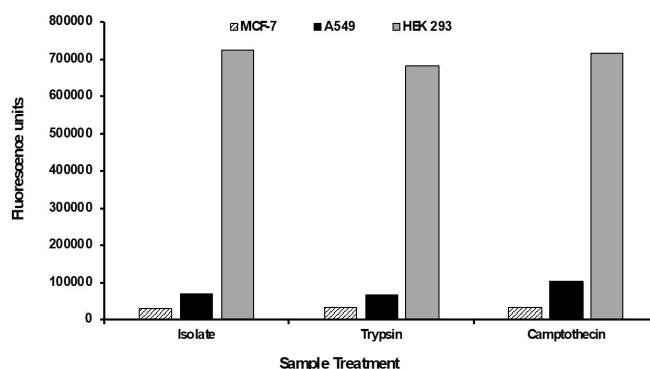


Figure 5. Caspase activity for MCF-7, A549 and HEK 293 cells treated with isolate, trypsin and camptothecin.

4 Conclusion

It was observed that *A. cruentus* protein isolate, employing three proteases (alcalase, pepsin and trypsin) was able to produce low intermediate and low mass size molecules. Proteolysis was found to increase the properties of the hydrolysates compared to *A. cruentus* protein isolate. Hydrolysates displayed better antioxidant potential in comparison to the isolate. Trypsin hydrolysate exhibited the best anticancer activity amongst all test samples.

Annexin V-FITC flow cytometry analysis and caspase 3/7 assay further confirmed this result. Further research needs to be

conducted to find the exact peptide responsible for the anticancer activity and the possible mechanism of action.

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