Anticancer and antioxidant potential of *Amaranthus cruentus* protein and its hydrolysates

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Reference Declaration

I, Ms. Shanece Ramkisson – 21325640 and Prof. JJ Mellem do hereby declare that in respect of the following dissertation – **Title: Anticancer and antioxidant potential of *Amaranthus cruentus* protein and its hydrolysates.**

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This study presents original work by the author. It has not been submitted in any form to another academic institution. Where use was made of the work of others, it has been duly acknowledged in the text. The research described in this dissertation was carried out in the Department of Biotechnology and Food Technology, Faculty of Applied Sciences, Durban University of Technology, South Africa, under the supervision of **Prof. John J. Mellem** and **Dr Sonja Venter**.

Student's signature

Dedication

I would like to dedicate this work to my parents, thank you for your sacrifice, dedication and encouragement during the pursuit of my Master's degree.

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Abstract

Traditionally, amaranth has been acknowledged to possess vital pharmacological properties with anticancer peptides having been found in Amaranthus cultivars. However, limited knowledge is available over the use of pepsin and alcalase enzymes to form hydrolysates. Thus, this study was aimed at comparing the *in vitro* anticancer effect of *Amaranthus cruentus* (grain) protein isolate and hydrolysates using alcalase, trypsin and pepsin. The safety of hydrolysates was investigated using the Ames mutagenicity and Brine Shrimp Lethality assay. Protein hydrolysates were thereafter investigated for their antioxidant potential using the FRAP, ABTS and DPPH assays. Subsequently, the protein hydrolysates were tested for their anticancer and apoptotic potential. The MTT assay was conducted to evaluate the cytotoxic potential of the protein hydrolysates using the HEK 293 (non-cancerous), A549 (cancerous) and MCF-7 (cancerous) cell lines. After that, morphological alterations were examined using the acridine orange and ethidium bromide double stain. Following this, the Annexin V apoptotic detection kit was used to quantify apoptosis together with the Glomax Caspase 3/7 kit to detect changes in the cell cycle.

Results show *A. cruentus* isolate and hydrolysates had no mutagenic response against *Salmonella typhimurium* TA 98 and TA 100 strains. The tested samples did not induce any significant increase in the death percentage of *Artemia* spp. in comparison to potassium dichromate (control). DPPH assay revealed that the hydrolysed samples had an enhanced scavenging activity compared to the unhydrolyzed sample, with pepsin having the greatest IC_{50} of 23.06 µg/ml. *Amaranthus cruentus* isolate (IC_{50} 17.57 µg/ml) was a greater scavenger of the Fe^{+} ions compared to the control glutathione (IC_{50} 79.81 µg/ml). For ABTS, all hydrolysates had a greater antioxidant scavenging potential compared to the isolate. The MTT cytotoxicity assay revealed that the isolate produced a greater cytotoxic effect on the MCF-7 and A549 cell line when compared to the control (camptothecin). For the non-cancerous cell line (HEK 293), trypsin hydrolysate had the highest toxicity. Apoptotic results revealed that trypsin hydrolysate was the most effective compared to the isolate, which was confirmed from morphological and Caspase 3/7 results. It may be concluded from the findings of this research that hydrolysates from food protein isolates have the potential for use as possible anticancer therapeutics. However, more research needs to be conducted to determine the peptides responsible for anticancer activity as well as the possible mechanism of action.

Chapter 1: Introduction

The body is continuously exposed to toxic extrinsic substances and physiological imbalances that disturb normal bodily functions, eventually resulting in the development of several health conditions (Udenigwe, 2012). "Reactive oxygen species (ROS) are usually small, short-lived, highly reactive molecules" (Galadari et al., 2017). Oxidative stress is defined as "a serious imbalance between the generation of ROS and antioxidant defences in favour of ROS, causing excessive oxidative damage" (Redza-Dutordoir and Averill-Bates, 2016). Excessive levels of ROS can cause damage to membranes, lipids, nucleic acids, lipids and organelles for example the mitochondria. The development of pathologies such as artherosclerosis, diabetes, amyotrophic lateral sclerosis (ALS), arthrosis, neurodegenerative disorders, for example, Alzheimer's and Parkinson's diseases as well as cancer has been associated with the enhanced production of ROS (Redza-Dutordoir and Averill-Bates, 2016).

Cancer has become one of the more common conditions and is deemed a serious health problem across the world (American Cancer Society, 2016). Cancer is defined as "a group of diseases characterised by the uncontrolled growth and spread of abnormal cells. If the spread is not controlled, it can result in death" (American Cancer Society, 2016). According to Al-Mamun et al. (2016), an estimated 14.1 million new cancer cases emerged, and 8.2 million deaths reported in 2012 with "South Africa ranked 50th on the World Cancer Research Fund's list of countries with the highest cancer prevalence rates" (Health24, 2014). The methods available to treat cancer include radiotherapy, surgery and chemotherapy which are associated with high costs and severe side effects which include hair loss, fatigue and damage to organs as well as the immune system (Howerton, 2012, Gaidhani et al., 2013). Due to the severity of these commonly used cancer treatments, there is a need to investigate possible alternative treatments (Gaidhani et al., 2013).

For the progression of numerous chronic diseases, such as cancer, food and nutrition are vitally important factors that exert a strong effect. In this regards, nutraceuticals and functional foods have emerged as an alternative or adjuvant to chemotherapy. They have been vitally imperative in the management and prevention of various human diseases (Quiroga et al., 2015). There is a profuse amount of literature on a variety of food protein hydrolysates and peptides with antioxidant properties in various oxidative reaction systems (Udenigwe, 2012).

These peptides have properties that include quenching or scavenging of free radicals/ROS and ROS inhibition of induced oxidation of biological macro-molecules, example, DNA, proteins and lipids (Malaguti et al., 2014). 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). Recognized examples of plant-derived peptides and proteins with known antitumour activity include lunasin and Bowman-Birk inhibitors from soybean as well as some plant lectins (Hernández-Ledesmaa et al., 2013). Initiation, promotion and progression are stages of cancer development on which peptides and proteins can exert their action using various mechanisms (Ortiz-Martinez et al., 2014).

Amaranth is a well-known pseudocereal belonging to the *Amaranthacea* family with numerous advantages including fast growth, drought resistance and nutritional properties. At the same time, its seeds are known to be rich in protein and present an excellent amino acid balance (Fritz et al., 2011; Orsini Delgado et al., 2011; Chauhan et al., 2015). Amaranth can be incorporated into the diet of healthy individuals as well as people allergic to cereal and patients who are coeliac. Amaranth 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 bioactive peptides have exhibited different biological activities which include antioxidant, antimicrobial and antihypertensive activity (Montoya-Rodríguez et al., 2015). Amaranth proteins have been associated with a potential hypocholesterolemic effect. It was reported that lunasin-like peptides were found in *Amaranthus hypchondriacus* glutelin fractions with potential antitumour properties (Quiroga et al., 2015). Barrio and Anon (2010) reported on *Amaranthus mantegazzianus* protein isolate, which presented antiproliferative activity and was able to reveal its putative mechanism of action in various non-tumour and tumour cell lines. However, there has been minimal work reported on the use of *Amaranthus cruentus* grain, hence the need to research it further. Therefore, this study aims to investigate the inhibitory potential of *Amaranthus cruentus* grain on selected cancer cell lines.

Chapter 2: Literature Review

2.1. General features of cancer

Cancer has become a serious health problem across the world and begins with damage/mutation of DNA in healthy cells (Figure 1). Healthy cells have the capacity to heal the bulk of mutations; however, if the cells can't repair mutations, this results in the formation of cancerous cells (Om et al., 2013). Cancer occurs when cell genes become abnormal resulting in cell division and uncontrolled growth (Figure 2).

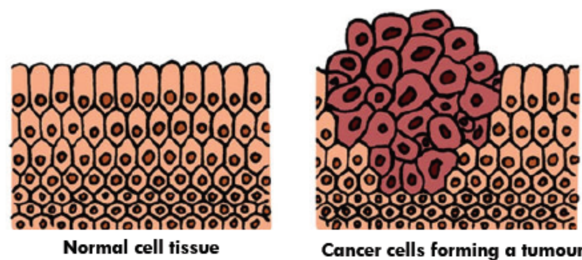

Figure 1: Normal cell tissue compared to cancer cells forming a tumour (Haleo, 2019).

The division of cells is a natural process, but when the process becomes uncontrollable, a mass of tissue is formed, often referred to as a tumour or growth (Dixit, 2010). Cancer cells can live beyond an average cell life span due to mutations, and this extended life span results in the accumulation of cancerous cells (Manoharan, 2010).

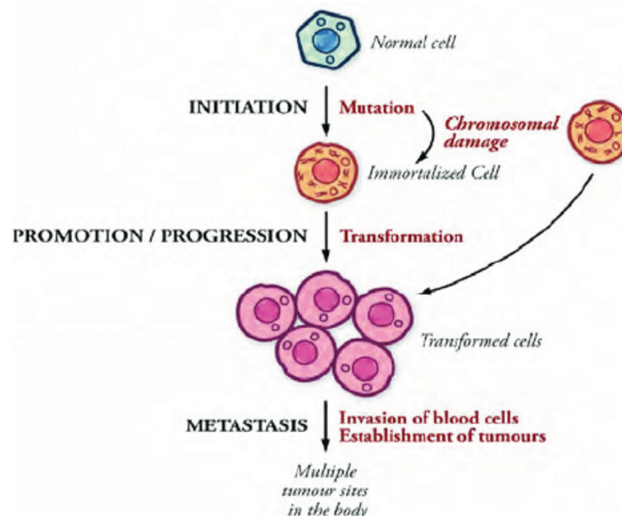

Figure 2: A schematic diagram showing the development of a cancer (Manoharan, 2010).

2.1.1. Cancer statistics

The World Health Organization (WHO) estimated a worldwide increase in cancer-related deaths, with an estimated 13.1 million deaths by 2030. In 2050, there are expected to be 27 million new cases of cancer and 17.5 million deaths from cancer worldwide (Senthilkumar et al., 2014). Table 1 depicts world cancer statistics for the most common types of cancers that occurred in 2012.

Table 1: World Cancer Statistics for most common Cancers in 2012 (Sultana et al., 2014)

Rank	Cancer	New cases diagnosed in 2010 (1000s)	Percent of all cancer excluding non-melanoma of skin
1	Lung	1825	13
2	Breast	1677	11.9
3	Colorectum	1361	9.7
4	Prostrate	1112	7.9
5	Stomach	952	6.8
6	Liver	782	5.6
7	Bladder	430	3.1
8	Non – Hodgkin lymphoma	386	2.7
9	Leukaemia	352	2.5
10	Pancreas	338	2.4
11	Kidney	338	2.4
12	Brain	256	1.8
13	Melanoma of skin	232	1.6
14	Gall bladder	178	1.3
15	Hodgkin Lymphoma	66	0.5

Amongst South African men, the number one cancer diagnosed is prostate cancer followed by lung, esophageal, colon and bladder cancer, as seen in Figure 3. Breast cancer is the most prevalent cancer amongst woman, followed by cervical, uterus, colorectal and esophageal cancer (Health24, 2014). Current statistics indicate that one in every 31 women across all ethnic groups in the country is likely to develop breast cancer (Dixit, 2010).

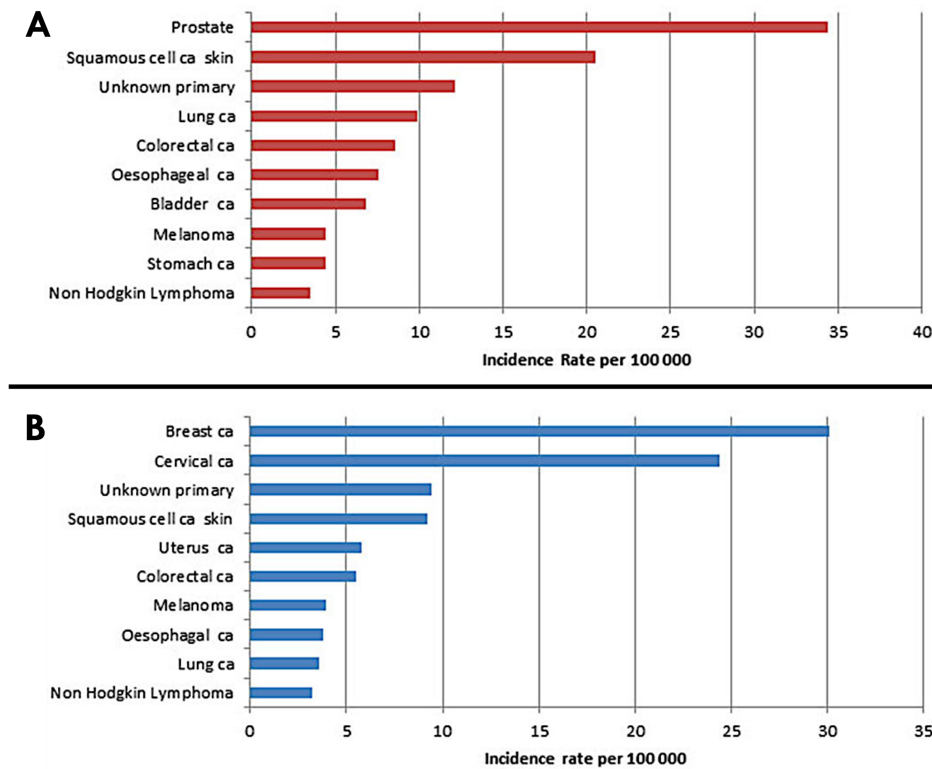

Figure 3: Ten top cancers among men (A) and women (B) in South Africa in 2004, African Cancer Registry Network (2019).

2.1.2. Types of Cancers

Cancer can affect almost any part of the body as all the cells share a majority of their functions and have similar structures (Manoharan, 2010). The categorization of cancers may be based on their locations and functions of the cells from which they originate (Manoharan, 2010). The following terms are used to distinguish tumours of different origin. Carcinoma – is a tumour, which is derived from epithelial cells. Sarcoma – is a tumour, which is derived from fat, connective tissue, muscle, bone and cartilage. Leukaemia – is a disease of white blood cells and/or their precursors. Lymphoma – is a cancer of bone marrow. Myeloma – is cancer which involves the white blood cells (Manoharan, 2010). Dixit (2010) reported that extensive research has contributed to the acceptance that 80-90% of human cancer is caused by environmental factors, which includes diet, infectious diseases, smoking and exposure to radiation and chemicals (Om et al., 2013)

2.1.3. Reactive oxygen species

Reactive oxygen species (ROS) are usually small, short-lived, highly reactive molecules (Redza-Dutordoir and Averill-Bates, 2016). Free radicals such as superoxide anion ($O2^{\bullet-}$), hydroxyl radical ($^{\bullet}OH$) and non-radical molecules such as hydrogen peroxide (H_2O_2) are the most common physiologically relevant ROS. The first time ROS was introduced, they were thought to be very toxic and associated only with various pathological conditions. A vast amount of research, connecting ROS to various physiological processes, has been published in recent years (Galadari et al., 2017). For normal physiological functions which may include cell cycle progression and proliferation, differentiation as well as migration, ROS are considered to be essential at low to modest doses. In the immune system and maintenance of redox balance, ROS plays a vital role (Figure 4) (Redza-Dutordoir and Averill-Bates, 2016). Oxidative stress is stated as "a serious imbalance between the generation of ROS and antioxidant defenses in favour of ROS, causing excessive oxidative damage" (Redza-Dutordoir and Averill-Bates, 2016). However, for the role ROS plays with diseases, clinical evidence supports an association rather than a causative role, and the molecular mechanism of ROS is not fully understood (Redza-Dutordoir and Averill-Bates, 2016, Apel and Hirt, 2004). In cells, ROS generation exists in symmetry with a range of antioxidant defenses such as catalase, peroxiredoxins, superoxide dismutases (SOD) and glutathione peroxidase including enzymatic and non-enzymatic scavengers (Redza-Dutordoir and Averill-Bates, 2016, Simon et al., 2000, Galadari et al., 2017).

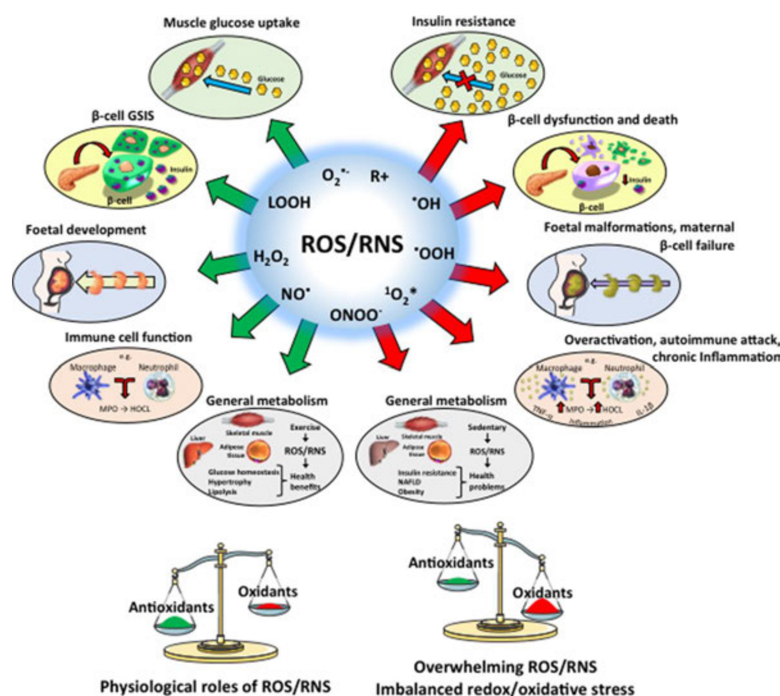

Figure 4: Systematic diagram of reactive oxygen species (Newsholme et al., 2016).

Both endogenous and exogenous stimuli can produce ROS in cells. Endogenous ROS, in general $O_2^{\bullet-}$, is primarily due to leakage during the activity of the mitochondrial electron transport chain. Complexes I and III of the respiratory chain are the two major sites for superoxide production. NADPH oxidases (Nox) which is localized at the membrane along with xanthine oxidases and enzymatic activation of cytochrome P450 reductases can also produce superoxide. When $O_2^{\bullet-}$ interacts with nitric oxide ($NO^{\bullet}$), a reactive nitrogen species (RNS), it produces the potent oxidant peroxynitrite ($ONOO^{-}$). Otherwise, SOD rapidly dismutates $O_2^{\bullet-}$ into O_2 and H_2O_2. $OH^{\bullet}$ is formed through the Fenton reaction when H_2O_2 is reduced by metal ions copper or iron. Biological macromolecules can be harmed due to the highly reactive nature of $OH^{\bullet}$. This can lead to the death of cells by apoptosis and/or necrosis (Figure 5) (Redza-Dutordoir and Averill-Bates, 2016, Galadari et al., 2017).

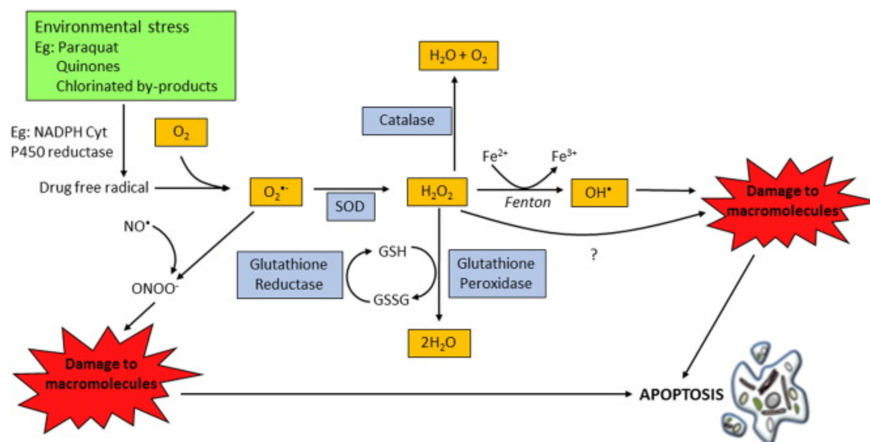

Figure 5: Generation of ROS due to environmental stress resulting in cellular damage and apoptosis (Redza-Dutordoir and Averill-Bates, 2016).

Oxidative damage and cancer

The primary structure of DNA (deoxyribonucleic acid), consists of three different chemical moieties: the cyclic five-membered deoxyribose sugar ring, the phosphate group, and the bases. DNA is a very long polymer with a repetitive backbone structure, in which phosphodiester linkages join the phosphate groups to the 3' and 5' hydroxyl groups of successive sugars along the chain. The DNA bases are planar aromatic groups, which are joined to the sugar atom by a glycosidic bond. The bases consist of the single-ring pyrimidines, cytosine (C) and thymine (T), and the double-ring purines, guanine (G) and adenine (A). For the DNA strand, there is no structural constraint on the sequence of bases; this allows the sequence to be the repository of genetic information for the cell.

The double helix is the most common biological form of DNA; it is formed when two strands are coiled around each other. The strands interact via hydrogen-bonding interactions between the bases to form base pairs between pyrimidine on one strand and a purine on the other. Thymine is usually paired with adenine to form a pair, with two hydrogen bonds. Cytosine and guanine form a second pair bond by three hydrogen bonds (Perona, 2001).

It has been widely accepted that damage to DNA by ROS is a major cause of cancer with an inherent elevated level of ROS found in cancer cells compared to healthy cells (Galadari et al., 2017). There has been an increased rate of oxidative DNA damage and, in some cases, deficient repair mechanisms in patients with cancer-related diseases (Waris and Ahsan, 2006). The experimental hypothesis of oxidative DNA damage as a major mutagenic and potentially carcinogenic factor is validated by human research (Waris and Ahsan, 2006). Mutations are caused when DNA and cell division with unpaired or mis-repaired cells are damaged by ROS (Galadari et al., 2017, Waris and Ahsan, 2006). Many ROS induced mutations tend to include changes in guanine, which leads to transversions of Guanine→Thymine. Initiation/progression can result if it relates to essential genes, for instance, tumour suppressor genes or oncogenes. Indeed, these species can participate in carcinogenesis in multiple stages.

ROS is now assumed to be engaged in both cancer initiation and progression (Galadari et al., 2017, Wiseman and Halliwell, 1996). Oxidative DNA damage caused by mutations includes several directly oxidized pyrimidines and purines, single-strand breaks, alkali labile sites and instability developed immediately or through the process of repair. Due to the multiplicity of DNA modifications produced by ROS, it is difficult to determine the frequency and extent of mutations triggered by dramatically induced lesions of individual oxygen molecules. Some of these modified bases have been found to possess mutagenic properties. Research has shown that although ROS modifies all four bases, mutations are typically associated with modification of Guanine-Cytosine base pairs. However, Adenine-Thymine base pairs are rarely associated with mutations (Waris and Ahsan, 2006).

2.1.4. Conventional cancer therapy

Conventional treatment includes chemotherapy and radiation. Chemotherapy uses antineoplastic drugs to kill cells that divide rapidly (Howerton, 2012). Chemotherapy, as well as other conventional treatments for the cure of cancer, are known to cause severe side effects, resulting in the failure to control the disease (Sultana et al., 2014).

Disadvantages associated with chemotherapeutic applications include fatigue, hair loss, easy bruising, bleeding, anemia, nausea and vomiting, appetite changes and constipation (American Cancer Society, 2016). According to the United States Nuclear Regulatory Commission (2015) fact sheet, biological effects of radiation on living cells results in three outcomes: cell death, damaged or injured cells and incorrect cell repair (United States Nuclear Regulatory Commission, 2015). The disadvantages of radiation therapy include damage to tissue, inability to kill all tumour cells, poor healing, inconvenience of radiation therapy (Emory Winship Cancer Institute, 2019).

2.2. Plants as antioxidant and anticancer agents

Throughout history, plants were used for conventional medicinal treatment for numerous diseases. In the past decade, scientific interest has increased due to medicinal herbs, which may be used as alternative or complementary medicines (Cragg, 2005). There may be an abundance of free radical scavenging molecules contained in plants (fruits, medicinal herbs, vegetables, etc.), these may include nitrogen compounds (amines, alkaloids, betalains), terpenoids (such as carotenoids), phenolic compounds (tannins, flavonoids, coumarins, phenolic acids, quinones, stilbenes, lignans), vitamins and other endogenous metabolites, which have powerful antioxidant activity (Cai et al., 2004).

Epidemiological research has indicated that several antioxidant compounds possess anti-mutagenic, antiviral, antitumour, antibacterial, anti-inflammatory or antiatherosclerotic properties to a greater or lesser extent. Natural antioxidant intake has been related to a reduced risk of cardiovascular disease, cancer, diabetes and other ageing-related diseases; however, there is still a substantial amount of disagreement in this area (Cai et al., 2004). In the fight against cancer, to improve the current and future health needs, therapeutic plants have become the focal point (Tagne et al., 2014). Around 60% of anticancer agents that are used currently originated from a natural source such as micro-organisms, plant and marine organisms (Williams, 2005). Plant natural compounds are isolated and fractionated using modern analytical and chemical techniques. It was projected that 5-15% of the 250 000 higher plant species were routinely tested for biological bioactive compounds (Svejda et al., 2010). With the detection and production of vinca alkaloids, vinblastine and vincristine and the isolation of cytotoxic podophyllotoxins, the exploration of plant-based anticancer drugs started in the 1950s. Thus, an extensive plant collection program was initiated by the National Cancer Institute (NCI) in the 1960s (Cai et al., 2004).

The program helped discover novel chemotypes which showed a variety of cytotoxic activities, which included camptothecin and taxanes; however, their advancement into clinical agents lasted over 30 years from the early 1960s to the 1990s. The plant collection program was concluded in 1982, and the collection of plants and other organisms revived in 1986 (Svejda et al., 2010).

A promising new class of molecules has arisen with several advantages in recent years (Felicio et al., 2017). For the development of potential drug candidates, peptide therapeutics has attracted considerable interest. Peptides are regarded as highly sensitive with good tolerability, safety and efficacy as well as exhibiting pharmacological profiles which are attractive. In existence, there are an estimated seven thousand biologically occurring peptides, that have been shown to offer a range of bioactivities that can consequently target various well-known diseases, for example, diabetes, cancer and cardiovascular disease. Sixty peptide-based drugs have been FDA-approved, while another 150 peptides are currently undergoing clinical and pre-clinical trials (Shoombuatong et al., 2018). In the future, the discovery of several tumour-related peptides/proteins receptors are expected to be more selective and effective anticancer drugs (Thundimadathil, 2012).

2.2.1. Anticancer peptides

Recently, the direct usage of peptides as a possible therapeutic agent for cancer treatment is gaining momentum. A variety of pathways that inhibit tumour growth are due to the anticancer activity of specific peptides (Thundimadathil, 2012). Kannan (2009) isolated and characterized novel anticancer pentapeptide derived from rice bran enzymatic hydrolysate. It was discovered that the gastrointestinal juices did not affect the peptide and breast, liver, colon and lung cancer cell lines showed cancer growth inhibition properties. He reported that there was over 60% cytotoxicity to A549 cells with the use of the <5 kDa fraction and the same fractions caused more than 80% cytotoxicity to HepG2 cells. The 5-10 kDa fraction caused around 50% on A549 but caused nearly 80% cytotoxicity to HepG2 cancer cells. A peptide (7.3 kDa) from *Phaseolus vulgaris* cv. "Spotted bean" was reported to display a robust antiproliferative activity on L1210 cells (leukaemia) and MBL2 cells (lymphoma) (Luna-Vital et al., 2015). In a study by Ortiz-Martinez et al. (2014) the isolated dimeric hemagglutinin caused MCF-7 (breast cancer cell line) proliferation to be suppressed cells [IC_{50}(concentration of an inhibitor where the response is reduced by half) of 0.2 µM].

Several changes such as cell cycle arrest of G2/Mphase, depolarization of mitochondrial membrane and phosphatidylserine externalization was shown in hemagglutinin-treated MCF-7 cells. By activating the death receptors mediated pathway, apoptosis was induced by the hemagglutinin. These pathways include caspase-8 activation, Fas lignands, p53 release, lamin A/C truncation, BID truncation and caspase-9 activation (Ortiz-Martinez et al., 2014).

Soybean was the first legume from which lunasin a 43 amino acid sequence peptide was discovered. As of late, lunasin has also been found in cereals and pseudocereals such as barley, rice, wheat, rye, amaranth and triticale (Hernández-Ledesmaa et al., 2013). Recently, lunasin has been the focal point for several experiments on anticancer peptides with its anti-cancer effects primarily against both chemical and viral oncogene-induced cancers, focused on the regulation of deacetylation and histone (H) acetylation pathways, mainly by inhibiting histone acetyl transferase (HAT). This causes cancer cells to block H3 and H4, repress the continuation of the cell cycle, and apoptosis. Its bioactive properties are due to its capacity to defend DNA against damage by oxidation, inhibit core histone acetylation in mammalian cells and arrest cell division in cancer cells (Malaguti et al., 2014). As an anticancer agent in cell cultures, lunasin shows excellent potential, however, questions are raised due to its large molecular size, which relates to absorption and the use of it as an orally bioavailable human promoting agent (Udenigwe, 2012).

Dia et al. (2009) reported that in human subjects who consume soy protein containing lunasin, 4.5% of lunasin was absorbed. It was also observed that other lunasin derived peptide sequences in plasma, could be attributed to degradation by gastrointestinal proteases and plasma peptidases. Another research study reported efficient absorption of lunasin from rye consumption into the kidney, blood, and tissue-derived extracts retained the anticancer HAT-inhibitory property of the parent molecule (Jeong et al., 2009). It was proposed that the presence of protease inhibitors in lunasin comprising whole foods resulted in lunasin's resistance to gastrointestinal digestion in comparison to its synthetic form (Hernandez-Ledesma et al., 2009). For anticancer therapy other soy protein-derived peptides apart from lunasin have shown promising activity. It was reported by Wang et al. (2008) that different soy hydrolysate cultivars inhibited the viability of L1210 (leukemia cells) cells IC_{50} 3.5 to 6.2 mg/mL, which was significantly lower than the activity of lunasin (IC_{50} of 0.078 mg/mL). Partially purified lunasin has shown *in vivo and in vitro* bioactivity (Ortiz-Martinez et al., 2014).

2.2.2 Mechanism in which peptides exert their anticancer activity

Throughout carcinogenesis, the induction of apoptosis is believed to play a central role in the production and growth of some cancers with a number of molecular mechanisms used by tumour cells to inhibit apoptosis (Elmore, 2007). Many signalling molecules, for instance, the antiapoptotic protein Bcl-2, the proapoptotic protein Bax and the tumour suppressor protein, *p53* are involved in apoptosis and are deregulated in cancer (Bhutia and Maiti, 2008). The *p53* protein is involved in cell cycle progression, DNA repair, cell growth regulation and apoptosis. TP53 gene mutations are the most common genetic alteration that results in the development of defective *p53* proteins that can allow genetically abnormal cells to survive and become malignant (Elmore, 2007). Over fifty percent of all human cancers that show *p53* is mutated, thereby proving its critical role. The *p53* protein can activate proteins that repair DNA when DNA has sustained damage; they can hold the cell cycle at the G_1/S DNA damage recognition point. They can initiate apoptosis if the DNA damage is irreparable (Elmore, 2007).

2.3. Apoptosis

Apoptosis, also referred to as programmed cell death, is regarded as a critical element of different processes such as proper development, turnover of healthy cells and immune system function, embryonic development, hormone-dependent atrophy and chemical cell death (Elmore, 2007). Apoptosis is characterized by several morphological changes in cells that include volume reduction, nuclear chromatin condensation, loss of specific surface structure, cytoplasmic organelle survival, and phagocytosis. Apoptosis can occur through two distinct signalling pathways, the intrinsic and extrinsic (de Mejia and Dia, 2010). Cytotoxicity mediated by T-cell and perforin-granzyme dependent killing of cells is an additional apoptosis pathway (Elmore, 2007). In response to strain, such as lack of growth factor deficiency and injury to DNA, the intrinsic pathway targets the mitochondrial membrane. “The extrinsic pathway is related to attaching apoptosis-inducing ligands to cell surface receptors like the Fas-associated death receptor, tumour necrosis factor-α (TNF-α) receptor and TNF-related apoptosis-inducing ligand receptor”. One of the hallmarks of cancer cells is its ability to resist apoptosis and contribute to tumour progression either by granzyme A or B, the perforin/granzyme pathway can induce apoptosis (de Mejia and Dia, 2010). The pathways of extrinsic, intrinsic, and granzyme B converge on the same terminal or pathway of execution and are the two major pathways of apoptosis.

This mechanism is initiated by caspase-3 cleavage and results in the breakdown of DNA, cytoskeletal degradation and nuclear proteins, protein cross-linking, the development of apoptotic bodies, the expression of ligands for receptors of phagocytic cells and finally the absorption by phagocytic cells (Figure 6) (Elmore, 2007).

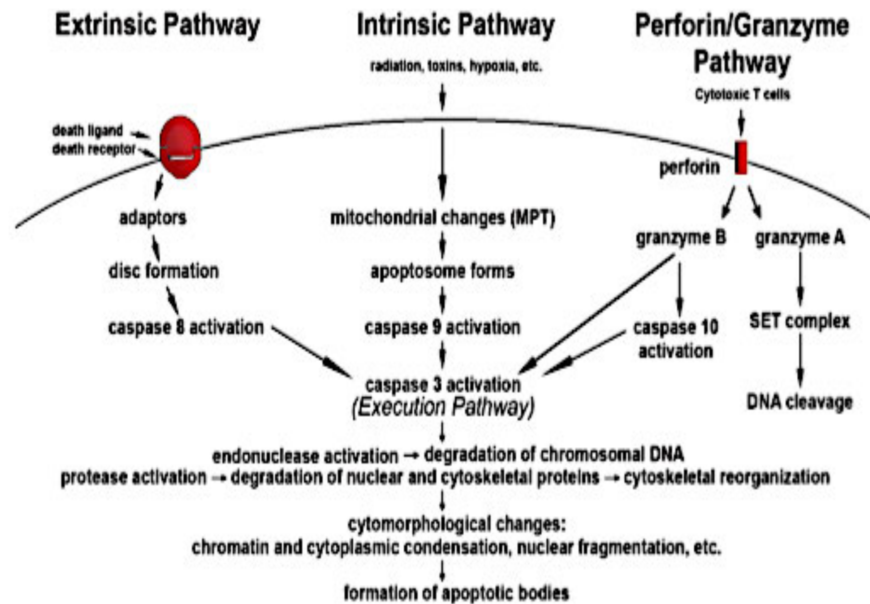

Figure 6: Systematic diagram of apoptosis (Elmore, 2007).

Barrio and Anon (2010) demonstrated *Amaranthus mantegazzianus* protein isolate antiproliferative activity and revealed its putative mechanism of action in various non-tumour and tumour cell lines. The executioner caspase-3 is activated by its own initiator caspase (8/9/10); however, granzyme A is caspase-independent. The pathway of execution results in characteristic cytomorphological characteristics that include condensation of chromatin, cell shrinkage, production of cytoplasmic blebs as well as apoptotic bodies, and finally phagocytosis of apoptotic bodies by adjacent parenchymal cells, neoplastic cells or macrophages (Elmore, 2007).

2.4. Characteristics of *Amaranthus cruentus*

2.4.1. History, Classification and botanical description

Amaranth is a pseudocereal (Figure 7), belonging to the order Caryophyllales group, *Amaranthaceae* family, subfamily *Amaranthoideae*, genus *Amaranthus* (Grobelnik - Mlakar et al., 2009). The *Amaranthaceae* family is known to have 70 genera and an estimated 80 species.

Three principal species of *Amaranth* that produce grains these are *Amaranthus caudatus* (native to Peru); *Amaranthus hypochondriacus* (native to Mexico) and *Amaranthus cruentus* (native to Guatemala and Mexico) (Sanz-Penella, 2013; Montoya-Rodríguez et al., 2015). Amaranth is known to be an herbaceous plant; its height is between 0.3 to 5 m with an erect stem and enormous inflorescence. (Montoya-Rodríguez et al., 2015).

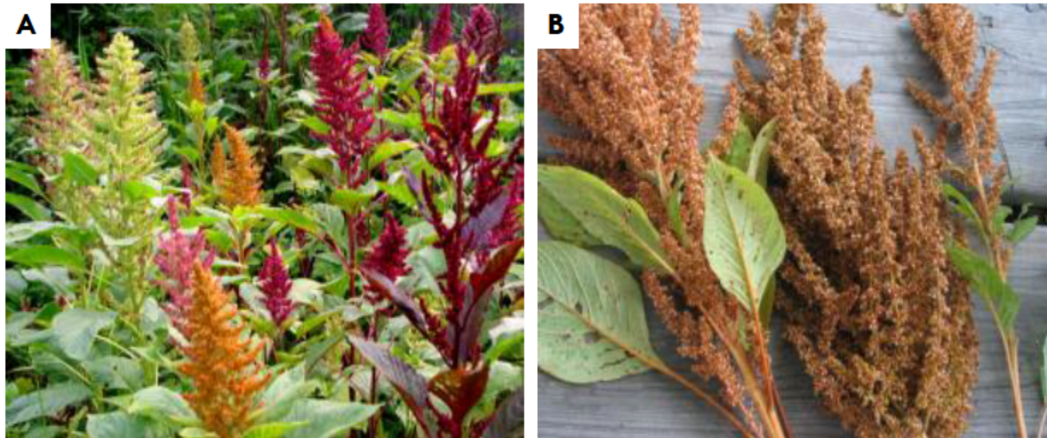

Figure 7: Amaranth growing in a field (A) and Amaranth once harvested (B) (Whole Grain Council, 2016).

2.4.2. Agronomical importance of amaranth

Over the past 20 years, the amaranth grain has gained interest due to its agricultural and nutritional features (Velarde-Salcedo et al., 2013). Amaranth is a known C4 plant, which means by transpiration, it loses less water and very efficiently uses carbon dioxide (CO_2) (Montoya-Rodríguez et al., 2015). The agronomical advantages of amaranth are it is drought tolerant, cultivated throughout the year, fast-growing plant and it grows in poor soil conditions (Fritz et al., 2011). Amaranth's numerous advantages make it a vital crop that may be utilized in areas where traditional crops cannot grow. Amaranth is currently cultivated in several regions of the world such as India, USA, Africa, China and South America (Aguilar et al., 2013).

2.4.3. Amaranth seed

The kernel of the amaranth is a dicotyledonous product that is comprised of a seed coat (thin layer of cells); the perisperm: rich in starch layer; the two cotyledons: rich in protein; the procambium: the endosperm; the radicle and the root (Grobelnik-Mlakar et al., 2009). The seed is tiny, measuring between 1-1.5 mm in diameter, and the number of seeds per gram can vary between 1000-3000 (Figure 8) (Painuli and Kumar, 2016).

The seeds have a round shape and may present nineteen colours, which include black, yellow, pink, white, gold and red (Montoya-Rodríguez et al., 2015).

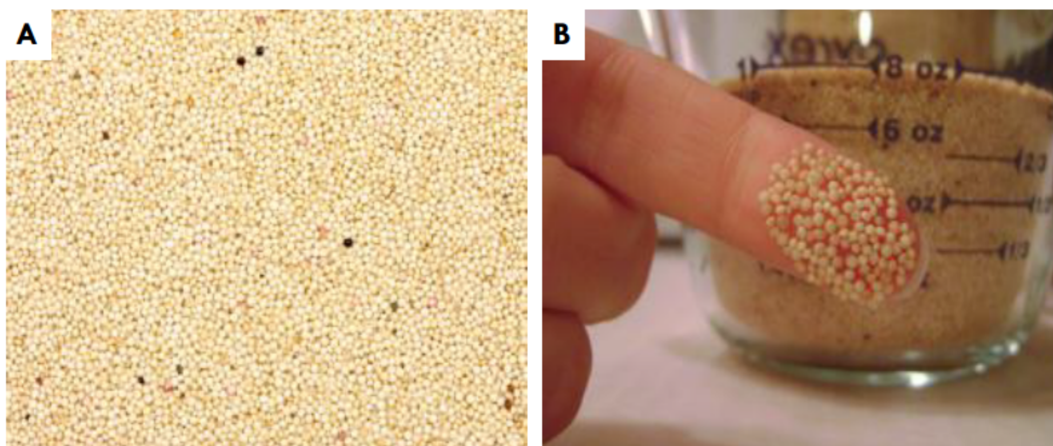

Figure 8: Amaranth grains (A) and Amaranth grain in detail (B) (Whole Grain Council, 2016).

2.4.4. Amaranth chemical composition

Compared to common cereals, amaranth grain possesses a superior protein content. The protein (13-19%) of the pseudocereal, is contained in the endosperm, which contains thirty-five percent of the total grain protein, the remaining protein is found in the germ and coat (Bressani et al., 1992). Amaranth also contains a good source of lipids between 5 to 13%, as well as minerals, for example, iron, potassium, phosphorus, sulphur, calcium, magnesium, sodium and manganese, and vitamins of B complex. Starch is the principal component of amaranth (62%), other than its nutritional features, amaranth grains contain bioactive components, which include phytosterols, flavonoids, tannins, phenolic acids and anthocyanins (Montoya-Rodríguez et al., 2015). The excellent nutritional profile and the high concentration of proteins are the principal characteristics of amaranth. Globulins and albumins are the major storage proteins in amaranth grain (Quiroga et al., 2012). Previous studies have reported amaranth's total dietary fibre (insoluble and soluble) to be in the range of 13.9 to 14.6% for unprocessed and extruded amaranth flour (Caselato-Sousa and Amaya-Farfan, 2012, Montoya-Rodríguez et al., 2015).

2.4.5. Amaranth uses

Grain amaranth has been incorporated into several foods. Stews, sauces, soups, souffles and porridges can be prepared using the whole grain. Grains that are boiled can be used like couscous and rice, which is usually prepared using wheat and semolina (Sanz-Penella, 2013).

During the boiling process of grain amaranth, the starch leaches out and is gelatinized. The gelatinization process causes water that is used for cooking to thicken and gelatinize. This happens when embryo – encircled gelatinous perisperms are separated during cooking (Rastogi, 2013). After processing, grain amaranth can also be utilized as an ingredient by an alkaline process, extrusion and germination. Different foods can be prepared using the entire plant; human food is prepared using the seed as the principal component. Flour can be obtained from the seed and used to prepare a variety of products. Animal feed is produced using the leaves and stems (Montoya-Rodríguez et al., 2015).

2.4.6. Traditional uses of Amaranth in South Africa

Amaranth is also known as cockscomb, hell's curse and pigweed in English, *hanekam misbredie and varkbossie* in Afrikaans, *umifino, unomdlomboyi,, umtyuthu, imbuya* in isiXhosa, *isheke, imbuya,indwabaza* in isiZulu, *theepe, thepe* in IsiPedi, Sesotho and Setswana, *isheke, umbuya* in Xitsonga, *mohwa* in Shona and *imbuya, tyutu* in Pondo. South Africa utilizes all different species of amaranth except for the arid South Western areas. In South Africa, the most widely used amaranth species include *Amaranthus hybridus*, *Amaranthus thunbergii, Amaranthus deflexus, Amaranthus greazicans, Amaranthus viridus, Amaranthus spinosus and Amaranthus hypochondriacus*. For use as a vegetable, whole seedlings are harvested and cooked. Amaranth has other uses as well with *A. spinosis* leaves and stems being dried and ground for the use of snuff in the Tzaneen area. In areas where salt access was limited in the past, such as parts of the Limpopo province, the entire dried plant of various amaranth species was burned to produce ash that was used as salt (Jansen van Rensburg et al., 2007). In South Africa, amaranth is generally not planted, but after first rains, it occurs as a voluntary invasive crop and harvested from the wild. Cultivation of this crop does not vary widely with the main cultivation reason associated with food security in households and seed bank replenishment (Jansen van Rensburg et al., 2007).

2.4.7. Medicinal uses of Amaranth

Based on their content of flavonoids and phenolic acids, amaranth varieties have been evaluated as a functional food in recent years (Aguilar et al., 2013, Al-Mamun et al., 2016). Pharmacological properties have been well documented in amaranth, which includes antioxidant and anti-inflammatory activities (Al-Mamun et al., 2016, Caselato-Sousa and Amaya-Farfan, 2012). Potential agents including dietary fibre, squalene, isoprenoid and tocotienol compound have been identified to reduce cholesterol levels.

Due to amaranth being gluten-free naturally, it's a vital food source for people with celiac disease (Aguilar et al., 2013). Most of these pharmacological properties are explained by the inclusion in methanol extracts of substances such as lunasin, an antitumour peptide as well as antihyperlipidemic, antidiabetic and antihelmintic compounds, in addition to those, antidiarrheal, antifungal and antimalarial properties were found in aqueous seed extracts. In amaranth seeds as well as sprouts specific flavonoids like rutin and some phenolic acids (vanillic, gallic and phydroxybenzoic) with antioxidant activity were found (Caselato-Sousa and Amaya-Farfan, 2012).

2.5. Amaranth proteins

2.5.1. Nutritional quality of Amaranth proteins

Amaranth grains are known to have proteins with a high-quality amino acid balance, which are known to be superior to that of cereals and selected legumes (Shevkani et al., 2013). Amaranth grains protein (13-19%) are known to be highly digestible (90%) and are abundant in the limiting amino acid lysine (4.9-6.1 g lys/100 g protein) (Grobelnik - Mlakar et al., 2009 , Painuli and Kumar, 2016). Amaranth protein contains a decent amount of sulfur and tryptophan-containing amino acids, which are usually limited in other grains (Montoya-Rodríguez et al., 2015, Inglett, 2015).

Sixty-five percent of amaranth proteins are contained in the embryo, while the remaining is found in the perisperm, compared to other grains, amino acids are located in the endosperm and have a poor quality of essential amino acids (Grobelnik - Mlakar et al., 2009). Table 2 depicts amaranth amino acid composition in comparison with other crop species such as wheat, maize and oats. Amaranth's amino acid composition is relatively close to the optimum protein reference pattern for the human's diet according to WHO/FAO requirements (Grobelnik - Mlakar et al., 2009 , Montoya-Rodríguez et al., 2015).

In amaranth, the limiting amino acids are leucine, isoleucine and valine. However, this is not a severe issue of concern as these amino acids are found in abundance in most common grains (Grobelnik - Mlakar et al., 2009). Amaranth's protein amino acid composition almost meets the daily requirements by FAO/WHO for adults, indicating that it can be used in a mixture or combination with cereals to boost protein quality and have a superior nutritional value (Montoya-Rodríguez et al., 2015).

A 50:50 maize-amaranth flour combination nearly reaches the 100 score, with amaranth protein also recognized as gluten-free (Fritz et al., 2011, Chauhan, 2015).

Table 2: Essential amino acid concentration in grains of different amaranth species in relation to other staple crops. Adapted from Montoya-Rodríguez et al. (2015).

Protein source	Amino Acids (g/100g of protein)									
	Trp	Met/Cys	Thr	Ile	Val	Lys	Phe/Tyr	Leu	LAA[A]	EAA[B]
Amaranth (average)	1.3	4.4	2.9	3.0	3.6	5.0	6.4	4.7	67	87
A. cruentus	-	4.1	3.4	3.6	4.2	5.1	6.0	5.1	84	89
A. cruentus	0.9	4.6	3.9	4.0	4	6.0	7.9	6.2	88	95
A. cruentus	-	4.6	3.9	4.0	4.5	6.1	8.5	6.1	87	96
A. cruentus	1.1	4.9	4.0	4.1	4.7	5.9	8.1	6.3	90	98
A. hypochondriacus	1.8	0.6	3.3	2.7	3.9	5.9	8.4	4.2	34	78
A. cruentus	1.4	4.1	3.4	3.6	4.2	5.1	6.0	5.1	73	91
Amaranth (average)	1.3	4.5	3.5	3.6	4.2	5.6	7.3	5.4	75	91
Barley	1.2	3.2	3.2	4.0	4.7	3.2	8.2	6.5	83	97
Buckwheat	1.4	3.7	3.9	3.8	5.2	5.9	5.8	5.8	83	97
Maize	0.6	3.2	4.0	4.6	5.1	1.9	10.6	13.0	35	86
Oat	1.2	3.4	3.1	4.8	5.6	3.4	8.4	7.0	62	92
Rice	1.0	3.0	3.7	4.5	6.7	3.8	9.1	8.2	69	94
Soya	1.4	3.1	3.9	5.4	5.3	6.3	8.1	7.7	89	98
Wheat	1.2	3.5	2.7	4.1	4.3	2.6	8.1	6.3	47	86

Note: A – relative value of limited amino acid according to FAO/WHO requirements. B – relative value of essential amino acids according to FAO/WHO requirements.

2.5.2. Globulins, glutelins and albumins

Solubility is one of the most common methods used to classify protein; glutelins, globulins and albumins are found in this category. In grain amaranth globulins and albumins are the main proteins found (Quiroga et al., 2012; Caselato-Sousa and Amaya-Farfan, 2012). Silva-Sanchez et al. (2008) reported that peptides present in amaranth grains were evaluated and the presence of glutelins, globulins and albumins noted. Montoya-Rodriguez et al. (2014) also reported the presence of globulins and albumins before and after extrusion. In amaranth isolates, globulins constitute the principle protein fraction with 11S globulin, known as Amaranthin; the principal constituent was characterized (Montoya-Rodríguez et al., 2015). This protein was found to have a molecular mass of 56 kDa and 501 amino acid residues. In another study, the globulin 11S was identified as amaranth's main storage protein (Condés et al., 2009). In amaranth isolates, 7S globulin is present but in a lower quantity than 11S; the 7S globulin is also less studied than the 11S (Montoya-Rodríguez et al., 2015). Quiroga et al. (2012) reported the 7S globulin has a molecular mass near 200 kDa and is formed by four subunits of 66, 52, 38 and 16 kDa.

2.6. Food protein derived bioactive peptides

In the development or modification of aggravating human diseases, it has long been recognized that the non-nutritive and nutritive components of food may aid in this regard. As an alternative or adjuvant to chemotherapy and radiotherapy functional foods and nutraceuticals have emerged. They have become fundamentally important in the management and prevention of various human diseases, as well as ensuring that the health state is maintained at its optimum (Udenigwe, 2012). The use of protein-derived peptides from foods have become a growing trend and interest as possible intervention agents against various human chronic diseases and ensuring health state is maintained (Moronta et al., 2016).

When food proteins are hydrolyzed using enzymes, peptides are produced, this is followed by post-hydrolysis processing where complex mixtures of inactive molecules are used for the isolation of bioactive peptides (BAPs) (Kannan, 2009). Endorphins, a naturally occurring BAPs are different from the above peptides as they are generated during proteolysis of food proteins (Hartmann, 2007). As seen in Figure 9, potent biological activities have been exhibited by food protein hydrolysates; these include antioxidant, antimicrobial, antihypertensive, anticancer, lipid-lowering activities and immunomodulatory. The constituent peptides are responsible for these activities (Sarmadi, 2010, Orsini Delgado et al., 2011, Malaguti et al., 2014).

Structural properties (length of the chain and amino acid residue, physiochemical characteristics) of food peptides is the main factor that determines specific bioactivity against a range of molecular disease targets (Sarmadi, 2010). In general, BAPs activity against molecular disease targets is generally considered to be weaker than their synthetic drugs and peptidomimetics. Many advantages are offered by the use of BAPs against human diseases; these include low health cost, natural product safety as well as the nutritive benefits of the peptides as sources of essential and beneficial amino acids (Udenigwe, 2012).

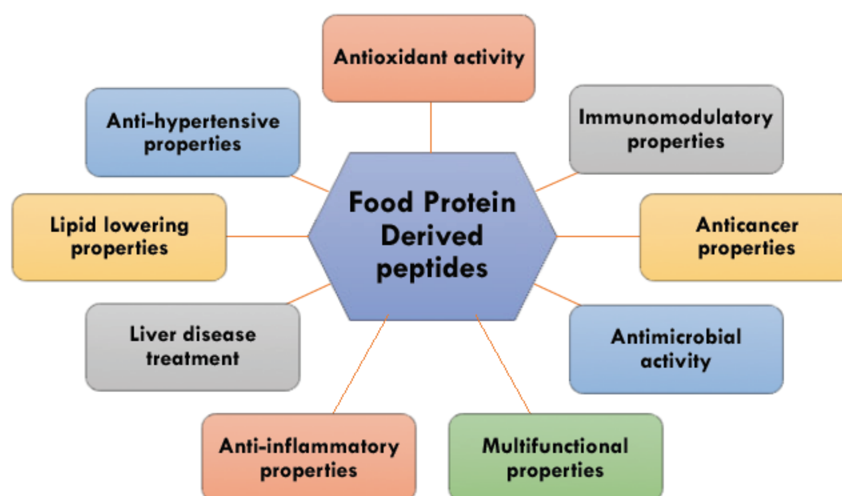

Figure 9: Bioactive properties associated with food protein-derived peptides for human health and disease prevention (Udenigwe, 2012).

2.6.1. Food protein – derived antioxidant peptides

Food antioxidants radical quenching activities are due to antioxidants ability to participate in the transfer reaction of single electrons (Udenigwe, 2012). Therefore, the large quantity of peptide amino acid residues that at physiological pH can pass electrons to free radicals can contribute to increased antioxidant properties. Other mechanisms of antioxidant activity in food peptides include ferric reducing power and transition metal chelating activity (Udenigwe, 2012). Antioxidant activity of food protein hydrolysates may be affected by some factors which include: degree of hydrolysis, specificity of the protease used for the hydrolysis reaction and structural properties of the peptides (amino acid composition, molecular size and hydrophobicity) (Sarmadi, 2010). It has been reported that the quantity of cysteine, proline, histidine and aromatic amino acids contribute to food peptides antioxidant activity (Malaguti et al., 2014). Using a variety of synthetic peptides, structure-function studies found that histidine peptide residue can chelate metal ions, scavenge OH and quench active O_2. Its imidazole group is responsible for these attributes, which participates in the transfer reactions of single electrons and hydrogen atoms. When found at the N-terminus of peptides, valine and leucine have been found to exert antioxidant properties. On the contrary to the above, when found at the C-terminus of peptides, tyrosine and tryptophan exert antioxidant properties (Malaguti et al., 2014). Hydrophobic amino acids are imperative for the enhancement of antioxidant properties of peptides as they are able to improve the availability of antioxidant peptides to hydrophobic cell targets, for instance, the polyunsaturated chain of biological membrane fatty acids (Malaguti et al., 2014).

When studying the oxidative effect of wheat peptide antioxidant activity level, heat and malondialdehyde (MDA) have been shown to oxidize wheat peptides, resulting in a loss of antioxidant activity. In addition, the authors mentioned that these oxidative conditions lead to an increase in *in-vitro* ROS production and peptide aggregation (Malaguti et al., 2014). The antioxidant function of wheat germ protein hydrolysates was investigated in-vitro using several assay methods; these include 1,1-diphenyl-2-picrylhydrazyl (DPPH), linoleic acid emulsion model system, ferrous ion-chelating activity, superoxide/hydroxyl radical-scavenging and reducing power. They also found that the antioxidant activity of the hydrolysates was comparable to α-tocopherol; hydrolysates also displayed scavenging activity against free radicals, for example, hydroxyl, DPPH and superoxide (Zhu et al., 2006). The precise role of specific amino acid residues to a peptide's antioxidant function is mainly dependent on the existence of ROS/free radicals and the medium of reaction. However, the contribution of these antioxidant amino acid residues to the antioxidant activity of food protein hydrolysates is not clear (Sarmadi, 2010).

2.6.2. Amaranth antioxidant capacity

Amaranth's antioxidant capacity, as well as those of other pseudocereals, can be compared to rice and soybean. Polyphenols are the principal compounds in amaranth grain that provide the antioxidant activity (Venskutonis, 2013). As radical scavengers, proteins are considered to play an important role. Barba de la Rosa et al. (2009) studied various cultivars of amaranth and reported phenols like rutin and isoquercitrin, as well as phenolic acids such as vanillic and syringic acids. These compounds reportedly exhibited antioxidant activity. Tiengo et al. (2009) demonstrated that amaranth grains are rich in amino acids like histidine, threonine, glycine, cysteine, lysine, proline, alanine, methionine, tryptophan, tyrosine and threonine, all are considered to possess antioxidant activity. In earlier studies, authors have shown the presence of antioxidant peptides in *Amaranthus mantegazzianus* seeds of organic peptides and polypeptides with linoleic, acid inhibition and free radical scavenging (Orsini Delgado et al., 2011). In the different protein fractions (globulin, glutenin and albumin), active molecules were distributed with the glutelin fraction having the highest activity. Furthermore, by releasing small peptides and/or free amino acids, alcalase hydrolysate was able to improve the scavenging activity (Orsini Delgado et al., 2011).

2.6.3. Amaranth as a source of bioactive peptides

Within the parent protein, BAPs are inactive. However, they may serve as a physiological metabolism modulator with enzymatic digestion or food processing. Peptides with biological activities (antithrombotic, antioxidant, anticancer and antihypertensive) have been reported in some studies with amaranth grain (Silva-Sanchez et al., 2008 , Barrio and Anon, 2010). *Amaranthus hypochondriacus* a lunasin containing glutelin fraction was digested with trypsin. The hydrolysate induced apoptosis (programmed cell death) in HeLa cells (cervical cancer) by 30 and 38% at 1 and 5 µg/ml, respectively. Whether the anticancer peptides were obtained from the primary lunasin sequence or other protein precursors within the fraction was not mentioned. Thus, BAPs with anticancer properties can be released by enzymatic hydrolysis of food proteins (Silva-Sanchez et al., 2008).

An antiproliferative profile on four cell lines (MC3T3E1, UMR106, Caco-2, and TC7) with different potencies was shown for *Amaranthus mantegazzianus* isolate. The most sensitive cell line was the UMR106 tumour cell (IC_{50} 1 mg/ml). Protease treatment enhanced the antiproliferative effect of the *A. mantegazzianus* isolate. In the UMR106 cell line, the *A. mantegazzianus* produced morphological changes, which resulted in a rearrangement of the cytoskeleton. They noted that the *A. mantegazzianus* inhibited cell adhesion and induced apoptosis as well as necrosis in UMR106 cells, in an attempt to elucidate the mechanism of action (Barrio and Anon (2010).

2.6.4. Production and processing of food derived BAPs

For the production of BAPs typical plant sources used include legumes (pea, bean, and soybean), cereals (rice, barley, wheat, and corn), pulses (chickpea, beans, pea, and lentils), pseudocereals (amaranth and buckwheat), brassica species and others (hemp seed, sunflower and flaxseed) (Malaguti et al., 2014). Based on current literature, potential sources of bioactive peptides are selected based on two primary criteria: (1) the use of value-added food industry by-products which are rich in protein or use of abundant underutilized proteins (2) the use of proteins which may contain amino acid residue of interest to the pharmacological industry or specific peptide sequences. While each of these criteria's are equally important, a combination of the two leads to a strategic selection of proteins that can produce high yields of a defined potent peptide sequence (Udenigwe, 2012).

Bioactive peptides are encrypted as inactive amino acid sequences within animal and plant proteins primary structure. Processing of food, fermentation and enzyme-catalysed proteolysis, may be used to release bioactive peptides (Hartmann, 2007, Moller et al., 2008, Kannan, 2009). In most cases, better bioactivity has been demonstrated by protein hydrolysates and peptides in comparison to their parent protein and has shown that in liberating potential peptides, hydrolysis of peptide bonds are necessary (Udenigwe, 2012).

The peptides bioactive properties are affected by several factors including processing conditions, choice of enzymes used in the hydrolysis reaction and the size of the resulting peptides, which greatly affects their bioavailability in target tissues and their absorption across the enterocytes. Most BAPs reported are produced through fermentation or enzymatic hydrolysis *in vitro* (Kannan, 2009, Udenigwe, 2012). Using single or multiple non – specific or specific proteases enzymatic hydrolysis is performed to release peptides of interest on an appropriately selected food protein (Kannan, 2009).

In the research of food protein-derived peptides, the challenge that is faced is the ability to achieve high yield peptides with potent bioactivity. This limitation in research results in conducting further processing of the bioactivity of peptides, after protein hydrolysis, the resulting peptide can be processed further based on structural and physicochemical properties of the constituent peptides. The properties of the peptides that are often considered include hydrophobicity, size and net charge (Pownall et al., 2010). In summary, the production and processing of BAPs that are commonly used are shown in (Figure 10).

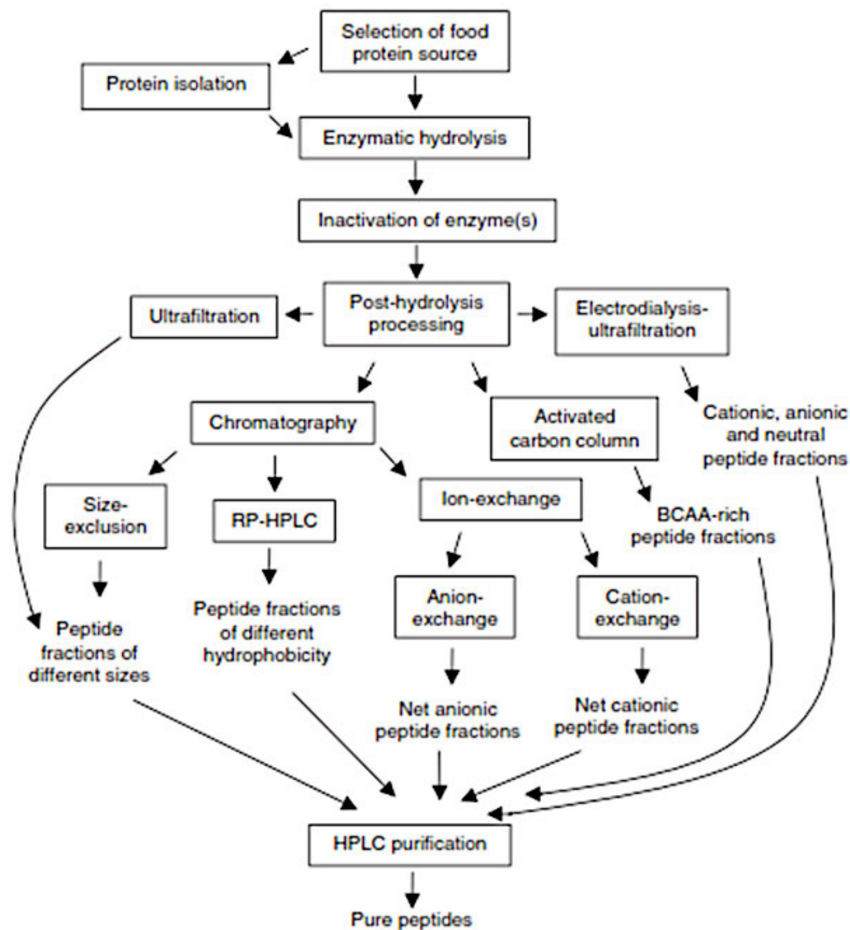

Figure 10: Systematic diagram showing steps involved in the production of food-protein based bioactive peptides (Udenigwe, 2012).

Enzymes used to produce peptides

Various gastrointestinal enzymes are found in the human body with pepsin and trypsin as the principle digesting enzymes (Kannan, 2009). Trypsin is produced as the inactive trypsinogen pro-enzyme in the pancreas. Trypsin cleaves peptide chains mostly on amino acids lysine and arginine's carboxyl side, except when followed by proline (Montoya-Rodríguez et al., 2015).

Alcalase is a non-specific serine-type of protease from *Bacillus licheniformis*, produced by Novozymes. At the active site, alcalase initiates the nucleophilic attack on the peptide (amide) bond using a serine residue (Montoya-Rodríguez et al., 2015). Six-point five to eight point five is its optimum pH range for catalysis. Alcalase is commonly utilized to produce hydrolysates from proteins with improved functional and nutritional properties than intact proteins (Yust et al., 2010). In the digestive system, pepsin is one of the principle protein-degrading enzymes.

These enzymes, each specialized in breaking down links between specific types of amino acids, work together during the digestion process to cut proteins into their components (free amino acids and peptides), which are easily absorbed by the intestinal lining and transferred into the circulatory system. Pepsin is most effective in cleaving hydrophobic peptide bonds and ideally, aromatic amino acids (tyrosine, phenylalanine and tryptophan) (Montoya-Rodríguez et al., 2015). While the complete bioavailability and absorption mechanism of different peptides are still being investigated, there is adequate evidence to suggest that bioactive peptides are bioavailable in food and can be absorbed into the body (Montoya-Rodríguez et al., 2015).

2.7. Aims and Objectives

Cancer is deemed a health problem across the world (Manoharan, 2010). To treat a cancer patient, the methods available mainly include surgery, radiotherapy and chemotherapy. These methods of treatment are known to be very costly and have severe side effects. Hence, there is a need to develop effective cancer therapeutic agents that should be effective, cost-effective, palatable, non-toxic, acceptable by the human population and highly efficacious against multiple cancers (Gaidhani et al., 2013). The discovery of several protein-based peptide receptors and tumour-related peptides and proteins are expected to create “a new wave” of more effective and selective anticancer drugs in the future, capturing the large share of the cancer therapeutic market (Thundimadathil, 2012). Anticancer activity of different peptides is attributed to the variety of mechanisms that restrict tumour growth. These mechanisms include protein–protein interactions, the inhibition of angiogenesis, proteins, enzymes, gene expression or signal transduction pathways.

2.7.1. Aim

To investigate the antioxidant activity and *in vitro* effect of Amaranth protein and its hydrolysates on cancer cell lines MCF-7 (Breast Cancer), A549 (lung cancer) and HEK 293 to inhibit the growth of cancer cells.

2.7.2. Objectives

- To produce hydrolysates from pepsin, alcalase and trypsin using *A. cruentus* isolate and determine the toxicity of Amaranth protein and its hydrolysates using the brine shrimp lethality test and Ames mutagenicity test.
- To evaluate the antioxidant activities of Amaranth protein and its hydrolysates using the DPPH, FRAP and ABTS methods.
- To determine cell viability of Amaranth protein and its hydrolysates using MCF-7 (Breast Cancer), A549 (lung cancer) and HEK 293 cell lines
- To determine the effect of the Amaranth protein and its hydrolysates on cell morphology using acridine orange and ethidium bromide
- To quantify apoptosis using the Annexin V-PE Apoptosis detection kit as well as Caspase-3/7 glowmax assay detection kit

Chapter 3: Methodology

3.1. Overview

Figure 11 provides an overview of the methodology carried out for this study; briefly, *Amaranthus cruentus* was soaked, drained and dried in an oven. The dried grains were then be ground into flour and the crude protein extracted. The crude protein was subjected to protein hydrolysis using pepsin, trypsin and alcalase. The toxicity of the compounds was investigated using the Ames mutagenicity assay and the Brine Shrimp lethality assay. The protein hydrolysates were then investigated for their antioxidant activity using the FRAP, ABTS and DPPH methods. After that, the anticancer activity of the protein hydrolysates was conducted using the MTT assay on cell lines MCF-7, A549 and HEK 293. Apoptosis was investigated using the BD Annexin V-PE kit. Morphological changes of treated cells were observed using acridine orange/ethidium bromide staining followed by the caspase 3/7 assay.

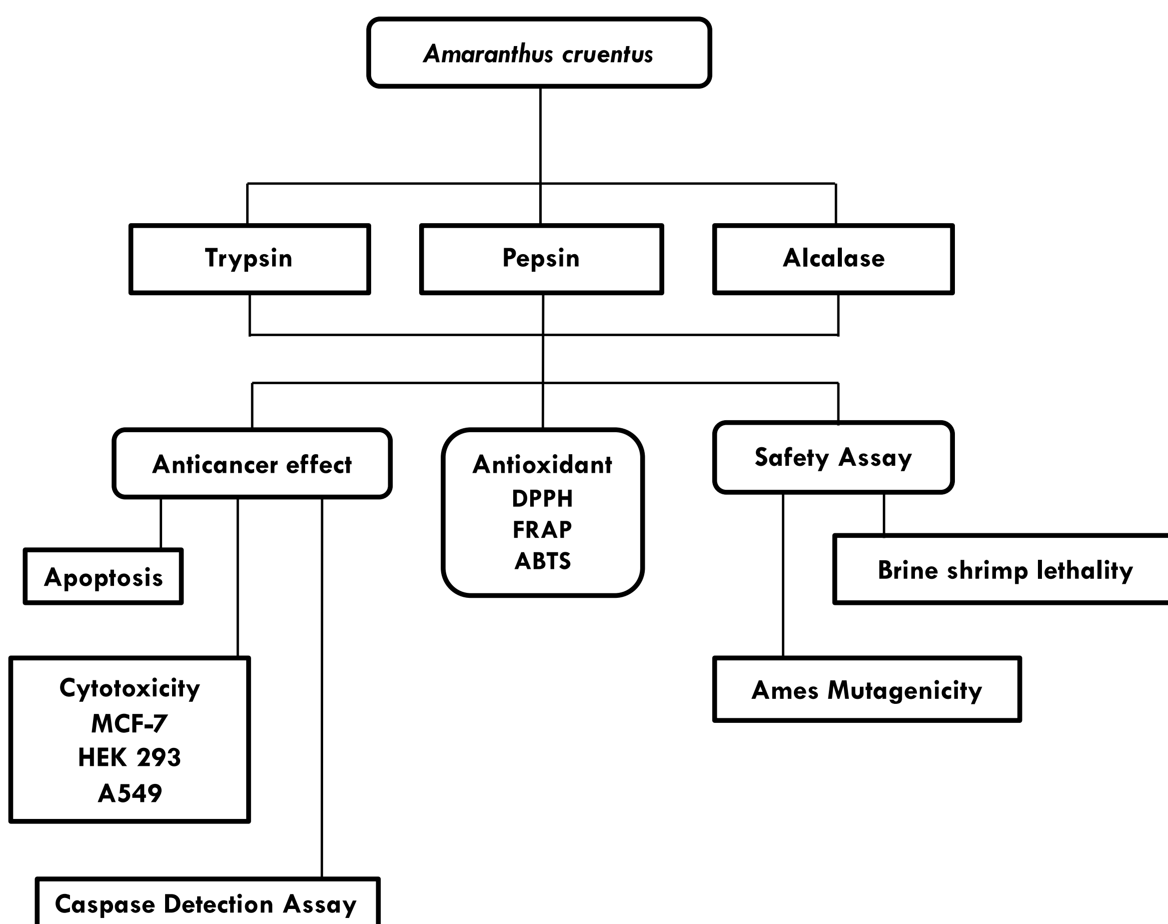

Figure 11: Overview of methodology.

3.2. Material and chemicals

Amaranthus cruentus grains were received from the Agricultural Research Council, Pretoria. *Amaranthus cruentus* seeds were cleaned, washed and ground into flour with the use of a grinder and then passed through a 180-micron mesh. Hexane was used to de-fat the flour at a flour/hexane ratio of 1:10 (w/v). The suspension of flour and hexane was stirred for a duration of 4 h at 4°C. The suspension was centrifuged for 20 min at 13000 x g, and the pellet dried at room temperature. Defatted *A. cruentus* flour was stored at 4°C until further use (Silva-Sanchez et al., 2008).

3.3. Grain Composition

Moisture, crude fat and total ash contents were determined using AOAC methods (AOAC, 2000) with analytical numbers 950.46, 960.39 and 920.153, respectively. The crude protein content (N x 6.25) was determined by the Kjeldahl method with No. 928.08. Total carbohydrate was calculated by difference. Under this approach, the other food constituents (fat, protein, ash, water) are determined individually, summed and subtracted from the total weight of the food. The following formula was used:

Carbohydrates = 100 (weight in grams (protein + fat + water + ash) in 100g of food)

3.4. Preparation of protein isolates

The extraction of protein from *A. cruentus* defatted flour was conducted according to Silva-Sanchez et al. (2008). Phosphate Buffered Saline (PBS) at 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 and extracted for 60 min, mixing every 10 min. The suspension was thereafter centrifuged at 4°C for 20 min at 13000 x g. Supernatants were collected and dialyzed at 4°C against distilled water. The dialyzed extract was freeze-dried and kept at a temperature of -20°C until further use.

3.5. Protein Concentration and yield

The protein concentration of *A. cruentus* protein isolates was evaluated using the standard Bio-rad protocol. Briefly, a BSA (Bovine Serum Albumin) stock solution was prepared (2 mg/mL) and *A. cruentus* isolate (1 mg/mL), from the stock BSA was made up into varying concentrations (2000, 1500, 1000, 750, 500, 250, 125, 0 µg/mL). Five microlitres of BSA for each concentration was pipetted into a 96-well plate, 250 µL of Bradford reagent was added to each well.

The plate was incubated for 5 min in the dark and thereafter read at 595 nm on a microplate reader (Thermoscientific Multiskan Go). The absorbance reading was plotted onto a graph to obtain a standard curve. Once the standard curve had been obtained, 5 µL of *A. cruentus* isolate was pipetted into a 96-well plate, 250 µL of Bradford reagent was added, and the plate was incubated for 5 min in the dark and thereafter read at 595 nm on a microplate reader. The protein concentration for *A. cruentus* was obtained by using the equation from the standard curve obtained for BSA (Bio-Rad Laboratories, 2018). "Yield of protein concentration was determined as the dry weight of protein concentrate after precipitation and solubilization, respectively, per weight of the defatted flour as shown below" (Arise, 2016). The Kjeldahl and Bradford methods were used to determine the protein content (N x6.25) of the defatted flour and the protein content of the concentrates, respectively.

$$Yield\ (\%) = \frac{Protein\ concentrate\ recovery\ \times protein\ content\ of\ concentrate\ (\%) \times 100}{Protein\ content\ of\ defatted\ flour}$$

3.6. Hydrolysis reaction

Hydrolysis of protein was conducted using a method by Arise (2016). Briefly, *A. cruentus* protein (5%, w/v) was dispensed in a reaction vessel in deionized water equipped with a stirrer; thereafter it was heated to the required temperature and pH adjusted (Pepsin pH 2.0, 37°C) (Alcalase pH 8.0, 50°C) (Trypsin pH 8.0, 37°C) before the proteolytic enzyme was added. Proteolytic enzymes were added at an enzyme to substrate ratio (E/S) of 1:100 to the *A. cruentus* protein. Protein isolate was digested for 4 h (pH kept constant). At the end of the proteolytic period, the suspension was heated for 10 min in boiling water to inactivate the action of the proteolytic enzyme. Using 2 M HCl, the pH was adjusted to pH 4.0 to precipitate the undigested protein.

The resulting suspension was centrifuged for 60 min at 8000 x g to remove the undigested protein. The supernatant was collected and freeze-dried. The freeze-dried protein was kept at a temperature of -20°C until further use.

3.7. Protein characterization - SDS PAGE

SDS-PAGE of *A. cruentus* protein isolate and hydrolysate was done according to Arise (2016). This was carried out under reducing (with mercapthoethanol) conditions. Polyacrylamide Tris - HCl gels (10-15%) were used in conjunction with the Bio-Rad Criterion cell under a constant voltage (200 V). A standard protein (10-250 kDa) mixture was used as the molecular weight markers. Coomassie Brilliant R-250 was used for staining of gels.

3.8. Safety Evaluation

3.8.1. Ames Mutagenicity Test

The *Salmonella* mutagenicity experiment was carried out using the modified method by Singh (2016) and Vijay et al. (2018). *Salmonella typhimurium* TA 98 and TA 100 strains were obtained on disc cultures from the Medical Research Council, Durban (SAMRC). Both strains were retrieved from disk cultures. With the aid of a sterile tweezer, the disk cultures were removed aseptically and then inoculated in a 250 mL flask (sterile) containing nutrient broth (Oxoid - 25 mL) as well as 78 µL of Ampicillin (8 mg/mL). The flask was kept on a shaking incubator (150 rpm) for 16 h at 37°C. This was performed to acquire an optical density ranging between 1.2 to 1.4 at 660 nm. Thereafter the cultures were streaked onto nutrient agar plates to obtain single fresh colonies. A single fresh colony of TA 98 and TA 100 was inoculated into nutrient broth (10 mL) and incubated for 10-12 h at 37°C in a shaker (120 rpm).

Amaranthus cruentus protein isolate and hydrolysates were dissolved in autoclaved distilled water (1 mg/mL). Sodium azide a potent chemical was used as the positive control, while autoclaved distilled water was used as the negative control. Eppendorf tubes (1.5 mL) were sterilized and 0.1 mL fresh salmonella culture, 0.2 mL histidine/biotin solution, 0.5 mL 0.2 M sodium phosphate buffer pH 7.4, 0.1 mL test sample and 0.1 mL autoclaved distilled water was added to the Eppendorf tubes. The contents were vortexed and poured onto minimal glucose plates; the content was spread using a glass spreader.

To protect the samples from photosensitive substances, the plates were covered with aluminium foil. After incubation for 48 h at 37°C, the colonies were counted, and the mutagenicity ration determined:

$$Mutagenicity\ ratio = \frac{Spontaneous\ revertants}{Spontaneous\ revertants\ (negative\ control)}$$

3.8.2. Brine Shrimp Lethality Assay

The Brine Shrimp lethality assay was conducted with the use of *Artemia salina* (brine shrimp). Artificial seawater (0.7 g KCl, 23 g NaCl, 1.3 g $CaCl_2.2H_2O$, 4 g Na_2SO_4, 11 g $MgCl_2.6H_2O$, in 1000 mL distilled water) was prepared and kept at the room temperature. The pH was adjusted to 9.0 using 0.1 M sodium carbonate solution to avoid the risk of death to the nauplii during the incubation period as a result of a decrease in pH at room temperature. Ten nauplii were counted and placed inside each well of a 6-well plate. Each well was filled to 5 mL total volume with artificial seawater containing 3 mg yeast. *Amaranthus cruentus* protein isolate and hydrolysates were dissolved in 2% DMSO. Potassium dichromate was used as the positive control. Suitable dilution of the sample (100 µL) was added to each well and incubated at 27°C for 24 h after which the dead and live nauplii were counted and death percentage determined (Singh, 2016).

3.9. Antioxidant Potential

3.9.1. DPPH radical scavenging assay

Antioxidant activity of *A. cruentus* protein and hydrolysates were evaluated using DPPH (α, α-diphenyl-β-picrylhydrazyl) radical scavenging assay, according to. DPPH was dissolved in methanol to a 0.1 mM concentration. *Amaranthus cruentus* protein isolate and hydrolysates were dissolved in sodium phosphate buffer pH 7.0 (2 mg/mL). Buffer was used as a blank and glutathione (positive control). Suitable dilutions of the sample (100 µL) were mixed with (100 µL) of DPPH. The plate was covered in foil and incubated for 30 min in the dark. Absorbance was read at 517 nm. Scavenging activity was calculated using the following formula:

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

3.9.2. FRAP assay

The FRAP (ferric reducing antioxidant power) was evaluated using the method by (Karamać et al., 2014). Hydrolysates (40 µl in different concentrations: 100, 200, 400, 600, 800, 1000 µg/mL) were dissolved in deionized water (1 mg/mL) or solutions of standard antioxidants glutathione, were applied onto a 96 well plate. Two hundred microlitres of reagent containing 0.3 M acetate buffer (pH 3.6), 10 mM 2,4,6-Tris (2-pyridyl)-s-triazine in 40 mM HCl and 20 mM $FeCl_3x6H_2O$ in a ratio of 5:1:1 (v/v/v) was heated to 37°C and added to the samples. Samples were incubated at 37°C for 30 min, and the absorbance read at 593 nm. The results were calculated using a standard curve for $FeSO_4$ X $7H_2O$ and expressed in mM of Fe (II) per g of hydrolysate.

3.9.3. ABTS assay

This assay is based on the percentage inhibition of the peroxidation of the ABTS (2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid) radical, which is observed as a discolouration of a blue-green colour (734 nm) (Alashi et al., 2014). The ABTS assay was performed using a modified method by (Re et al., 1999) and (Karamać et al., 2014). ABTS (7 mM) was dissolved in water. ABTS radical cation ($ABTS^+$) was generated by the reaction of ABTS stock solution with potassium persulfate (2.45 mM) and allowing the mixture to stand for 12-16 h in the dark at room temperature. 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. Hydrolysates were dissolved in 0.1 M sodium phosphate buffer pH 7.0 in the concentration of 2 mg/mL. Then 10 µL of sample at various concentrations (100, 200, 400, 800, 1200, 1600, 2000 µg/mL) was added to the 96-well plate, and 200 µL of ABTS solution was added. The absorbance was read out after 6 min at 734 nm. The results were expressed in mM of glutathione per g of hydrolysate.

3.10. Antiproliferative effect of *A. cruentus* protein and its hydrolysates

3.10.1. Cell line

The MCF-7 (Breast cancer), HEK 293 (Human embryonic kidney), A549 (adenocarcinomic human alveolar basal epithelial) were used in this study with all cell lines supplied by Department of Physiology (UKZN). For studying cell growth and behaviour of cells *in vitro*, primary stem, lineage-specific and progenitor cells are the gold standards.

However, an unreliable supply, the difficulty of performing *in vitro* isolation and culture procedures, and the loss of phenotype with increasing time in culture can hinder the use of primary cells (Swain et al., 2010). Cell lines are often used as models for primary cells in order to overcome these limitations. These cells are usually generated from cancerous tissue or through retroviral transfection or transduction immortalization of primary cells. In general, cell lines are easier to cultivate than primary cells as they have a high rate of proliferation and a long lifespan and maintain their culture phenotype. The main drawback of cell lines, however, is that the phenotype they express may not be consistent with the exact phenotype of their primary counterparts (Swain et al., 2010).

MCF-7

Breast cancer is among the most widely diagnosed cancer and the leading cause of cancer mortality in women; thus, research in this area is vital to address both economic and psychological burdens (Comșa et al., 2015). Cells were isolated from a sixty-nine-year-old woman with metastatic disease at the Michigan Cancer Foundation (Levenson, 1997). MCF-7 is among the most commonly used breast cancer cell line in anticancer research. MCF-7 cells are known to be non-invasive and poorly aggressive, with low metastatic potential. It has become apparent in recent years that breast cancer is not a single disease but rather a variety of molecularly differentiated tumours originating from the breast's epithelial cells. As far as breast cancer is concerned, MCF-7 cells are important candidates as they are used omnipresently in estrogen receptor (ER)-positive breast cancer cell experiments and many sub-clones that have been established which represent different classes of ER-positive tumours with varying levels of expression of nuclear receptors (Comșa et al., 2015).

A549

The human adenocarcinoma cell line A549 was used as a model for ATII (primary pulmonary alveolar type II) cells in the biology of lung cells. These highly specialized cells produce surfactant, a multifunctional lubricant that reduces surface tension during ventilation and prevents alveolar collapse. Giard and colleagues derived the A549 cell line in 1972 from a type II pneumocyte lung tumour. Since then, A549 cells have been used for surfactant production and surfactant system regulation for *in vitro* studies (Swain et al., 2010). For almost four decades, this cell line has been the cornerstone of respiratory research (Cooper et al., 2016).

HEK 293

In Alex van der Eb laboratory in the Netherlands, a human embryonic kidney cell line was derived by exposing the human primary embryonic kidney cell culture of an aborted embryo to the mechanically sheared DNA of adenovirus type 5 (AD5) (Stepanenko and Dmitrenko, 2015). The fast-growing HEK-293 cells were established after several months of cultivation. HEK293 cells are a famous cell line in academic research due to their ease of growth and transfection. Additionally, the high transfection efficiency of HEK293 cells enables exogenous proteins or viruses to be developed for pharmaceutical and biomedical research. For example, during the discovery process, HEK293 cells are often used to express possible biological drugs and/or therapeutic targets. After HeLa cells in cell biology and after CHO in biotechnology experiments, HEK293 and its variants are the most commonly used cells. Due to its versatility for transfection studies, HEK293 cells have recently gained interest (Yuan et al., 2018).

3.10.2. Cell maintenance

All cell culture experiments were conducted in the laminar flow cabinet (Scientific Engineering, INC) in order to maintain an aseptic/sterile environment. The laminar flow cabinet was sterilized by exposing it to UV-light as well as frequently swabbing with 70% ethanol (Merck, South Africa) before performing cell culture experiments. HEK 293, A549 and MCF-7 cells were grown as two separate monolayers in Dulbecco`s Modified Eagle Medium (DMEM-comprised of glucose 4.5 g/L, 1 mM L-glutamine and 1 mM sodium pyruvate) (Sigma-Aldrich, Inc). The DMEM was supplemented with 10% heat inactivate foetal calf serum (FCS) and 1% antibiotic (penicillin/streptomycin) solution (Sigma-Aldrich, Inc). HEK 293, A549 and MCF-7 were sub-cultured every 2 days, once the flasks had become 80% confluent to ensure they are in the exponential growth phase. During sub-culturing, supplemented DMEM was removed from flasks, and the monolayer of cells was washed with 5 mL Phosphate Saline Buffer (PBS). After that, an aliquot of 1 mL trypsin was added to the flasks. The monolayer of cells was incubated at 37°C in the humidified incubator with a 5% CO^2 environment for 3 min. The flasks were tapped on the side for 30 seconds to detach the monolayer. A final aliquot of 20 mL DMEM was added to each flask, and this was followed by incubation at 37°C in a humidified incubator that contained a 5% CO^2 atmosphere. The cells were monitored for contamination daily by noting medium colour and turbidity changes during the incubation. Cell growth was examined with an inverted microscope (Nikon, Japan).

3.10.3. Storage of cells

Cell culture flasks that are 80% confluent were washed with 5 mL PBS and thereafter subjected to treatment with trypsin, as described during sub-culturing. An aliquot of 10 mL DMEM was thereafter added to each flask, and the cells were subsequently transferred to 50 mL tubes. The 50 mL tubes were centrifuged at 1 500 rpm for 10 min in order to collect pellets of cells. Two millilitres of cryo-protective medium (comprised of DMSO and FBS 1:4) were added to re-constitute the pellet. An aliquot of 1 mL cryo-protective/cell-containing solution was thereafter added to the cryotubes (Corning, South Africa) and transferred to a biofreezing vessel for storage at -80°C.

3.10.4. Cell regeneration

If there were no freshly incubated cells available for immediate use, cells were removed from storage at -80°C and quickly thawed (placed in a 37°C water bath), and the cryo-protecting agent removed by centrifugation with PBS. Thereafter, the pelleted cells were transferred to a 20 mL prewarmed supplemented-DMEM in 75 cm^2 tissue culture flasks. These cells-containing flasks were incubated at 37°C in a humidified incubator containing a 5% CO^2 atmosphere.

3.10.5. Cell Enumeration

Cells were enumerated using trypan blue an exclusion dye used for counting viable cells. This staining technique is based on the principle that viable (or intact) cells would not use the trypan blue dye, while non-viable cells (cells in which membrane integrity is altered) will use the dye. Trypan blue staining allows scientists to visualize the morphology of cells since viable cells remain translucent, while non-viable cells appear blue. In this study, 100 µL trypan blue (Bio Whittaker, Walkersville, USA) was mixed with 100 µL cell suspension cultures in centrifuge tubes. These tubes were incubated at room temperature for approximately 1 min. Thereafter, an aliquot of 10 µL trypan-suspended cell cultures was loaded into both chambers of the Neubauer haemocytometer. The cells within the middle square and in the four 1 mm corner squares of the two chambers were counted. The following equation will be used to determine the number of cells in the suspension:

Total cell count = 16 squares x 4
= Cell counts in 4 sets of 16 squares
16 squares = 2 x 10^4/mL
Therefore, cells per ml = total cell count 4 x 2 x 10^4 per mL
= cells per ml

3.10.6. Cytotoxicity assay of *A. cruentus* proteins and its hydrolysates

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] cytotoxicity assay was used to evaluate the cytotoxic effect of the *A. cruentus* protein and its hydrolysates on MCF-7, A549 and HEK cell lines. The MTT cytotoxicity assay was conducted according to (Dwarka et al., 2017). The assay was carried out in flat-bottomed microlitre plates (96-well) (Cellstar, Greiner, Germany). *Amaranthus cruentus* isolate, and hydrolysates were dissolved in 2% DMSO. Cells (50 µL; $\pm 1.2 \times 10^3$), media (50 µL) and *A. cruentus* hydrolysate and isolates were added to respective wells in a twofold dilution factor (1,000-7.8 µg/mL). In the control wells, cells (50 µL) only and camptothecin (1,000-7.8 µg/mL) were added respectively. The plates were incubated (37°C) in a humidified incubator (5% CO_2 atmosphere) for 24 h. Thereafter MTT (20 µL) reagent was added and the plates further incubated (4 h; 37°C) in a humidified incubator (5% CO_2 atmosphere). Finally, DMSO (100 µL) were added and incubated for 1 h, and the absorbance read at 570 nm on an ELISA plate reader (Thermoscientific Multiskan Go). Percentage viability was determined using the following formula:

$$\%\,Cell\ viability\ = \frac{Absorbance\ of\ treated\ cells}{Absorbance\ of\ untreated\ cells} \times 100$$

3.10.7. Quantification of apoptosis

The Annexin V-PE Apoptosis detection kit (BD Biosciences) was used as per the manufacturer's protocol. The cells were seeded in plates (24-well) and left to adhere overnight. They were then treated with *A. cruentus* isolate (MCF-7: 3.55, A549: 249.40 and HEK: 8.57 µg/mL), trypsin hydrolysate (MCF-7: 3.87, A549: 14.10 and HEK: 10.03 µg/mL and camptothecin (MCF-7: 9.35, A549: 304.90 and HEK: 9.10 µg/mL). These values were obtained after the MTT as the optimum concentrations. After 24 h, the cells were trypsinized, washed twice with PBS and resuspended in binding buffer (1 x at 50 000 cells/mL). FITC Annexin V (5 µL) and propidium iodide (PI) (5 µL) were added, vortexed and incubated (15 min) in the absence of light. Thereafter, binding buffer (400 µL) was added to each tube and the results analysed by flow cytometry (BD FACS Aria).

3.10.8. Assessment of morphology in apoptosis

The membrane changes of apoptosis were observed using acridine orange and ethidium bromide. A stock solution (100x) was made up by adding ethidium bromide (50 mg) and acridine orange (15 mg). This was dissolved in ethanol (1 mL; 95 %) and distilled water (49 mL). This was then mixed well and divided into aliquots (1 mL). The working stock solution (1x) was made up of an aliquot (1 mL) of the 100x stock solution and diluted with PBS (99 mL). The cells were seeded in 24-well plates and left to adhere overnight. They were then treated with *A. cruentus* isolate (MCF-7: 3.55, A549: 249.40 and HEK: 8.57 µg/mL), trypsin hydrolysate (MCF-7: 3.869 µg/ml, A549: 14.10 µg/ml and HEK: 10.03 µg/ml and camptothecin (MCF-7: 9.35, A549: 304.90 and HEK: 9.10 µg/mL). After 24 h, the media was removed, and 1 mL of acridine orange-ethidium bromide solution was added to each well, left for 5 min then removed. Thereafter, the plate was placed on the fluorescence microscope set up to excite for fluorescein (i.e., with a 495 nm primary and 515 nm secondary filter). Cells were viewed under the 10x objective where viable, healthy cells stained bright green with intact structured nuclei, and viable apoptotic cells stained green with highly condensed or fragmented nuclei. Non-viable healthy cells were identified as those with chromatin stained bright orange and with an organized structure. In contrast, nonviable with apoptotic nuclei were observed to have highly condensed and fragmented chromatin (Dwarka et al., 2017).

3.10.9. Caspase-3 Fluorimetric Assay Detection

Involvement of caspase-3 in cell death was determined according to Caspase-Glo 3/7 assay kit (Promega, Cat No. G8090). Cells were seeded in 96-well plates at a density of 1×10^3 cells/well for 24 h. *Amaranthus cruentus* isolate (MCF-7: 3.55, A549: 249.40 and HEK: 8.57 µg/mL), trypsin hydrolysate (MCF-7: 3.87, A549: 14.10 and HEK: 10.03 µg/mL and camptothecin (MCF-7: 9.35, A549: 304.90 and HEK: 9.10 µg/mL) was added. After 24 h cells were removed from the incubator and allowed to equilibrate to room temperature, 100 µL of Caspase-Glo 3/7 reagent was added to each well and incubated for an hour. Untreated cells were considered the assay's negative control. The luminescence of samples was measured using a GlowMax luminescence 96 well plate reader.

3.11. Statistical analysis

For this study means of replicate data (n=3) were analyzed and data subjected to analysis of variance (ANOVA) for determination of significant differences ($p<0.05$).

Chapter 4: Results

4.1. Grain composition

The proximate composition of *A. cruentus* is presented in Table 3. The results indicated that the defatting significantly increased the protein and carbohydrate contents of the flour. The defatting of *A. cruentus* flour removed most of the lipids; however, a part of it (0.97%) remained in the flour. This residual lipid might be starch-bound lipids that could not be removed using hexane. It was reported that in three Amaranth cultivars 0.16% to 0.28% starch-bound lipids were not removed during the defatting process. Additionally, the presence of polar lipids interacted with proteins cannot be ruled out (Shevkani et al., 2014).

Table 3: Proximate composition (dwb) of *A. cruentus* defatted flour

	Moisture	Ash	Protein	Fat	Carbohydrates
A. cruentus	10.04 ± 0.17^{a}	2.90 ± 0.16^{b}	15.18 ± 0.18^{c}	0.97 ± 0.06^{d}	70.91 ± 0.13^{e}

Results are expressed as mean±standard deviation (n=3). Values with different superscripts are significant different at $p \leq 0.05$.

4.2. Protein concentration and yield

The protein concentration and yield of *A. cruentus* protein isolate and hydrolysates are depicted in Table 4. *Amaranthus cruentus* isolate had a higher protein concentration (61,52 %) than that obtained after the hydrolysis reaction, with the trypsin hydrolysate having the lowest (55,59 %). In respect to yield, the unhydrolyzed sample had the highest yield.

Table 4: Protein content and yield of *Amaranthus cruentus* protein isolate and hydrolysate

	Protein concentration (%)	Yield (%)
***A. cruentus* isolate**	61.52 ± 0.35^{a}	26.04
Pepsin hydrolysate	56.49 ± 0.15^{b}	2.74
Alcalase hydrolysate	58.09 ± 0.15^{c}	2.53
Trypsin hydrolysate	55.59 ± 0.25^{d}	2.46

Data represents mean±standard deviation (n=3). Means values with different superscripts are significant different at $p \leq 0.05$.

4.3. SDS PAGE

The profile of *Amaranthus cruentus* isolate showed well-defined bands with molecular weights between 57-67 kDa corresponding to the AB sub-units and the P-54 polypeptide, 30 and 43 kDa corresponding to type A polypeptides naturally free in the amaranth seed and between 20 and 30 kDa that correspond to type B polypeptides (Figure 12). A very faint band can also be seen between 10-15 kDa corresponding to the albumin fraction. For pepsin hydrolysate, a distinct 20 kDa band can be seen with lower molecular weight bands, below 10 kDa also visible. The higher molecular weight bands are extremely faint, suggesting that these proteins were broken down. The alcalase hydrolysate revealed a distinct 20 kDa band, which can also be seen for all hydrolysates. Two new bands can be seen between 15-20 kDa and 25-37 kDa. The higher molecular weight protein can be seen, but they are extremely faint, suggesting the proteins were hydrolysed using alcalase. Trypsin hydrolysate revealed faint higher molecular bands, the distinctive 20 kDa band and faint bands below 15 kDa, suggesting that the bands are below 10 kDa.

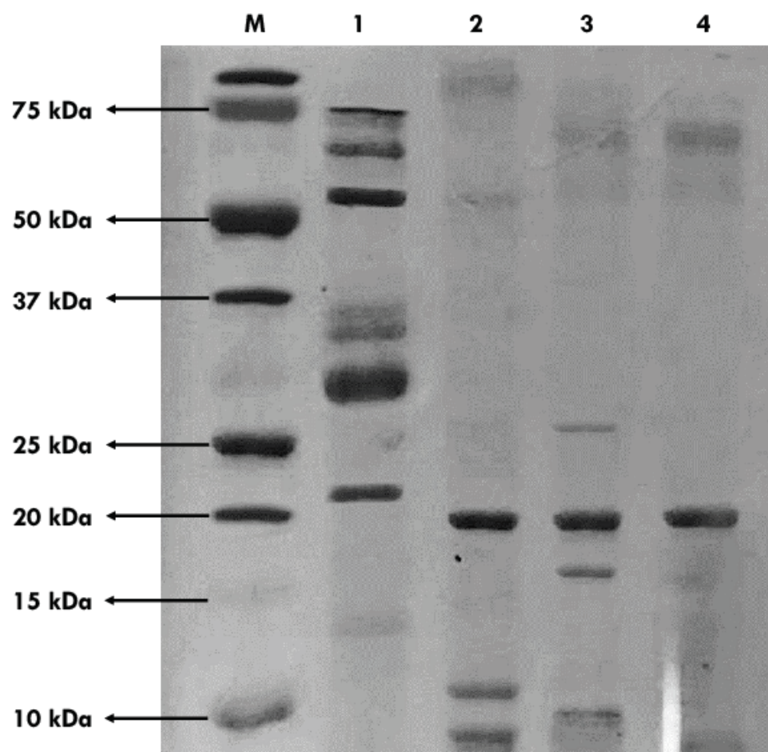

Figure 12: SDS - Page of *A. cruentus* isolate and hydrolysates under reducing conditions. Lane M - molecular weight marker, lane 1 - isolate, lane 2 - pepsin hydrolysate, lane 3 - alcalase hydrolysate and lane 4 - trypsin hydrolysate.

4.4. Toxicity Evaluation

4.4.1. Ames mutagenicity test

The mutant frequency caused by the amaranth protein isolates and hydrolysates in *S. typhimurium* TA 98, TA 100 is shown in Table 5. The mutant frequency was calculated as a fraction of the quotient of the number of revertant colonies divided by the number of colonies in the negative control sample. Mutant frequency higher than two was considered mutagenic. None of the tested samples induced any significant increase in the number of revertant colonies in comparison to the control (sodium azide). For TA 98, alcalase hydrolysate had the lowest IC_{50} of 10,77 µg/ml, while sodium azide had the highest of 598,6 µg/ml. The IC_{50} values for *A. cruentus* isolate, pepsin and trypsin hydrolysate were 11.66 µg/ml, 38.69 µg/ml and 20.46 µg/ml respectively. The IC_{50} values for TA 100 ranged between 9,069 to 597,3 µg/ml with the alcalase hydrolysate having the lowest IC_{50} value and trypsin hydrolysate having the highest. *Amaranthus cruentus* isolate, pepsin hydrolysate and sodium azide had IC_{50} values of 28.75 µg/ml, 21.16 µg/ml and 327.2 µg/ml respectively.

Table 5: Ames mutagenicity response caused by *A. cruentus* isolate and hydrolysates on *S. typhimurium*

	Mutant frequency of revertants at different concentrations (µg/mL)				
	10	**100**	**500**	**800**	**1000**
Amaranth isolate	0.01 ± 0.02^{a}	0.31 ± 0.06^{a}	0.31 ± 0.15^{a}	0.38 ± 0.15^{a}	0.58 ± 0.09^{a}
	N	0.19 ± 0.08^{c}	0.49 ± 0.11^{c}	0.47 ± 0.26^{c}	0.75 ± 0.08^{c}
Pepsin hydrolysate	0.06 ± 0.03^{a}	0.37 ± 0.07^{a}	0.37 ± 0.09^{a}	0.56 ± 0.05^{a}	0.73 ± 0.05^{a}
	0.05 ± 0.05^{c}	0.31 ± 0.20^{c}	0.37 ± 0.03^{c}	0.64 ± 0.08^{c}	0.59 ± 0.23^{c}
Alcalase hydrolysate	0.06 ± 0.03^{a}	0.23 ± 0.03^{a}	0.31 ± 0.06^{a}	0.54 ± 0.07^{a}	0.69 ± 0.09^{a}
	N	0.09 ± 0.08^{c}	0.37 ± 0.08^{c}	0.70 ± 0.06^{c}	0.56 ± 0.15^{c}
Trypsin hydrolysate	0.03 ± 0.05^{a}	0.32 ± 0.11^{a}	0.24 ± 0.21^{a}	0.59 ± 0.11^{a}	0.70 ± 0.02^{a}
	0.02 ± 0.03^{c}	0.12 ± 0.06^{c}	0.39 ± 0.11^{c}	0.70 ± 0.08^{c}	0.73 ± 0.19^{c}
Sodium Azide	0.86 ± 0.06^{b}	1.32 ± 0.11^{b}	2.03 ± 0.15^{b}	2.70 ± 0.17^{b}	3.93 ± 0.30^{b}
	0.95 ± 0.16^{d}	1.48 ± 0.14^{d}	2.97 ± 0.31^{d}	3.41 ± 0.23^{d}	4.20 ± 0.21^{d}

Data represents mean±standard deviation (n=3). Means values with different superscripts are significant different at $p\leq0.05$. [Shaded values represent TA 98 and unshaded TA 100, N – no activity]

4.4.2. Brine shrimp lethality assay

At 10, 100 and 500 µg/ml, alcalase hydrolysate had the highest death percentage of 20, 23.333 and 30% respectively (Figure 13). *Amaranthus cruentus* isolate induced the highest percentage death of 16,667% for the 800 µg/ml sample. At 1000 µg/ml, pepsin induced the highest percentage death of 13,333 %. It was observed that A. *cruentus* hydrolysates were not significantly different ($p < 0.05$) from *A. cruentus* isolate. IC_{50} values ranged from 34.92 to 309.70 µg/ml, with the alcalase hydrolysate having the lowest IC_{50} value and potassium dichromate the highest. *Amaranthus cruentus* isolate, pepsin and trypsin hydrolysate had IC_{50} values of 115.9, 46.56 and 59.43 µg/ml respectively.

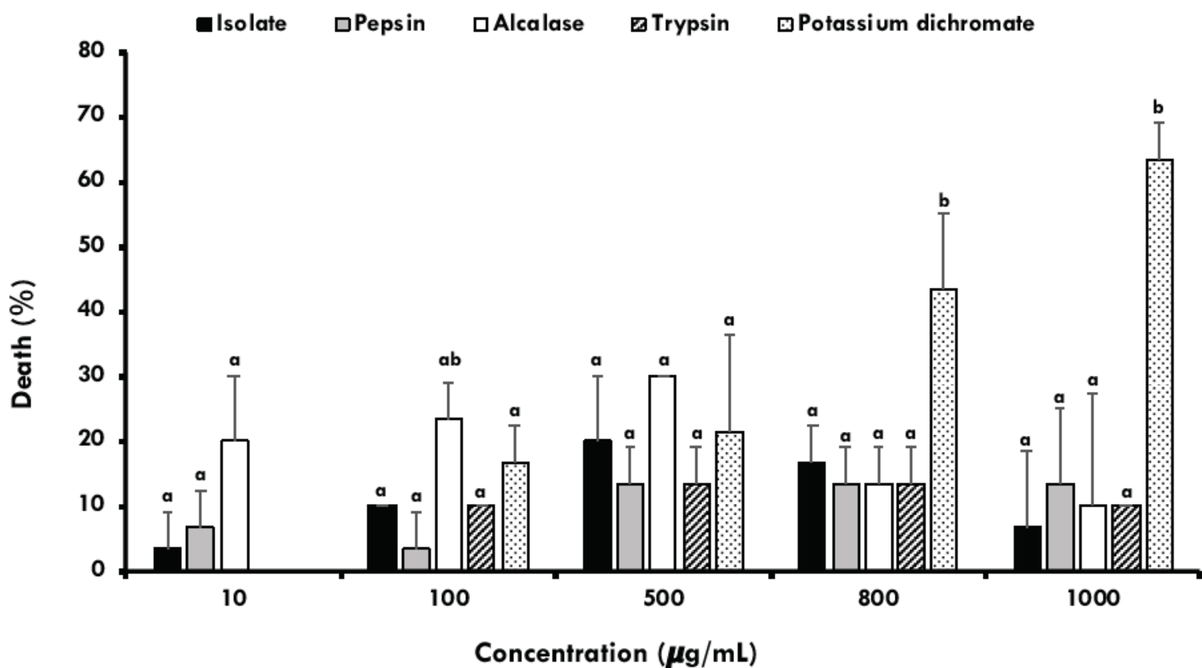

Figure 13: Percentage death of *Artemia spp.* against *A. cruentus* isolate and hydrolysates. Data represents mean±standard deviation (n=3). Mean values with different letters are significantly different at p≤0.05.

4.5. Antioxidant assay

4.5.1. DPPH

As seen in Figure 14 before hydrolysis, the unhydrolyzed protein isolate showed low DPPH radical scavenging percentage (14-18.3%), and after hydrolysis, the values increased with all tested hydrolysates (12-26.8%). Glutathione (control) had the highest DPPH radical scavenging percentage (64-70%). Glutathione (control) was significantly different ($p<0.05$) compared to *A. cruentus* isolate and hydrolysates for all concentrations. Pepsin hydrolysate had the lowest IC_{50} values of 23.06 μg/ml while trypsin hydrolysate had the highest of 34.41 μg/ml. *Amaranthus cruentus* isolate, alcalase hydrolysate and glutathione had IC_{50} values of 29.48, 26.21 and 26.06 μg/ml respectively. During pepsin digestion, the peptide bonds between hydrophobic and preferably aromatic amino acids, including phenylalanine, tryptophan, and tyrosine, were cleaved, which resulted in increased hydrophobicity, thus allowing *A. cruentus* pepsin hydrolysate to react appropriately with DPPH radicals in the methanol system (Phongthai et al., 2018). However, this ability was reduced with further digestion by trypsin, because it selectively cleaves at basic amino acids (lysine and arginine). The increased polarity of *A. cruentus* trypsin hydrolysate made it more difficult to react with the oil-soluble DPPH radicals (Phongthai et al., 2018).

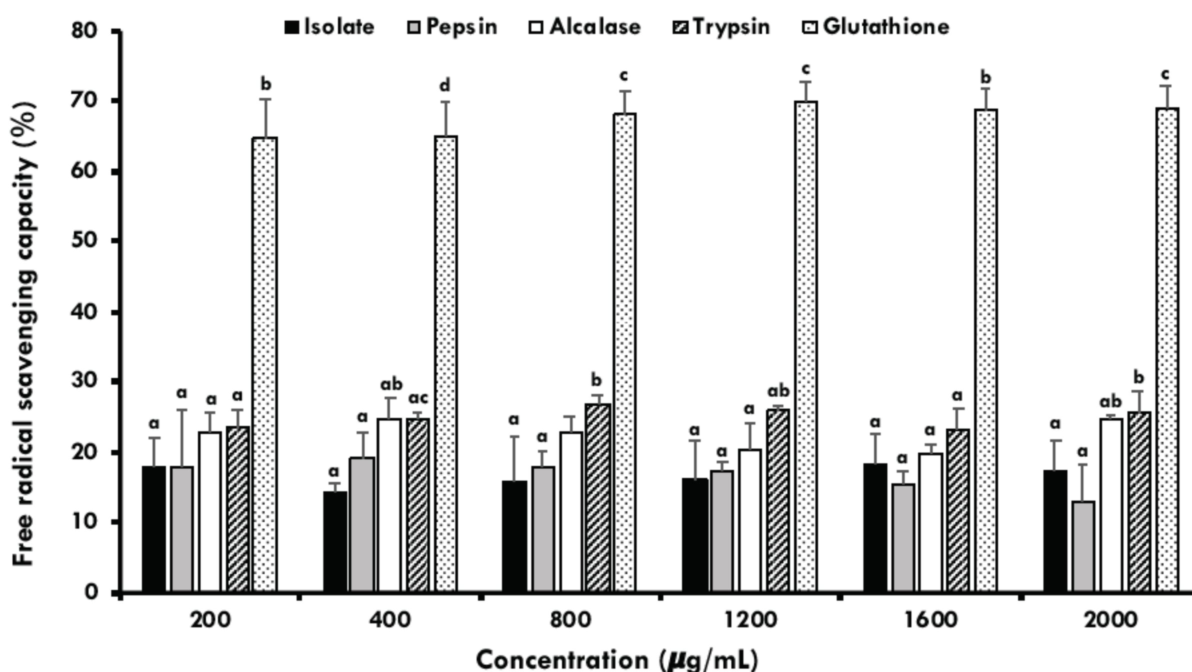

Figure 14: DPPH radical scavenging activity of *A. cruentus* protein isolates and hydrolysates compared to the control (Glutathione). Data represents mean±standard deviation (n=3). Mean values with different letters are significantly different at p≤0.05.

4.5.2. FRAP assay

The reducing power of *A. cruentus* protein isolate and its hydrolysates are shown in Figure 15. Glutathione had the highest $FeSO_4$ concentration amongst all samples (0.8 – 1.2 µmol/g $FeSO_4$). Before hydrolysis, A. *cruentus* isolate showed low $FeSO_4$ concentration (0.6 – 0.8 µmol/g); however, after hydrolysis, the $FeSO_4$ concentration increased for all hydrolysates (0.6 – 0.92 µmol/g). Trypsin hydrolysate was found to have the highest $FeSO_4$ concentration for most concentrations. According to literature, smaller size peptides exhibit better-reducing ability in comparison to high molecular weight peptides Karamać et al. (2014). This was confirmed by SDS PAGE (Figure 12) which shows that trypsin hydrolysate produced several low molecular weight peptides. *Amaranthus cruentus* isolate had the lowest IC_{50} value of 17.57 µg/ml, while glutathione had the highest of 79.81 µg/ml. Pepsin, alcalase and trypsin hydrolysates had IC_{50} values of 28.28 µg/ml, 23.92 µg/ml and 25.29 µg/ml respectively.

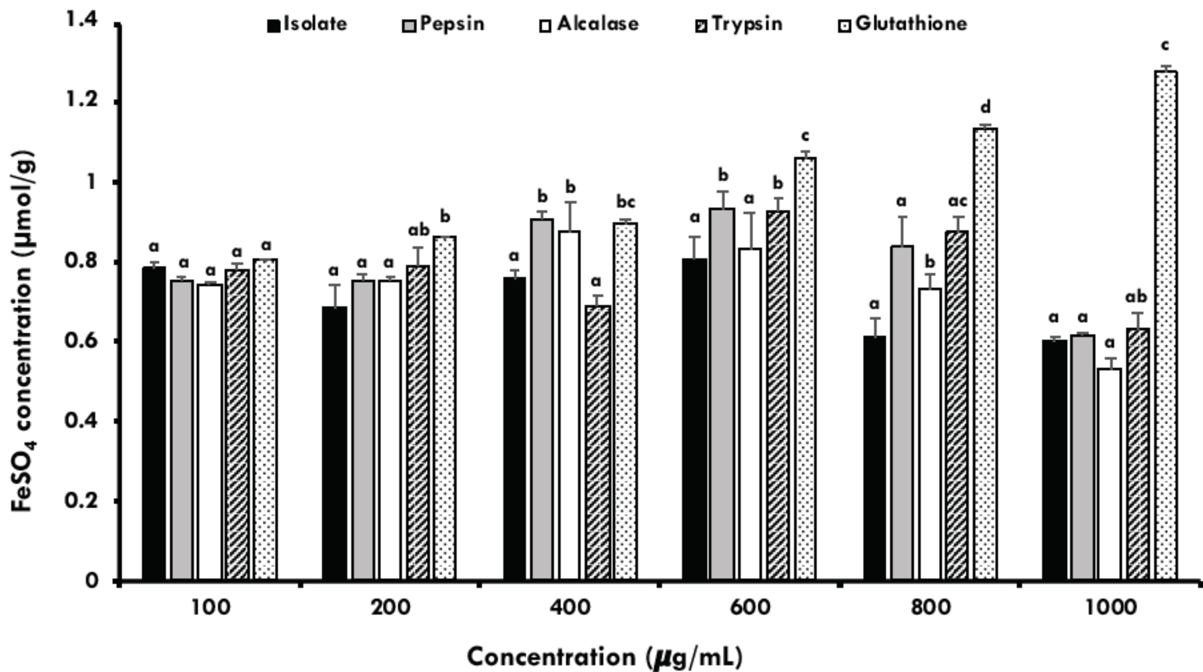

Figure 15: FeSO4 concentration of *A. cruentus* protein isolates and hydrolysates compared to the control (Glutathione). Data represents mean±standard deviation (n=3). Mean values with different letters are significantly different at p≤0.05.

4.5.3. ABTS Assay

The glutathione equivalent of A. *cruentus* isolate and hydrolysate are shown in Figure 16. 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 equivalent for most concentrations. The higher glutathione content for the hydrolysates compared to the isolate showed that antioxidant peptides were released from *A. cruentus* isolate during enzyme hydrolysis. This is an indication that the peptide was able to donate hydrogen atoms for $ABTS^{+}$ reduction. This may suggest that the release of peptides that are able to scavenge ABTS radical cations depends on the specificity of the proteases used in the hydrolysis of *A. cruentus* proteins and on the amino acid composition of proteins. For most concentrations, no significant difference ($p<0.05$) was observed. In respect of IC_{50} values, trypsin hydrolysate had the lowest IC_{50} value of 114 µg/ml, while the alcalase hydrolysate had the highest of 206.6 µg/ml. *Amaranthus cruentus* isolate, and pepsin hydrolysate had IC_{50} values of 119 µg/ml and 145.6 µg/ml, respectively.

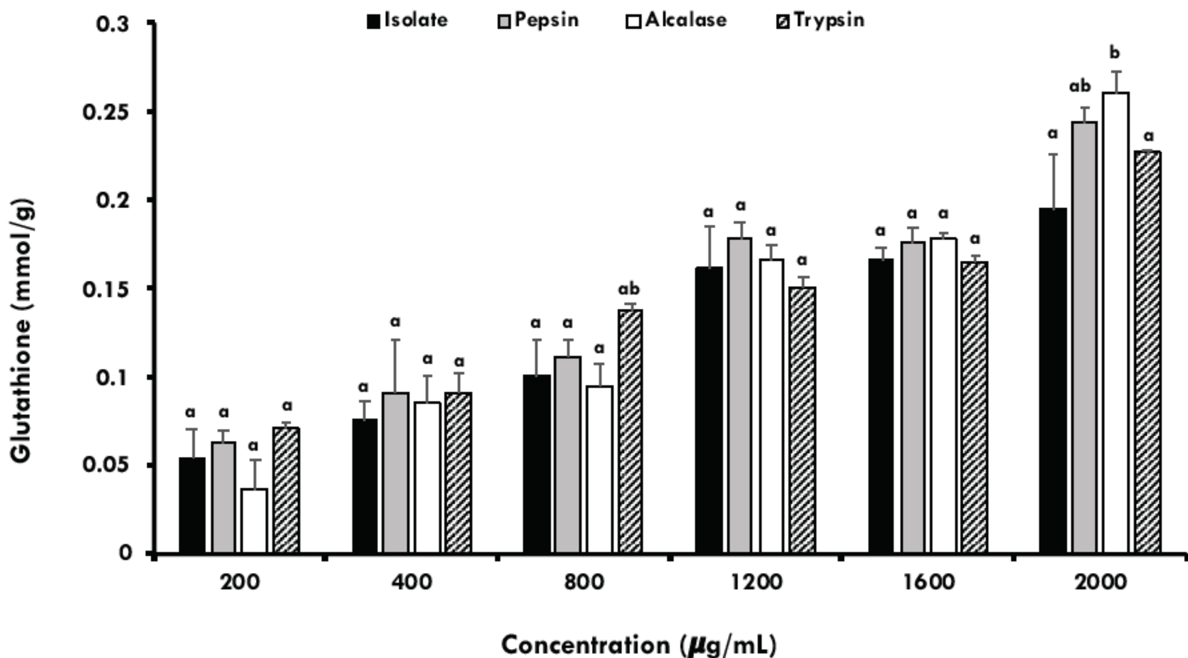

Figure 16: Glutathione equivalent of *A. cruentus* protein isolates and hydrolysates. Results are represented as mean ± SD. Data represents mean±standard deviation (n=3). Mean values with different letters are significantly different at p≤0.05.

4.6. Cytotoxicity assay

4.6.1. MCF-7

Cell viability for the MCF-7 cell line is shown in Figure 17. Camptothecin (control) had the lowest cell viability amongst all the samples (42-70%). However, for the protein samples, trypsin hydrolysate had the lowest cell viability amongst all concentrations (60-71%). Pepsin had the highest cell viability for most concentrations (74-111%). *Amaranthus cruentus* isolate had the lowest IC_{50} value of 3.55 µg/ml while alcalase hydrolysate had the highest of 965.50 µg/ml. Trypsin hydrolysate, pepsin hydrolysate and camptothecin had IC_{50} values of 3.87 µg/ml, 173 µg/ml and 9.35 µg/ml respectively.

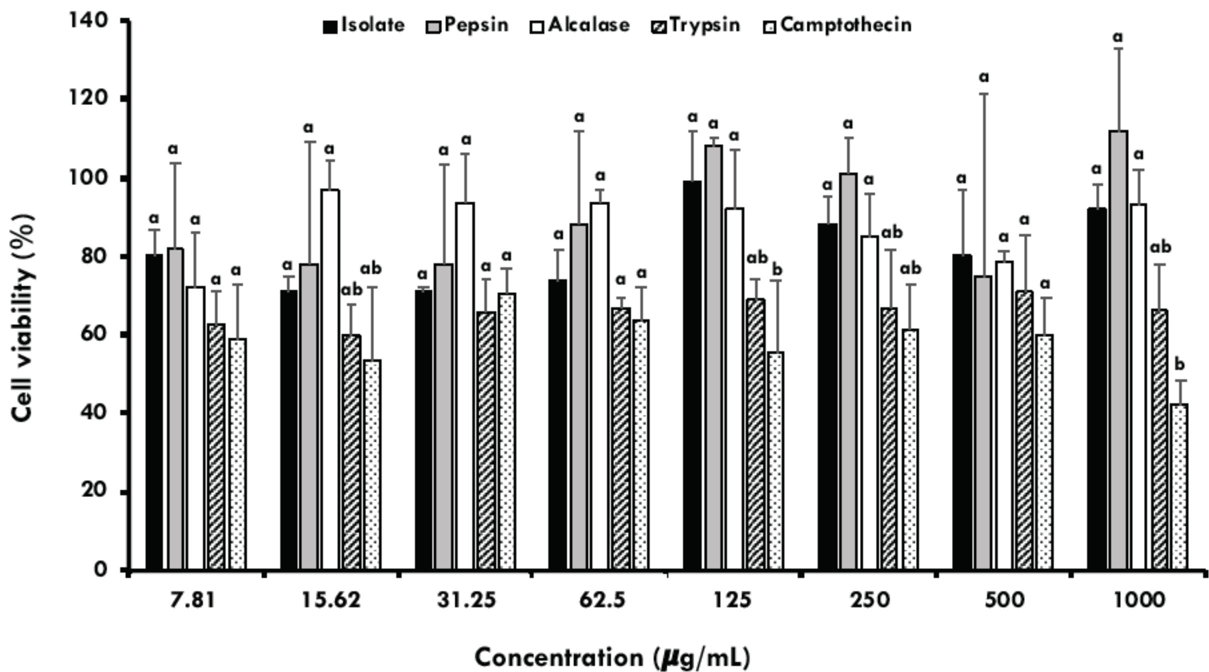

Figure 17: Cell viability (%) of MCF-7 cell line against *A. cruentus* isolate and hydrolysate in comparison to camptothecin. Data represents mean±standard deviation (n=3). Mean values with different letters are significantly different at $p \leq 0.05$.

4.6.2. A549

Cell viability for cell line A549 is shown in Figure 18. 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 cell viability for most concentrations (19-57.1%). The cell viability for camptothecin ranged between 20-99.9%. Trypsin hydrolysate had the lowest IC_{50} value of 14.10 µg/ml, while camptothecin had the highest of 304.9 µg/ml. The IC_{50} values for *A. cruentus* isolate, pepsin and alcalase hydrolysate were 249.40, 38.57 and 58.05 µg/ml respectively.

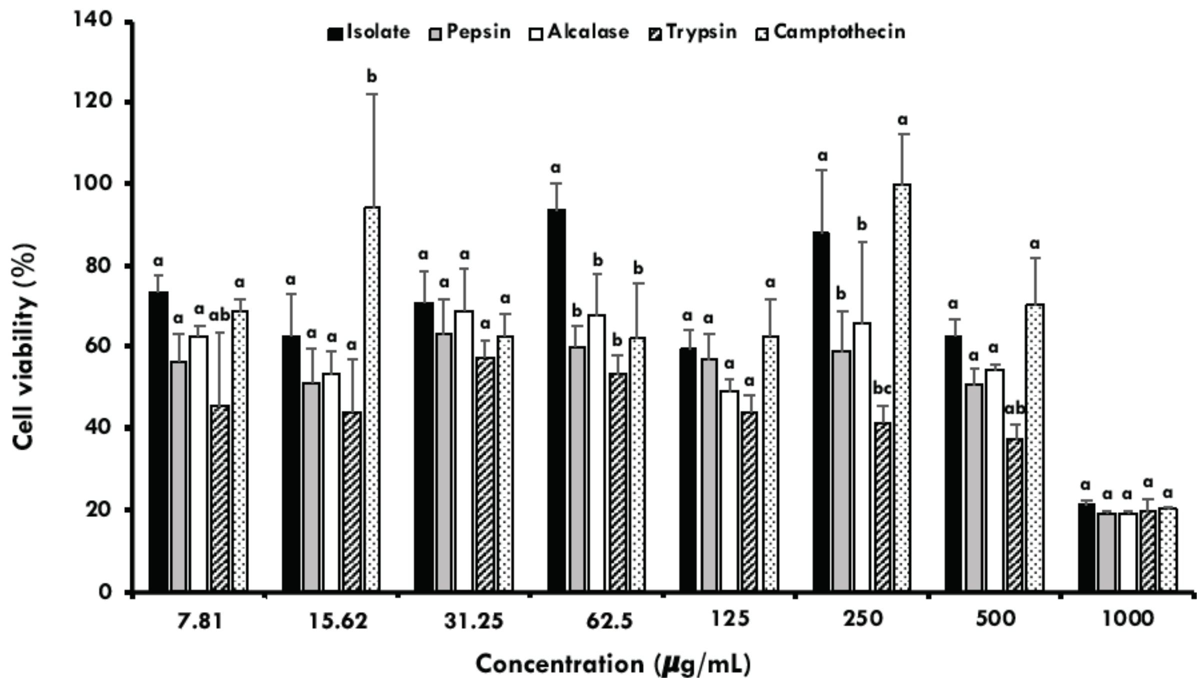

Figure 18: Cell viability (%) of A549 cell line against *A. cruentus* isolate and hydrolysate in comparison to camptothecin. Data represents mean±standard deviation (n=3). Mean values with different letters are significantly different at p≤0.05.

4.6.3. HEK 293

Cell viability for the HEK 293 cell line is shown in Figure 19. 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%). Trypsin hydrolysate had the lowest cell viability for most concentrations (56-86%). It was observed that *A. cruentus* isolate was not significantly different ($p<0.05$) compared to *A. cruentus* hydrolysates. *Amaranthus cruentus* isolate had the lowest IC_{50} value of 8.57 µg/ml, while alcalase hydrolysate had the highest of 11.07 µg/ml. Camptothecin had an IC_{50} value of 9.10 µg/ml.

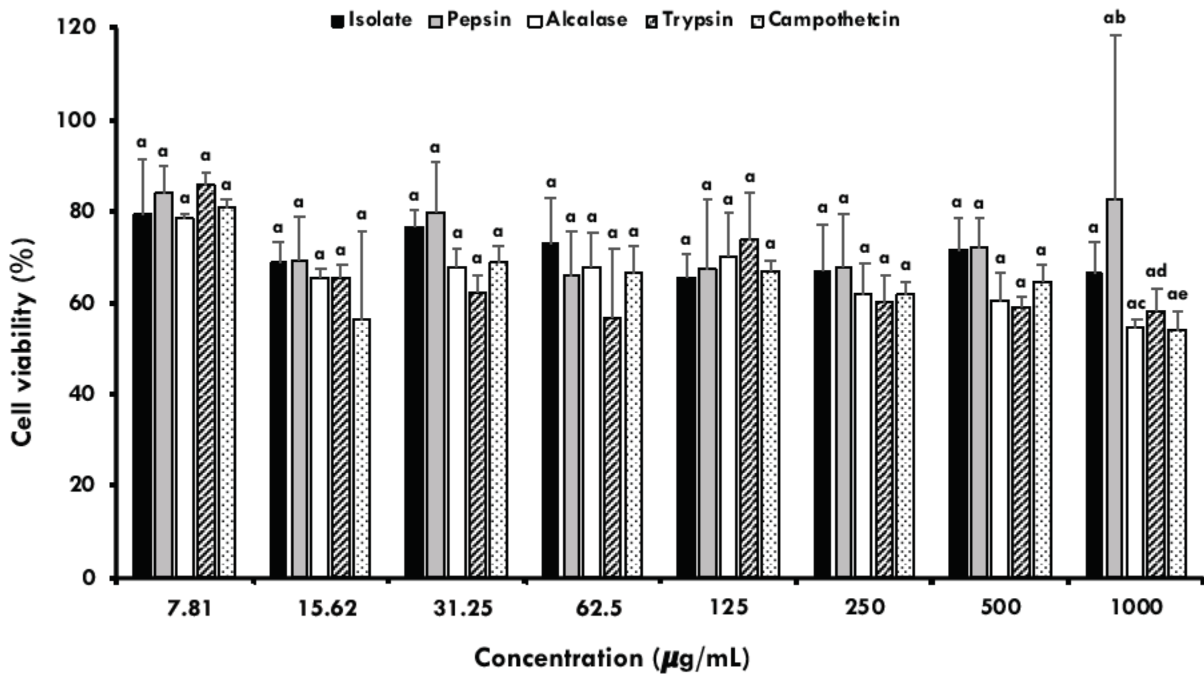

Figure 19: Cell viability (%) of HEK 293 cell line against *A. cruentus* isolate and hydrolysate in comparison to camptothecin. Data represents mean±standard deviation (n=3). Mean values with different letters are significantly different at p≤0.05.

4.7. Annexin V

Apoptosis

The Annexin V kit using flow cytometry was used to investigate the potential apoptotic effects of A. *cruentus* isolate and trypsin hydrolysate (Figure 20). Annexin PI is based upon externalization of phospholipid phosphatidyl serine Annexin-V staining, and permeabilization of nuclear membrane for PI staining. The methodology differentiates living (Annexin-V−/PI−), dying (early apoptosis: Annexin-V+/PI−) and dead cells (late apoptosis/necrosis: Annexin-V+/PI+). Control (cells only) profiles showed that most of the cells remained alive (Q4). In contrast, 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 was shown to be the most effective, with 38.50 and 28.70 % of early apoptotic cells for MCF-7 and A549, respectively.

Morphology

Figure 21 depicts morphological changes of cells with *A. cruentus* isolate, trypsin hydrolysate, camptothecin and untreated cells. For MCF-7, these cells exhibited an epithelial morphology with a mixture of well strained regular and irregular-shaped nuclei and a well-defined vacuole-contain cytoplasm. Slight condensation was observed after 24 h of incubation. In addition, fragmented nuclei can be observed as well as bright green spots suggesting the cells were undergoing early apoptosis. No late apoptotic or necrotic cells can be observed because the media was removed prior to staining (necrotic cells float in the media) The structure analysis of A549 cell morphology indicated a pronounced effect on membrane fragmentation and cytoplasm disorganisation. For A549 cells exposed to camptothecin, a notable change in morphology can be noted, from cobblestone shaped to an elongated spindle shape. No late apoptotic or necrotic cells were observed. For HEK 293, distinctive morphological changes can be seen as well as bright green spots, which are a sign for early apoptosis. Condensation can be observed in the nucleus and cytoplasm. No late apoptotic or necrotic cells can be seen in either of the cell lines; this is because dead cells detach and float in the media, the media was removed before viewing.

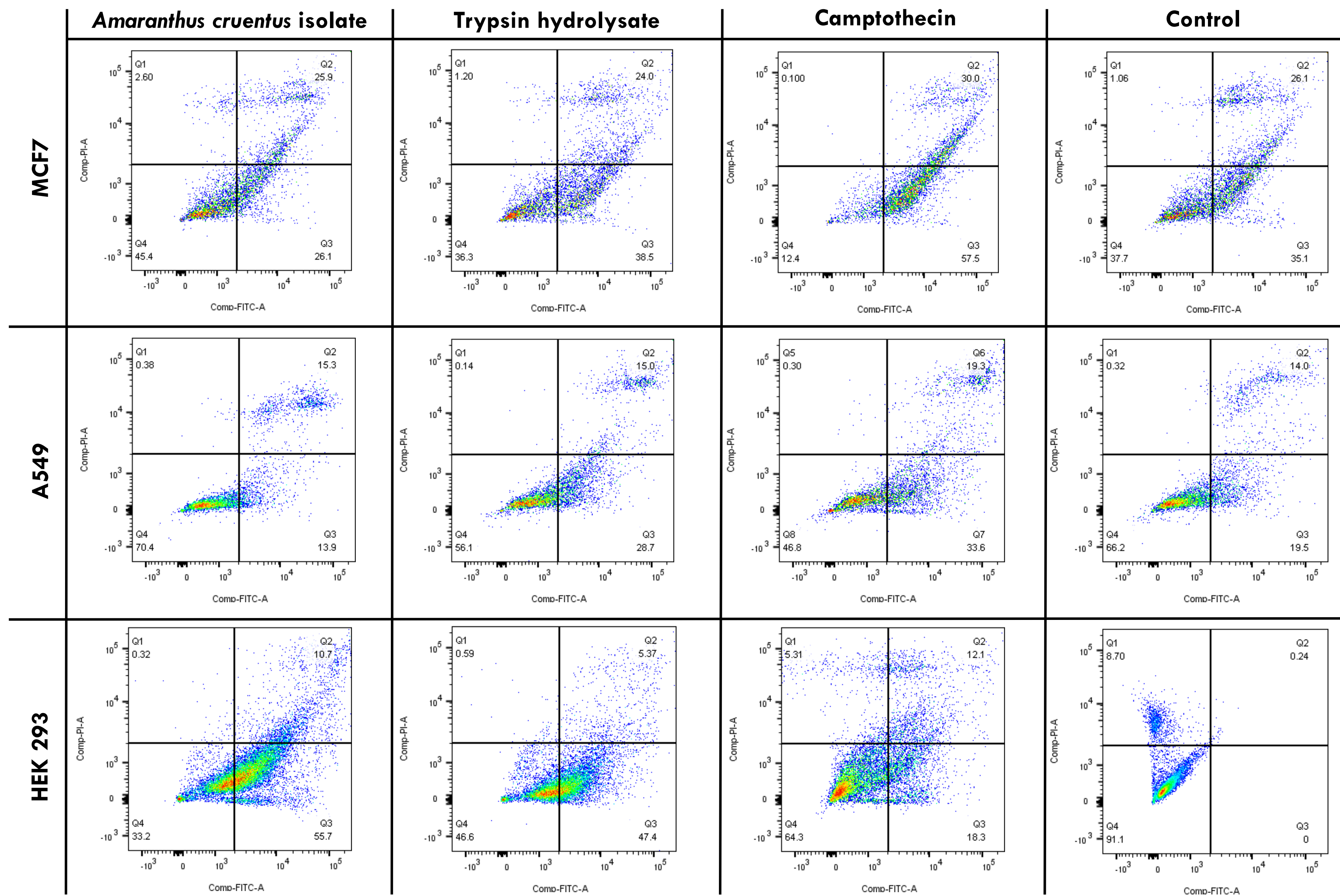

Figure 20: Flow cytometry of MCF-7, A549 and HEK 293 cells treated and untreated after 24 h. [Control - untreated cells]

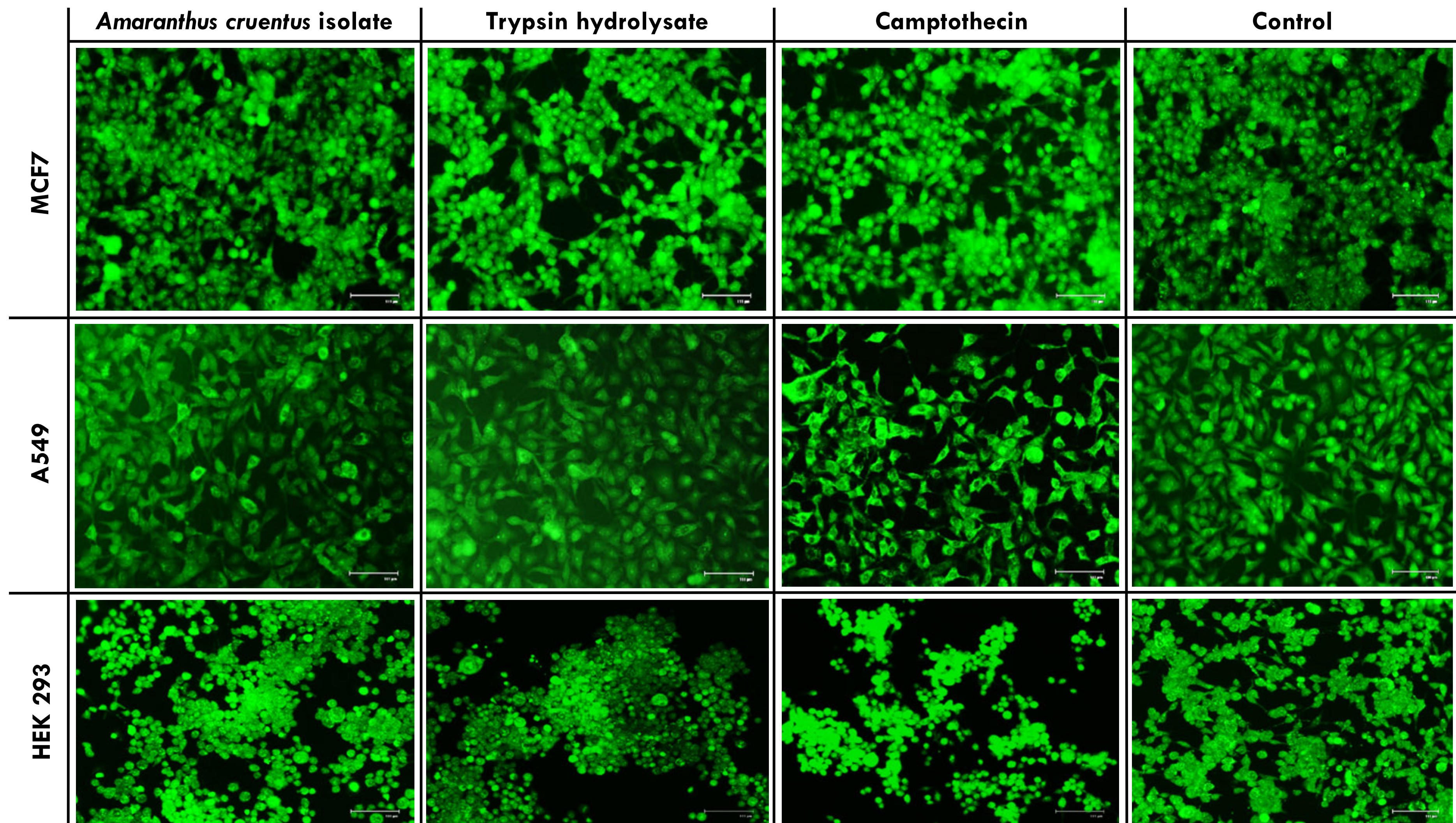

Figure 21: Fluorescent microscopic observation of MCF-7, A549 and HEK 293 cells treated and untreated after 24 h. [Control - untreated cells]

4.8. Caspase 3/7 Activity

Luminescence is proportional to the amount of caspase activity present (Promega, 2019). All treatments for all cell lines expressed higher caspase-3/7 compared with the control. It can be observed that A. *cruentus* isolate had slightly higher fluorescence units compared to trypsin hydrolysate (Figure 22) with HEK 293 exhibiting the highest fluorescence units amongst all the cell lines. Since luminescence is proportional to caspase activity, it can be noted that HEK 293 showed the highest caspase activity. Since the cells had to be transported in order for readings to be captured and the high caspase reading is probably due to stress of the cells, since HEK 293 cells are extremely sensitive. Morphology results for HEK 293 further confirmed the caspase results as it can be observed that the cells are stressed, and due to this, they had begun to undergo apoptosis.

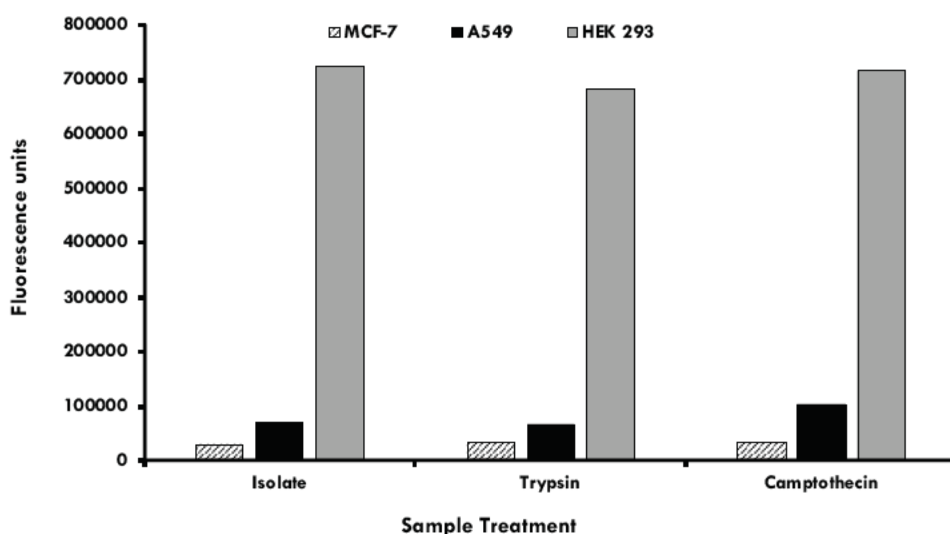

Figure 22: Caspase activity for MCF-7, A549 and HEK 293 cells exposed to isolate, trypsin and camptothecin. Data represents mean±standard deviation (n=3).

Chapter 5: Discussion

5.1. Grain composition

Results for grain composition of *A. cruentus* flour depicted in Table 3 indicated that defatting significantly increased the protein and carbohydrate content of the flour. The defatting of *A. cruentus* flour removed most of the lipids, however, a part of it (0.97%) remained in the flour. This residual lipid may be starch-bound lipids that could not be removed using hexane. For three amaranth cultivars, 0.16% to 0.28% starch-bound lipids were reported. Additionally, the presence of polar lipids interacted with proteins cannot be ruled out (Shevkani et al., 2014). Shevkani et al. (2014) reported similar results after defatting for *A. hypochondriacus* flour. Barba de la Rosa et al. (2009) reported similar results for two new commercial varieties of *A. hypochondriacus.*, Tiengo et al. (2011) revealed similar results for *A. cruentus* (Algerian variety) defatted flour. Caselato-Sousa and Amaya-Farfan (2012) revealed similar results for Amaranthus varieties. Therefore, the grain composition results obtained for *A. cruentus* defatted flour in this research is in agreement with literature.

5.2. Protein concentration and yield

The reduction in protein concentration between the isolate and hydrolysates is due to the centrifugation step (Table 4) that eliminates the undigested protein, and the addition of HCl and NaOH to adjust the pH during the hydrolysis process (Tiengo et al., 2009). Adebowale et al. (2007) reported a lower yield for mucuna bean protein concentrate prepared by salt solubilisation. However, Barba de la Rosa et al. (1992) and Arise (2016) reported similar results for salt extracted *A. hypochondriacus* fractions and Bambara protein isolates. A similar decrease in protein concentration has been reported by Tiengo et al. (2009). The low protein yield for hydrolysates can be attributed to the fact that only one gram of protein isolate was used to produce each hydrolysate. Therefore, the protein yield for *A. cruentus* isolate and hydrolysates is in agreement with literature for salt extracted proteins.

5.3. SDS PAGE

The electrophoretic pattern (SDS-PAGE) of *A. cruentus* isolate, and hydrolysates are presented in Figure 12 with other studies showing similar findings for amaranth isolates of different cultivars (Silva-Sanchez et al., 2008 , Tiengo et al., 2009, Barrio and Anon, 2010, Sabbione et al., 2015, Sabbione et al., 2016). For pepsin hydrolysate, a distinct 20 kDa band can be seen with lower molecular weight bands, below 10 kDa were also visible.

The higher molecular weight bands are extremely faint, suggesting that these proteins were broken down. Bejosano and Corke (1999) revealed similar findings for an amaranth pepsin hydrolysate. The alcalase hydrolysate revealed a distinct 20 kDa band, which can also be seen for all hydrolysates. Two new bands can be seen between 15-20 kDa and 25-37 kDa. The higher molecular weight protein can be seen, but they are extremely faint, suggesting the proteins were hydrolysed using alcalase. Quiroga et al. (2017) showed similar results for an alcalase hydrolysate from *A. hypochondriacus*. It was also shown that the 20 kDa band is a basic globulin subunit that is resistant to enzymatic treatment. It was further revealed that after 6 h of enzymatic treatment the band remained. These findings correlate with the 20 kDa band, which can be seen for all hydrolysates. Trypsin hydrolysate revealed faint higher molecular bands, the distinctive 20 kDa band and faint bands below 15 kDa, suggesting that the bands are below 10 kDa. Condés et al. (2009) presented similar results for a trypsin hydrolysate in *A. hypochondriacus*.

5.4. Toxicity Evaluation

5.4.1. Ames mutagenicity test

The mutation in TA 100 codes for the first enzyme of histidine biosynthesis (hisG). This mutation, determined by DNA sequence analysis, substitutes proline (-GGG-) for leucine (-GAG-) in the wild type organism. The TA 100 detect mutagens which cause base-pair substitutions generally at one of these G-C pairs. The mutation in TA 98 codes for histodinol dehydrogenase. TA 98 detects various frameshift mutagens in repetitive sequences as 'hot spots' resulting in a frameshift mutation (Vijay et al., 2018). The mutagenicity of protein isolates and protein hydrolysate are not well known. Table 5 shows the mutagenic response of *A. cruentus* isolate, and its hydrolysates against *S. typhimurium* strains TA 98 and TA 100. Sodium azide, the positive control, exhibited a mutagenic potential with increasing concentration. Furthermore, with an increase in the concentration of *A. cruentus* isolate and hydrolysate, there was an increase in the number of revertant colonies. In principle, the higher the number of revertant colonies, the better the frequency of mutations the compound causes. Kasumbwe (2015) reported that mutagenic potential could be expected if the mutant frequency is greater than 2. However other possibilities indicate that a probable mutagenic potential can be assumed if the mutant frequency ranges between 1.7 and 1.9 µg/mL, and that no mutagenic potential can be considered if the mutant frequency is lower than 1.6 µg/mL.

Cattaneo et al. (2014) reported similar results for a *Prosopis alba* protein isolate and hydrolysate; however, the study did not calculate mutagenic response and only reported the number of revertant colonies. None of the preparations was mutagenic in strains TA 98 or TA 100 under the conditions used in this assay which indicates the inexistence of mutagens or pro-mutagens that cause base-pair substitution (detected in TA 100) and frameshift (detected in TA 98) mutations.

5.4.2. Brine shrimp lethality assay

Artemia salina, commonly known as brine shrimp, is an invertebrate of the saline aquatic ecosystem (Janaćković et al., 2016). The brine shrimp test represents a rapid, inexpensive and straightforward bioassay for testing the lethality of extracts, which in most cases correlates reasonably well with cytotoxic and antitumour properties. According to Kasumbwe (2015) for the assay to be classified as non-lethal, it has to demonstrate the brine shrimp death of lower than 50%. Percentage death of brine shrimp for *A. cruentus* isolate and hydrolysates are represented in Figure 13. The toxicity of plant proteins and hydrolysates are not known. None of the tested samples induced any significant increase in the death percentage of *Artemia spp* in comparison to the control (potassium dichromate). This study found that the sample protein isolate and hydrolysates had a low toxicity effect against *Artemia* spp.

5.5. Antioxidant assay

5.5.1. DPPH

DPPH radical scavenging assay has been widely used to evaluate antioxidant capacity. The DPPH compound is a stable free radical due to its resonance stability and special blockade of benzene rings (Liu et al., 2016). When the DPPH molecule encounters a substance that donates a proton (H^+) such as an antioxidant, the radical is scavenged (the rich purple chromogen radical of the DPPH solution is reduced by antioxidant compounds to a pale yellow colour hydrazine) (Liu et al., 2016), the DPPH then becomes a stable diamagnetic molecule because the radical has accepted an electron and the absorbance is reduced (Alashi et al., 2014). Figure 14 represents the results of DPPH radical scavenging ability of *A. cruentus* isolate and its hydrolysates at various concentrations. The IC_{50} (the concentration of an inhibitor where the response is reduced by half) is a parameter widely used to measure the antiradical efficiency. The lower the IC_{50}, the higher the scavenging activity of free radicals (Zhu et al., 2006).

Pepsin hydrolysate had the lowest IC_{50} 23.06 μg/ml; trypsin had the highest IC_{50} value of 26.06 μg/ml. It was reported that wheat germ protein hydrolysates showed dose-dependent DPPH radicals scavenging activity and an EC^{50} (concentration that provides half-maximal results) value of 1.30 mg/ml (Zhu et al., 2006). Liu et al. (2016) reported that the scavenging activities of DPPH radical by walnut neutrase and papain hydrolysates increased with increasing concentration. Abu-Salem et al. (2013) demonstrated with increasing peptide concentrations of soy protein isolates and hydrolysates, the scavenging effect on DPPH radical was increased until about 70%, thereafter reaching a plateau. However, *A. cruentus* isolate and hydrolysates did not show dose-dependent scavenging activity for DPPH.

In contrast, Alashi et al. (2014) revealed that an alcalase hydrolysate and its derived fractions from Australian canola meal protein isolates scavenged the radical to a 50% inhibition at a range of 0.5-0.9 mg/ml for all the hydrolysate and fractions. Pazinatto et al. (2013) demonstrated the *in vitro* digestion with gastrointestinal enzymes of defatted *Amaranthus cruentus* flour (BR Algeria variety), amaranth protein concentrate and alcalase hydrolysate resulted in a significant increase in the antioxidant capacity, especially for the amaranth protein concentrate and the alcalase hydrolysate, which increased more than 7 times. This study is line with the results found by Arise (2016) and Phongthai et al. (2018) who revealed higher scavenging activity for a pepsin and trypsin hydrolysate from Bambara protein. Phongthai et al. (2018) reportedly found that the digestion by pepsin and pepsin-trypsin increased the DPPH radical scavenging activity of rice bran protein concentrate by about 3.1-4.9 fold.

It was revealed that low-molecular-weight hydrolysates appear, on average, to have higher DPPH scavenging activities than high-molecular-weight fractions (Abu-Salem et al., 2013). The results showed that *A. cruentus* isolate and its hydrolysates are free radical inhibitors, as well as a primary antioxidant that reacts with free radicals, which may limit the occurrence of free radical damage in the human body. It was also demonstrated that differences in the enzyme-substrate combination are responsible for different antioxidant activities of the hydrolysed and unhydrolyzed proteins. These differences could be attributable to the specificity of these enzymes on proteins, releasing peptides with different sizes, amino acid sequences, degree of hydrolysis and antioxidant activities (de Castro and Sato, 2014). However, it is difficult to make a direct comparison between different studies due to the lack of antioxidant standards and considerable influence by radical concentration and experimental conditions.

5.5.2. FRAP

The antioxidant activity of compounds is calculated not only by their radical scavenging behaviour, but also by their ability to participate in redox reactions and, more importantly, by their ability to reduce them (Karamać et al., 2014). In the body, iron is the most abundant transition element, with the capacity of catalysing the generation of reactive oxygen species. Ferric reducing power of antioxidants of food samples or extracts refers to their ability to convert ferric ions from ferricyanide complex to more stable ferrous ions and could be used as a measure of antioxidant potentials of such foods (Oyedeji, 2018). Literature states that a direct correlation is noted between antioxidant activity and the reducing power of protein hydrolysates (Pownall et al., 2010).

The ferric reducing antioxidant power (FRAP) of *A. cruentus* isolate, and hydrolysates at various concentrations are presented in Figure 15. Trypsin hydrolysate had the highest µmol/g $FeSO_4$ for most concentrations amongst the hydrolysed and un-hydrolysed samples. The standard antioxidants reduced glutathione acted significantly stronger. These results were comparable to results found by Karamać et al. (2014) who found that hydrolysate fractionation produced smaller peptides with greater reducing ability than large molecular weight fractions. In a study by Oyedeji (2018) it was found that an increase in peptide size caused a decrease in reducing ability for African Yam bean alcalase hydrolysate. It was noted that regardless of the type of protease used to produce hydrolysates, they acted as stronger reducing agents than the protein isolates (Karamac et al., 2016). Moure et al. (2006) found hydrolysates to increase FRAP values in respect to those of the original fractions for soybean protein concentrates. It was also revealed that enzyme concentration and hydrolysis time barely influenced the antioxidant activity of the peptides from soybean protein concentrates. Phongthai et al. (2018) reported rice bran protein hydrolysates digested by pepsin-trypsin provided the highest TRAP values (125.09 ± 1.45 mmol $FeSO_4$ equivalent/g sample), while rice bran protein concentrate had the lowest value. Arise (2016) found that Bambara protein hydrolysates and their membrane fractions revealed higher absorbance values of 0.04-0.07 compared to glutathione which had the lowest absorbance value of 0.03. Trypsin hydrolysate was found to have had the highest absorbance 0.08 among the hydrolysates.

The antioxidant ability of peptides depends on size, the composition of amino acids, sequence, and structural characteristics; these factors may explain the significant differences between hydrolysates derived from different sources of protein as well as different functional conditions (i.e. different proteases or hydrolysis extents) (Orsini Delgado et al., 2015).

From these results, it could be concluded that hydrolysis of the protein isolate improved the reducing ability of *A. cruentus* hydrolysates. An additional supply of electrons and protons to maintain a high redox potential was provided by the increased free amino acids and peptide fragments.

5.5.3 ABTS

The peroxidase substrate ABTS has become a popular substrate for estimating total antioxidant capacity, forming a relatively stable radical (ABTS.+) after one-electron oxidation. The ABTS method detects compounds capable of producing hydrogen to neutralize the free radical that has performed. Adding antioxidants to the radical cation of the preformed chromophore decreases absorption depending on the sample's antioxidant strength, concentration, and exposure period. The degree of discolouration associated with the antioxidant ability is, therefore dependent on concentration and time (Sabbione et al., 2016). Due to ABTS' sensitivity the antioxidative ability of protein hydrolysate samples can be determined at lower inhibition concentrations (Liu et al., 2016). ABTS' antioxidant activity of hydrolysates is expressed in Trolox equivalents, according to Re et al. (1999) procedure. Peptides are the potential antioxidant compounds in the case of protein hydrolysates; hence the findings of the ABTS experiment in our sample are expressed in reduced glutathione equivalents as an example of an antioxidant with a peptide chemical structure.

As seen in Figure 16, *A. cruentus* isolate and hydrolysates were slightly dose-dependent. The results obtained in this study are similar to studies by Arise (2016), who reported that Bambara protein hydrolysates had higher $ABTS^{\bullet+}$ scavenging ability in comparison to the unhydrolyzed Bambara protein isolate. She found that trypsin hydrolysate was found to be a better scavenger (EC_{50} 22 µg/ml) compared to alcalase and pepsin hydrolysate. Orsini Delgado et al. (2011) and Sabbione et al. (2016) reported dose-dependent activity for *Amaranthus mantegazzianus* isolate and *Amaranthus hypochondriacus*, respectively. Karamać et al. (2014) revealed similar results for flaxseed protein hydrolysates with different degrees of hydrolysis, expressed as glutathione and Trolox equivalent. Flaxseed protein hydrolysates showed greater radical scavenging ability than the flaxseed protein isolate (Karamac et al., 2016). Phongthai et al. (2018) revealed that rice bran protein hydrolysates digested by pepsin (243.78 mmol Trolox/g) and pepsin-trypsin (247.84 Trolox/g) were significantly higher than that of rice bran protein concentrate. Liu et al. (2016) revealed with increasing concentration, walnut neutrase and papain hydrolysates exhibited increased ABTS radical scavenging activities, and their scavenging activity were 66.41 and 76.14%, respectively.

The IC_{50} value of walnut neutrase was 80.36 μg/ml, while for walnut papain, the IC_{50} value was 62.22 μg/ml. In contrast, Alashi et al. (2014) revealed that canola meal protein hydrolysates have higher $ABTS^{\bullet+}$ scavenging ability in comparison to the unhydrolyzed canola meal protein isolate, chymotrypsin and alcalase hydrolysates were found to perform better as ABTS scavengers with lower EC_{50} values than pancreatin, trypsin and pepsin hydrolysates. Before hydrolysis, Dryáková et al. (2010) discovered that all samples (unhydrolyzed whey protein) had low antioxidant activity (7-19.8%) and that during 180 minutes of hydrolysis, the values increased significantly with all hydrolysates tested (40-54.2%). Alcalase hydrolysate was found to be the most active in scavenging ABTS.

Low antioxidant activity can be due to the specific cleavage of the enzyme which did not produce ABTS-scavengeable peptides. It has been noted that higher chain length peptide mixtures (16 of the amino groups in the chain) were not effective scavengers of ABTS (Dryáková et al., 2010). These variations can be attributed to enzymes specificity, peptides released with different sizes, the sequence of amino acids, degree of hydrolysis and antioxidant activities (de Castro and Sato, 2014). Although *A. cruentus* isolate and hydrolysates showed good glutathione equivalent results, due to the variety of methodologies used to measure ABTS, it is difficult to compare the antioxidant potential directly with data available in the literature.

5.6. Cytotoxicity assay

This method (MTT) by principle, is centred on the ability of a specific type of mitochondrial dehydrogenase - known as succinate dehydrogenase to cleave the tetrazolium ring structure of diphenyltetrazolium bromide in viable (living) cells. The cleavage reaction produces formazan crystals by reducing yellow coloured MTT to purple-coloured formazan crystals. The amount of crystals generated from this reduction reaction is taken as being a measure of the number of metabolically active (or surviving) cells. It has been indicated that the amount of formazan formed in turn, is an indication of the degree of cytotoxicity induced/caused by a compound (Kasumbwe, 2015). The effect of *A. cruentus* isolate, and hydrolysates on cell lines MCF-7, A549 and HEK 293 is shown in Figures 17, 18 and 19, respectively. After 24 h of treatment, isolate and hydrolysates showed an antiproliferative effect on the cell lines. *Amaranthus cruentus* isolate and hydrolysates were not dose-dependent and different sensitivities were observed for the three cell lines. Proteolytic hydrolysis improved the inhibitory effect for the A549 cell line.

Using the crystal-violet bioassay Barrio and Anon (2010) showed the effect of *A. mantegazzianus* isolate and *A. mantegazzianus* alcalase hydrolysate ultra-filtration on cell proliferation. After 24 h of culture, *A. mantegazzianus* isolate, and *A. mantegazzianus* alcalase hydrolysate had an antiproliferative effect on both cell lines. This growth inhibition was more distinct on the neoplastic UMR106 (IC_{50} 1.0 mg/ml) line compared to the MC3T3-E1 cells (IC_{50} 2.5 mg/ml). They also further observed the growth-inhibitory effect of the *A. mantegazzianus* isolate on other cell lines derived from human-colon tumours (Caco-2 and TC7), *A. mantegazzianus* isolate had IC_{50} values of 1.5 and 2.5 mg/ml, respectively. It was also observed that MPI had different sensitivities for the four cell lines, and proteolytic hydrolysis enhanced the inhibitory effect of two protein sources.

Kannan et al. (2008) fractionated rice bran peptide hydrolysates into > 50, 10-50, 5-10, and < 5 kDa sizes and stated that peptide fractions of < 5 and 5-10 kDa inhibited growth ~ of Caco-2 cells by 80 percent, while HepG2 cell growth was inhibited by < 5 kDa fraction by 50 percent compared to controls. Xue et al. (2009) evaluated the antitumour activity of protein hydrolysate from rapeseed using an *in vivo* S180 tumour-bearing Kunming mouse model. They confirmed that the protein hydrolysate from rapeseed significantly reduced tumour weight (44 and 53%) in the 100 and 150 mg/kg/day groups, respectively. Glu-Gln-Arg-Pro-Arg, purified and isolated anticancer pentapeptide from rice bran caused growth inhibition (84%) of colon cancer cells (Caco-2, HCT-116), 80% growth in breast cancer cells (MDA-MB-23, MCF-7) and 84% growth in hepatic cancer cells (HepG-2) at 600-700 µg / ml (Kannan et al., 2010).

Girón-Calle et al. (2010) stated that protein hydrolysates from chickpeas produced with the enzyme's pancreatin and pepsin inhibited the proliferation of THP-1 cells up to 78% depending on culture conditions and degree of hydrolysis. Xue et al. (2010) demonstrated that rapeseed peptides from flavourzyme and alcalase digestion had an antiproliferative effect on human cervical cancer cell line (HeLa) by apoptosis initiation, DNA disruption, and S-phase cycle arrest. Xue et al. (2012) studied the antitumour effects of chickpea albumin hydrolysate in H-22 tumour-bearing mice. They found that the administration of chickpea hydrolysate improved the tumour inhibition rate significantly and decreased tumour sizes. Peptides derived from corn also demonstrated anti-proliferative action in HepG2 cells by cell cycle arrest and cleaved-caspase-3 and p53 activation (Li et al., 2013). Joanitti et al. (2010), researched anticarcinogenic protease inhibitor Bowman-Birk from *Vigna unguiculata* seeds on MCF-7 cells and the findings showed a substantial dose-dependent reduction in cell viability. In contrast, normal human breast MCF-10A cell viability was not affected.

Vilcacundo et al. (2018) have observed that other food-derived proteins, such as quinoa proteins, can exert a dose-dependent antiproliferative effect on colon cancer HT-29 cells with an IC50 value of 0.212±0.002 mg protein / mL with MTT assay. Recently, Sabbione et al. (2019) performed the MTT assay on *A. mantegazzianus* isolate, and *A. mantegazzianus* simulated gastrointestinal digested isolate to determine whether the isolates were able to inhibit proliferation of HT-29 cancer cells. At a 24 h treatment time and a concentration range of 0.15-2.5 mg soluble protein/ml, cell viability was measured. Tumour cell proliferation inhibition was found at different concentrations in both samples tested. It was found that *A. mantegazzianus* simulated gastrointestinal digested isolate presented the highest inhibition of proliferation with an IC_{50} value of 0.30±0.07 mg soluble protein/ml. In contrast, *A. mantegazzianus* protein isolate showed a moderate effect with an IC_{50} value of 1.35±0.12 mg soluble protein/ml.

Pepsin has been reported to be one of the most active in the manufacture of food protein anticancer peptides (Chalamaiah et al., 2018). However, in this study *A. cruentus* trypsin hydrolysate was found to be the most efficient, due to having lower optimum concentrations. Food generated peptides ' anticancer function is dependent on their structural characteristics such as the composition of amino acids, sequence, size, total charge/hydrophobicity. Hydrophobic amino acids, for instance, glycine, leucine, proline, alanine and one or more residues of serine, tyrosine, lysine, glutamic acid, arginine and threonine are the primary amino acids in food protein-based anticancer peptides. Hydrophobic amino acids can intensify interactions between anticancer peptides and tumour cell membrane bilayers ' outer leaflets, and thus exert higher and stronger cytotoxic activity against cancer cells (Chi et al., 2015, Pan et al., 2016). Studies have shown that the compositions of bilayers of the cell membrane and the distribution of phospholipids could determine the peptides ' cell selectivity and susceptibility to lysis. The presence of charged (glutamic acid) and heterocyclic amino acid (proline) in the chain sequence has been shown to contribute to the peptide's anti-cancer properties. The shorter peptides with higher molecular mobility and diffusivity may be better able to interact with components of cancer cells and thus show greater efficacy against cancer (Chalamaiah et al., 2018).

5.7. Quantification of Apoptosis

Apoptosis is distinguished by specific morphological characteristics, including loss of plasma membrane asymmetry and binding, cytoplasm and nucleus condensation, and DNA internucleosomal cleavage. One of the early symptoms is the degradation of the plasma membrane (Elmore, 2007). The membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer plasma membrane leaflet in apoptotic cells, thereby exposing PS to the outer cell environment. While externalization of PS happens in earlier apoptosis stages, apoptosis can be detected at an earlier stage through FITC Annexin V staining (BD Biosciences, 2019). Staining with FITC Annexin V is used in conjunction with propidium iodide (PI), which allows for identification of late apoptotic and dead cells. Viable cells with intact membranes do not utilise the PI, whereas the membranes of dead and damaged cells are permeable to PI" (BD Biosciences, 2019).

Flow cytometry results are represented in Figure 20, where it can be observed that trypsin hydrolysate was more effective than *A. cruentus* isolate for MCF-7 and A549 cell lines. Similar results have been reported in literature. Silva-Sanchez et al. (2008) discovered a peptide able to induce apoptosis against HeLa cells after trypsin proteolysis contained in amaranth seeds that matched more than 60% with the sequence of lunasin. After 24 h *Amaranthus mantegazzinus* increased the population of apoptotic cells in a dose-dependent manner (Barrio and Anon, 2010). Hsieh et al. (2010) reported that a mixture of lunasin (10 μM) with aspirin (2 mM) caused a significant increase in early-stage (7%) and late-stage (12%) apoptosis of MDA-MB-231 cells. Li et al. (2013) reported that HepG2 cells induced apoptosis in cells (11.18%) when treated with corn protein isolate (160 μg/ml) for 48 h (2.22% early apoptosis; 8.96% late apoptosis), in 15.38% of cells treated with corn protein isolate (640 μg/ml) (3.87% early apoptosis; 11.60% late apoptosis/necrosis) and in 55.17% of cells treated with corn protein isolate (2560 μg/ml) (47.84% early apoptosis; 7.33% late apoptosis/necrosis) were noted in a dose-dependent manner. Xue et al. (2010) demonstrated that rapeseed peptide-induced concentration-dependent cell cycle arrest in the S-phase (38.75-54.17%). In a study by Sabbione et al. (2019) it was revealed that amaranth protein isolate produced 46.23±8.08 and 4.59±1.19% of late apoptosis and early apoptosis, respectively; whereas the same concentration of peptides from simulated gastrointestinal digestion induced cell death to a higher extent, 54.72±6.22 and 22.77±4.07% of late apoptosis and early apoptosis for HT-29 cells at 2mg/ml. It can be observed that the results stated in literature are higher than the results obtained for *A. cruentus* isolate and trypsin hydrolysate. However, it must be noted that in this assay, the minimum inhibitory concentration was used.

5.8. Morphology

Acridine orange/ethidium bromide (AO/EB) double staining combines the differential uptake of fluorescent DNA binding dyes acridine orange and ethidium bromide and the morphological aspect of chromatin condensation in the stained nucleus, allowing one to distinguish between viable, apoptotic (early or late stages) and necrotic cells. The acridine orange is taken up by both viable and non-viable cells. It emits either green fluorescence, as a result of intercalation into double-stranded nucleic acids (mainly DNA) or red fluorescence as a result of binding to nucleic acids of single-strand (RNA).

The ethidium bromide is only taken up by non-viable cells and emits red fluorescence by intercalation into DNA. Thus, a viable cell would have a uniform bright green nucleus and orange cytoplasm; an early apoptotic cell, whose membranes are still intact but has started to cleave its DNA would still have a green nucleus, but chromatin condensation becomes visible in the form of bright green patches; a late apoptotic cell would have bright orange areas of condensed chromatin in the nucleus (ethidium bromide predominates over acridine) and a necrotic cell will have a uniform bright orange nucleus (Leite et al., 1999, Kasibhatla et al., 2006). The morphologic alterations caused by *A. cruentus* isolate and trypsin hydrolysate (Figure 21) involved distinctive changes in the characteristics of the cell nucleus and cytoplasm.

Barrio and Anon (2010) and Quiroga et al. (2015) revealed similar morphological results for a UMR 106 cell line after the addition of amaranth protein isolate. Barrio and Anon (2010) noted at 2 mg/ml, cellular pyknosis and fragmented nuclei typical of apoptotic cells were observed, and the cell borders were also poorly defined. The cells exhibited a dense nucleus surrounded by a very small and highly condensed cytoplasm. Joanitti et al. (2010) noted morphological changes in MCF-7 cells after the addition of Bowman-Birk protease inhibitor from *Vigna unguiculata* seeds. Maldonado-Cervantes et al. (2010) and Jeong et al. (2009) revealed how a lunasin-like peptide from *Amaranthus hypochondriacus* and rye internalised and localized mostly in the nucleus of mouse and rat NIH-3T3 cells within 3-6 and 20 h respectively after administration. Li et al. (2013) observed similar results for a corn peptide; it was noted that the number of survival cells decreased significantly when compared with the control.

The nucleus appeared fragmented and condensed in the treated cells, which was typical of apoptotic cells. Xue et al. (2010) reported observed similar results for HeLa cells treated with rapeseed peptide. Many apoptotic bodies were noted in cells that were treated.

It was found that round, compact granular masses appeared near the centre of the nucleus, and there was a reduction in nuclear volume, the cytoplasm also displayed condensation. Recently, Sabbione et al. (2019) revealed morphological changes in HT-29 cells after the addition of amaranth protein isolate and peptides.

5.9. Caspase 3/7 activity

Caspases are a family of proteases that have been implicated to play a crucial role in apoptosis. Among them, caspase-3 is a frequently activated death protease, required for some typical hallmarks of apoptosis (Sabbione et al., 2019). Resazurin is a cell-permeable compound that undergoes an enzymatic reduction in metabolically active cells, a process thought to occur via the mitochondria. Viable cells continuously convert resazurin to resorufin, producing a fluorescent signal (7-amino-4-methylcoumarin (AMC), which is proportional to the number of viable cells. Caspase-3/7 activity is then analysed using a DEVD-based lumogenic assay. DEVD is an amino acid sequence (Asp-Glu-Val-Asp) cleaved by caspase-3. When this sequence is coupled to a lumogenic substance, upon activation of intracellular caspase-3/7 and subsequent cleavage of DEVD substrate, the luminescent product is released. This reaction is proportional to caspase activity and thus to the induction of apoptosis. As dead cells cannot produce caspase (Butterick et al., 2014, Promega, 2019), extrinsic and intrinsic apoptosis pathways activate caspases, which are responsible for the activation of endonucleases contributing to the chromatin condensation and DNA fragmentation. These molecules also degrade cytoskeleton proteins, altering cell morphology (Joanitti et al., 2010). Figure 22 shows the effect of *A. cruentus* isolate, trypsin hydrolysate and camptothecin caspase-3/7 activity of MCF-7, A549 and HEK 293 cancer cells after a 24 h administration at two minimum inhibitory concentrations.

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, thereby implying that one of the mechanisms of corn proteins pro-apoptotic is achieved by increasing the expression of caspase-3. Recently, 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. Considering the results obtained in the present work, *A. cruentus* isolate, and trypsin hydrolysate could contain a peptide sequence which would increase apoptosis through the caspase-3 mechanism. However, further research needs to be conducted.

Chapter 6: Conclusion

This study demonstrated the potential use of *A. cruentus* protein isolate and hydrolysates as an anticancer therapeutic agent. 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 antioxidant and anticancer properties of the hydrolysates compared to *A. cruentus* protein isolate. Hydrolysates displayed better antioxidant potential in comparison to the isolate. For cytotoxicity, trypsin hydrolysate exhibited the best anticancer activity amongst all test samples, this was due to trypsin hydrolysate having the lowest IC_{50} values for the A549 and HEK-293 cancer cell lines. Annexin V-FITC flow cytometry showed that there was an increase in the number of early apoptotic and late apoptotic cells in comparison to the control sample. Acridine orange/ethidium bromide fluorescence staining and caspase 3/7 assay further confirmed the Annexin V-FITC reults. In this thesis, it is shown for the first time the anticancer potential of three hydrolysates on two cancerous and one non-cancerous cell line, which was found to be significantly higher than other amaranth cultivars. This work supports the idea of using *A. cruentus* isolate and hydrolysates as a potential functional food with health-promoting benefits. Further research needs to be conducted to find the specific peptide responsible for the anticancer activity and the possible mechanism of action.

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