Antioxidant and anticancer properties of bioactive peptides from *Lablab purpureus*

Submitted in complete fulfilment for the Degree of Doctor of Food Science and Technology in the Department of Biotechnology and Food Science, Durban University of Technology, Durban, South Africa

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Reference declaration

I, Shivon Sipahli – 20902315 and Prof John Mellem do hereby declare that in respect of the following dissertation:

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Author's declaration

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 Science, Faculty of Applied Sciences, Durban University of Technology, South Africa, under the supervision of **Prof J.J. Mellem and Prof E.O. Amonsou**.

Student's signature

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Research Outputs

Publications

1. **Sipahli, S.,** Dwarka, D., Amonsou, E. and Mellem, J. 2022. *In vitro* antioxidant and apoptotic activity of *Lablab purpureus* (L.) Sweet isolate and hydrolysates. *Food Science and Technology (Campinas)* 42, e55220 https://doi.org/10.1590/fst.55220

2. **Sipahli, S.,** Dwarka D., Amonsou E. O., Mellem J. J. 2023. *In vitro* apoptotic activity of *Lablab purpureus* (L.) Sweet low molecular weight peptides. *Journal of Food Science and Technology* [Recommended for acceptance (under second review – Manuscript ID CTA 2022 1249)]

Conference outputs

1. **Sipahli, S.,** Dwarka, D., Amonsou, E. and Mellem, J. Evaluation of antioxidant and antiproliferative bioactive peptides from *L. purpureus*. Food Quality and Safety Symposium, Garden Court, Durban South Africa 28 November 2019

2. **Sipahli, S.,** Dwarka, D., Amonsou, E. and Mellem, J. *In vitro* Antioxidant and Apoptotic Activity of *Lablab purpureus* (L.) Sweet Isolate and Hydrolysates. 24th SAAFoST Biennial International Virtual Congress, from 20th - 22nd September 2021

Preface

The following dissertation is organised into six chapters and is presented as follows:

Chapter 1: Introduction (describes problem statement, aims, and contribution to knowledge relating to research).

Chapter 2: Literature Review (review of previous related studies and potential knowledge gaps).

Chapter 3: *In vitro* antioxidant and apoptotic activity of *Lablab purpureus* (L.) Sweet isolate and hydrolysates.

Chapter 4: *In vitro* apoptotic activity of *Lablab purpureus* (L.) Sweet low molecular weight peptides

Chapter 5: General Discussion

Chapter 6: Conclusion (summary, limitations, recommendations, and future work).

Table of Contents

List of Figure

List of Tables

Abstract

Cancer can be described as a non-communicable disease that develops from defective cells in the human body and grows uncontrollably. Globally in 2020, statistics revealed that the disease had affected approximately 19.3 million people. With about 51% of these cases resulting in death. Cancer treatments usually comprise surgery, chemotherapy, radiotherapy, or a combination of the three. Traditional therapies such as chemotherapy and radiotherapy drugs are effective at shrinking tumours. However, a key disadvantage is that these drugs are unable to distinguish between cancerous and healthy cells. Subsequently, the human body experiences many adverse side effects such as hair loss, vomiting, lowered immunity, and a general deterioration of health. Drug resistance and rejection are also major disadvantages of these traditional therapies. Alternative therapies are required to mitigate these drawbacks. The vital factor to consider for alternative treatments should be to selectively target cancer cells thereby alleviating the unwanted side effects. Compounds derived from non-toxic edible plants have shown to have bioactive potential. These plants are regarded as non-toxic to the human body therefore they would be able to target the tumour cells alone. Plant compounds also provide additional protection such as their antioxidant abilities and apoptotic potential. Evidence suggests that bioactive peptides derived from legumes can act as both anticancer agents and strong antioxidants. This study investigated the bioactive potential of peptides derived from *Lablab purpureus*.

This investigation began by assessing the antioxidant capacity (2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic (ABTS), superoxide radical scavenging and Ferric Reducing Antioxidant Power (FRAP) assays) and antiproliferative abilities (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)) of *L. purpureus* protein isolate and hydrolysates (alcalase, trypsin and pepsin). The hydrolysate and fractions of interest were selected based on the MTT assay with the pepsin hydrolysate selected for further apoptotic studies (caspase-3 and -7, and annexin V-PI). Thereafter, the pepsin hydrolysate was fractionated by ultrafiltration (molecular weight cut-off: <1, 3, 5, 10, >10 kDa). The 3 kDa fraction was further fractionated by

RP-HPLC. Five peaks appeared on the chromatogram, however, fraction 2 was selected, for apoptotic investigations (caspase -3 and -9, p53 and annexin V-PI).

Antioxidant studies are a good measure of the isolate or hydrolysate's ability to perform as a bioactive compound. The 50% inhibitory concentration (IC_{50}) observed for the respective antioxidant studies showed the radical scavenging ability of the isolate and hydrolysates to be 1.81-4.47 mg/mL (DPPH), 1.73-2.42 mg/mL (ABTS), 1.36-4.4 mg/mL (superoxide radical scavenging) and 19.20-21.94 mg/mL (FRAP).

Anticancer activity was substantiated by the peptides' ability to induce apoptosis. The pepsin hydrolysate was selected using the MTT assay (IC_{50} values of A549, 119.6; MCF-7, 9.80 and HEK293, 13.86 µg/mL). Pepsin hydrolysate inhibited cancerous cells (A549 and MCF-7) while causing minimal damage to healthy cells (HEK293). Thereafter apoptotic markers, caspase 3/7 and annexin V-PI were quantified. Visualisation of cells in different stages of apoptosis was investigated by Annexin V-PI staining quantified by flow cytometry. During early apoptosis; A549, 42%; MCF-7, 17%; HEK293, 34%. Caspase 3/7 assay verified that the pepsin hydrolysate caused an increase in apoptotic activity.

Caspase-3 and -9 activity of cells, determined by ELISA showed that Fraction 2 treated cancer cells (A549 - 0.067 ng/mL, 21.966 ng/mL, and MCF-7 - 0.137 ng/mL, 0.205 ng/mL respectively) had a greater caspase concentration over camptothecin (A549 - 0.029 ng/mL, 20.486 ng/mL and MCF-7 - 0.051 ng/mL, 0.112 ng/mL respectively). Tumour suppressor protein, p53, acts as a protective mechanism by initiating apoptosis in 'suspicious' cells. The A549 cell line showed the greatest p53 expression compared to MCF-7 and HEK293. Increased p53 can regulate signalling pathways leading to targeted apoptosis. Finally, annexin V-PI confirmed that Fraction 2 did induce apoptosis in the cells (cells in early apoptosis, A549, 85%; MCF-7, 90%; HEK293, 94%). Results from this study have shown that peptides derived from *L. purpureus* (specifically fraction 2) have potential anticancer abilities which may be attributed to their antioxidant and apoptotic abilities.

Chapter 1: Introduction

The 21st century has seen an increase in terminal and chronic diseases such as heart disease, cancer, diabetes, and hypertension. Lifestyle and environmental factors are the main contributors to deterioration of human health. Conversely, nutritionally rich diets have been linked with a reduced incidence of chronic diseases (Sánchez-Chino et al., 2015, Chen et al., 2017).

Globally, 1 in 6 people die from cancer each year, with lifestyle and dietary factors contributing to a third of these deaths (WHO, 2020). Conventional cancer treatments, chemotherapy and radiotherapy, are associated with side effects such as lowered immunity, memory loss, nausea, vomiting, and general deterioration of well-being (Avilés-Gaxiola et al., 2020; Sharma et al., 2020). The cytotoxic drugs used in conventional treatments are delivered to cancerous cells, however, this approach is not targeted ultimately resulting in the death of healthy cells (Thundimadathil, 2012; Sharma et al., 2020). Apart from selectivity, patients can be resistant to chemotherapy thereby negating the efficacy of these drugs (Barari et al., 2017). The use of bioactive peptides as potential anticancer agents would provide a more targeted approach to cancer treatment with minimal side effects thereby enhancing a patient's recovery and overall quality of life (Avilés-Gaxiola et al., 2020).

For alternative cancer treatments to be successful, they are required to target cancerous cells whilst leaving healthy cells relatively unaffected subsequently preventing unwanted side effects. Furthermore, chronic diseases are triggered by the presence of free radicals. Free radicals produced by cancerous cells can result in growth stimulation, cell survival, and inflammation. Therefore, antioxidants, especially those derived from plants, are advantageous in preventing and aiding in the treatment of cancers (Chi et al., 2015; Athreya and Xavier, 2017).

Numerous research articles associated with traditional knowledge systems have shown how populations have long used edible plants for disease prevention and treatment (Gupta, 2020). Modern science has provided evidence for the medicinal value of traditional medicine leading to the notion of bioactive foods or nutraceuticals. A bioactive food is identified as

any substance, either a food or food component that delivers medicinal benefits (Gupta, 2020). One such group of plants with traditional medicinal value is leguminous crops. Proteins and peptides derived from legumes have been shown to exhibit protective abilities against chronic diseases (such as diabetes, cancer, and hypertension) (Korhonen and Pihlanto, 2006, Udenigwe and Aluko, 2012, Hernández-Ledesma and Hsieh, 2013, Singh et al., 2014, Barnes et al., 2015 Erbersdobler et al., 2017).

Lablab purpureus is a legume found to be a good source of protein (18-25%) making it a prospective source for bioactive peptides (Subagio, 2006; Hossain et al., 2016). Bioactive peptides are protein fragments that provide increased bioactive potential when released from the sequence of their source protein (Udenigwe and Aluko, 2012; Hernández-Ledesma and Hsieh, 2013). The aim of this study was therefore to investigate the anticancer and antioxidant ability of bioactive peptides derived from *L. purpureus*. Specific objectives were to:

- Determine the cytotoxic effect (MTT assay) of three hydrolysates (alcalase, pepsin and trypsin) on selected cancer cell lines (A549 and MCF-7) and a healthy cell line (HEK293).
- Assess the antioxidant effect of the three hydrolysates using DPPH, FRAP, ABTS and superoxide radical scavenging assays.
- Determine the apoptotic potential of the most active hydrolysate by morphological changes (ethidium bromide staining), Annexin V-PI (flow cytometry), and caspase 3/7 (fluorescence spectroscopy) analysis.
- Evaluate the cytotoxic effect (MTT assay) of ultrafiltration fractions derived from the most active hydrolysate.
- Purify the most active peptide fraction by RP-HPLC and determine the cytotoxic effect (MTT assay) of the collected fractions.
- Determine the apoptotic potential of the most active fraction derived from RP-HPLC by Caspase3/9 (ELISA), Annexin V-PI (flow cytometry) and p53 (ELISA) analysis.

Chapter 2: Literature review

2.1. Prevalence of cancer

Cancer can be defined as a non-communicable illness where cells grow uncontrollably subsequently altering the structure of neighbouring tissue (Jan and Chaudhry, 2019). In 2020, worldwide statistics have estimated that 19.3 million people were diagnosed with various types of cancers and approximately 10 million people succumbed to the disease (Sung et al., 2021). In South Africa, specifically, it was reported that in 2020, there were 108 168 diagnosed cases and 56 802 deaths (WHO, 2020).

Statistically, cancers are diagnosed in approximately 20% (1 in 5 people) of people aged 75 years and younger while 10% (1 in 10 people) of those cancers result in death. Globally, breast and lung cancers are the most diagnosed types of cancer with mortality most prevalent in lung, liver, and stomach cancer patients (Ferlay et al., 2021). Women in South Africa are at risk (1 in 7 women) of breast, cervical, colorectal, uterus and lung cancer. In the case of men, they are more at risk (1 in 6 men) of prostate, lung, colorectal, non-Hodgkin's lymphoma and bladder cancers (CANSA, 2021).

Primarily, cancer is caused by mutation of DNA that controls normal cellular functions such as cellular growth, maturation, and apoptosis or programmed cell death (Kharb et al., 2012). Several factors are responsible for mutations of cells, including genetic predisposition, chronic diseases of infectious origin (e.g., viral hepatitis and HIV), the environment (exposure to carcinogens in the air and water), and lifestyle choices (smoking, excessive alcohol consumption and poor dietary habits) (Kharb et al., 2012; Sánchez-Chino et al., 2015). The common thread among these factors is exposure to carcinogenic substances. Many of these substances are also found naturally e.g., ultraviolet radiation from the sun and aflatoxins present in certain varieties of nuts (Sánchez-Chino et al., 2015).

Carcinogens linked with the immune system's inability to trigger apoptosis in cancerous cells at the beginning of the life cycle eventually result in the formation of tumours (Kharb et al., 2012; Sánchez-Chino et al., 2015). Altered cells multiply faster than healthy tissue without executing their intended functions. Normal cells that have been exposed to carcinogens are unable to

trigger apoptosis or programmed cell death, creating neoplastic growths and subsequently tumours (Luna-Vital et al., 2015).

During tumour development, metastasis can occur, where cancerous cells can invade blood vessels and lymphatic nodes resulting in cells being transported to peripheral organs (Luna-Vital et al., 2015). Figure 2.1 depicts the process of tumour formation and eventual metastasis. Targeted treatments can be used to control and treat cancers which would in turn limit the invasion of cells that ultimately result in metastasis (Zubair and Ahmad, 2017).

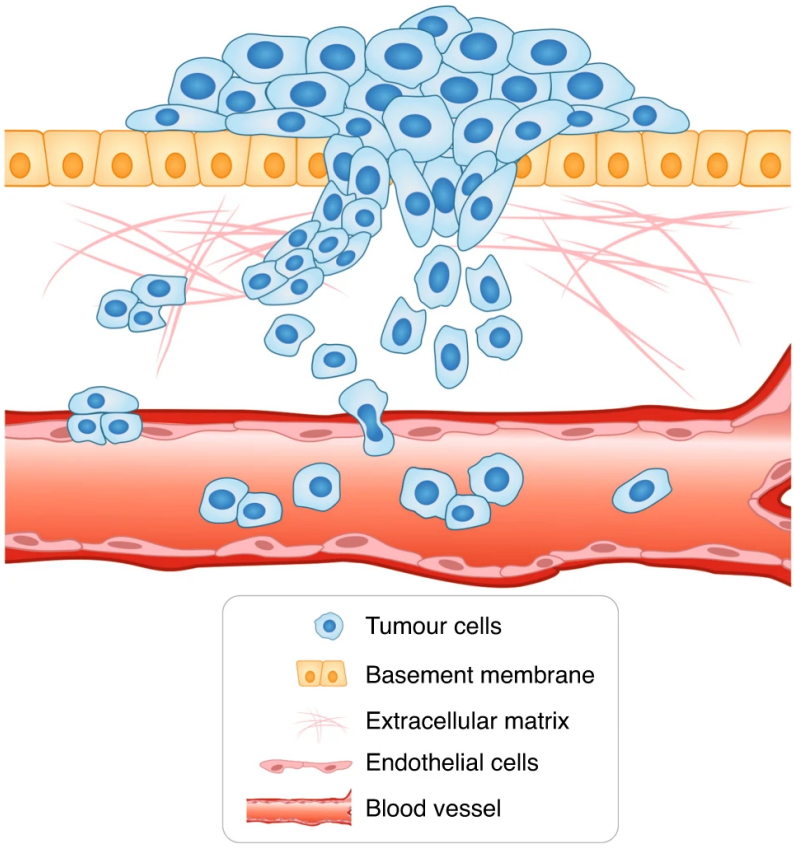

Figure 2.1: Cancerous cells have multiplied and formed a mass. Some of the tumour cells are freed from the mass and end up in blood vessels that carry the cells to other parts of the body resulting in metastasis (Novikov et al., 2021)

2.2. Physiology of cancer

In cancer cases, cells begin to proliferate rapidly causing several mutations to key genes that are responsible for cell proliferation and survival. Steps involved in mutation include (1) deactivation of DNA repair genes, (2) mutation of the protooncogene creating an oncogene and (3) mutation of other tumour suppressor genes resulting in a tumour (Gupta, 2020).

Tumours can be classified as benign or malignant. Benign tumours are usually confined to their original location and do not spread to surrounding tissue. They are not usually life-threatening except when they grow in a restricted space such as the brain (Cooper, 2000; Alison, 2001). However, these tumours can become malignant and therefore should be surgically removed (Cooper, 2000; Alison, 2001). Conversely, malignant tumour cells replicate at a faster pace and invade surrounding tissue via circulatory and lymphatic systems, resulting in metastasis, (Cooper, 2000; Alison, 2001). Malignant tumours are life-threatening due to their ability to invade and metastasise.

2.3. Classification of cancers

Cancers are divided into four main categories: carcinoma, sarcoma, leukaemia, and lymphoma. Figure 2.2 showed the morphology of the cancer types.

2.3.1. Carcinoma

Carcinomas are formed in the epithelial cells and are the most common form of cancer that usually forms solid tumours (Lin et al., 2019; CTCA, 2022). Carcinomas are classified into; (1) *in situ*, tumour cells have not invaded surrounding tissue; (2) invasive, tumour cells have invaded the surrounding tissue; and (3) metastatic, tumour cells have now disseminated to tissue and organs throughout the body (CTCA, 2022). Common types of carcinomas include basal cell carcinomas (developed in the basal cell layer of the skin or the lowest layer of the epidermis) and squamous cell carcinoma (tumour cells start in the outmost layer of the epidermis).
In ductal carcinoma, the most common form of breast cancer, cells are restricted to the lining of the milk ducts and have not spread into duct walls and nearby breast tissue (Lin et al., 2019; CTCA, 2022). Some of the risk factors associated with carcinomas are excessive exposure to ultraviolet radiation and chemicals as well as inherited genetic conditions and

gene mutations (CTCA, 2022). Conventional treatments for carcinoma include surgery, chemotherapy, and radiation (CTCA, 2022).

2.3.2. Sarcoma

Sarcomas are a type of cancer that advances in the bones and soft tissue including; fat, muscles, blood vessels and fibrous tissue. Soft tissue sarcomas are frequently found in the legs or arms and can occur in both children and adults. Bone sarcomas, also referred to as bone tumours are commonly prevalent in children (JohnsHopkinsMedicine, 2022). There are no specific causes of sarcomas however there are associated risk factors. Including previous exposure to radiation therapy, genetic disorders, exposure to toxic chemicals (such as dioxin and arsenic, and lymphedema or long term swelling) (JohnsHopkinsMedicine, 2022). Early symptoms of soft tissue sarcomas are presented as lumps or swelling. Later symptoms include extreme pain on and around the areas of the tumour site. Symptoms of bone sarcomas include pain and/or swelling, hindered range of motion, fever and weaker bone density resulting in breakages (JohnsHopkinsMedicine, 2022). Although sarcomas are rare in human beings, they are traditionally treated by a combination of chemotherapy, radiation, and surgery (JohnsHopkinsMedicine, 2022).

2.3.3. Leukaemia

Leukaemia also known as cancer of the blood begins in the bone marrow. There are four types of leukaemia namely acute lymphocytic, acute myelogenous, chronic lymphocytic and chronic myelogenous (ClevelandClinic, 2022). Leukaemia results when there is a mutation in bone marrow cells (ClevelandClinic, 2022). Risk factors for developing leukaemia are mutations in DNA that have arisen from previous chemotherapy and/or radiation therapy and genetic disorders such as neurofibromatosis and Klinefelter syndrome (ClevelandClinic, 2022). Common symptoms include tiredness, frequent infections, tenderness in bones and joints and swollen lymph nodes (ClevelandClinic, 2022).

Treatments include chemotherapy, immunotherapy, targeted therapy, radiation, hematopoietic cell transplant (replacing cancerous cells with new hematopoietic cells that can form new blood marrow and blood cells) and chimeric antigen receptor T-cell therapy (a novel approach that adapts T-cells to fight the cancer cells) (ClevelandClinic, 2022).

2.3.4. Lymphoma

Lymphoma begins in the lymph system (the tissue and organs that produce, store, and carry white blood cells). Hodgkin's and Non-Hodgkin's lymphoma are the two types of lymphomas that can occur in children, teenagers, and adults. Hodgkin's lymphoma spread systematically from one lymph node to the next while Non-Hodgkin's spreads within the lymph nodes in a haphazard manner (CDC, 2018). Risk factors associated with lymphoma include HIV infection, family history and exposure to high levels of ionising radiation (CDC, 2018). Some of the symptoms presented in patients with lymphoma include swollen lymph nodes and unexplained tiredness and weight loss (CDC, 2018). Treatment options include chemotherapy, radiotherapy, and monoclonal antibody therapy (a targeted approach) (NHS, 2022).

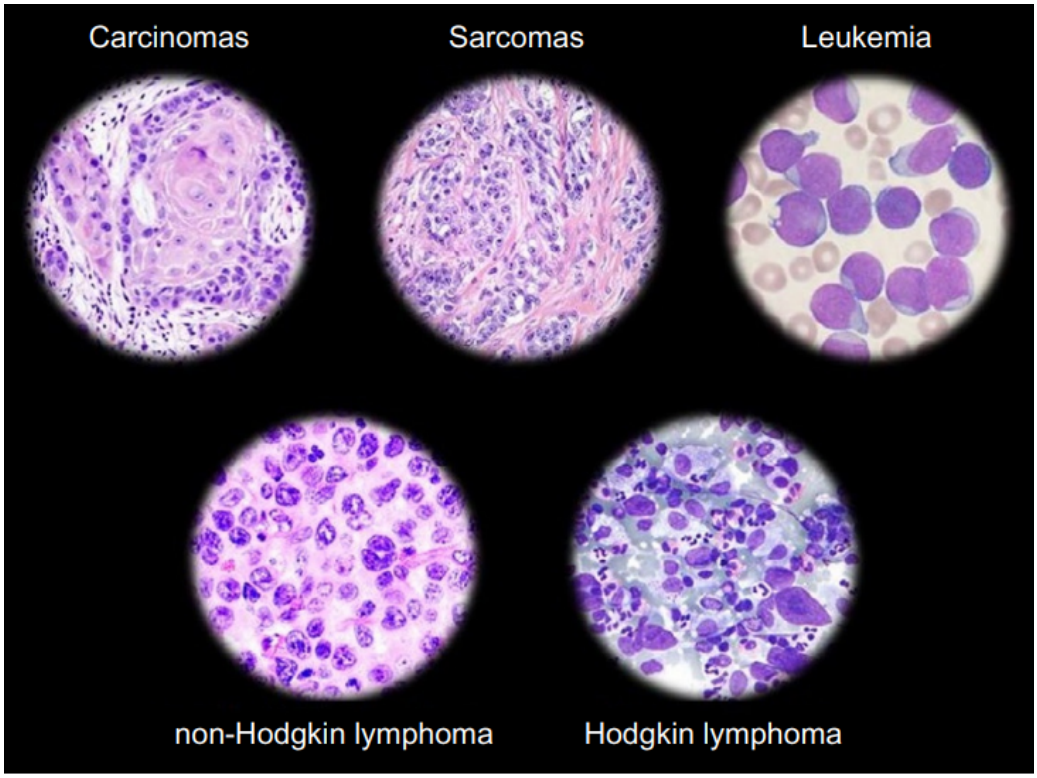

Figure 2.2: Morphologies of cancer types (Lin et al., 2019)

2.4. Conventional cancer treatment and their disadvantages

Conventional oncology uses many different strategies for cancer therapy (Quintal-Bojórquez and Segura-Campos, 2021). Common treatments for cancer are surgery, chemotherapy, and radiotherapy (Marqus et al., 2017). Surgery is the first line of treatment for most benign, solid tumours, largely used for diagnosis and tumour removal. Radiation therapy uses photons (i.e., gamma rays and x-rays) or particles (i.e., protons, neutrons, and electrons) to generate negatively charged molecules (free radicals) when they interact with cells or tissue. The resulting free radicals cause damage to the DNA of cancer cells thereby preventing replication. Chemotherapy uses toxins that target tumour cells ultimately resulting in cell death (Alison, 2001; Huang et al., 2011; Kharb et al., 2012). Chemotherapy drugs include alkylating agents (affects reproduction by damaging its DNA), antimetabolites (interferes with cell replication), antitumour antibiotics (hinders the DNA of the cell preventing cell division), topoisomerase inhibitors (restricts topoisomerase enzymes), and mitotic inhibitors (mitosis/ cell division inhibitors) (Stengler and Anderson, 2018).

Although chemotherapy and radiation therapy are effective against cancer cells, the treatment is nonspecific resulting in many adverse side effects experienced by patients. Some of the healthy cells that are commonly affected include hair follicles, mucous membranes, erythrocytes and leukocytes (Alison, 2001; Rejhova et al., 2018). Side effects not only greatly impact the patient's quality of life but also have an adverse effect on treatment and prognosis (Rejhova et al., 2018; Quintal-Bojórquez and Segura-Campos, 2021). Apart from the mentioned side effects, chemotherapy has the ability to fail due to adverse reactions, drug resistance and target specificity of the drugs (Aung et al., 2017; Gupta, 2020). Therefore, there is growing interest in alternative therapies from natural sources (Aung et al., 2017; Quintal-Bojórquez and Segura-Campos, 2021). Essentially, a successful anticancer agent should have the ability to trigger cell death in cancerous cells while causing minimal harm to healthy cells (Kharb et al., 2012; Letai, 2017).

2.5. *In vitro* cancer cell lines

Cancer develops when there is atypical proliferation of any type of cell in the body hence the different distinct types of cancers. Outlined in Table 2.1 are some of the more common types of human cancers and the cell lines associated with them. When cancers are investigated *in vitro*, immortal cell lines are often used. Using cell lines in research is cost-effective, they offer an unrestricted source of material, and are easy to use and maintain in a laboratory environment. The use of these cell lines also allows for studies to be conducted without the need for ethical clearance which is normally associated with *in vivo* studies (Kaur and Dufour, 2012). In this study Lung cancer cells (A549), Breast cancer cells (MCF-7) and a healthy cell line, human embryonic kidney cells (HEK293) were chosen to be used as lung and breast cancer are some of the more prevalent cancers.

2.5.1. Lung cancer cells (A549)

The human non-small cell lung cancer cell line, A549, developed in 1972 is a common cell line used in research and drug. Pulmonary carcinoma tissue derived from the tumour of a 58-year-old Caucasian male was removed and cultured to obtain the A549 cell line (Synthego, 2022a). A549 cells are inherently squamous and allow diffusion of substances like water and electrolytes across the alveoli of the lungs. When grown under *in vitro* conditions, the cells are adherent and grow in a single layer. The A549 cells are hypotriploid human cells with a modal chromosome number ranging between 64 and 67. Tested and approved by the FDA, the A549 cell line has been used in adenovirus production, disease modelling, drug development and CRISPR delivery (Synthego, 2022a).

2.5.2. Breast cancer cells (MCF-7)

The MCF-7 breast cancer cell line was isolated in 1973 by Dr Soule and his team at the Michigan Cancer Foundation. The MCF-7 cell line was derived from the pleural effusion of the breast adenocarcinoma of a 69-year-old female (Comsa et al., 2015). The cell line has a modal chromosome number ranging between 60 and 140, depending on the variant. The MCF-7 cell line is primarily used in research *in vitro* for the development of anticancer drugs and the understanding of drug resistance (Comsa et al., 2015).

2.5.3. Human embryonic kidney cells (HEK293)

The Human embryonic kidney cells, HEK293, were developed by Alex Van der Eb and Frank Graham in the 1970's. The cell line was isolated from a single healthy female foetus of unknown parentage that was electively terminated. The cells were immortalised by adding Ad5 E1A and E1B genes to the HEK genome. The hypohaploid human cell line has a modal chromosome number of 64. When grown under *in vitro* conditions these cells grow in a monolayer and are adherent. The HEK293 cell line has been applied in protein production, the heterologous expression of cell membrane receptors and ion channels use this cell line to study the signalling pathways of G protein-coupled receptors which are involved in diseases and cancer research, cells have been observed for markers that exhibit anticancer potential (Synthego, 2022b).

Table 2.1: Types of cancer and the associated cell lines

Cancer type	Description	Cell line (s)
Breast cancer	- 2.26 million people (WHO, 2020) Ductal carcinoma begins in the ductal cells and is known as the most common form of breast cancer. Other types of breast cancer can start in the lobules and other breast tissue (NIH, 2017).	MCF-7 MDA-MB-231 BT549
Prostate cancer	- 1.41 million people (WHO, 2020) Most prevalent in men. Triggered by cells that produce and secrete mucus. Prostate cancer affects males older than 65 years (NIH, 2017).	PC3 LNCaP DU-145
Leukemia	- 475 thousand people (WHO, 2020) Leukaemia is formed when immature blood cells become cancerous and eventually overwhelm healthy blood cells in bone marrow. Lymphoblastic leukaemia affects the lymphoblast cells, white blood cells that are responsible for fighting infections. White and red blood cells can be affected; however, leukaemia of white blood cells is the most common. More prevalent in people older than 55 and younger than 15 years (NIH, 2017)	THP-1
Lung cancer	- 2.21 million people (WHO, 2020) Categorised into two main types, namely, non-small cell lung cancer and small cell lung cancer. Differentiated when the cancerous cells are observed under a microscope. Most often lung cancer is a result of lifestyle choices such as smoking. Currently, no known treatments can cure this cancer (NIH, 2017)	A549 H-1299

2.6. Natural therapies

Approximately 80% of the global population use medicinal plants to treat a vast array of ailments with dietary supplements derived from natural sources having earned traction over the years as nutraceuticals (Aung et al., 2017). When carbohydrates, lipids, proteins, vitamins, or minerals are consumed they are metabolised into monosaccharides, amino acids, or peptides. These metabolites are then absorbed by the intestine and utilised by the body for benefits beyond basic nutrition (Quintal-Bojórquez and Segura-Campos, 2021). Some of the bioactive metabolites derived from these macronutrients include antioxidants.

Oxidative therapies employ oxidants to stimulate bodily processes that have the capability to fight cancer cells and heal tissue concurrently. Antioxidants such as glutathione can be used in conjunction with chemotherapy and radiation to minimise side effects (Stengler and Anderson, 2018). While resveratrol has demonstrated antiproliferative abilities on prostate and bowel cancer cells (Stengler and Anderson, 2018).
Curcumin derived from *Curcuma longa* or turmeric, suppresses proliferation and selectively triggers apoptosis in tumour cells (Gupta, 2020). L-asparaginase is an enzyme that has been utilised in the treatment of lymphoblastic leukaemia, acute myeloid leukaemia, and non-Hodgkin's lymphoma (Sebastian et al., 2020). The common trend for natural therapies is for apoptosis or programmed cell death to be triggered in a cell (Pfeffer and Singh, 2018). In a normal cell cycle, apoptosis controls cell numbers and proliferation through checkpoints or repair defects (Brown and Attardi, 2005; Zivny et al., 2010). When there is a defect in the normal apoptotic mechanisms of cells, the development of tumours is allowed (Alison, 2001; Zivny et al., 2010; Letai, 2017; D'Arcy, 2019).

Figure 2.3 depicts the effects that protons and hydrogen therapies have on oxidative stress and inflammation in oncogenesis. The reactive oxygen species or ROS through oxidative stress and inflammation stimulate oncogenesis in cells that have defective signalling pathways. The interconnection between oxidative stress and inflammation is associated with the activation of NRF2 and NF-κB.
Protons provoke distress in gene expression thereby inhibiting cancer cells from evading progression, while hydrogen reduces oxidative stress, employs anti-inflammatory effects, and acts as a modulator of apoptosis (Rochette et al., 2021).

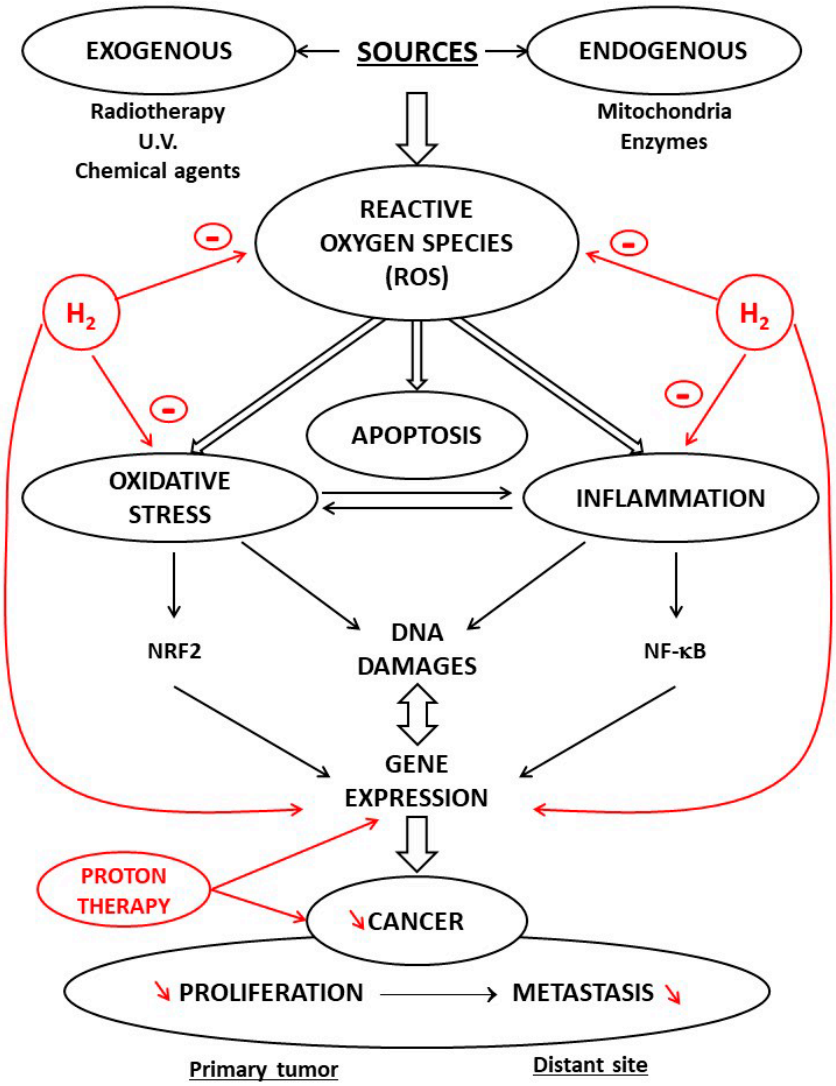

Figure 2.3: The effects of proton and hydrogen therapies on oxidative stress and inflammation in oncogenesis (Rochette et al., 2021)

2.7. Mechanisms of cell death

For tissue development and homeostasis of cells, timely death of the cell is a crucial process in their lifecycle (Marqus et al., 2017; An et al., 2019; Jan and Chaudhry, 2019). Apoptosis and necrosis are two mechanisms involved in cell death (Barrio and Anon, 2010; Zivny et al., 2010; Marqus et al., 2017; Jan and Chaudhry, 2019; Sabbione et al., 2019).

2.7.1. Apoptosis

Conversely, apoptosis is a controlled process that does not induce inflammatory responses (Zivny et al., 2010). Apart from cell regulation, apoptosis can be triggered by damage and external stress on cells. Apoptosis is characterised by cell shrinkage, membrane blebbing, chromatin material condensation, fragmentation of DNA in the nucleus, and loss of adhesion to extracellular matrices (Figure 2.4) (Jan and Chaudhry, 2019). Biochemical changes in a cell include externalisation of phosphatidylserine, and activation of caspases resulting in cell death (Liu et al., 2011; Jan and Chaudhry, 2019). Apoptosis is an essential mechanism that regulates the balance between survival and death in cells for the prevention of cancer (Jan and Chaudhry, 2019).

Chemotherapy is mainly based on promoting apoptosis however cancer cells have created evasion mechanisms for survival. Cancer cells inhibit the activation of apoptotic signals by increasing the expression of the anti-apoptotic gene, decreasing the expression of the pro-apoptotic genes and/or causing the stabilisation or destabilisation of the anti-apoptotic and pro-apoptotic genes respectively (Fernald and Kurokawa, 2013).

Apoptosis is moderated by intrinsic and extrinsic signalling pathways which are mediated by the cell's environment and intrinsic signals (An et al., 2019; Jan and Chaudhry, 2019; Avilés-Gaxiola et al., 2020). Intrinsic pathways involve mitochondria mediated pathways while extrinsic pathways involve the death receptor pathway. Both pathways are linked and can influence one another (Zivny et al., 2010; An et al., 2019).

The extrinsic pathway initiates apoptosis when the death ligand and the death receptor bind. Ligations of the death receptor's cell surface with the tumour necrosis factor (TNF) receptor

superfamily (such as CD95L, TRAIL and TNF-α), cause the receptor-ligand to activate caspase-8 or -10. Exciting the transcription factor nuclear, factor-B (NF-κB) and mitogen-activated protein kinases (MAPKs). These activities take place in the adaptive, non-apoptotic signalling pathways associated with the regulation of developmental and inflammatory processes, resulting in apoptosis (An et al., 2019).

The intrinsic pathway involves the discharge of apoptotic factors like cytochrome c from the mitochondrial membrane (Avilés-Gaxiola et al., 2020). Apoptosis can be triggered by a positive or negative pathway (D'Arcy, 2019). The negative signal occurs when the cells lack survival signals, cytokines, hormones, and growth factors. Consequently, the pro-apoptotic molecules (puma, noxa, and bax) that are usually inhibited in the cells become activated thereby inducing apoptosis (D'Arcy, 2019). Positive signals that stimulate apoptosis include hypoxia, toxins, radiation, and oxidative stress (D'Arcy, 2019).

Peptide sequence, GEGSGA, from *Phaseolus vulgaris* beans and *Gloriosa superba* hydrolysate, encouraged apoptosis by p53 upregulation against colorectal cancer. The apoptotic effect of GEGSGA was attributed to the decreased expression of tumour necrosis factor receptor type 1, a transmembrane receptor that induces the activation of anti-apoptotic proteins through subunit nuclear factor-kB p65 signalling (Avilés-Gaxiola et al., 2020). Vglycin, a polypeptide derived from pea seeds and sweet potato (*Ipomoea batatas*) hydrolysates reported apoptosis through increased expression levels of Bax (apoptotic protein). Consequently, decreased expression levels of anti-apoptotic proteins, Bcl-2 and Mcl-1, were also observed (Gao et al., 2017; Avilés-Gaxiola et al., 2020). Soybean lunasin and amaranth (*Amaranthus mantegazzianus*) hydrolysates promoted caspase-3 activation and cleavage of Poly (ADP-ribose) polymerase by functioning as an adapter molecule (Avilés-Gaxiola et al., 2020).

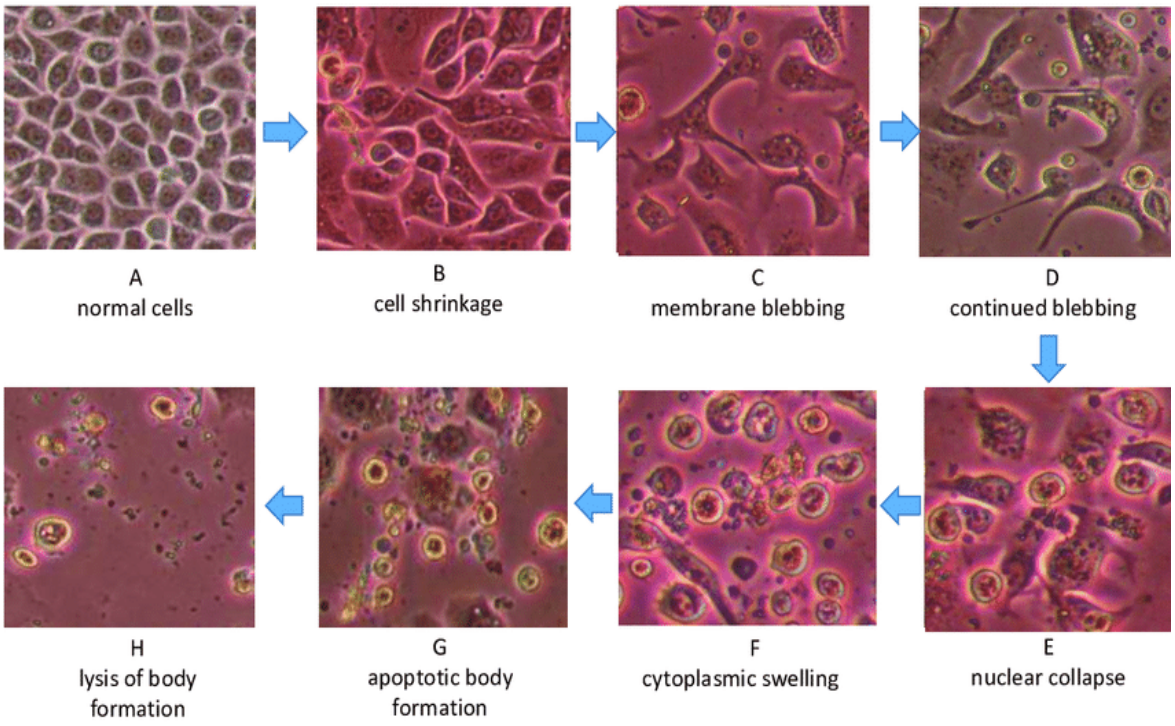

Figure 2.4: Stages of apoptosis in a cell (Amini-Sarteshnizi et al., 2014)

2.7.2. Necrosis

Necrosis is an uncontrolled cellular process that discards cells that are irreversibly damaged, consequently causing inflammatory reactions. Necrosis occurs when cells are irreversibly injured resulting in premature death (Zivny et al., 2010; Xu et al., 2019). The process is usually stimulated by factors such as toxic trauma or physical injury. Morphologically, necrosis can be characterised by the swelling of the cytoplasm and organelles, particularly the endoplasmic reticulum and mitochondria (Jan and Chaudhry, 2019).

The release of lactate dehydrogenase (LDH) into cell media is a common marker for necrotic cell death *in vivo*. LDH is detected when cells are harmed, subsequently damaging the integrity of the membrane and cell lysis (Barrio and Anon, 2010; Marqus et al., 2017; Sabbione et al., 2019).

2.7.3. Apoptotic and necrotic pathways

Necrotic pathways are being explored as an alternative to overcome cancer cell resistance as opposed to apoptotic mechanisms (D'Arcy, 2019). In a study by Sabbione et al. (2019), it was reported that amaranth peptides were able to promote necrosis in colon cancer cells, however further research was recommended on the mechanism of action. Anti-apoptotic proteins of the Bcl-2 family showed inhibition of both apoptotic and necrotic pathways. The two pathways are not independent and share some common messengers, activators, and inhibitors (Figure 2.5). Specifically, an apoptotic response could turn necrotic if intracellular ATP is depleted (Jan and Chaudhry, 2019).

Apoptosis and necrosis can be quantified by flow cytometry where cells are labelled with Annexin V and Propidium Iodide (PI). This protocol is based on the externalisation of phospholipid phosphatidylserine by Annexin-V staining and permeabilization of nuclear membrane for PI staining. The lifecycle of the cell can then be differentiated i.e., healthy (Annexin-V: positive/PI: negative), dying (early apoptosis: Annexin-V: positive/PI: negative) and dead cells (late apoptosis/necrosis: Annexin-V: positive/PI: positive) (D'Arcy, 2019; Sabbione et al., 2019).

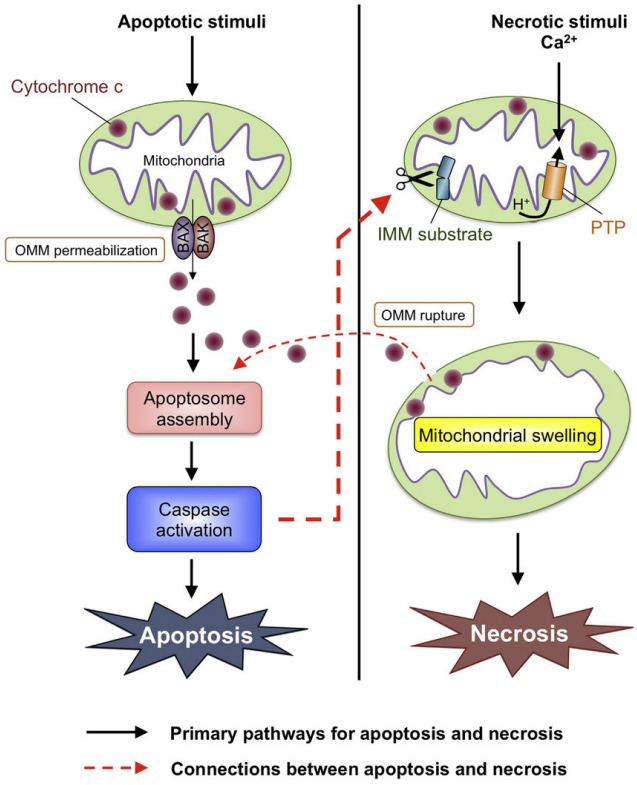

Figure 2.5: Pathways of apoptosis and necrosis (Amgalan et al., 2017)

2.8. Caspases

The activation of caspases from cysteinyl aspartate-specific protease family is also responsible for the triggering of apoptosis (An et al., 2019; D'Arcy, 2019). Caspases are categorised into initiator caspases (caspase-2, -8, -9 -10 and -12) and effector caspases (caspase-3, -6 and -7), both pathways are illustrated in Figure 2.6 (Saunders et al., 2000; An et al., 2019; D'Arcy, 2019). Initiator caspases such as caspase-9 are activated from the inactive procaspases when cell death is detected, consequently, executioner caspases (Caspase-3 and -7) are then activated (Zivny et al., 2010; D'Arcy, 2019). This activation is fundamental for DNA fragmentation, chromatin condensation, plasma membrane blebbing and ultimately cell death (Brauchle et al., 2014).

Tumour cells are protected from apoptosis by overexpression of anti-apoptotic proteins and have been noted in many human cancers such as prostate (Raffo et al., 1995), neuroblastoma (Castle et al., 1993), kidney (Gobe et al., 2002), and breast cancer (DelBufalo et al., 1997).

Activation of caspases is an effective approach used in the treatment of cancer and is especially important when developing alternative treatment protocols (An et al., 2019).

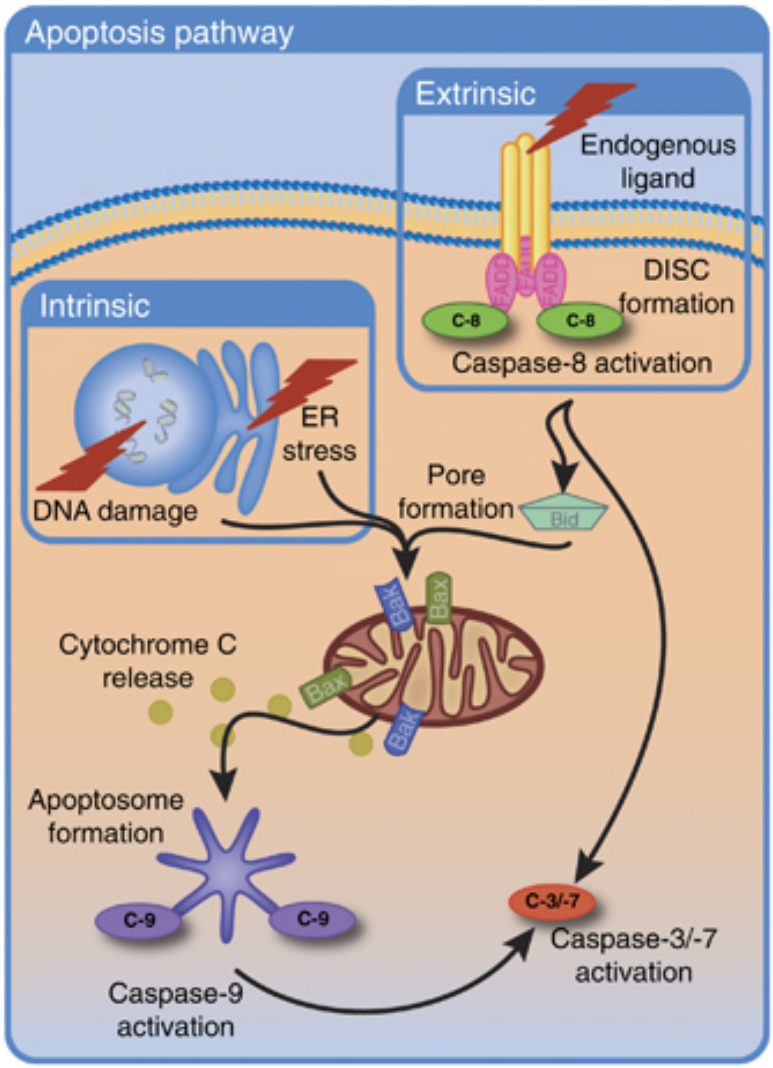

Figure 2.6: Caspase activation through intrinsic and extrinsic apoptotic pathways (Boland et al., 2013).

Caspase activation can be measured by Enzyme-linked Immunosorbent Assays (ELISA), fluorescent spectrophotometry and western blot. ELISA uses caspases labelled with biotinylated inhibitors captured on an ELISA plate coupled with a caspase specific antibody. The active caspase is detected by horseradish peroxidase (HRP)-streptavidin complex that attaches to the biotinylated inhibitor which is covalently bound to the enzyme (Saunders et al.,

2000). Fluorescent spectrophotometry measures activated caspases, the cleavage of the substrate between DEVD (the tripeptide substrate specific to caspase-3) and AMC fluorophore (7-Amino-4-methylcoumarin) generates extremely fluorescent AMC. Excitation of AMC is identified at 380 nm and emission between 420-460 nm. Caspase activation determined by western blot makes use of antibodies that can detect full-length caspases. Activation is detected by the decrease in band length when compared to the full length pro-caspase as well as the appearance of smaller cleaved products (Chehade et al., 2021).

2.9. Tumour suppressor proteins (p53)

Tumour suppressor protein, p53, takes on a protective role in decreasing the development of cancers by initiating apoptosis in 'suspicious' cells (Brown and Attardi, 2005; Letai, 2017; Marqus et al., 2017; Xu et al., 2019). However, a mutation in the gene makes the protective mechanism useless against tumorigenesis resulting in different outcomes (Figure 2.7) (Brown and Attardi, 2005; Jan and Chaudhry, 2019; Xu et al., 2019). Analysis of the DNA binding capacity of p53 in cancer cells can be done by electrophoretic mobility shift assay (EMSA) on polyacrylamide/ agarose gels, DNA footprinting and ELISA, with the latter being commonly used to quantify the p53 protein present in cell samples.

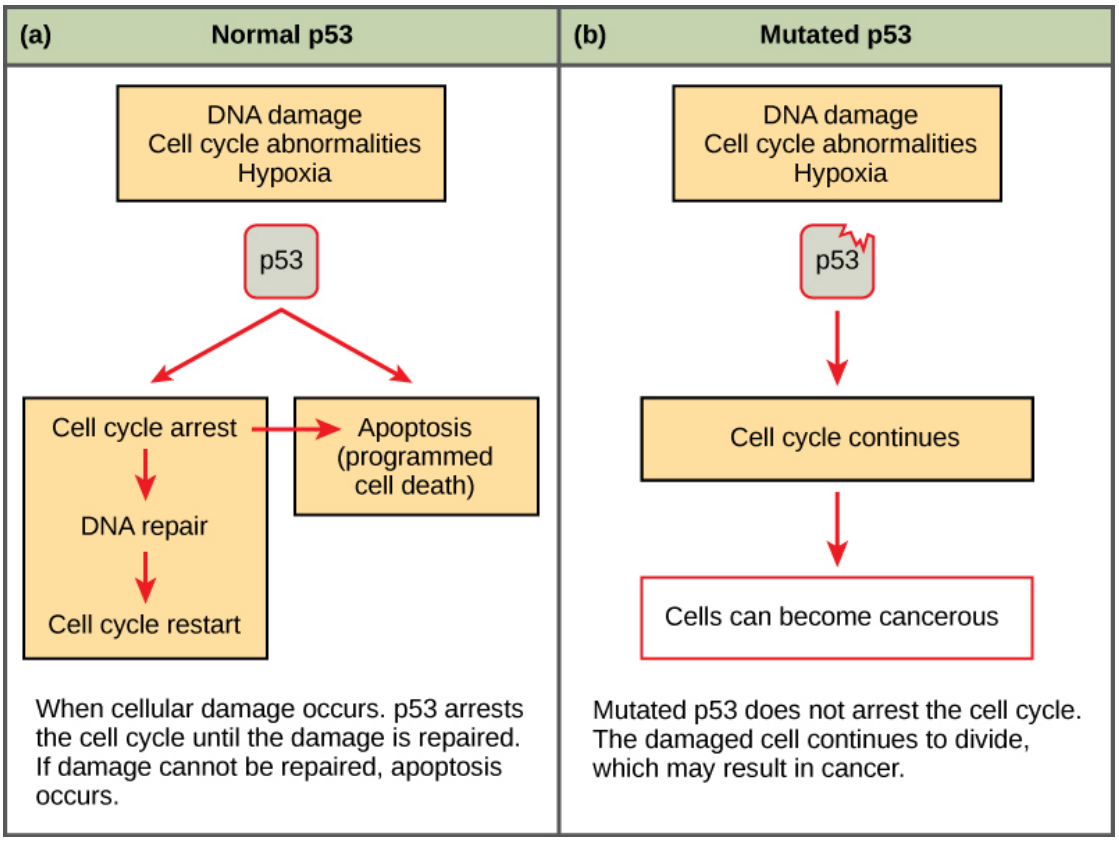

Figure 2.7: (a) Normal p53 triggering repair mechanisms when abnormal cells are discovered. (b) Mutated p53 allows abnormal cells to continue growing resulting in cancerous cells (Rye et al., 2016).

DNA damage, hypoxia, and expression of oncoproteins (e.g. Myc and Ras) trigger the p53-dependent apoptotic pathway. Subsequently, pro-apoptotic factors such as Bax are activated while suppressing anti-apoptotic factors like Bcl-2 (Xu et al., 2019). Intrinsic and extrinsic stress signals can shift dormant p53 to an active form thereby promoting p53 accumulation in a cell nucleus (Jan and Chaudhry, 2019). The main role of p53 is to stimulate apoptosis in a transcription-dependent and transcription-independent approach. This function is performed by transcription activation of pro-apoptotic Bcl-2 family proteins and transcription inhibition of anti-apoptotic Bcl-2 family proteins. p53 also has the ability to interact directly with Bax which in turn stimulates the release of cytochrome C by way of mitochondrial outer membrane permeabilization (MOMP) and assists in initiating apoptosis (Jan and Chaudhry, 2019).

p53 levels are usually low due to rapid degradation by ubiquitin-dependent proteolysis. Increased p53 levels occur as a response to cellular stress and significant cell damage, ultimately triggering apoptosis. The main cellular opponent of p53, murine double minute 2 (MDM2) oncogene, attaches to p53 and subsequently suppresses its activity as a transcription factor. Peptides designed from the amino group of p53 were aimed to prevent interaction between MDM2 and p53 thereby, inhibiting the swift degradation of p53 (Marqus et al., 2017). The TIP peptide created from the N-terminal MDM2-binding domain region was shown to hinder the interaction between p53 and MDM2. This resulted in accumulation of p53 in the cells and stimulation of p53 transcription factor (Marqus et al., 2017).

Three peptides comprising 12 and 26 amino acid residues were synthesised from the MDMD2-p53 domain: PNC-27, PNC-21, and PNC-28. These peptides showed *in vitro* cytotoxicity in human metastatic colon adenocarcinoma cells, transformed rat brain capillary endothelial cells, human cervical carcinoma, human metastatic breast carcinoma cells, human non-small cell lung carcinoma and human osteosarcoma. However, they did not have a cytotoxic effect on noncancerous cells such as rat pancreatic acinar cells (Marqus et al., 2017).

Cytotoxicity of PNC-27 was investigated in human breast cancer cell lines, namely, MDA-MB-468 (mutant p53); MCF-7 (overexpressed wild type p53), and MDA-MB-157 (null p53). Necrosis was induced in the three cell lines followed by cell lysis. In the null p53 cell line, K563, treated with PNC-27 it was shown that the peptide caused pore formation in the membrane thereby resulting in cell death (Marqus et al., 2017). Penetratin joined in the C terminal end of PNC-28 induced necrosis in human pancreatic cell lines. PNC-28 on its own, allowed cells to undergo apoptosis by deploying caspase-3 and -7 (Marqus et al., 2017).

2.10. Antioxidants

Cellular damage can be caused by an excessive amount of free radicals or oxidants in the human body (Singh et al., 2014). Free radicals in the form of reactive oxygen species (ROS) and reactive nitrogen species (RNS), are naturally created in the human body through processes such as normal respiration (Olagunju et al., 2018; Orona-Tamayo et al., 2018). In some instances, free radicals are regarded as useful in cells for facilitating processes such as cell proliferation, apoptosis, and defence against infections (Chahal et al., 2018; Orona-

Tamayo et al., 2018; Chen et al., 2019). Under normal physiological conditions, cells control ROS and RNS levels via scavenging systems (Chi et al., 2015). However, when cells undergo oxidative stress, the production or accumulation of ROS and RNS has the ability to overload antioxidant defences resulting in damage to cellular proteins, lipids, and DNA (Chi et al., 2015).

Accumulation of ROS and RNS has the ability to trigger chronic illnesses such as hypertension, cancer, cardiovascular disease and diabetes (Orona-Tamayo et al., 2018). Oxidative stress in the human body performs the role of promoter rather than the initiator of chronic diseases (Sarmadi and Ismail, 2010). The presence of free radicals in the body is attributed to environmental (e.g. pollutants, heavy metals and UV radiation) and lifestyle (e.g. poor diet and smoking) factors (Sarmadi and Ismail, 2010; Jahanbani et al., 2016). Although the human body provides some defence against oxidative stress, it is still insufficient to fully protect the body (Olagunju et al., 2018). Diets rich in antioxidants are required to offer higher levels of protection against oxidative stress and consequently, chronic diseases associated with ROS (Chi et al., 2015; Olagunju et al., 2018).

Synthetic and natural antioxidants are therefore required for the prevention of oxidative stress. Although synthetic antioxidants have advantages such as cost-efficiency and efficacy, they are also associated with toxic and hazardous effects. An example of a potentially hazardous synthetic antioxidant is butylated hydroxytoluene (BHT) which is added to foods as a preservative (Sarmadi and Ismail, 2010; Torres-Fuentes et al., 2015; Roy et al., 2021). Although BHT shares some structural similarities to BHA (butylated hydroxyanisole) which has shown potential toxigenic effects more research is required to substantiate claims as both BHT and BHA are known additives within the food sector (Felter et al., 2021).
Natural antioxidants such as vitamins C and A as well as herbal and tea considered a safer option as they are associated with little or no hazardous effects (Sarmadi and Ismail, 2010; Torres-Fuentes et al., 2015; Roy et al., 2021). Studies have reported that the consumption of fruits and vegetables can lower the risk of diseases related to oxidative stress as plants are one of the leading natural sources of antioxidants (Torres-Fuentes et al., 2015). Plants are also regarded as an abundant source of natural protein that may possess the ability to exert antioxidant activity due to associated amino acids.

Antioxidants are often used to supplement cancer treatment (Chahal et al., 2018). Oxidative stress ensues when there is an imbalance between ROS being produced and the defence antioxidants. The imbalance results in the overproduction of ROS thereby bringing about oxidative stress causing inhibition of normal functioning of cellular lipids, proteins, DNA and RNA being inhibited. Studies on application of natural antioxidant compounds for efficient removal of free radicals are of growing interest (Gulcin, 2012). Defence mechanisms of antioxidants are established by hydrogen-donating, electron-donating, metal-ion-chelating and radial-scavenging abilities of the peptides (Matemu et al., 2021).

Amino acids from plants can act as metal chelating and hydrogen donors (Torres-Fuentes et al., 2015). Control of oxidative stress can be a vital action in decreasing the progression of illnesses and could be used to prevent complications that may arise (Sarmadi and Ismail, 2010). Peptides can be found in food sources that have high protein content (Paula et al., 2020). Antioxidants are naturally present in legume seeds (López-Cortez et al., 2016). Legume proteins derived from soybean, chickpea, lentil, mung bean and cowpea have been discovered as safer options than synthetic antioxidants (Matemu et al., 2021).

2.11. Antioxidant peptides

Antioxidant peptides from food sources are regarded as safe and healthy compounds. Antioxidant peptides can be categorised as endogenous and exogenous peptides (Jakubczyk et al., 2020; Wong et al., 2020). Endogenous peptides are naturally occurring in cells and can exert cytoprotection through the detoxification of free radicals (Jakubczyk et al., 2020; Wong et al., 2020). These include glutathione, carnosine, and human tripeptide GHK (Jakubczyk et al., 2020; Wong et al., 2020).

Exogenous peptides are derived from food proteins which have undergone enzymatic hydrolysis (Jakubczyk et al., 2020). Antioxidants from food usually have a lower molecular weight, higher activity, and easier absorption (Sarmadi and Ismail, 2010). Enzymatic hydrolysis of proteins results in the exposure of more active amino acid R groups resulting in the higher antioxidant activity of a peptide compared to a protein (Singh et al., 2014; Wong et al., 2020). The properties of a peptide are dependent on the amino acids present in its

structure and hydrophobicity (Sarmadi and Ismail, 2010; Jakubczyk et al., 2020; Paula et al., 2020).

Amino acids with antioxidative properties include tyrosine, tryptophan, methionine, lysine, cysteine, and histidine (Sarmadi and Ismail, 2010; Wong et al., 2020). Aromatic amino acids can donate protons to radicals that require an electron consequently providing the potential to improve the radical scavenging activity of the amino acid (Sarmadi and Ismail, 2010). Antioxidant peptides can be applied in food additives, health-promoting supplements, and therapeutic agents with approximately 50 peptide drugs having already been developed and marketed globally (Wong et al., 2020).

Antioxidant peptides derived from plant sources have also been studied *in vitro* using cellular models and oxidative stress treatments (Wong et al., 2020). These studies assess a peptide's capacity to reduce ROS, prevent oxidative stress and protect cellular redox balance (Wong et al., 2020). Cancer cell lines hepatocarcinoma (HepG2), intestinal (Caco-2) and cervical (HeLa) have been used to analyse the effects of antioxidant peptides (Wong et al., 2020).

Soybean derived peptides, IYVVDLR and IYVFVR, showed a reduction in ROS generation in a dose-dependent approach against hydrogen peroxide treated Caco-2 cells. The results are in line with the ability of the peptides to improve cellular glutathione content and the activities of catalase and glutathione peroxidase (Zhang et al., 2019).

2.12. Antioxidant activity assays

There are numerous methods for the determination of radical scavenging ability. Assays for measuring antioxidant activity can be categorised into two groups namely, hydrogen atom transfer (HAT) and single electron transfer (SET). HAT assays utilise a competitive reaction, antioxidants and substrates contend for thermally created peroxyl radicals. The SET method measures the capacity of an antioxidant in the reduction of an oxidant. It also behaves as the probe for monitoring the reaction and indicator of the reaction endpoint (Sarmadi and Ismail, 2010). Several methods are therefore required to determine the mechanisms involved in free radical scavenging capacity of a compound (Thumbrain et al., 2020).

2.12.1. 2,2-dipheny-2-picrylhydrazyl Assay (DPPH)

The DPPH assay is rapid, simple, reliable and does not require any specialised equipment. DPPH is a stable synthetic radical that does not deteriorate in water, methanol, or ethanol. The DPPH free radical, at a maximum wavelength of 517 nm, can receive a hydrogen or an electron easily from antioxidants. A DPPH radical made up in methanol is converted to DPPH-H (diphenylhydrazine) molecules in the presence of an antioxidant agent. The resulting discolouration of the DPPH demonstrates the radical scavenging activity (Aksoy et al., 2013).

A study by Thumbrain et al. (2020) assessed protein isolates from cowpea cultivars where it was found that the cultivars, Vegetable cowpea 3 (78.95%) and Embu buff (74.21%) had comparable DPPH radical scavenging activity to the control, ascorbic acid (85.84%). In other studies, *Amaranthus cruentus* protein isolates and hydrolysates showed that radical scavenging activity increased after hydrolysis. Among the isolates, pepsin showed the lowest IC_{50} value of 23.06 ug/mL while trypsin showed the greatest of 34.41 ug/mL (Ramkisson et al., 2020). Pigeon Pea isolate, and hydrolysates showed EC_{50} values ranging between 0.91 and 2.44 mg/mL (Olagunju et al., 2018). The authors also reported higher activity on their fractions suggesting that ultrafiltration improves DPPH radical scavenging activity (Olagunju et al., 2018). The results observed by the authors indicate that hydrolysates and fractions derived from legumes possess potent radical scavenging abilities.

2.12.2. Ferric Reducing Antioxidant Power Assay (FRAP)

FRAP, is a colourimetric assay that evaluates the capability of plasma to decrease the intense blue ferric tripyridyltriazine complex to its ferrous form. The FRAP assay is simple, relatively easy to use and inexpensive (Badarinath et al., 2010). In a study looking at antioxidant capacity of protein hydrolysates, the IC_{50} values of cowpea cultivars showed the following results: Vegetable cowpea 3: 5.89; Vegetable cowpea 2: 3.22; Makhatini: 2.46; Embu buff: 1.71; Glenda: 1.13 and Ascorbic acid: 2.59 mg/mL.
The Glenda cultivar had the best activity indicated by the lowest IC_{50} value (Thumbrain et al., 2020). When compared with a known antioxidant, ascorbic acid, the cultivars proved to have strong antioxidant potential.

2.12.3. 2,2'azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) Assay (ABTS)

ABTS measures an antioxidant's, hydrogen or electron donor, suppression of the radical cation ABTS•+ based on single electron reduction of the reasonably stable radical cation ABTS•+ that was created by an oxidation reaction. When added to phosphate buffered saline (PBS) medium that contains ABTS•+, the proteins in the hydrolysate and peptide fractions probably acted as electron donors, altering this radical cation into the nonradical ABTS (Hernández-Ledesma and Hsieh, 2013). Walnut protein peptides showed decreased antioxidant activity of the peptide fractions compared to the hydrolysate (Jahanbani et al., 2016). These results show that smaller peptide fractions are not necessarily more potent that the hydrolysates. The hydrolysate may cause a synergistic effect when exhibiting its radical scavenging abilities. Bambara protein hydrolysates however showed superior antioxidant activity compared to the isolate and glutathione (Arise, 2016). Glutathione is a potent antioxidant, therefore the results observed imply that the Bambara protein isolate would also be a potent antioxidant agent. The authors of these studies have shown that hydrolysates have a high potential as radical scavenging agents.

2.12.4. Superoxide radical scavenging activity

The superoxide radical is a toxic free radical that can be created through many biological reactions (Olagunju et al., 2018). *In vivo*, the superoxide radical has the capability to encourage oxidative reactions because it can reduce transitional metals and react with hydroxyl radicals subsequently causing damage to vital components in a cell (He et al., 2013) Bambara protein hydrolysates and peptide fractions showed lower superoxide radical scavenging when compared to the standard, glutathione (Arise et al., 2016). Pigeon pea ultrafiltration fractions showed improved radical scavenging over crude hydrolysates (Olagunju et al., 2018). It is vital to compare the protein isolates and hydrolysates with known antioxidants such as ascorbic acid, gallic acid and glutathione to show the potential of the sample. The Bambara hydrolysates proved to be less potent while the pigeon pea ultrafiltration fractions were observed to be a good antioxidant agent.

2.13. Amino acids and proteins

Amino acids are commonly referred to as building blocks of proteins. The basic structure (Figure 2.8) consists of a carboxyl group (COO^-) and an amino group (NH_3^-) on either end of the α-carbon atom and an 'R' group or side chain with the side chain giving the amino acid its specific properties. These include whether the amino acid is acidic, basic, polar or nonpolar (Yada, 2004; Buxbaum, 2007).

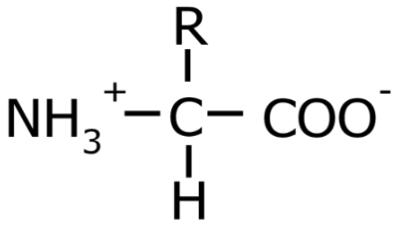

Figure 2.8: General structure of an amino acid.

There are over 300 naturally occurring amino acids found in plants and animals however, food proteins are composed of 20 amino acids (Yada, 2004). Amino acids are linked together by peptide bonds developed between α-amino and α-carboxylic acid groups of adjacent amino acids in the polypeptide sequence (Yada, 2004).

Proteins are a vital component of food and are essential for the functioning of the body (Orona-Tamayo et al., 2018). They are a group of complex organic compounds, composed of amino acids (Yada, 2004). Food proteins contain several biologically active peptides. Peptides can make use of positive physiological reactions in the body, apart from the basic nutritional roles of providing nitrogen and essential amino acids (Hernández-Ledesma and Hsieh, 2013). Legumes are regarded as a superior source of macronutrients when compared to cereals due to their lower glycaemic indexes and fat, and higher protein, fibre, and carbohydrate contents (Maphosa and Jideani, 2017).

2.14. Bioactive peptides

Bioactive peptides are short-chain amino acid chains usually consisting of 20 to 50 amino acid residues (Lopez-Barrios et al., 2014; Marqus et al., 2017; Avilés-Gaxiola et al., 2020). When released from their parent protein, the bioactive potential of peptides have been shown to become heightened (Korhonen and Pihlanto, 2006; Lopez-Barrios et al., 2014; Montoya-Rodríguez et al., 2015; Daliri et al., 2017; Salampessy et al., 2017; Hsieh et al., 2020). Peptides as therapeutic agents compared to proteins and antibodies hold many key advantages. They are small, therefore able to penetrate the cell membrane and are easier to synthesise. They also have a high affinity (the degree to which a substance tends to combine with another), strong specificity for targets, lower toxicity (low/no accumulation in organs such as kidneys or liver) and good penetrating ability of tissue (Hernández-Ledesma and Hsieh, 2013; Luna-Vital et al., 2015; Marqus et al., 2017; Avilés-Gaxiola et al., 2020; Thumbrain et al., 2020). The toxicity of peptides is low as degradation would yield amino acids which are innately used by cells (Avilés-Gaxiola et al., 2020).

The type of bioactive peptide released from an individual protein is reliant on factors such as the main sequence of the parent protein together with specificity of the enzymes used to produce the peptide (Hernández-Ledesma and Hsieh, 2013; Chalamaiah et al., 2018). Peptides composed of two or three residues have been reported to be useful after release by proteolytic enzymes (Orona-Tamayo et al., 2018). Many biologically active peptides have been determined through hydrolysis of plant proteins such as opioid, antihypertensive, antioxidant, immunomodulatory or antimicrobial peptides (Hernández-Ledesma and Hsieh, 2013). Accurate characterisation of peptides is done by isolating and initial fractionation of hydrolysates from the food source. Thereafter purification and characterisation with proteomic techniques are essential in allocating the peptide to its specific bioactive properties (Kannan et al., 2010).

Peptides that have been released due to hydrolysis are not the same as naturally occurring bioactive peptides like endorphins because they are produced by proteolysis of the parent food proteins (Udenigwe and Aluko, 2012). Bioactive peptides can play a role in broadening the use of crops and animal products that exceeds their basic nutritional uses. Peptides could then be incorporated into food products to provide additional health benefits (Udenigwe and Aluko, 2012). The bioactivity of a peptide against a targeted disease will depend upon the

structure of the peptides such as chain length and physiological properties of the amino acid residues (e.g., molecular charge, hydrophobicity, and side-chain bulkiness) (Udenigwe and Aluko, 2012; Chalamaiah et al., 2018).

The group of amino acids from food-derived anticancer peptides are hydrophobic in nature. Hydrophobic amino acids include proline, leucine, glycine, and alanine as well as one or more residues of lysine, arginine, serine, glutamic acid, threonine, and tyrosine (Chalamaiah et al., 2018). Hydrophobic amino acids are advantageous in anticancer studies as they can improve interactions between anticancer peptides and the outer membrane bilayer of tumour cells, resulting in better selectivity and stronger cytotoxic activity (Chalamaiah et al., 2018). The selectivity and susceptibility of cells to rupture when treated with peptides can be determined by the composition of cell membrane bilayers and the distribution of phospholipids (Chalamaiah et al., 2018). *In vitro* and *in vivo* investigations have shown that many naturally occurring compounds found in diets could potentially lower cancer risk and even sensitise tumour cells against anticancer therapies.

2.14.1. Bioactive peptides from food proteins

Peptides derived from food proteins have gained popularity because of their added benefits to human health (Korhonen and Pihlanto, 2006; Martínez-Leo et al., 2019). Milk, seafood, and leguminous crops are some of the protein sources that have been reported to exhibit bioactive potential (Girón-Calle et al., 2010; Chang et al., 2014; Lopez-Barrios et al., 2014; Kamran and Reddy, 2018). Some of the reported biological activities possessed by peptides include anticancer, antioxidant, antihypertensive, antidiabetic, antimicrobial and antithrombotic activities (Lopez-Barrios et al., 2014; Rocha et al., 2014; López-Cortez et al., 2016).

Twelve peptides were identified from mung bean protein hydrolysates with significant antioxidant ability (Sonklin et al., 2021). Sesame seed protein hydrolysed by pepsin and pancreatin showed hydrolysed protein to have enhanced antioxidant abilities compared with the unhydrolyzed protein isolate (Idowu et al., 2021). Maize peptides also demonstrated that strong antioxidants assist in inhibiting cancer cell proliferation (Díaz-Gómez et al., 2017). Peptides derived from oysters inhibited cell growth and showed apoptotic morphological changes and oxidative DNA damage (Umayaparvathi et al., 2014). Rohu egg protein

hydrolysates exhibited antiproliferative activity on colon cancer cell line, Caco-2 (Chalamaiah et al., 2018).

2.14.2. Bioactive peptides from legumes

For centuries, legume crops have been a staple food in many developing countries. These crops are cost-effective, easily adaptable and a source of both protein and calories to the consumer (Lopez-Barrios et al., 2014). Studies have observed that populations that consume a larger amount of legumes in their diets have a decreased incidence of certain cancers (Carbonaro et al., 2015). Soy, chickpea, cowpea, and lentils are some common legumes that have been researched for their bioactive potential (Kamran and Reddy, 2018). Soy protein that was hydrolysed by alcalase showed inhibition of liver, lung, and colon cancer cells (Chatterjee et al., 2018).

2.14.3. Mechanism of action of legume derived peptides

In essence, effective alternate therapies should target cancer cells without causing excessive harm to healthy cells (Huang et al., 2011). Positively charged peptide membranes interact with the negatively charged membranes of cancer cells subsequently destroying them by micelle formation. Healthy cells should not be affected by peptides as they are neutrally charged (Barari et al., 2017; Avilés-Gaxiola et al., 2020; Sharma et al., 2020).

The anticancer potential of a peptide is also reliant on its amino acid sequences (Avilés-Gaxiola et al., 2020). Peptides with hydrophobic amino acids interact intracellularly as they are soluble across cancer cell membranes. Additionally, aromatic and negatively charged amino acids can aid peptides in the penetration of cancer cells (Avilés-Gaxiola et al., 2020).

2.14.4. Current Status of Bioactive Peptides Studies

Most *in vitro* studies assess cytotoxicity using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Cell morphology is also a tool used for visually depicting the cell, with dyes such as ethidium bromide, Hoechst and DAPI stains allowing for cells to be viewed under a microscope. The mechanisms of apoptosis such as Annexin V-PI, caspases-3, -7 and/or -9 and p53 can also be quantified. Outlined in Table 2.2 are some of the recent

studies associated with the inhibition of cancer cells using proteins/ peptides from different leguminous sources. In a study by Thumbrain et al. (2020), protein isolate derived from cowpea was shown to inhibit A549 and MCF-7 cancerous cell lines while minimally affecting the healthy HEK293 cell line. The isolate induced apoptosis was supported by caspase 3/7, Annexin-V FITC and morphology. *Amaranthus cruentus* hydrolysates were also able to induce apoptosis on A549, MCF-7 and HEK293 (Ramkisson et al., 2020). *Amaranthus mantegazzianus* isolates, MW>3 kDa (MPI) and MW<3 kDa (MPI-UF) were able to inhibit MC3T3-E1, UMR106, Caco-2, TC7 cells (Barrio and Anon, 2010).

Table 2.2: Current studies on the apoptotic activity of protein isolates and peptides from legumes

Source	Cell lines	Evidence	Reference
Cowpea (*Vigna anguicuilata*) var. *Embu buff* isolate	HEK293, A549, MCF-7	**Cytotoxicity (MTT)** exhibited consistent inhibition (IC_{50}) on A549 (245.6 µg/mL) and MCF-7 (16.63 µg/mL) while having the least harmful effect on HEK293 (317.90 µg/mL). **Cell morphology (acridine orange staining).** Untreated controls appeared well-shaped while treated cells appeared rounded. Necrotic cells were not observed. **Annexin-V FITC by flow cytometry.** Camptothecin was effective against MCF-7 cells due to the lowered apoptotic activity however toxicity to normal cells is a primary reason for alternative treatments. *Embu buff* isolate proved to have a better effect on MCF-7 cells rather than A549 cells. **Caspase 3/7 activity by fluorescence spectrophotometry.** Normal cells (HEK293) showed a higher caspase 3/7 activation as compared to cancerous cells (MCF-7 and A549). The disproportionality of normal cells being sensitive to change in growth parameters resulting in cells being stressed while cancerous cells are more resistant to change. *Embu buff* isolates possess anticancer activity on cancerous cells and a protective effect on normal cells.	(Thumbrain et al., 2020)
***Amaranthus cruentus* isolate and hydrolysates**	HEK293, A549, MCF-7	**Cytotoxicity (MTT).** IC_{50} of MCF-7 cells treated with Isolate (3.55 µg/mL), alcalase hydrolysate (965.50 µg/mL), Trypsin hydrolysate (3.87 µg/mL), pepsin hydrolysate (173 µg/mL) and camptothecin (9.35 µg/mL). A549 cells treated with Trypsin hydrolysate IC_{50} of 14.10 µg/mL and camptothecin 304.9 µg/mL. IC_{50} of HEK293 cells treated with isolate (8.57 µg/mL), alcalase hydrolysate (11.07 µg/mL) Camptothecin (9.10 µg/mL). **Annexin-V FITC by flow cytometry.** The majority of untreated cells were alive. Cells treated with the isolate and trypsin hydrolysate showed an increase in early apoptotic (cells are apoptotic but still alive) and late apoptotic cells (dead cells). Trypsin hydrolysate treated MCF-7 (38.5%) and A549 (28.7%) during early apoptosis. **Caspase 3/7 activity by fluorescence spectrophotometry.** Higher caspase activity was exhibited by the isolate and trypsin hydrolysate when compared to the control, camptothecin. HEK293 cells showed greater sensitivity to environmental changes.	(Ramkisson et al., 2020)
***Amaranthus mantegazzianus* isolates, MW > 3 kDa (MPI) and MW <3 kDa (MPI-UF)**	MC3T3-E1, UMR106, Caco-2, TC7	**Cell proliferation (crystal-violet bioassay).** MPI and MPI-UF treated cells revealed an antiproliferative effect on both cell lines. However, growth inhibition was shown to be greater with UMR106 cells (IC_{50} 1.0 mg/mL) compared to MC3T3-E1 cells (IC_{50} 2.5 mg/mL). Caco-2 and TC7 cells treated with MPI showed IC_{50} of 1.5 and 2.5 mg/mL, respectively. **Cell morphology (Giemsa staining).** UMR106 cells treated with MPI appeared as multilateral morphology with well-stained irregular-shaped nuclei and a well-defined vacuole-containing cytoplasm before incubation. After 24 h, condensation was noted MPI at 1 mg/mL. At 2 mg/mL, cells appeared to be undergoing apoptosis due to the observation of cellular pykonosis, fragmented nuclei and undefined cell borders. **Inhibition of cell adhesion (PROBIT).** UMR106 cells treated with MPI showed 50% inhibition at a 0.5 mg/mL concentration. **Annexin-V PI and LDH.** Lactose dehydrogenase (LDH) activity was increased as the MPI concentration increased (up to 7-fold at 2 mg/mL). Annexin-V PI labelled cells treated with MPI showed an increase in apoptotic cells as the concentration increased (up to 5-fold at 2 mg/mL)	(Barrio and Anon, 2010)
Soybean (SB), Black soybean (BSP), Adzuki bean (ABP), and mung bean (MBP) isolates	SKOV3, SMMC-7721	**Cell proliferation (MTT).** SKOV3 cell-treated SP, BSP, ABP, and MBP showed IC_{50} values 431.0, 327.9, 720.4, and 505.1 µg/mL, respectively. SMMC-7721 cells treated with isolates were 143.5, 88.9, 391.0, and 323.6 µg/mL, respectively.	(Chen et al., 2017)

2.15. Protein and Peptide isolation, purification, and identification

2.15.1. Protein extraction

Plant proteins are extracted through methods such as isoelectric precipitation, salt extraction and acid/ alkaline extraction-isoelectric precipitation (IEP). Acid/ alkaline extraction-isoelectric precipitation (IEP) employs acidic or alkaline conditions to dissolve proteins followed by isoelectric precipitation (Han and Hamaker, 2002). Isoelectric precipitation is a widely used protocol where plant proteins are solubilised at alkaline pH conditions (pH 8-10) and thereafter precipitated at isoelectric point (pH 3-5) (Wong et al., 2020). Water and salt extraction methods are based upon ionic strength to dissolve and fractionate storage proteins, albumin, and globulin (Boye et al., 2010). Once proteins are isolated, hydrolysis is carried out to break amino acid bonds and liberate peptide sequences.

2.15.2. Enzyme hydrolysis

Controlled enzymic hydrolysis is one of the common methods employed for the release of peptides from their parent protein (Rayaprolu et al., 2017; Jakubczyk et al., 2020; Wong et al., 2020). Microbial fermentation, food processing and chemical synthesis are alternative methods (Jakubczyk et al., 2020; Yeo and Shahidi, 2021). Proteases, alcalase, flavourzyme, pepsin, trypsin and pancreatin are enzymes commonly used for hydrolysis (Wong et al., 2020; Quintal-Bojórquez and Segura-Campos, 2021). They can be utilised both individually or in combination, depending on the type of peptides required (Hernández-Ledesma and Hsieh, 2013; Wong et al., 2020; Yeo and Shahidi, 2021).

Hydrolysis requires appropriate reaction conditions such as time, temperature and enzyme-substrate ratio for peptide chains to be adequately released from their parent protein (Yeo and Shahidi, 2021). Shorter chain peptides can be obtained by longer cleavage times and the use of broad-spectrum proteases. Shorter chained peptides are favoured as they hold many advantages such as inexpensive synthesis, ease of modification and superior bioactivity, selectivity, and specificity (Apostolopoulos et al., 2021). However, a drawback of enzyme hydrolysis is that peptide sequences and sizes can differ even when the same protease is used (Tacias-Pascacio et al., 2020). Therefore the potential of bioactive peptides is dependent on amino acid sequence and peptide length (Karami and Akbari-adergani, 2019; Yaghoubzadeh et al., 2020).

Alcalase, produced from *Bacillus licheniformis*, is a broad-spectrum protease thereby hydrolysing most peptide bonds. However, preference is given to aromatic and hydrophobic amino acids like phenylalanine, tyrosine, tryptophan, leucine, isoleucine, valine and methionine (Hernández-Ledesma and Hsieh, 2013; Olagunju et al., 2018). Flavourzyme, a fungal protease complex, also has broad-spectrum specificity (Hernández-Ledesma and Hsieh, 2013).

Pepsin hydrolyses peptide bonds with hydrophobic amino acid residues, particularly aromatic amino acid residues such as phenylalanine, tryptophan, and tyrosine. During hydrolysis, bonds are cleaved, releasing the bioactive hydrophobic peptides veiled within the inner core of the protein (Chalamaiah et al., 2018; Taniya et al., 2020). Trypsin cleaves proteins at the C-terminal of the amino acids lysine and arginine (Luna-Vital et al., 2015). Pancreatin is a serine protease released by the pancreas in the small intestine such as trypsin, chymotrypsin, and elastase (Olagunju et al., 2018).

The resultant peptides are reported to be unaffected by pancreatin thereby suggesting that they could be absorbed by digestive epithelial cells in the small intestine (Hernández-Ledesma and Hsieh, 2013). Peptides achieved from hydrolysis can be purified and identified by techniques such as selective protein precipitation, membrane filtration, ion exchange, gel filtration and liquid chromatography (Hernández-Ledesma and Hsieh, 2013).

2.15.3. Purification and identification

Identification of peptide structure and amino acid sequence can give valuable insight into their functional properties (Rayaprolu et al., 2017). Analytical separation and purification techniques can be used to distinguish important biological modulators (Rayaprolu et al., 2017). Separation techniques like microfiltration, ultrafiltration, nanofiltration and reverse osmosis are used to separate peptides according to defined molecular weight ranges (Rayaprolu et al., 2017; Wong et al., 2020; Yeo and Shahidi, 2021). Reverse Phase High Performance Liquid Chromatography (RP-HPLC) with ion exchange, size exclusion or gel filtration chromatography can be utilised for peptide purification (Lopez-Barrios et al., 2014; Singh et al., 2014; Li-Chan, 2015; Rayaprolu et al., 2017; Wong et al., 2020).

RP-HPLC can also be used for fractionating peptides based on their hydrophilic/hydrophobic properties (Yeo and Shahidi, 2021). However, the most efficient method for determining peptide purity is liquid chromatography coupled with mass spectrometry (LC-MS) (Rayaprolu et al., 2017). Ionisation techniques can also be employed, electrospray and matrix-assisted laser desorption/ionisation (MALDI) can be used to determine the purity of pure peptides and fragmenting patterns (Lopez-Barrios et al., 2014; Singh et al., 2014; Li-Chan, 2015; Rayaprolu et al., 2017; Matemu et al., 2021). Low selectivity when separating biomolecules of similar sizes or properties creates a challenge when purifying peptides (Wong et al., 2020).

2.16. Legumes

Globally, 33% of dietary plant proteins are provided by leguminous crops (Matemu et al., 2021). Legumes are characterised by edible seeds grown in pods (López-Cortez et al., 2016). The crop is cost-effective, easily adaptable and a source of both protein and calories for the consumer (Lopez-Barrios et al., 2014; López-Cortez et al., 2016). The average protein content of legumes ranges between 20 to 40% protein in dry weight while cereals contain 10-15% (Lopez-Barrios et al., 2014; Erbersdobler et al., 2017; Matemu et al., 2021). In the modern world, food is not just a means of calories. Healthy diets have a part in the prevention of diseases (Orona-Tamayo et al., 2018). A German clinical study assessed the effect of diabetes and the consumption levels of legumes such as lentils, peas, beans, and chickpeas. The group that consumed a legume-rich diet had a lower incidence of diabetes (Erbersdobler et al., 2017). Proteins derived from legumes can counteract the oxidative effects of free radicals (López-Cortez et al., 2016).
The growing world population calls for more plant-derived proteins (Erbersdobler et al., 2017). Plant-based proteins are an alternative to animal proteins which assist with food insecurity because of the increasing world population.

2.17. *Lablab purpureus*

Lablab purpureus commonly known as lablab, hyacinth bean, bonavist, Pharao, shink or Indian bean is a drought-tolerant leguminous crop (Subagio, 2006; Naiker et al., 2020a; Roy et al., 2021). Consumed for thousands of years, the crop is mostly cultivated in the Middle East, Asia, Africa, Europe and North and South America (Subagio, 2006). In Bangladesh, the crop is the third most cultivated legume with cultivated lands spanning 48 000 ha (Maass et al., 2010). *Lablab purpureus* is a climbing herbaceous plant that

grows 3-6 m in height. Seeds are white, dark brown or black in colour and oval-shaped (Figure 2.9).

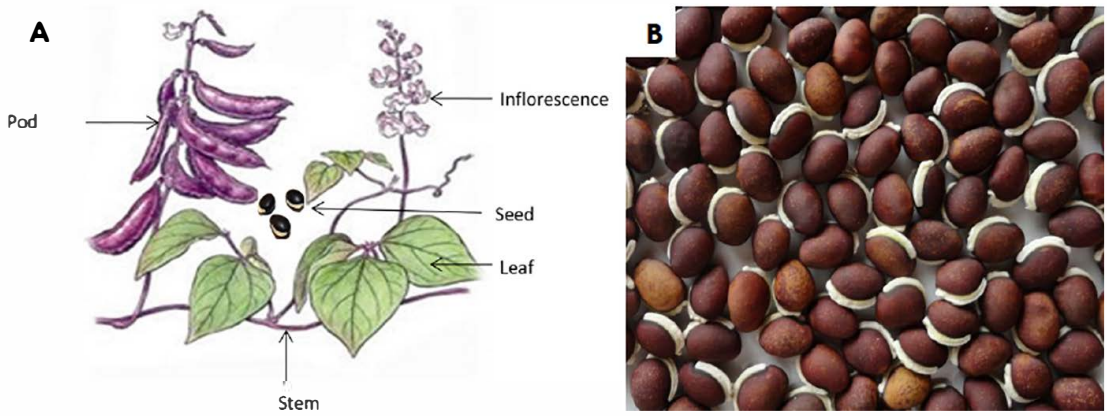

Figure 2.9: *Lablab purpureus* plant [A] (Vaijayanthi and Chandrakant, 2019) and seed [B] (Feedipedia, 2016).

2.17.1. Nutritional profile of *Lablab purpureus*

The legume is regarded as a suitable source of functional protein, containing 18-25% protein. The legume is also comprised of 54-63% carbohydrates and 1.1% lipids (Roy et al., 2021). *Lablab purpureus* has a good balance of amino acids, particularly, lysine and leucine (Subagio, 2006). A study by Naiker et al. (2020b), reported *L. purpureus* protein isolates contained relatively higher amounts of the essential amino acids leucine, phenylalanine and valine (10.20, 6.26 and 4.69 g/100 g respectively), all of which are hydrophobic amino acids. This crop is regarded as a suitable source of edible protein because of its amino acid composition and enhanced level of protein bioavailability (Roy et al., 2021).

2.17.2. Uses of *Lablab purpureus*

Lablab purpureus can be described as a multipurpose legume as all of its components are consumed. The undeveloped seeds, pods and young leaves are consumed as vegetables (Subagio, 2006; Hossain et al., 2016). As an additive, the protein isolate has been used to improve the quality of cakes (Subagio and Morita, 2008). *Lablab purpureus* has been used traditionally for its medicinal properties. Therapeutic analysis has highlighted phytochemical, antibiotic and flavonoid abilities on various human diseases (Naeem et al., 2020). However, little attention has been given to the nutraceutical and pharmaceutical

abilities of protein isolates and peptides derived from the crop (Hossain et al., 2016; Roy et al., 2021). Genistein flavonoid has shown potential in cancer prevention (Umayaparvathi et al., 2014). While, Verma and Singh (2020) studied the apoptotic cell death pathway, mitochondrial membrane potential and expression levels of apoptosis inducing proteins of extracted phytochemicals. Morris (2009) showed vitamin C concentrations of extracted phytochemicals to reach 1063 ppm. Roy et al. (2021) reported greater radical scavenging ability in the *L. purpureus* protein hydrolysate compared to the flour.

Chapter 3: *In vitro* antioxidant and apoptotic activity of *Lablab purpureus* (L.) Sweet isolate and hydrolysates

Abstract

Cancer is a disease that invades the lives of millions of people each year. Chemotherapy is currently the most effective treatment however resulting in many adverse effects on the human body. Alternative treatments are being explored to overcome this obstacle. Peptides that possess radical scavenging activity and bioactive properties can be advantageous in the prevention and treatment of chronic diseases. In this study, *Lablab purpureus* isolate and its hydrolysates (trypsin, pepsin and alcalase) were analysed for radical scavenging potential (DPPH, ABTS, superoxide radical scavenging and FRAP) and antiproliferative activity. Antiproliferative activity was confirmed with the peptides' ability to induce apoptosis (Caspase 3/7 activity and Annexin V-PI). The lowest inhibitory concentrations (IC_{50}) for DPPH, ABTS and Superoxide radical scavenging ranged between 1.81-4.47, 1.73-2.42 and 1.36-4.41 mg/mL, respectively. FRAP ranged from 19.20 to 21.94 mg/mL. Generally, it is considered that a good antioxidant encompasses antiproliferative potential. Cell lines, A549, MCF-7 and HEK293, treated with pepsin hydrolysate showed (IC_{50} values of 119.6, 9.80 and 13.86 µg/mL). The isolate and pepsin were chosen for apoptotic studies. The pepsin hydrolysate showed the highest inhibition in the cancerous cell lines (A549 and MCF-7) without greatly affecting normal cells (HEK293), and the isolate was selected for comparative analysis. Annexin V-PI staining showed cells in different stages of apoptosis (cells during early apoptosis; A549, 42%; MCF-7, 17%; HEK293, 34%). Caspase 3/7 assay demonstrated that the peptide causes an increase in caspase activity. Peptides have the potential to act as chemopreventative agents due to their antioxidant and apoptotic abilities.

Practical application

Bioactive peptides derived from the hydrolysis of *Lablab purpureus* protein induces apoptosis in cancer cells. Peptides can target cancer cells due to their small size thereby allowing them to enter the cell and trigger apoptosis serving as an effective antiproliferative agent capable of inducing apoptosis in cancer cells while leaving healthy cells relatively unharmed.

Keywords

Lablab purpureus, apoptosis, antioxidants, caspase activity

3.1. Introduction

The World Health Organisation (WHO) reports that cancer cases will rise from 14 million in 2012 to 22 million within two decades (Chalamaiah et al., 2018, Sharma et al., 2020). Chemotherapy is still regarded as the most effective treatment for cancers even though it is costly and has many unavoidable side effects (Chen et al., 2019). Antioxidants can be described as an agent that can protect cells from the damaging effects caused by free radicals. Free radicals are natural by-products of cell metabolism, ageing and environmental factors (Badarinath et al., 2010, Jahanbani et al., 2016). Diets rich in antioxidants have been reported to reduce the risk of many developing diseases (Jahanbani et al., 2016, Chen et al., 2019). Legumes with high antioxidant potential like; *Phaseolus vulgaris* L. (common beans), *Glycine max* (L.) *Merr*. *Fabaceae* (Soybean) and *Phaseolus lunatus* L. (Lima beans) have been reported to be beneficial in inhibiting cancers (López-Cortez et al., 2016; Guleria et al., 2020).

Cancer can be triggered or catalysed by the presence of free radicals; therefore, antioxidants can be useful in the prevention and treatment of cancer (Athreya and Xavier, 2017, Chi et al., 2015). An effective antiproliferative agent should be able to induce apoptosis in cancerous cells while leaving healthy cells relatively unharmed. Apoptosis in multicellular organisms is demarcated as the process of programmed cell death (Wang et al., 2012). Effector caspases (caspase 3/7) are responsible for morphological and biochemical changes during the degradation phase of apoptosis (Brentnall et al., 2013). Alternative treatments from natural sources such as food proteins could provide a better option to induce apoptosis and treat cancer cells efficiently.

Legumes have been regarded as a significant source of plant-based protein, containing between 20-40% protein in dry weight (Erbersdobler et al., 2017, Lopez-Barrios et al., 2014). Apart from being a cost-effective and adaptable crop, in recent years, legumes have been utilised for more than their nutritional benefits (Lopez-Barrios et al., 2014). Legume proteins and peptides have been studied for their effects against chronic diseases such as diabetes, inflammation, hypertension, and cancer (Barnes et al., 2015, Erbersdobler et al., 2017, Korhonen and Pihlanto, 2006). Numerous reports specify that diets rich in legume crops are correlated with lower cancer mortality rates (Duranti, 2006; Sánchez-Chino et al., 2015).

Proteins and peptides from legume sources such as soybean and black Jamapa bean have been reported by Torres-Fuentes et al. (2015) and Carrasco-Castilla et al. (2012), to have potent antioxidant capacity. Soybean peptides have been reported to have approximately 3 - 5 times more antioxidant activity as compared to the parent protein (Kamran and Reddy, 2018). The authors isolated and characterized a novel anticancer pentapeptide derived from rice bran hydrolysate. The peptide is resistant to gastrointestinal juices and possesses inhibitory properties on colon, breast, lung, and liver cancer cell lines. Peptides are specific protein fragments that are inactive within the sequence of the parent protein (Hernández-Ledesma and Hsieh, 2013, Udenigwe and Aluko, 2012). These peptides can be liberated from the parent proteins by fermentation, food processing and enzyme hydrolysis (Udenigwe and Aluko, 2012). Girón-Calle et al. (2010) assessed peptides derived from chickpea hydrolysed by pepsin and pancreatin. The hydrolysates inhibited colon and leukaemia cell lines.

Lablab purpureus also referred to as hyacinth bean or Lablab is regarded as an adaptable and drought-tolerant crop that grows throughout the year. This legume contains between 18-25% protein (Hossain et al., 2016, Subagio, 2006). Lablab is regarded as a rich source of essential amino acids, particularly lysine and leucine (Hossain et al., 2016). There is continuous interest in peptides for their use as nutraceuticals, particularly in cancer therapy (Chen et al., 2019). The protein content and under-utilisation of Lablab would make this a suitable source for peptides. Therefore, this study aimed to determine the antioxidant effects of *L. purpureus* isolate and hydrolysates. It is generally considered that a good antioxidant encompasses antiproliferative potential. Hence, this study also assessed the antiproliferative effect of the isolate as well as the hydrolysate on cancerous and non-cancerous cell lines.

3.2. Materials and Methods

3.2.1. Sample preparation

Lablab purpureus seeds were collected in Durban, Kwazulu Natal, South Africa. *Lablab purpureus* seeds were soaked in water (16-20 h), dehulled and dried for 20 h at 50°C. Seeds were milled into flour and sieved through a 180 µm sieve and then defatted with hexane at a ratio of 1:5 (flour: hexane; w/v). The defatted flour was stored in a cool, dry environment until required for analysis.

3.2.2. Protein extraction

Protein was extracted according to the method of He et al. (2013) with minor modifications. Briefly, defatted flour was reconstituted in distilled water (1:5; w/v) and the pH was adjusted to pH 10. The solution was stirred for 2 h at 37°C and centrifuged at 10 000 x g for 45 min (Eppendorf, AG, Germany). Thereafter the supernatant was adjusted to pH 5 (pellet discarded) and centrifuged at 10 000 x g for 45 min (Eppendorf, AG, Germany). The supernatant was discarded, and the pellet was resuspended in distilled water. Finally, the pH was adjusted to pH 7 and the protein extract was freeze-dried.

3.2.3. Preparation of protein hydrolysates

Protein hydrolysates were prepared using the method of Tang et al. (2009) with some modifications. Three proteases were used; pepsin (Sigma Aldrich - 561 U/mg): 37°C; pH 2, alcalase (Sigma Aldrich - ≥ 0.75 U/mL): 50°C; pH 8 and trypsin (Sigma Aldrich - 13 000-20 000 BAEE U/mg): 37°C; pH 8. The substrate was prepared by reconstituting protein isolates in distilled water (1:20; w/v). For all reactions, enzymes were added at 5% of the substrate ratio on dry basis. Each substrate was incubated for 4 h, with the pH monitored regularly and adjusted when required. Thereafter, the substrate was heated at 100°C for 10 min, to stop the reaction, and subsequently cooled on ice to 37°C and centrifuged (10 000 x g for 30 min) with the supernatant then freeze dried.

3.2.4. SDS-PAGE

The SDS-PAGE of *L. purpureus* isolate and hydrolysates were analysed according to Invitrogen (2010) under reducing conditions. Novex™ Bolt Bis-Tris gels were used with the Invitrogen Mini Gel tank under a constant voltage (200 V).

3.2.5. Antioxidant potential

3.2.5.1. DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay

DPPH radical scavenging assay was done according to the method of He et al. (2013) with modifications. Samples were prepared in distilled water containing 1% triton X-100 (1-5 mg/mL). Briefly, 100 µL of DPPH (100 µM) was added to 100 µL of the sample in a 96-well plate.

The plate was then incubated in the dark for 30 min and the absorbance read at 517 nm (Multiscan Go, Thermo Scientific, USA) using glutathione as the positive control with the blank containing distilled water and DPPH. The free radical scavenging capacity was calculated using the following equation:

$$Radical\ Scavenging\ activity = \frac{Absorbance\ (blank) - Absorbance\ (sample)}{Absorbance\ (blank)} \times 100 \quad \text{.....[1]}$$

3.2.5.2. ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) radical scavenging assay

The ABTS radical scavenging assay was conducted according to the method of Lee et al. (2015) with modifications. Briefly, 7 mM ABTS and 2.45 mM potassium persulfate were combined in a ratio of 1:1 (v/v) and stirred for 16-20 h under dark conditions. The ABTS stock solution was diluted with 10 mM potassium phosphate buffer (pH 7.4) to an absorbance of 0.700±0.020 (734 nm). Thereafter, 100 µL ABTS reagent was added to 100 µL of sample (1-5 mg/mL) in a 96-well plate and incubated at 30°C for 4 min. The absorbance was read at 734 nm (Multiscan Go, Thermo Scientific, USA). The positive control was glutathione and buffer was used as the blank. The scavenging capacity was calculated using the following formula:

$$Scavenging\ capacity = \frac{Absorbance\ (blank) - Absorbance\ (sample)}{Absorbance\ (blank)} \times 100 \quad \text{.....[2]}$$

3.2.5.3. Superoxide radical scavenging assay

The superoxide radical scavenging activity was assessed using the method of He et al. (2013) with modifications. The samples and standards (1-5 mg/mL) were prepared in 0.1 M NaOH. Briefly, 80 µL of 50 mM Tris-HCl (pH 8.3) containing 1 mM EDTA was added to 80 µL of the sample. Thereafter, 40 µL of 1.5 mM pyrogallol prepared in 10 mM HCl was added and the absorbance read at 420 nm (Multiscan Go, Thermo Scientific, USA) within 4 min using glutathione as the positive control and 50 mM Tris-HCl as the blank. Scavenging capacity was calculated using the following formula:

$$Superoxide\ radical\ scavenging\ activty\ (\%) = \frac{\Delta Abs_{control} - \Delta Abs_{sample}}{\Delta Abs_{control}} \times 100 \quad \text{.....[3]}$$

3.2.5.4. Ferric Reducing Ability of Plasma (FRAP) assay

The FRAP assay was conducted according to the method of Benzie and Strain (1996) with modifications. The FRAP reagent was prepared by combining 300 mM acetate buffer, 10 mM TPTZ prepared in 40 mM HCl and 20 mM ferrous chloride in a ratio of 10:1:1 (v/v/v). Briefly, 3 mL of FRAP reagent was added to 100 µL of the sample (1-5 mg/mL) and incubated at 37°C for 4 min. The absorbance was then read at 593 nm (Multiscan Go, Thermo Scientific, USA) at 0 and 4 min respectively. The FRAP value was determined by an iron sulphate standard curve with glutathione as the positive control.

3.2.6. Cell culture

The cells of human embryonic kidney (HEK293 - P10), breast cancer (MCF-7 - P9) and human lung cancer (A549 - P10) were obtained from the Department of Human Physiology at the University of KwaZulu-Natal, Westville campus. The cells were grown at 37°C, 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal bovine serum (FBS) and antibiotics (penicillin; 10 000 U/mL and streptomycin sulphate; 10 000 U/mL).

3.2.6.1. Cytotoxicity

Cytotoxicity was determined by the 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Dwarka et al., 2017). Briefly, cells (1x10^6 cells/mL) in 50 µL DMEM were seeded in a 96-well flat bottom plate and incubated for 24 h at 37°C, 5% CO_2. Cells were then treated with 50 µL of isolate and hydrolysates prepared in 5% DMSO (1000-7.8 ug/mL) and incubated for 24 h. Camptothecin was used as the positive control with the negative control containing untreated cells. A 20 µL aliquot of MTT (5 mg/mL) solution prepared in PBS was added to the cells and incubated for 4 h at 37°C, after which, 100 µL of DMSO was added to solubilise the formazan salt formed. The absorbance was read at 570 nm on a microplate spectrophotometer (Multiscan Go, Thermo Scientific, USA) and percentage viability was determined using the following formula:

$$\%\ Cell\ viability\ = \frac{Absorbance\ of\ treated\ cells}{Absorbance\ of\ untreated\ cells} \times 100 \quad \text{.....[4]}$$

3.2.6.2. Annexin V-PI

The FITC Annexin V apoptosis detection kit II (BD Bioscience) assay was conducted according to the manufacturer's specifications. Briefly, cells were seeded into a 24-well flat bottom plate ($1x10^6$ cells/mL) and incubated overnight to adhere. Cells were then treated with 100 µL of isolate (IC_{50} of 255.0, 31.5 and 49.0 for A549, MCF-7 and HEK293 respectively) and pepsin (IC_{50} of 119.6, 9.8 and 13.9) for each of the cell lines. Camptothecin was used as the positive control. After 24 h, cells were trypsinised and centrifuged to obtain a pellet. Cells were then resuspended in 100 µL of 1X binding buffer. Thereafter, 5 µL of FITC Annexin V and 5 µL PI were added and incubated for 15 min at 25°C in the dark. Finally, 400 µL of 1X Binding buffer was added. Samples were analysed using flow cytometry (BD FACSAria II, BD Bioscience) within 1 h.

3.2.6.3. Caspase-Glo 3/7 assay

The Caspase-Glo 3/7 kit [Promega Corporation (2019) Cat no. G8090] was used to determine the presence of caspase 3/7 with the assay conducted according to the manufacturer's protocol. Cells were seeded ($1x10^6$ cells/mL) and incubated for 24 h. Cells were then treated with isolate (IC_{50} of 255.0, 31.5 and 49.0 for A549, MCF-7 and HEK293 respectively and pepsin (IC_{50} of 119.6, 9.8 and 13.9) for each of the cell lines respectively). For the reaction, 100 µL of Caspase-Glo reagent was added and incubated for 1 h. The blank contained 5% DMSO and DMEM while the negative control contained treated cells and the positive control was camptothecin. Samples were analysed using a fluorescence spectrophotometer (GloMax, Promega).

3.2.7. Statistical analysis

Results were analysed by ANOVA (Graph Pad Prism software, San Diego, CA, USA). All analysis was done in triplicate and mean $\pm$ standard deviation was calculated. IC_{50} was also calculated using Graph Pad Prism. The lower the IC_{50} concentration on the cancerous cells, the more potent the isolate is as a therapeutic agent.

3.3. Results and Discussion

3.3.1. SDS-PAGE

Lablab purpureus isolate and hydrolysates ranged between 198 and 14 kDa (Figure 3.1) with the SDS-PAGE result confirming that samples were hydrolysed. Naiker et al. (2020b) assessed *L. purpureus* isolates and reported bands between 200 and 24 kDa with dominant bands at approximately 55 kDA. These findings were consistent with the proteins that were hydrolysed in this study.

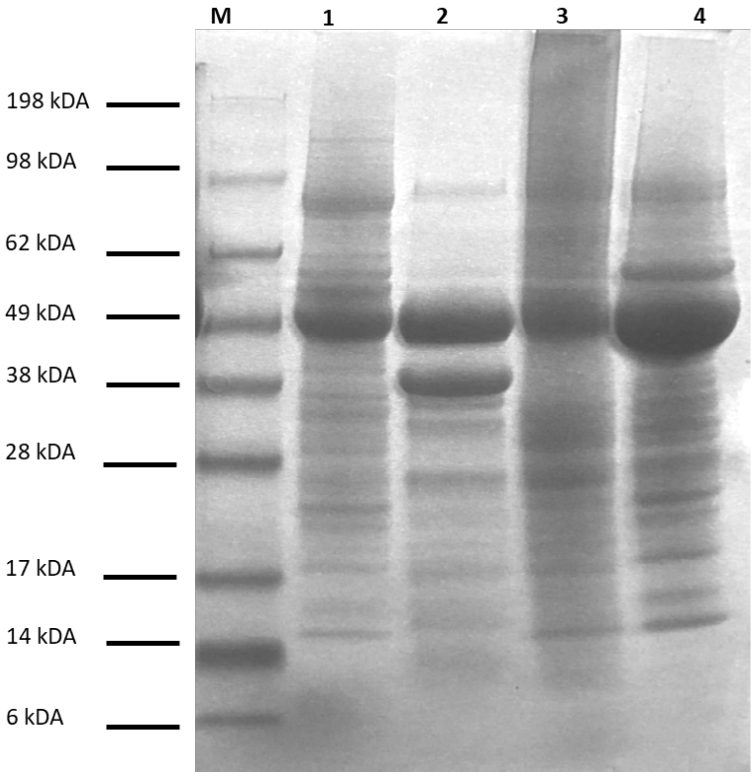

Figure 3.1: SDS-PAGE of *Lablab purpureus* isolate and hydrolysates under reducing conditions. Lane M-Molecular weight marker, 1-Isolate, 2-Pepsin hydrolysate, 3-Trypsin hydrolysate and 4-Alcalase hydrolysate.

3.3.2. Antioxidant activity

The inhibitory concentration (IC_{50}) of DPPH, ABTS and superoxide radical scavenging activities of *L. purpureus* isolate and hydrolysate are shown in Table 3.1. IC_{50} values indicate the lowest inhibitory concentration of the samples. The DPPH radical scavenging activity showed that the isolate has the lowest IC_{50} (1.81 mg/mL) which was significantly different ($p<0.05$) to the hydrolysates.

Glutathione, a known antioxidant was 1.8-fold greater than the isolate. IC_{50} of hydrolysates ranged between 2.76-4.47 mg/mL. *Lablab purpureus* isolate was similar in comparison with the radical scavenging activity of soybean protein (IC_{50} 2.11 mg/mL) and mung bean protein (IC_{50} 1.47 mg/mL) (Chen et al., 2017). The DPPH assay favoured the isolate which contradicts most studies that have found that hydrolysates have higher antioxidant activity while the ABTS assay showed greater activity in the hydrolysates (Singh, 2011; Ramkisson et al., 2020). This could be attributed to the mechanisms employed by each assay. Therefore, their radical scavenging capabilities could be different (Thumbrain et al., 2020). The ABTS radical scavenging activity resulted in IC_{50} for *L. purpureus* isolate and hydrolysates ranging from 1.73-2.42 mg/mL. The pepsin and alcalase hydrolysates had higher activity with IC_{50} values of 1.73 mg/mL and 1.72 mg/mL respectively, as compared to the isolate (IC_{50} 2.03 mg/mL). When compared to glutathione, the samples showed greater antioxidant potential (2.6-3.7-fold greater). Similar findings were reported by a study conducted on Bambara groundnut (Arise et al., 2016) and Okra (Sbroggio et al., 2016).

The IC_{50} values corresponding with the superoxide radical scavenging activity ranged between 1.36 and 4.51 mg/mL. Alcalase was significantly ($p<0.05$) more potent against the superoxide radical (IC_{50} 1.36 mg/mL). Glutathione had significantly lower antioxidant activity (IC_{50} 2.74 mg/mg) when compared with the alcalase hydrolysate indicating that the hydrolysate has good antioxidant potential. Higher peptide chain lengths can result in lower antioxidant activity making them less effective in scavenging the ABTS radical (Ramkisson et al., 2020).

The FRAP assay showed no significant ($p<0.05$) difference between the isolate and hydrolysates (Table 3.2). Results ranged between 19.20 and 21.94 mg/mL. Lower activity was reported for pigeon pea (Sekhon et al., 2017), lentil, pea, and cowpea (Marathe et al., 2011).

A strong reducing power of protein hydrolysate could be attributed to the increase in available hydrogen ions because of the breaking of peptide bonds (Sbroggio et al., 2016). Antioxidant activity is generally used for preliminary screening of bioactivity. Overall, the isolate and hydrolysates proved to be good antioxidants as they possessed activity comparable to glutathione.

Table 3.1: Radical scavenging activity (IC_{50}) of *Lablab purpureus* isolate and hydrolysates was determined by DPPH, ABTS and Superoxide radical scavenging activity

Sample	DPPH radical scavenging activity IC_{50} (mg/mL)	ABTS radical scavenging activity IC_{50} (mg/mL)	Superoxide radical scavenging IC_{50} (mg/mL)
Isolate	$1.81^{b} \pm 2.94$	$2.03^{e} \pm 1.75$	$3.17^{ab} \pm 1.09$
Trypsin	$4.47^{de} \pm 0.14$	$2.42^{d} \pm 2.41$	$3.79^{ab} \pm 0.83$
Pepsin	$2.76^{e} \pm 0.14$	$1.73^{cd} \pm 0.50$	$4.51^{ab} \pm 0.56$
Alcalase	$3.81^{ce} \pm 0.29$	$1.82^{b} \pm 0.39$	$1.36^{ab} \pm 0.81$
Glutathione	$0.98^{a} \pm 1.08$	$6.44^{a} \pm 0.35$	$2.74^{b} \pm 0.30$

Data represent mean±SD (n=3). Values with different superscripts are significantly different ($p<0.05$).

Table 3.2: Ferric reducing ability of *Lablab purpureus* isolate and hydrolysates determined by FRAP

Sample	mM $FeSO_4$/mg protein
Isolate	$19.20^{ab} \pm 5.49$
Trypsin	$20.57^{ab} \pm 4.11$
Pepsin	$21.94^{ab} \pm 3.88$
Alcalase	$27,43^{ab} \pm 3.88$
Glutathione	$855,71^{b} \pm 24.68$

Data represents mean ± SD (n = 3). Values with different superscripts are significantly different ($p<0.05$).

3.3.3. Cytotoxicity

The MTT colourimetric assay was conducted to assess whether *L. purpureus* isolate and hydrolysates had the ability to inhibit the proliferation of cancerous cell lines A459 and MCF-7 and healthy cells HEK293 (Figure 3.2). A549 cells treated with pepsin hydrolysate had the lowest IC_{50} of 119.60 µg/mL which was 2.5-fold greater than camptothecin, a chemopreventative agent. Cell viability of pepsin and camptothecin treated at a concentration of 125 µg/mL was 48.37% and 57.11% respectively. MCF-7 cells treated with pepsin had the lowest IC_{50} of 9.8 µg/mL while the isolate had the highest IC_{50} of 31.54 µg/mL. Cell viability respectively was 55.34% and 51.56% at a concentration of 31.25 µg/mL. Peptides had lower IC_{50} values in MCF-7 treated cells suggesting sensitivity in these cells as compared to A549 and HEK293 (Fan et al., 2016; Khangebam and Chakrabarti, 2018).

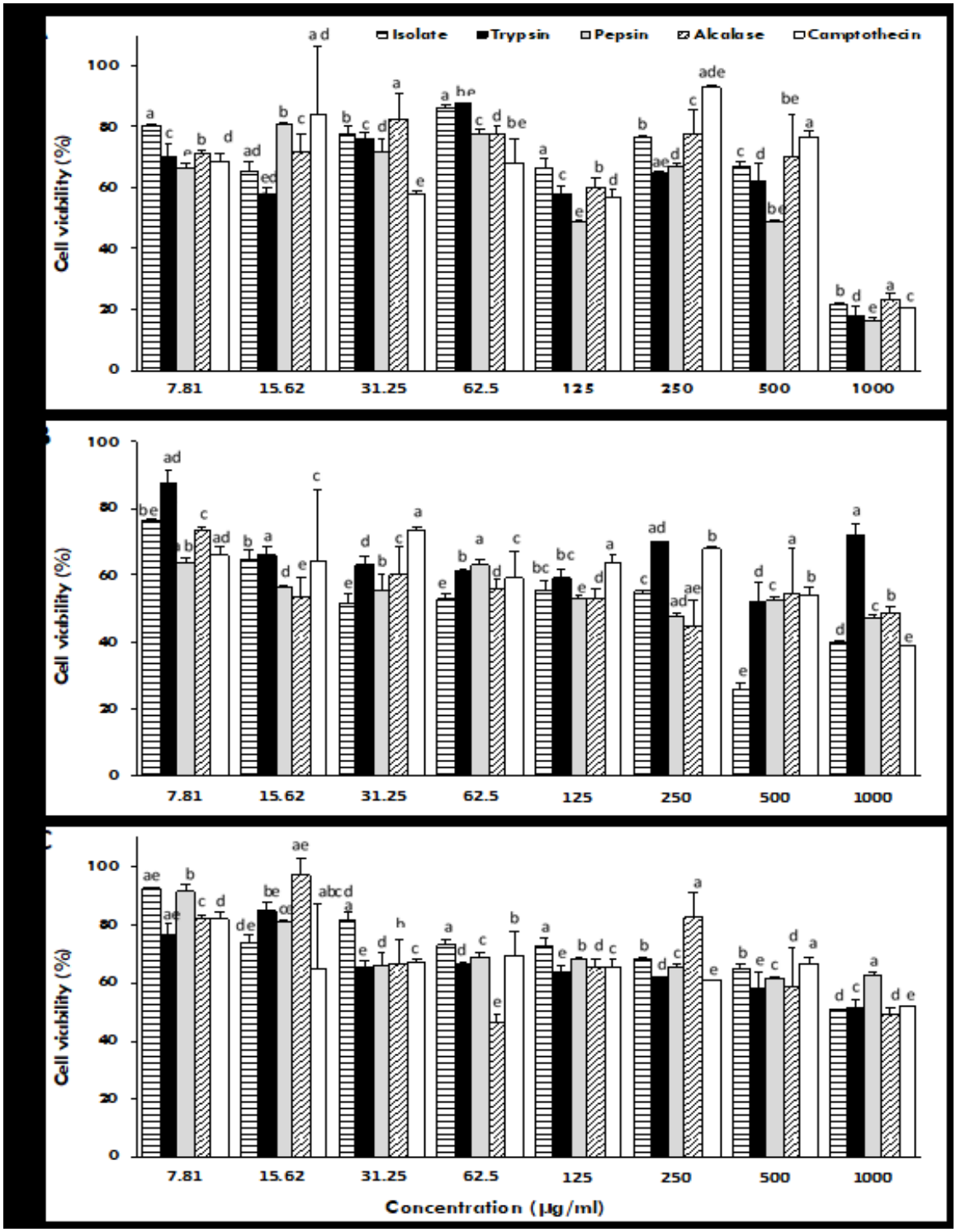

Figure 3.2: Cell viability of A549 [A], MCF-7 [B] and HEK293 [C] cell lines treated with *Lablab purpureus* isolate and hydrolysates as determined by MTT cytotoxicity assay. Data represent mean±SD (n=3). Values with different superscripts are significantly different ($p<0.05$).

HEK293 cells treated with Pepsin had the lowest IC_{50} of 13.86 µg/mL. Cell viability of pepsin ranged between 62.77 and 91.65% across the concentrations. The cell viability of the cancerous cell lines was lower than that of the healthy cell line. The isolate and pepsin hydrolysate were chosen for apoptotic studies as the pepsin hydrolysate showed the highest inhibition in the cancerous cell lines (A549 and MCF-7) while not affecting normal cells (HEK293) immensely, with the isolate selected for comparative analysis.

3.3.4. Quantification of apoptosis

Annexin V-PI staining separates cells according to stages of apoptosis i.e., living cells or healthy (Q4), dying or early apoptotic cells (Q3) and dead or late apoptotic (Q2) and necrotic cells (Q1) (Sabbione et al., 2019). Assessment of early apoptosis in A549 cells treated with the isolate (26.4%) and pepsin (41.5%) could be compared with camptothecin (31.6%). MCF-7 cells that were treated with the isolate and pepsin showed 33.4 and 17.4%, respectively, in early apoptosis while late apoptosis showed 18.4 and 17.9% (Figure 3.3). Overall, the pepsin hydrolysate was more effective in initiating apoptosis in cancerous cell lines. A study by (Fan et al., 2016) reported that 13% and 18.2% of MCF-7 cells treated with a peptide conjugated with a viral protein were in late apoptosis. The isolate and pepsin treated HEK293 cells showed healthy cells of 77.2 and 64% as depicted in Figure 3.3. Studies by Dia and Mejia (2010), Dia and Gonzalez (2011); McConnell et al. (2014) showed that anticancer activities of different peptides induce pro-apoptotic activity on selected cell lines.

The induction of apoptosis was also evaluated by measuring caspase 3/7 activation (Figure 3.4). Cell viability is indirectly proportional to fluorescence units (FU) (Butterick et al., 2014). HEK293 treated cells showed the greatest fluorescence units, this result is largely due to cells undergoing stress during transportation for analysis. Cancerous cell lines are hardier than healthy cells which are very sensitive to environmental changes. The cell lines treated with pepsin hydrolysate showed no significant difference ($p<0.05$) when compared with camptothecin. Thereby confirming that the pepsin hydrolysate is inducing apoptosis. These results are supported by Annexin V-PI. Burz et al. (2009) confirmed that bioactive peptides exert antitumor activity via apoptosis induction which involves the activation of specific caspases. Studies on whey isolates and fractions (Castro et al., 2009) and amaranth proteins (Sabbione et al., 2019) both showed higher caspase 3 activity compared to the control.

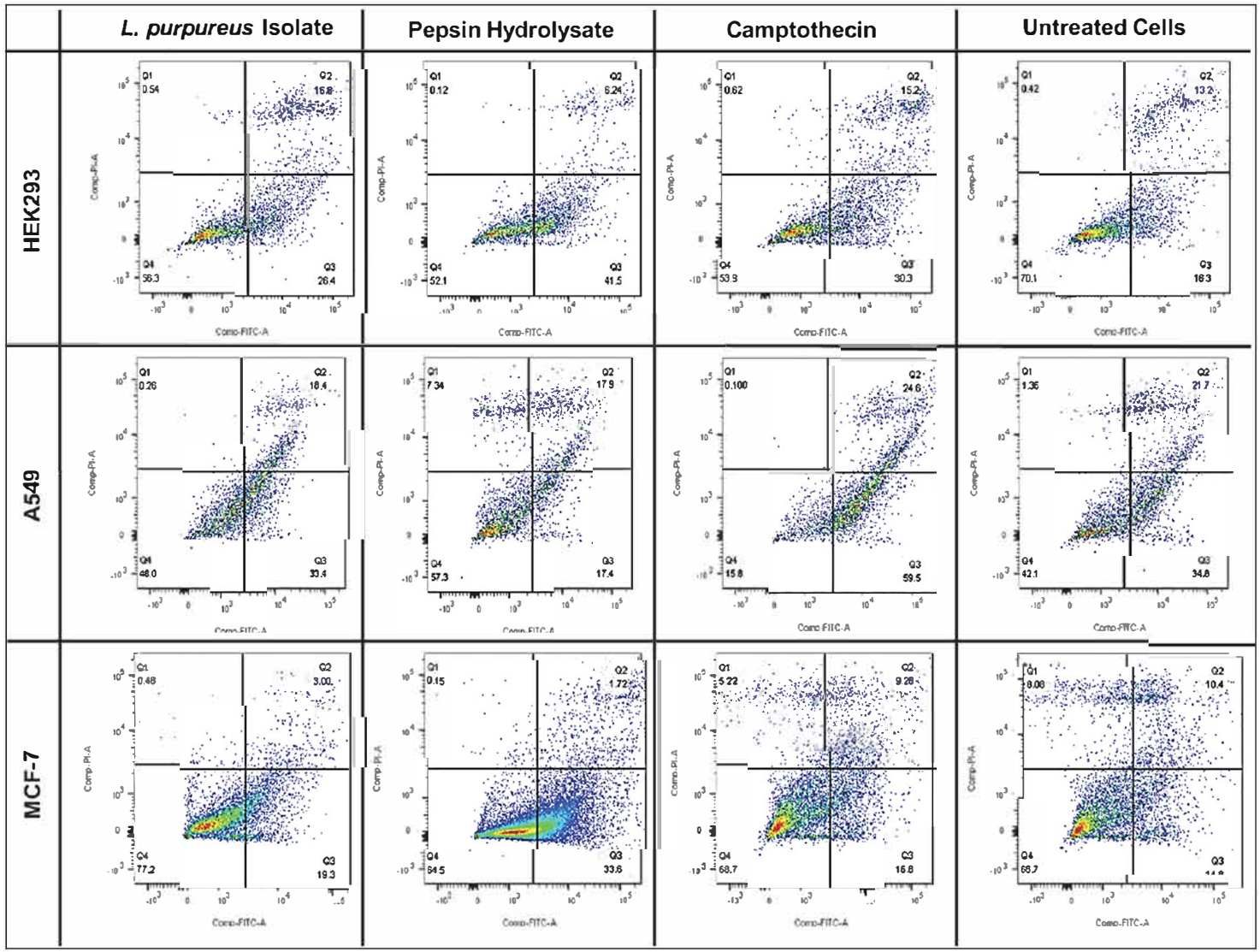

Figure 3.3: Flow cytometry analysis showing the externalisation of phosphatidylserine in A549, MCF-7 and HEK293 cells treated with *Lablab purpureus* isolate, pepsin hydrolysate, Camptothecin and untreated cells at IC_{50} values determined by the MTT assay.

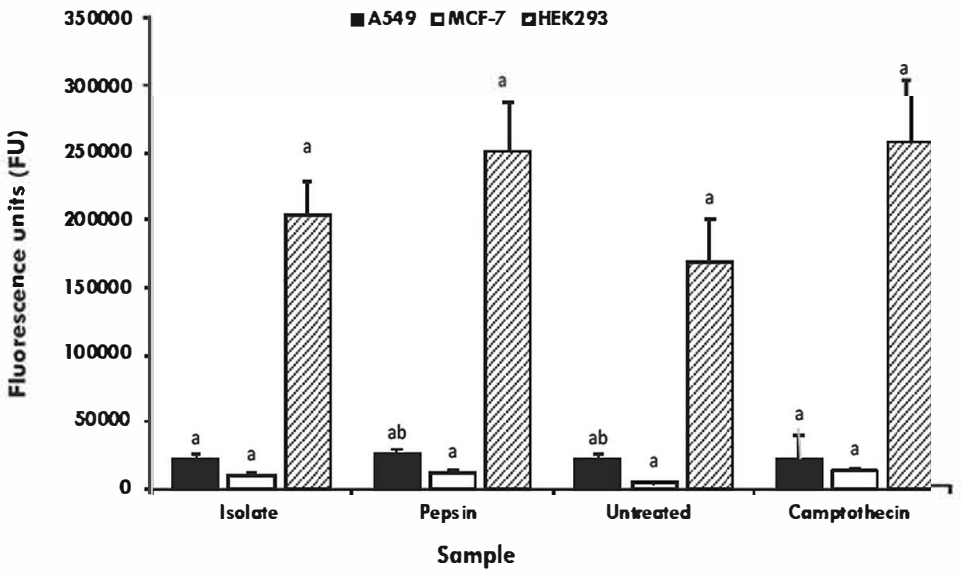

Figure 3.4: Caspase 3/7 activity measured as fluorescence units emitted by the cleavage of peptide DEVD. Cell lines, A549, MCF-7 and HEK293 were treated with *Lablab purpureus* isolate and pepsin hydrolysate at IC_{50} values obtained by MTT assay. Camptothecin was the positive control and untreated cells were the negative control. Data represent mean±SD. Values with different superscripts are significantly different ($p<0.05$).

3.4. Conclusions

Peptides have attracted attention as drug candidates for cancer therapy because they offer certain key advantages over alternative molecules. In contrast to traditional drugs, peptides have high affinity, strong specificity, low toxicity, and adequate tissue penetration. This study showed that protein isolates and hydrolysates from *L. purpureus* had positive antioxidant potential, especially when compared to glutathione, a known antioxidant. As a potential chemopreventative agent, the isolate and pepsin hydrolysate exhibited the best antiproliferative activity at the lowest concentrations. Results were confirmed by apoptotic studies (Annexin V-PI and Caspase 3/7 analysis). Further research must be performed to determine the peptides that provide antiproliferative effects.

Chapter 4: *In vitro* apoptotic activity of *Lablab purpureus* (L.) Sweet low molecular weight Peptides

Abstract

Cancer prevalence is a growing concern globally with a rapid increase in cases. Radiotherapy and chemotherapy are current treatment protocols however they have adverse effects on the human body. Peptides from leguminous protein are known for exhibiting positive biological activity. This study assesses the apoptotic ability of peptide fractions derived from *Lablab purpureus* by identifying apoptotic markers (caspase-9 and -3, p53 and annexin V-PI). Caspase-9 and -3 showed activity to a greater degree in cancerous cell line A549 (2.3-fold and 1.1-fold) and MCF-7 (2.7-fold and 1.8-fold), respectively, compared to camptothecin (control). Expression of p53 on cancerous cell lines demonstrated greater ability over HEK293 and camptothecin with A549 showing the highest activity at 29.923 µg/mL. Finally, annexin V-PI staining sorted cells into phases of apoptosis. Treated A549 and MCF-7 cells detected 85.4% and 89.6%, respectively, of cells undergoing apoptosis. Overall, Fraction 2 triggered apoptosis in cancer cell lines while minimally harming healthy cells.

Keywords

Lablab purpureus, caspase-3, caspase-9, p53

4.1. Introduction

Globally, cancer affects approximately 19.3 million people and it is one of the leading causes of death (Rayaprolu et al., 2017; WHO, 2020; Quintal-Bojórquez and Segura-Campos, 2021). Traditional cancer therapies, chemotherapy, and radiotherapy are effective at attacking cancer cells. However, side effects are experienced because the drugs also affect normal cells in the body (Huang et al., 2011; Orona-Tamayo et al., 2018; Chen et al., 2019; Quintal-Bojórquez and Segura-Campos, 2021). An alternative anticancer agent should therefore have a targeted approach and be nontoxic to healthy cells (Sipahli et al., 2022). Apoptosis or programmed cell death is a normal process that occurs in the lifecycle of a healthy cell. Damaged cells evade this process resulting in the development of tumours (Alison, 2001; Zivny et al., 2010; Letai, 2017; D'Arcy, 2019).
Tumour suppressor protein, p53, plays a protective role in decreasing the development of cancers by initiating apoptosis in 'suspicious' cells (Brown and Attardi, 2005; Letai, 2017;

Marqus et al., 2017; Xu et al., 2019). Subsequently, the expression of pro-apoptotic proteins (annexin v and caspases) is induced (Bowne et al., 2007). Caspases from the group of cysteinyl aspartate-specific proteases are responsible for the disassembly of cells during apoptosis (An et al., 2019; D'Arcy, 2019). Annexin V, from the phospholipid-binding annexin family, can bind to a marker of apoptosis, phosphatidylserine, therefore Annexin V-PI staining determines apoptotic cells by their ability to bind to phosphatidylserine (Logue et al., 2009). Nutraceuticals have been studied for their ability to induce apoptosis in cancer cells (Gupta and Bhagyawant, 2021). Bioactive peptides derived from legumes have been associated with lower risk cancers (Barman et al., 2018; Orona-Tamayo et al., 2018). A study according to Tak et al. (2021) suggests that bioactive peptides can halt cancer development by hindering specific pathways or inhibiting inflammation and cell proliferation thereby inducing apoptosis. *Lablab purpureus* is an underutilised crop cultivated in Africa. The legume has a protein content of 18-25% with a good balance of essential and nonessential amino acids (Cheng et al., 2019; Naiker et al., 2020b).

Enzymatic hydrolysis is commonly preferred for the extraction of bioactive peptides as toxic secondary metabolites are not produced during the process. It also stimulates gastrointestinal digestion subsequently allowing for a shorter reaction time (Quintal-Bojórquez and Segura-Campos, 2021). Pepsin hydrolysate from *L. purpureus* exhibited potential chemopreventative activity. Lung cancer cell line, A549, showed 2.5-fold greater cytotoxicity compared with the chemopreventative agent, camptothecin (Sipahli et al., 2022). The pepsin hydrolysate was also found to be more effective in initiating apoptosis in the A549 and MCF-7 cancer cell lines. Annexin V-PI staining showed that 41.5% (A549) and 33.4% (MCF-7) of cells experienced early apoptosis while the control (camptothecin) showed 31.6%. The healthy cell line (HEK293) showed 77.2% of cells in early apoptosis indicating that healthy cells were least affected by the pepsin hydrolysate (Sipahli et al., 2022). The present study assesses the apoptotic activity of the pepsin hydrolysates from *L. purpureus* that have been fractionated using ultrafiltration and RP-HPLC.

4.2. Materials and Methods

4.2.1. Sample preparation

Lablab purpureus (L.) Sweet samples were collected in Durban (KZN, South Africa), with seeds dehulled, dried, milled and sieved (180 µm sieve). Flour prepared from the seeds was then defatted with hexane at a 1:5 (w/v) ratio for 16-24 h. Excess hexane was removed, and flour was left to dry under a fume hood overnight and stored at 4°C until analysis.

4.2.2. Protein extraction

Protein isolate samples were prepared using defatted flour reconstituted in distilled water (1:5; w/v) and adjusted to pH 10 (He et al., 2013). The solution was then stirred at 37°C for 2 h and centrifuged (Eppendorf, Hamburg, Germany) at 10 000 x g (45 min at 4°C). The supernatant was then adjusted to pH 5 and centrifuged and the pellet resuspended in distilled water (pH 7). The protein isolate was subsequently freeze-dried and stored in a biofreezer at -80°C until required.

4.2.3. Preparation of protein hydrolysates and fractions

Protein isolate was reconstituted in distilled water (1:20; w/v), adjusted to pH 2, preincubated for 20 min at 37°C, thereafter pepsin was added at 5% of the substrate (dwb) (Tang et al., 2009). Substrate-enzyme solution was then incubated at 37°C for 4 h, with the pH monitored and maintained at pH 2. Thereafter, to stop the reaction, the solution was heated (100°C; 10 min), cooled on ice to 37°C and centrifuged at 10 000 x g [30 min at 4°C]. The supernatant was fractionated by ultrafiltration stirred cell Amicon 8400 (Merck Group, Darmstadt, Germany). Collected fractions (<1, 3, 5, 10 and >10 kDa) were freeze dried, with samples stored in a biofreezer at -80°C until required for analysis.

4.2.4. Cell culture

The cell lines used in this study (Human embryonic kidney (HEK293), breast cancer (MCF-7) and human lung cancer (A549)) were grown at 37°C in a 5% CO_2 incubator using Dulbecco's modified Eagle's medium (DMEM) supplemented with Foetal Bovine Serum (FBS) (Dwarka et al., 2017).

4.2.5. Cytotoxicity assay

Cytotoxicity was measured using the MTT assay with 50 µL of cells (1x10^6 cells/mL) in DMEM supplemented with Foetal Bovine Serum (FBS) seeded in a 96-well flat-bottom plate and incubated for 24 h at 37°C in 5% CO_2. Cells were then treated with 50 µL of the sample prepared in 5% DMSO (1000-7.8 µg/mL) and incubated for 24 h with camptothecin was used as a positive control and untreated cells served as the negative control. An aliquot of MTT solution (20 µL; 5 mg/mL) was then added to cells and incubated for 4 h at 37°C in 5% CO_2. One hundred microlitres (100 µL) of DMSO was then added and absorbance read at 570 nm (Multiscan Go, Thermo Scientific, Waltham, MA, USA). Percentage viability was determined using Equation 1 with the most active ultrafiltration fraction further purified by RP-HPLC.

$$\%\ Cell\ viability \quad \frac{Absorbance\ of\ treated\ cells}{Absorbance\ of\ untreated\ cells} \times 100 \quad \text{.....[1]}$$

4.2.6. Peptide fractionation using Reverse Phase-High Pressure Liquid Chromatography (RP-HPLC)

RP-HPLC (Shimadzu Corporation, Kyoto, Japan) coupled with a Photodiode array detector was used to further fractionate the selected sample. Sample was passed through a Zorbax 300SB-C8 column (9.4 x 250 mm ID, 5 µm particle size, 300-Å pore size) (Agilent Technologies, Santa Clara, CA, USA). Mobile phase A contained 0.1% Trifluoroacetic acid (TFA) in water while mobile phase B contained 0.1% TFA in acetonitrile. Flow rate was maintained at 1 mL/min with a fixed column temperature of 32°C. Separation was performed with a stepwise gradient, 10-60% of mobile phase B (increasing 10% every 10 min) over 60 min. Fractions at each major peak were collected, freeze dried, and stored at -20°C until required for analysis.

4.2.7. Caspase -3 and -9 assay

The Human Caspase-3 and -9 ELISA kits (Cat no. E-EL-H0663 and E-EL-H0017, Elabscience, Wuhan, China) were used according to the manufacturer's protocol. Briefly, cells were seeded (1x10^6 cells/mL) into a T25 cell culture flask and incubated at 37°C in 5% CO_2 for 24 h. The selected fraction and camptothecin were then used to treat cells at IC_{50} concentrations determined by the MTT assay and incubated at 37°C in 5% CO_2 for

24 h. Treated cells were lysed using the freeze-thaw method and analysed according to the manufacturer's protocol with the absorbance read at 450 nm.

4.2.8. p53 ELISA

The p53 Human SimpleStep ELISA kit (Lot no. GR3221726-1, Abcam, Cambridge, UK,) was used according to the manufacturer's protocol. Cells were seeded ($1x10^6$ cells/mL) into a T25 cell culture flask, incubated at 37°C in 5% CO_2 for 24 h and subsequently treated with the selected fraction and camptothecin at IC_{50} concentrations determined by the MTT assay and incubated at 37°C in 5% CO_2 for 24 h. Treated cells were lysed using an extraction buffer and analysed according to the manufacturer's protocol with the absorbance read at 450 nm.

4.2.9. Annexin V-PI

The FITC Annexin V apoptosis detection kit I (Cat no.556547, BD Bioscience, Franklin Lakes, NJ, USA) assay was conducted according to the manufacturer's specifications. Cells were seeded into a 24-well flat bottom plate ($1x10^6$ cells/mL), incubated overnight, and treated with 100 µL of the selected fraction for each of the cell lines and analysed using flow cytometry (BD LSRFortessa, BD Bioscience, Franklin Lakes, NJ, USA).

4.2.10. Reverse Phase - Liquid Chromatography Mass Spectroscopy

Purity and molecular weight of the selected fraction were analysed by RP-HPLC. The Prominence-i LCMS coupled with a Refractive Index Detector (RID-20A) (Shimadzu Corporation, Kyoto, Japan) was used with a Phenomenex Yarra 3µm SEC 3000 (300 x 7.8 mm) column (Torrance, CA, USA). The mobile phase comprised 50 mM Na phosphate buffer with 300 mM NaCl [pH 6.8] at a flow rate of 1 mL/min and a fixed column temperature of 30°C. Molecular weight standards were used to achieve a standard curve which was then used to calculate the unknown molecular weight.

4.2.11. Statistical analysis

Data was analysed using One-way and Two-way ANOVA (GraphPad Prism software, San Diego, CA, USA) with all analyses in triplicate and results presented as mean±standard deviation. Inhibitory concentrations (IC_{50}) were also determined using GraphPad Prism

4.3. Results and Discussion

4.3.1. Cell viability (MTT Assay) of peptide fractions

An effective anticancer agent should inhibit cancer cells at the 50% inhibitory concentration (IC_{50}) while leaving healthy cells relatively unharmed. The MTT assay confirms that the ultrafiltration fractions do not have a cytotoxic effect on the cells (Figure 4.1). At concentrations between 7.81 and 250 µg/µL cells remained 90-50% viable across the cell lines. Indicating that the fractions were non-toxic to the cells. The IC_{50} values were then established to determine the concentration at which 50% of the cells were inhibited. Lower molecular weight fractions showed better inhibition of cancer cells as seen by the low IC_{50} values while minimally affecting healthy cells as observed by the higher IC_{50} values (Table 4.1).

Compared with the control, camptothecin, the 3 kDa fraction exhibited lower IC_{50} values in cancer cells, A549 (13.6 µg/µL) and MCF-7 cells (20.48 µg/µL). Cell viability for the cells was 55.49% and 56.11%, respectively, at a concentration of 15.63 µg/µL. The healthy cell line, HEK293 (145.10 µg/µL), was 2.5-fold greater than camptothecin. The cell viability was 84.31% and 68.16% at concentrations of 15.63 and 125 µg/µL respectively. Chen et al. (2019), corroborated that lower molecular weight peptides showed improved anticancer activity. Their <4 kDa derived from black soybean had the highest anticancer activity compared to their larger factions (4-6 kDa and >6 kDa). The 4 kDa fraction displayed 2.28, 5.91 and 1.96-fold inhibition on HepG2, MCF-7 and Hela cells respectively (Chen et al., 2019). This study selected the 3 kDa fraction for further purification by RP-HPLC because it showed the best fit for each of the cell lines.

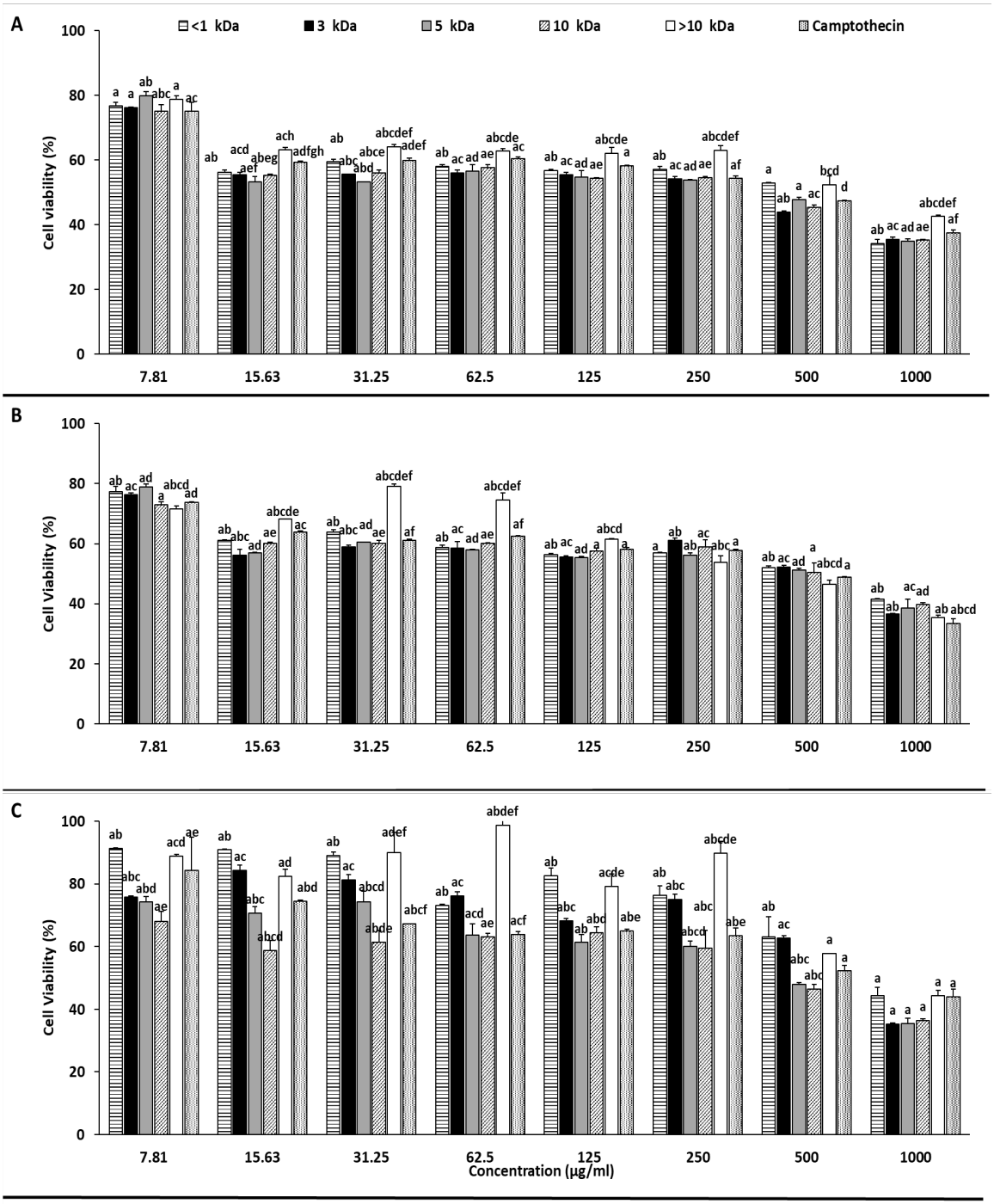

Figure 0.1: Cell viability of A549 [A], MCF-7 [B] and HEK293 [C] cell lines treated with *Lablab purpureus* fractions. Data represent mean±SD (n=3). Values with different superscripts are significantly different ($p<0.05$).

Table 4.1: Inhibitory concentrations (IC_{50}) of A549, MCF-7 and HEK293 cell lines treated with *Lablab purpureus* fractions

Fraction	A549 (µg/µL)	MCF-7 (µg/µL)	HEK293 (µg/µL)
<1 kDa	20.20[a]	16.45[a]	181.60[a]
3 kDa	13.60[a]	20.48[a]	145.10[a]
5 kDa	15.27[a]	14.29[a]	36.30[a]
10 kDa	14.12[a]	15.54[a]	19.76[a]
<10 kDa	18.45[a]	51.40[a]	168.2[a]
Camptothecin	19.73[a]	44.60[a]	40.51[a]

Data represents mean±SD (n=3). Values with different superscripts are significantly different ($p<0.05$).

4.3.2. Cell viability (MTT) RP-HPLC Fractions

Cells treated with Fraction 2 exhibited IC_{50} values for A549 and MCF-7 of 141.80 and 250.00 µg/µL, respectively (Table 4.2). The A549 cell line showed a significant ($p<0.05$) increase from the control, camptothecin (Figure 4.2). The healthy cell line, HEK293, exhibited an IC_{50} of 179.00 µg/µL compared to camptothecin which was 126.80 µg/µL. The purified kla-TAT peptide showed the lowest IC_{50} value on the A549 cell line while the highest IC_{50} was observed on the healthy cell line HaCat. This supports the findings in this study as the cancerous cell line was more sensitive to the peptide and had lower toxicity than the healthy cell line (Chen et al., 2019). Fraction 2 was selected for further analysis because the results showed the best fit for each of the cell lines. After RP-HPLC, collected fractions were run on SDS-PAGE, and no bands were visible in the lanes with fractions. The low molecular weight of the peptides could have run out of the gel (Taniya et al., 2020).

Table 4.2: Inhibitory concentrations (IC_{50}) of A549, MCF-7 and HEK293 cell lines treated with *Lablab purpureus* RP-HPLC fractions

Sample	A549 (µg/µL)	MCF-7 (µg/µL)	HEK293 (µg/µL)
Fraction 2	141.80[a]	250.00[a]	179.00[a]
Fraction 3	542.60[a]	235.00[a]	299.90[a]
Fraction 4	712.70[a]	265.00[a]	190.70[a]
Fraction 5	373.30[a]	350.90[a]	174.30[a]
Camptothecin	255.20[a]	239.40[a]	126.80[a]

Data represents mean±SD (n=3). Values with different superscripts are significantly different ($p<0.05$).

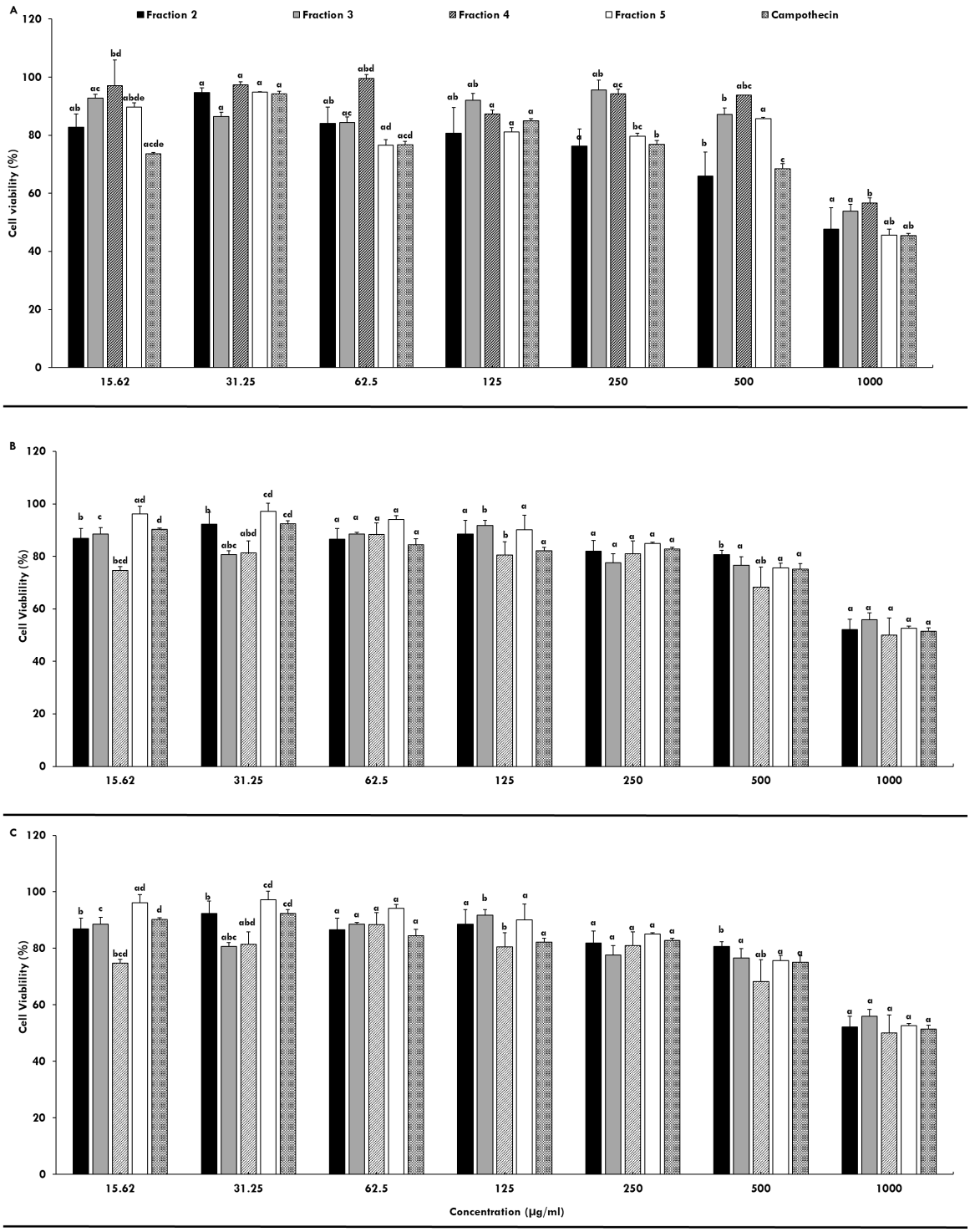

Figure 0.2: Cell viability of A549 [A], MCF-7 [B] and HEK293 [C] cell lines treated with *Lablab purpureus* RP-HPLC fractions. Data represent mean±SD (n=3). Values with different superscripts are significantly different ($p<0.05$).

4.3.3. Caspases -3 and -9

When caspase-9 is triggered, caspase-3 is activated thereby signalling a caspase cascade ultimately leading to apoptosis (Jan and Chaudhry, 2019). Cells treated with Fraction 2 were analysed for their caspase 3 and 9 activity by ELISA (Figure 4.3). Caspase 3, the executioner caspase, showed the highest caspase concentration in MCF-7 cells (0.137±0.0015 ng/mL). Cell lines, A549 and MCF-7 showed 2.3-fold and 2.7-fold greater activity compared to camptothecin. Conversely, camptothecin showed 0.63-fold greater activity than the healthy cell line (HEK293). Analysis of caspase 9 activity produced a similar trend by HEK293 cells as they exhibited 0.9-fold lower activity. It can therefore be deduced here that Fraction 2 did not harm the cells to a great degree. The cancer cells, MCF-7 and A549, showed 1.8-fold (0.205 ng/mL) and 1.1-fold (21.966 ng/mL) greater caspase-9 activity. Comparatively, purified peptides from kidney beans were also able to induce apoptosis in MCF-7 cells via the extrinsic pathway. This involved the upregulation of caspase 3 and 9 in cells (Rao et al., 2018).

Table 4.3: Caspase 3/9 activity on A549, MCF-7 and HEK293 cells treated *with Lablab purpureus* RP-HPLC Fraction 2 derived from pepsin hydrolysate

Sample	MCF-7		A549		HEK293	
	Caspase 3 (ng/mL)	**Caspase 9** (ng/mL)	**Caspase 3** (ng/mL)	**Caspase 9** (ng/mL)	**Caspase 3** (ng/mL)	**Caspase 9** (ng/mL)
Treated	0.137^{ab}±0.015	0.205^{a}±0.065	0.067^{a}±0.032	21.966^{a}±0.302	0.076^{a}±0.003	0.191^{a}±0.064
Camptothecin	0.051^{ab}±0.002	0.112^{a}±0.014	0.029^{a}±0.017	20.486^{a}±0.172	0.121^{ab}±0.015	0.220^{a}±0.036
Untreated	0.041^{a}±0.002	0.155^{a}±0.020	0.016^{a}±0.007	20.148^{a}±0.496	0.012^{ab}±0.010	0.249^{a}±0.050

Data represents mean±SD (n=3). Values with different superscripts are significantly different ($p<0.05$).

4.3.4. Annexin V-PI

Based on results, cell death induced by Fraction 2 was studied for apoptotic activity by observing phosphatidylserine (PS) translocation using the annexin V-FITC/PI assay. Quadrants (Figure 4.3) are labelled as viable (lower left), early apoptosis (lower right), late apoptosis/dead (upper right) and necrotic (upper right). Cancerous cell lines, A549 and MCF-7, treated with peptide Fraction 2 reported 85.4% and 89.6% of cells in early apoptosis respectively. Early apoptosis is distinguished by translocation of PS from the inner layer of the plasma membrane to the outer surface (Kwan et al., 2016).

Cells in late apoptosis were 14.2% and 10.4% respectively for each of the cancer cell lines. The healthy cell line comparatively showed a larger population in early apoptosis of 93.9% however 0.3% of the cells were still viable. The cancer cell lines did not show any viable cells. When compared to camptothecin, the cell lines showed equal or better ability than the peptide Fraction 2. The cell lines did not exhibit necrosis thereby indicating that the peptide Fraction 2 and camptothecin were not toxic to the cells.

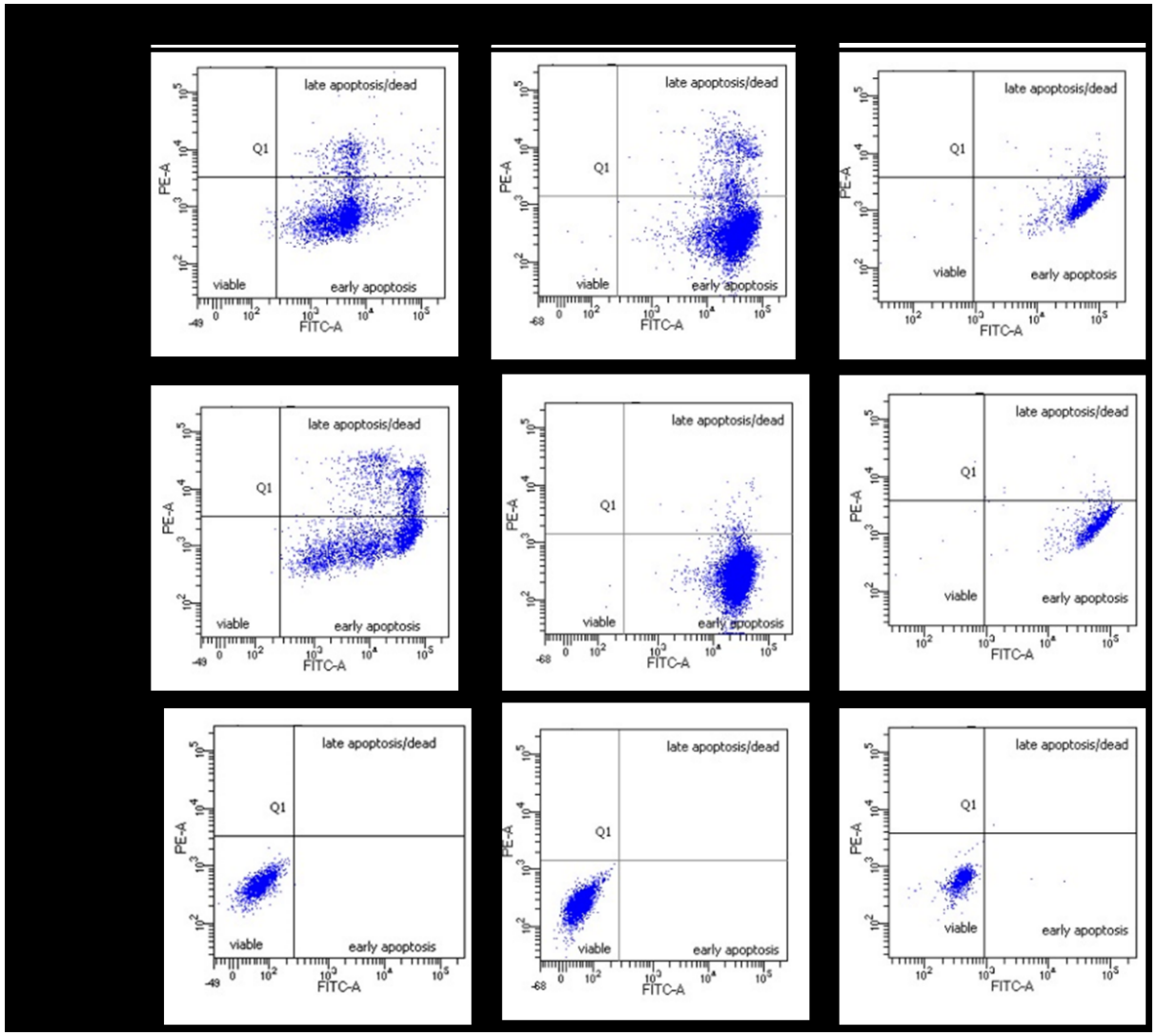

Figure 0.3: Flow cytometry analysis showing the externalisation of phosphatidylserine in A549, MCF-7 and HEK293 cells treated with *Lablab purpureus* peptide Fraction 2, camptothecin and untreated cells.

4.3.5. p53

The p53 ELISA kit was used to verify the apoptotic ability of the fractions and to determine whether the peptide sequence can specifically bind to p53 (Xue et al., 2015). When p53 becomes activated in cancer cells, apoptosis is also activated by inducing and activating the expression of pro-apoptotic proteins (Bowne et al., 2007). Bioactive compounds have been reported to reduce p53 degradation and assist in the activation of p53 (Xue et al., 2015). The synthetic peptide derived from chickpea exhibited an increase in p53 protein compared to its control (Xue et al., 2015). This study also observed higher p53 expression levels in cells treated with the peptide fraction compared to the controls, camptothecin and untreated cells. MCF-7 and A549 showed a 1.03 and 1.97-fold increase, respectively, when compared with camptothecin. Under normal conditions, p53 levels are low because of ubiquitin-dependent proteolysis. However, levels increase when cells undergo stress and eventually lead to apoptosis (Marqus et al., 2017). Results show a similar trend. Therefore, the increase of p53 could regulate signalling pathways resulting in targeted apoptosis and mitochondrial oxidative phosphorylation (Quintal-Bojórquez and Segura-Campos, 2021). Heathy cell (HEK293) observed low p53 levels.

Table 4.4: Human p53 activity on A549, MCF-7 and HEK293 were treated with *Lablab purpureus* RP-HPLC Fraction 2 derived from pepsin hydrolysate

Sample	MCF-7 (ng/mL)	A549 (ng/mL)	HEK293 (ng/mL)
Treated	$9.021^{a}\pm0.024$	$29.923^{ab}\pm1.661$	$0.762^{a}\pm0.216$
Camptothecin	$8.697^{a}\pm0.252$	$15.195^{ab}\pm0.128$	$0.650^{a}\pm0.152$
Untreated	$6.776^{a}\pm0.080$	$21.888^{ab}\pm0.735$	$0.538^{a}\pm0.008$

Data represents mean±SD. Values with different superscripts are significantly different ($p<0.05$).

4.3.6. RP LCMS

The purity of Fraction 2 was determined by passing the sample through RP LCMS (Figure 4.4). The fraction seems to be fairly pure as there is one large peak that appears on the chromatogram at the retention time of 11.85 min when detected at 214 nm. Fraction 2 was achieved by further purifying the 3 kDa fraction by RP-HPLC. The determined molecular weight of the largest peak was 13.01 Da. Further analysis would be required to determine the peptide sequence and structure.

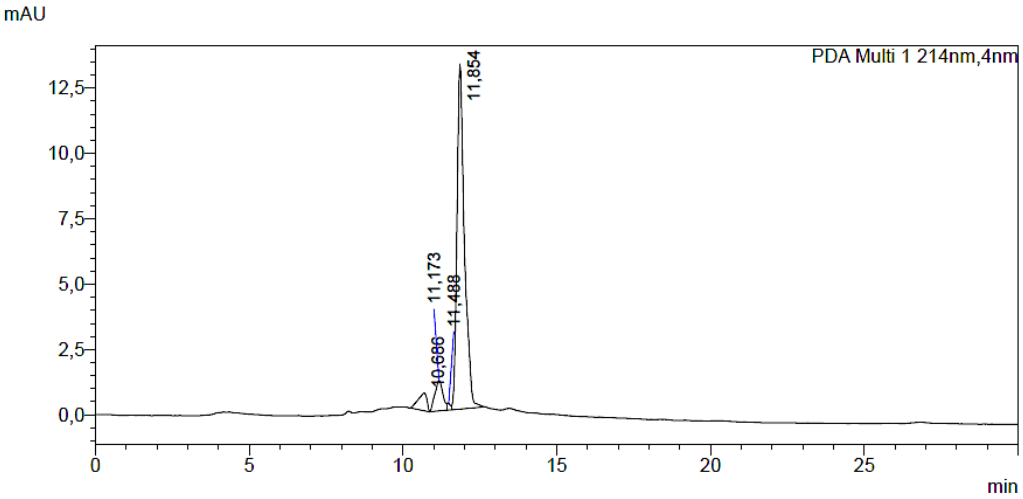

Figure 4.0: LCMS chromatogram of Fraction 2 from *Lablab purpureus* purified by RP-HLPC

4.4. Conclusions

The development of a new class of anticancer agents that is not toxic to healthy cells is of utmost importance. Food derived peptides, particularly, have become desirable as nutraceuticals because of their enhanced bioactive abilities and their low toxicity. The peptide fraction derived from *L. purpureus* has demonstrated the ability to induce apoptosis. Caspases-3 and -9 are markers of apoptosis which were observed by the cancerous cell line A549 and MCF-7. The healthy cell line, HEK293, showed lower caspase-3 and -9 activities. Similar findings were observed for the presence of p53. Finally, annexin V-PI staining confirmed that Fraction 2 induced apoptosis in the cancer cells. Although this fraction can induce apoptosis in cancer cells and has minimal impact on healthy cells, further analysis is required to determine the peptide sequence and the structure of this peptide.

Chapter 5: General Discussion

Legumes form an essential part of the human diet, regarded as an excellent source of proteins (16-50%), vitamins, minerals, and bioactive compounds (Hou et al., 2019; Matemu et al., 2021). Globally, legumes provide approximately 33% of dietary proteins to millions of people, especially in developing countries (Matemu et al., 2021). Human diets are slowly moving away from consumption of animal-based proteins and toward a plant rich protein diet as an alternative. Apart from basic nutrition, legumes also serve as a source of bioactive components. Antioxidants are one such class of compounds naturally present in legumes. Proteins and peptides from food legumes are known to have good antioxidant potential. Peptides liberated during enzymatic hydrolysis of proteins have been reported to enhance antioxidant abilities (López-Cortez et al., 2016). Peptides derived from the enzymatic hydrolysis of varieties of *Phaseolus vulgaris* L. beans, chickpeas, lentils and mung beans have demonstrated antioxidant ability (López-Cortez et al., 2016).

Although antioxidant activity has been reported as a common factor in peptides with multifunctional abilities, most peptides are studied for their singular bioactivity (Daliri et al., 2017). Peptides with multifunctional abilities should be able to provide more than one bioactive role. A peptide derived from chickpea albumin has been shown to possess both strong antioxidant and anticancer abilities in MCF-7 cancer cell line (Daliri et al., 2017). Low molecular weight peptides, in general, have been shown to exert multifunctional bioactive activity (Daliri et al., 2017). The results of *L. purpureus* Fraction 2 which was derived from this study have proved to possess both antioxidant and anticancer abilities.

Enzymatic hydrolysis is the most common method used for the breakdown of proteins (Yeo and Shahidi, 2021). Enzymatic hydrolysis leads to structural changes, reduction of molecular size, higher accessibility of hydrophobic regions and the generation of ionisable groups (Yeo and Shahidi, 2021). The resultant structural changes subsequently lead to a wide spectrum of bioactivities (Yeo and Shahidi, 2021). Specific and nonspecific proteases are used for the hydrolysis of crude proteins to release bioactive peptides with consistent molecular weights and peptide composition (Olagunju et al., 2018). This study observed the *in vitro* anticancer and antioxidant capabilities of peptides derived from pepsin hydrolysis.

Chickpea proteins hydrolysed with neutrase protease recorded a higher degree of hydrolysis over 4 h when compared with trypsin, alcalase and papain proteases. The relatively high degree of hydrolysis suggests that smaller peptides are cleaved (Wali et al., 2021). SDS-PAGE serves as a visual representation that proteins have been hydrolysed as well as their respective molecular weights. Figure 3.1 confirms that *L. purpureus* peptides have been hydrolysed where it was observed that the pepsin hydrolysate had a greater number of bands at lower molecular weights. Pepsin is a broad-spectrum enzyme and therefore the resultant peptide chain will be shorter thereby resulting in many bands.

Oxidative stress has been noted to promote cell proliferation in different types of cells, consequently playing a role in the carcinogenic process. Therefore ROS-eliminating approaches have been developed to decrease ROS levels in cancer cells. This can be actioned by employing antioxidant-based anticancer agents (Chi et al., 2015). Bioactive peptides derived from dietary sources and possessing antioxidant potential have an increased physiological response to reducing the risk of developing diseases caused by oxidative stress (Heredia-Rodríguez et al., 2016). Low molecular weight peptides, in particular, have been observed to hold stronger antioxidant activity (He et al., 2013). The antioxidant activity of the peptide is also associated with its amino acid composition, structure, and molecular weight (Wali et al., 2021; Sosalagere et al., 2022).

Half-maximal inhibitory concentration or IC_{50} is used to measure the efficacy of a drug (Aykul and Martinez-Hackert, 2016). It is indicative of the concentration of drug required to inhibit a biological process by 50% thereby measuring the potency of the drug (Aykul and Martinez-Hackert, 2016). The lowest inhibitory concentration (IC_{50}) values and radical scavenging ability (%) were used to evaluate the radical scavenging abilities of the isolate and hydrolysates. Radical scavenging ability is inversely proportional to the IC_{50} values.
he hydrolysates exhibited greater ABTS radical scavenging ability, among the three hydrolysates, the pepsin hydrolysate proved to have a higher radical scavenging ability. These findings could be attributed to the mechanisms of action used by DPPH and ABTS to scavenge free radicals. DPPH measures the scavenging capacity of antioxidants towards DPPH and ABTS evaluates the decomposition of the ABTS cation in the presence of an antioxidant agent.

Olagunju et al. (2018) reported that hydrolysates with a higher degree of hydrolysis exhibit increased exposure to hydrophobic side chains resulting in increased DPPH radical scavenging activity. However, findings in this study contradict most other studies as *L. purpureus* hydrolysates were shown to possess superior radical scavenging ability. Changes to the composition and amino acid sequence of the peptide can affect the antioxidant activity (Hamzeh et al., 2016). The isolate showed 0.5-fold greater DPPH radical scavenging activity than the pepsin hydrolysate (the highest radical scavenging ability among the hydrolysates). Adzuki bean protein isolate also reported higher radical scavenging activity compared to hydrolysates. The authors attributed their findings to the presence of hydrophobic amino acid residues (Chen et al., 2017)The authors also suggest that hydrogen atoms were readily donated to neutralise DPPH. However, the strength is dependent on the peptide size as well as the enzyme used for hydrolysis (Olagunju et al., 2018). The higher radical scavenging activity could also be attributed to a synergistic effect of the proteins present in the isolate (Floegel et al., 2011).

The ABTS radical scavenging assay found that the pepsin hydrolysate showed greater radical scavenging ability over the other hydrolysates and the isolate. Similar findings were observed on pepsin hydrolysates derived from chickpeas (Faridy et al., 2020). Compared with a strong antioxidant, glutathione, the pepsin hydrolysate showed a 2.6-fold greater ability. The comparison between glutathione, a known powerful antioxidant, is indicative that a compound would be a good antioxidant. Thereby proving the potential of the pepsin hydrolysate as a strong antioxidant. While the pepsin hydrolysate was observed to have greater antioxidant activity overall, the other hydrolysates also showed superior ability over glutathione.

The superoxide radical is a toxic radical species that can be created through biological reactions. They are also precursors to other reactive species such as hydrogen peroxide and hydroxyl radicals (Olagunju et al., 2018). Amongst the hydrolysates, the alcalase hydrolysate proved to have higher radical scavenging activity. This hydrolysate showed 2-fold greater activity than glutathione.

The FRAP assay evaluates the antioxidants' ability to donate hydrogen atoms or electrons (Olagunju et al., 2018). The ability to donate hydrogen atoms is usually dependent on the presence of antioxidant activity (Olagunju et al., 2018). This assay differs from the other assays as it directly measures the antioxidants or reductants present in the sample (Payne et al., 2013). Unlike other assays, free radicals are not involved during the reaction. Rather

the reduction of ferric iron to ferrous iron is observed (Floegel et al., 2011). There was no significant difference observed between the isolate and hydrolysates. Although one hydrolysate did not show dominance over the others, the hydrolysates still showed superior radical scavenging ability over glutathione. This observation is indicative that *L. purpureus* isolate and hydrolysates are strong antioxidants. While the antioxidant ability was determined, the radical scavenging ability was not employed as the deciding factor when selecting the hydrolysate that would be further fractionated. Rather, the MTT assay was utilised as the determining factor.

Based on the MTT assay, the pepsin hydrolysate was selected for apoptotic studies (i.e., morphology (ethidium bromide staining), caspase-3 and -7 and annexin V-PI). The pepsin hydrolysate was thereafter fractioned by ultrafiltration to separate the hydrolysate into molecular weight fractions. The 3 kDa ultrafiltration fraction was then further fractionated by RP-HPLC. Five main peaks were observed in the RP-HPLC chromatogram (Appendices Figure 7.2). Each peak was collected and analysed for the fraction with the best cell viability. Each of the collected fractions was passed through RP-HPLC to determine their purity (Appendices Figures 7.3-7.7). Finally, fraction 2 was selected for apoptotic analyses and analysed by RP-LCMS (Figure 7.8, appendix 1). The calculated molecular weight of Fraction 2 was 13.01 Da. Common bean cultivars that were cultivated in regions of Mexico and Brazil identified peptides by HPLC-MS/MS with molecular weights between 300 and 1950 kDa (López-Cortez et al., 2016; Yaghoubzadeh et al., 2020).

The MTT colourimetric assay was used to determine whether *L. purpurues* isolate and hydrolysates were able to inhibit the proliferation of cancerous cell lines, A549 and MCF-7, while resulting in minimal harm to the healthy cell line, HEK293. The assay also determined whether the hydrolysates and fraction were toxic to the cells. The toxicity of the compounds would be established by the immediate death of the cells, especially at the lowest concentration. Again, the most active hydrolysate was determined by the process of elimination. The process of elimination determined by the MTT assay was carried through when selecting the prominent ultrafiltration and RP-HPLC fractions. The selection was based on the hydrolysate/fraction with the lowest IC_{50} value for cancer cells and the highest for the healthy cell line.

The lowest and highest IC_{50} values were not always observed by the same hydrolysate/fraction therefore the hydrolysate/fraction with the best fit was selected for

further analysis. This is due to cancer treatment not following a uniform approach. Peptides differ in their ability to inhibit cancerous cells. Differences include the type of cells as well as the treatment conditions (Heredia-Rodríguez et al., 2016). Peptides derived from hydrolysis showed enhanced bioactive abilities compared to their parent proteins (Hsieh et al., 2020). The A549 and MCF-7 cell lines treated with the pepsin hydrolysate showed 2.5-fold greater inhibition compared to camptothecin, the anticancer agent that served as the control. This comparison shows that the pepsin hydrolysate has the potential to be a strong anticancer agent.

The whole concept of alternative anticancer agents is to induce apoptosis in cancerous cells while causing the least amount of harm to healthy cells. As stated earlier, traditional cancer treatments induce apoptosis or inhibit cell proliferation in cancerous cells however the drugs are also toxic to normal cells. Consistently through this study, it was observed that the hydrolysate and fractions did not cause great harm to the healthy cell line, HEK293. The compounds however did inhibit proliferation in both cancer cell lines.

The mechanism used in alternative treatments is also required to be investigated to determine to give more insight into the agent. Although not extensively reviewed, the anticancer potential of plant peptides can be exerted through many mechanisms such as inducing apoptosis, cell cycle arrest and damage to the cell cycle (Heredia-Rodríguez et al., 2016; Hsieh et al., 2020). Chickpea protein hydrolysed by pepsin exhibited inhibitory effects against colorectal cancer cells (Caco-2). Mung bean isolates showed antiproliferative activity on MCF-7 cells (Rao et al., 2018; Hou et al., 2019). However, the specific regulatory mechanisms related to the inhibition of cancer cells have not been fully understood (Hou et al., 2019). This study revealed that caspases -3, -7 and -9 and p53 markers were present during apoptosis of the cancer cells to some degree or the other and to a lesser extent in the HEK293 cell line.

Cancerous cells are characterised by a large nucleus, irregular size and shape and predominant nucleoli (Baba and Câtoi, 2007). Apoptosis occurs naturally in the lifecycle of a cell, the cell stops all essential functions and consequently, dies. This process is triggered by activating genetic self-destruction programs present in the cell's genome (Baba and Câtoi, 2007).
Characterised by biochemical and morphological processes like chromatin concentration, poly-nucleosomal DNA fragmentation and the fragmentation of the cell into apoptotic

bodies (Baba and Câtoi, 2007). Visually, cells undergo morphological changes such as shrinkage, blebbing of the plasma membrane, cell detachment, externalisation of phosphatidylserine and finally DNA fragmentation (Brauchle et al., 2014). These morphological changes can be seen in Figure 7.1 (Appendices)

Although the mechanisms of inhibition are not fully understood, apoptosis is the key marker for an effective anticancer agent. Five peptides derived from the hydrolysis of common bean exhibited antiproliferative effects against human colon cells; HCT-116, RKO and KM12L4 through the modification of proteins that are involved in cell cycle arrest or induction of apoptosis (Quintal-Bojórquez and Segura-Campos, 2021). Peptides derived from rapeseed protein showed potent antiproliferative activity against MCF-7 cells (Yeo and Shahidi, 2021).

p53, the tumour suppressor gene or oncogene, has a significant role in the prevention of tumours (Liang et al., 2013). The oncogene works by blocking mitogenic signals in the nucleus of a cell. The expression of p53 is stimulated when cells are stressed, there is damage to DNA or when reacting to oncogene activation (Jan and Chaudhry, 2019). While normal cells in normal conditions are deficient in p53 (Jan and Chaudhry, 2019). Intracellular and extracellular stress signals activate dormant p53 causing p53 accumulation in the cell nucleus (Jan and Chaudhry, 2019). DNA mutations occur in over 50% of approximately every type of tumour (Jagelska et al., 2002; Liang et al., 2013; Marqus et al., 2017). The disruption of normal cell functions is usually a prerequisite for the initiation and/or progression of tumours (Liang et al., 2013). Fraction 2 showed higher p53 expression levels in both the cancer cell lines while lower levels were noted in the healthy cell line. The increased p53 expression levels in the cancer cell lines show that fraction 2 was targeting cancerous cells. The isolate and pepsin hydrolysate observed more viable cells in the HEK293 cell line. This trend was also noted in fraction 2. Again, this indicated that peptide fraction 2 has more of an effect on cancerous cells.

During apoptosis, caspases are triggered. Firstly, caspase-9, the initiator caspase is activated and later caspase-3 and -7, the executioner caspases, leading to the cleavage of many cellular targets ultimately resulting in cell death (Marqus et al., 2017; Mohamed et al., 2017).
Initiation of the executioner caspase 3 is responsible for the cleavage of many cellular proteins which leads to biochemical and morphological characteristics of apoptosis (Boland

et al., 2013). When cells undergo stress, apoptosis is mainly facilitated by caspase-3 (Boland et al., 2013). Mohamed et al. (2017) reported that their compounds induced apoptosis through the upregulation of caspase-3.

Badmus et al. (2015) reported an increase in caspase-3 activity in Hela cells in a concentration-dependent manner while MCF-7 and HT-29 cells had decreased activity. Caspase-9 on the other hand had decreased activity in all three of the cell lines. The increased caspase activity in Hela cells can be attributed to apoptosis being triggered by caspases while the decrease in MCF-7 and HT-29 cells could involve the degradation of proteases.

Caspase-3 and -7 levels were lower in A549 and MCF-7 cells that were treated with the isolate and pepsin hydrolysate than HEK293 cells that were treated in the same manner. Although lower levels were observed in the cancerous cell lines, when compared with camptothecin, the cells showed no significant difference in activity thereby showing that the isolate and pepsin hydrolysate were capable of inducing apoptosis in cells. Higher levels of caspases in HEK293 cells were attributed to these cells being more sensitive than cancerous cells. After fractionation, the caspase -3 and -9 activity was more than two times greater in cancer cells treated with fraction 2 compared to camptothecin. The A549 cell line responded to fraction 2 to a greater degree (more than 100-fold) than the MCF-7 cell line.

Once markers of apoptosis are established, annexin V-PI staining can be conducted to confirm whether the peptide fraction, although initiating apoptosis, is not toxic to the cell as well as to differentiate the stages of apoptosis. A single anticancer agent is unable to target every type of cancer. Therefore, fraction 2 may have a better affinity to lung cancer cells (A549). This finding is attribute can be confirmed by the caspase-3 and -9 and p53 activity of the A549 cell line showing increased activity over the MCF-7 cell line. However, overall, it appears that *L. purpureus* derived fraction 2 has the ability to induce apoptosis in the cancer cell lines while leaving the healthy cells relatively unharmed.

Future perspectives from peptides as therapeutic adjuvants for cancer

Alternative therapies have many supporting benefits such as improved organ or tumour permeability, lowered production and synthesis costs, lower toxicity since the principal components are amino acids. Peptides are also less immunogenic and more stable at room temperature allowing for a longer shelf-life.

Many peptides exhibit anticancer and antioxidant potential. However, the drawbacks of peptide therapies include the lack of appropriate and scalable production methods, satisfactory analysis of mechanisms of action and only a small number of these peptides reach clinical trials (Quintal-Bojórquez and Segura-Campos, 2021; Sosalagere et al., 2022). Further studies are necessary to define the future of bioactive peptides for their use as chemopreventative agents (Quintal-Bojórquez and Segura-Campos, 2021). In-depth, studies are required for a better understanding of the mechanisms.

Conclusion and Recommendations

The most important concept when developing a new class of anticancer agents is for the agent to be nontoxic to healthy cells. The purified peptide derived from *L. purpureus* was able to achieve this outcome. Furthermore, peptides that have not been used for their anticancer potential will be utilised by the body as an antioxidant. An anticancer agent with antioxidant abilities makes a good treatment protocol for cancers. Understanding the antioxidant potential of fractions *in vitro* would be advantageous.

The enhanced antioxidant ability of both the isolate and hydrolysates provided a foundation for exploring the anticancer potential of the hydrolysates. As it is generally accepted that good antioxidants would make effective anticancer agents. The pepsin hydrolysate while non-toxic, was able to induce apoptosis in the cancer cell lines while leaving healthy cells minimally harmed. After further fractionation, fraction 2, demonstrated the ability to induce apoptosis in cancerous cells. The cancer cell lines observed a higher level of caspases while the healthy cell line observed low levels. A similar trend was observed for p53. The peptides' ability to selectively downregulate anti-apoptotic proteins and consequently upregulate pro-apoptotic proteins and p53 in cancerous cells verify the potential as an anticancer agent. Annexin V-PI staining confirmed that cells were undergoing apoptosis in the cancer cells. Overall, peptide Fraction 2 showed low toxicity in healthy cell lines and exhibited antiproliferative abilities on cancerous cells.

Chapter 6: References

AKSOY, L., KOLAY, E., AGILONU, Y., ASLAN, Z. & KARGIOGLU, M. 2013. Free radical scavenging activity, total phenolic content, total antioxidant status, and total oxidant status of endemic *Thermopsis turcica*. *Saudi Journal of Biological Sciences,* 20, 235 - 239.

ALISON, M. R. 2001. Cancer. *Encyclopedia of life sciences*. London, UK: Nature Publishing Group.

AMGALAN, D., PEKSON, R. & KITSIS, R. N. 2017. Troponin Release Following Brief Myocardial Ischemia Apoptosis Versus Necrosis*. *Basics To Transitional Science,* 2.

AMINI-SARTESHNIZI, N., ZAHRI, S., JAFARI-GHAHFAROKHI, H., HAFSHEJANI, F. K. & TEIMORI, H. 2014. Morphological changes of apoptosis and cytotoxic effects induced by Caffeic acid phenethyl ester in AGS human gastric cancer cell line. *Journal of HerbMed Pharmacology,* 3, 77-82.

AN, W., LAI, H., ZHANG, Y., LIU, M., LIN, X. & CAO, S. 2019. Apoptotic Pathway as the Therapeutic Target for Anticancer Traditional Chinese Medicines. *Frontiers in Pharmacology,* 10.

APOSTOLOPOULOS, V., BOJARSKA, J., CHAI, T., ELNAGDY, S., KACZMAREK, K., MATSOUKAS, J., NEW, R., PARANG, K., LOPEZ, O. P., PARHIZ, H., PERERA, C. O., PICKHOLZ, M., REMKO, M., SAVIANO, M., SKWARCZYNSKI, M., TANG, Y., WOLF, W. M., YOSHIYA, T., ZABROCKI, J., ZIELENKIEWICZ, P., ALKHAZINDAR, M., BARRIGA, V., KELAIDONIS, K., SARASIA, E. M. & TOTH, I. 2021. A Global Review on Short Peptides: Frontiers and Perspectives *Molecules,* 26, 430.

ARISE, A. K. 2016. *Composition and functional bioactive properties of bambara groundnut protein and hydrolysates*. Doctor of Philosophy Durban University of Technology.

ATHREYA, K. & XAVIER, M. F. 2017. Antioxidants in the Treatment of Cancer. *Nutrition and cancer,* 69, 1099-1104.

AUNG, T. N., QU, Z., KORTSCHAK, R. D. & ADELSON, D. L. 2017. Understanding the Effectiveness of Natural Compound Mixtures in Cancer through Their Molecular Mode of Action. *International Journal of Molecular Science,* 18.

AVILÉS-GAXIOLA, S., GUTIÉRREZ-GRIJALVA, E. P., LEÓN-FELIX, J., ANGULO-ESCALANTE, M. A. & HEREDIA, J. B. 2020. Peptides in Colorectal Cancer: Current State of Knowledge. *Plant Foods for Human Nutrition.*

AYKUL, S. & MARTINEZ-HACKERT, E. 2016. Determination of half-maximal inhibitory concentration using biosensor-based protein interaction analysis. *Analytical Biochemistry,* 508, 97-103.

BABA, A. I. & CÂTOI, C. 2007. Tumor Cell Morphology. *Comparative Oncology*. Bucharest: The Publishing House of the Romanian Academy.

BADARINATH, A. V., RAO, K. M., CHETTY, C. M. S., RAMKANTH, S., RAJAN, T. V. S. & GNANAPRAKASH, K. 2010. A Review on In-vitro Antioxidant Methods: Comparisions, Correlations and Considerations. *International Journal of PharmTech Research,* 2, 1276 - 1285.

BADMUS, J., EKPO, O., HUSSEIN, A., MEYER, M. & HISS, D. 2015. Antiproliferative and Apoptosis Induction Potential of the Methanolic Leaf Extract of *Holarrhena floribunda* (G. Don). *Evidence-Based Complementary and Alternative Medicine.*

BARARI, L., ABEDIAN, Z., ASADI, A., ELMI, F. & ELMI, M. M. 2017. Anti-proliferative Effect of Chickpea Extract on Hela, MCF-7, Saos and Fibroblast Cell Lines. *British Journal of Pharmaceutical Research,* 15, 1-9.

BARMAN, A., MARAK, C. M., BARMAN, R. M. & SANGMA, C. S. 2018. Nutraceutical Properties of Legume Seeds and Their Impact on Human Health. *In:* JIMENEZ-LOPEZ, J. C. (ed.) *Legume Seed Nutraceutical Research*. IntechOpen.

BARNES, M., URUAKPA, F. & UDENIGWE, C. 2015. Influence of Cowpea (Vigna unguiculata) Peptides on Insulin Resistance. *Journal of Nutritional Health & Food Science,* 3, 1-3.

BARRIO, D. A. & ANON, M. C. 2010. Potential antitumor properties of a protein isolate obtained from the seeds of *Amaranthus mantegazzianus*. *European Journal of Nutrition* 49.

BENZIE, I. & STRAIN, J. J. 1996. The Ferric Reducing Ability of Plasma (FRAP) as a Measure of "Antioxidant Power": The FRAP Assay. *Analytical Biochemistry,* 239.

BOLAND, K., FLANAGAN, L. & PREHN, J. H. M. 2013. Paracrine control of tissue regeneration and cell proliferation by Caspase-3. *Cell Death and Disease,* 4.

BOWNE, W. B., MICHL, J., BLUTH, M. H., ZENILMAN, M. E. & PINCUS, M. R. 2007. Novel peptides from the RAS-p21 and p53 proteins for the treatment of cancer. *Cancer Therapy,* 5B, 331-344.

BOYE, J., ZARE, F. & PLETCH, A. 2010. Pulse proteins: Processing, characterization, functional properties and applications in food and feed. *Food Research International,* 43, 414-431.

BRAUCHLE, E., THUDE, S., BRUCKER, S. Y. & SCHENKE-LAYLAND, K. 2014. Cell death stages in single apoptotic and necrotic cells monitored by Raman microspectroscopy. *Scientific Reports.*

BRENTNALL, M., RODRIGUEZ-MENOCAL, L., GUEVARA, R. L. D., CEPERO, E. & BOISE, L. H. 2013. Caspase-9, Caspase-3 and Caspase-7 have distinct roles during intrinsic apoptosis. *BMC Cell Biology,* 14.

BROWN, J. M. & ATTARDI, L. D. 2005. The role of apoptosis in cancer development and treatment response. *Nature reviews,* 5, 231-237.

BURZ, C., BERINDAN-NEAGOE, I., BALACESCU, O. & IRIMIE, A. 2009. Apoptosis in cancer: key molecular signaling pathways and therapy targets. *Acta Oncologica,* 48, 811-821.

BUTTERICK, T., DUFFY, C., LEE, R., BILLINGTON, C., KOTZ, C. & NIXON, J. 2014. Use of a Caspase Multiplexing Assay to Determine Apoptosis in a Hypothalamic Cell Model. *Journal of Visualized Experiments,* 86.

BUXBAUM, E. 2007. *Fundamentals of Protein Structure and Function,* Portsmouth, Dominica, Springer.

CANSA. 2021. *South African Cancer Statistics* [Online]. Available: cansa.org.za/south-african-cancer-statistics/ [Accessed 04 October 2021].

CARBONARO, M., MASELLI, P. & NUCARA, A. 2015. Structural aspects of legume proteins and nutraceutical properties. *Food Research International,* 76, 19-30.

CARRASCO-CASTILLA, J., HERNÁNDEZ-ÁLVAREZ, A. J., JIMÉNEZ-MARTÍNEZ, C., JACINTO-HERNÁNDEZ, C., ALAIZ, M., GIRÓN-CALLE, J., VIOQUE, J. & DÁVILA-ORTIZ, G.

2012. Antioxidant and metal chelating activities of peptide fractions from phaseolin and bean protein hydrolysates. *Food Chemistry,* 135, 1789-1795.

CASTLE, V. P., HEIDELBERGER, K. P., BROMBERG, J., OU, X., DOLE, M. & NUNEZT, G. 1993. Expression of the Apoptosis-Suppressing Protein bcl-2, in Neuroblastoma Is Associated with Unfavorable Histology and N-myc Amplification. *American Journal of Pathology,* 143, 1543-1550.

CASTRO, G. A., MARIA, D. A., BOUHALLAB, S. & SGARBIERI, V. C. 2009. *In vitro* impact of a whey protein isolate (WPI) and collagen hydrolysates (CHs) on B16F10 melanoma cells proliferation. *Journal of Dermatological Science,* 56, 51-57.

CDC. 2018. *Lymphoma* [Online]. USA: Centers for Disease Control and Prevention. Available: https://www.cdc.gov/cancer/lymphoma/index.htm [Accessed 18-09-2022 2022].

CHAHAL, A., SAINI, A. K., CHHILLAR, A. K. & SAINI, R. V. 2018. Natural Antioxidants As Defense System Against Cancer. *Asian Journal of Pharmaceutical and Clinical Research,* 11, 38-44.

CHALAMAIAH, M., YU, W. & WU, J. 2018. Immunomodulatory and anticancer protein hydrolysates (peptides) from food proteins: A review. *Food Chemistry,* 245, 205-222.

CHANG, S. K., ISMAIL, A., YANAGITA, T., ESA, N. M. & BAHARULDIN, M. T. H. 2014. Biochemical characterisation of the soluble proteins, protein isolates and hydrolysates from oil palm (*Elaeis guineensis)* kernel. *Food Bioscience,* 7, 1-10.

CHATTERJEE, C., GLEDDIE, S. & XIAO, C. 2018. Soybean Bioactive Peptides and Their Functional Properties. *Nutrients,* 10.

CHEHADE, H., FOX, A., MOR, G. G. & ALVERO, A. B. 2021. Determination of Caspase Activation by Western Blot. *In:* ALVERO, A. B. & MOR, G. G. (eds.) *Detection of Cell Death Mechanisms Methods and Protocols*. New York: Springer Protocols.

CHEN, Z., LI, W., SANTHANAM, R. K., WANG, C., GAO, X., CHEN, Y., WANG, C., XU, L. & CHEN, H. 2019. Bioactive peptide with antioxidant and anticancer activities from black soybean [Glycine max (L.) Merr.] byproduct: isolation, identification and molecular docking study. *European Food Research and Technology*.

CHEN, Z., WANG, J. & CHEN, H. 2017. Physicochemical characterization, antioxidant and anticancer activities of proteins from four legume species. *Journal of Food Science and Technology,* 54, 964-972.

CHENG, A., RAAI, M. N., ZAIN, N. A. M., MASSAWE, F., SINGH, A. & WAN-MOHTAR, W. A. I. Q. I. 2019. In search of alternative proteins: unlocking the potential of underutilized tropical legumes. *Food Security,* 11, 1205-1215.

CHI, C., HU, F., WANG, B., LI, T. & DING, G. 2015. Antioxidant and anticancer peptides from the protein hydrolysate of blood clam (*Tegillarca granosa*) muscle. *Journal of Functional Foods,* 15, 301-313.

CLEVELANDCLINIC. 2022. *What is Leukaemia* [Online]. Cleveland, Ohio, USA: Cleveland Clinic. Available: https://my.clevelandclinic.org/health/diseases/4365-leukemia [Accessed 17-09-2022 2022].

COMSA, S., CIMPEAN, A. M. & RAICA, M. 2015. The Story of MCF-7 Breast Cancer Cell Line: 40 years of Experience in Research. *Anticancer Research,* 35, 3147-3154.

COOPER, G. 2000. The cell: A molecular approach. 2nd ed. Sunderland (MA): Sinauer Associates.

CTCA. 2022. *What is carcinoma* [Online]. Cancer Treatment Centers of America. Available: https://www.cancercenter.com/carcinoma [Accessed 19-09-2022 2022].

D'ARCY, M. S. 2019. Cell death: a review of the major forms of apoptosis, necrosis and autophagy. *Cell Biology International,* 43, 582-592.

DALIRI, E. B., OH, D. H. & LEE, B. H. 2017. Bioactive Peptides. *Foods,* 6.

DELBUFALO, D., BIROCCIO, A., LEONETFI, C. & ZUPI, G. 1997. Bcl-2 overexpression enhances the metastatic potential of a human breast cancer line. *Federation of American Societies for Experimental Biology,* 11, 947-953.

DIA, V. P. & GONZALEZ, M. E. 2011. Lunasin induces apoptosis and modifies the expression of genes associated with extracellular matrix and cell adhesion in human metastatic colon cancer cells. *Molecular Nutrition and Food Research, 55,* 623-634.

DIA, V. P. & MEJIA, E. G. 2010. Lunasin promotes apoptosis in human colon cancer cells by mitochondrial pathway activation and induction of nuclear clusterin expression. *Cancer Letters,* 295, 44-53.

DÍAZ-GÓMEZ, J. L., CASTORENA-TORRES, F., PRECIADO-ORTIZ, R. E. & GARCÍA-LARA, S. 2017. Anti-Cancer Activity of Maize Bioactive Peptides. *Frontiers in Chemistry, 5*.

DURANTI, M. 2006. Grain legume proteins and nutraceutical properties. *Fitoterapia 77*, 67 - 82.

DWARKA, D., THAVER, V., NAIDU, M., KOORBANALLY, N. & BAIJNATH, H. 2017. *In vitro* chem-preventative activity of *Stelitzia nicolai* aril extract containing bilirubin. *African Journal of Traditional Complementary and Alternative Medicines,* 14, 147-156.

ERBERSDOBLER, H. F., BARTH, C. A. & JAHREIS, G. 2017. Legumes in human nutrition. *Ernaehrungs Umschau international,* 9.

FAN, L., DU, G., LI, P., LI, M., SUN, Y. & ZHAO, L. 2016. Improved breast cancer cell-specific intracellular drug delivery and therapeutic efficacy by coupling decoration with cell penetrating peptide and SP90 peptide. *Biomedicine & Pharmacotherapy,* 84, 1783-1791.

FARIDY, J. M., STEPHANIE, C. M., GABRIELA, M. O. & CRISTIAN, J. 2020. Biological Activities of Chickpea in Human Health (*Cicer arietinum* L.). A Review. *Plant Food for Human Nutrition*.

FEEDIPEDIA. 2016. *Lablab (Lablab purpureus)* [Online]. [Accessed 30 August 2017].

FELTER, S. P., ZHANG, X. & THOMPSON, C. 2021. Butylated hydroxyanisole: Carcinogenic food additive to be avoided or harmless antioxidant important to protect food supply? *Regulatory Toxicology and Pharmacology*.

FERLAY, J., COLOMBET, M., SOERJOMATARAM, I., PARKIN, D. M., PIÑEROS, M., ZNAOR, A. & BRAY, F. 2021. Cancer statistics for the year 2020: An overview. *Cancer Epidemiology,* 1, 778-789.

FERNALD, K. & KUROKAWA, M. 2013. Evading apoptosis in cancer. *Trends in Cell Biology,* 23, 620-633.

FLOEGEL, A., KIM, D., CHUNG, S., KOO, S. I. & CHUN, O. K. 2011. Comparison of ABTS/DPPH assays to measure antioxidant capacity in popular antioxidant-rich US foods§. *Journal of Food Composition and Analysis,* 24, 1043-1048.

GAO, C., SUN, R., XIE, Y., JIANG, A., LIN, M., LI, M., CHEN, Z., ZHANG, P., JIN, H. & FENG, J. 2017. The soy-derived peptide Vglycin inhibits the growth of colon cancer cells *in vitro* and *in vivo*. *Experimental Biology and Medicine,* 242, 1034-1043.

GIRÓN-CALLE, J., ALAIZ, M. & VIOQUE, J. 2010. Effect of chickpea protein hydrolysates on cell proliferation and *in vitro* bioavailability. *Food Research International,* 43, 1365-1370.

GOBE, G., RUBIN, M., WILLIAMS, G., SAWCZUK, I. & BUTTYAN, R. 2002. Apoptosis and Expression of Bcl-2, Bcl-XL, and Bax in Renal Cell Carcinomas. *Cancer Investigation,* 20, 324-332.

GULCIN, I. 2012. Antioxidant activity of food constituents: an overview. *Archives Toxicology,* 86, 345–391.

GULERIA, P., KUMAR, V. & LICHTFOUSE, E. 2020. *Sustainable Agriculture Reviews,* Switzerland, Springer.

GUPTA, N. & BHAGYAWANT, S. S. 2021. Bioactive peptide of Cicer arietinum L. induces apoptosis in human endometrial cancer via DNA fragmentation and cell cycle arrest. *3 Biotech,* 11.

GUPTA, P. 2020. *Targeted cancer therapy with bioactive foods and their products,* United Kingdom, Academic Press.

HAMZEH, A., BENJAKUL, S. & SENPHAN, T. 2016. Comparative study on antioxidant activity of hydrolysates from splendid squid (*Loligo formosana*) gelatin and protein isolate prepared using protease from hepatopancreas of Pacific white shrimp (*Litopenaeus vannamei*). *Journal of Food science and Technology,* 53, 3615-3623.

HAN, Z. & B.R. HAMAKER 2002. Partial leaching of granule-associated proteins from rice starch during alkaline extraction and subsequent gelatinization. *Starch–Starke,* 54, 454-460.

HE, R., GIRGIH, A., MALOMO, S., JU, X. & ALUKO, R. 2013. Antioxidant activities of enzymatic rapeseed protein hydrolysates and the membrane ultrafiltration fractions. *Journal of Functional Foods,* 5, 219-227.

HEREDIA-RODRÍGUEZ, L., GARZA, A. L. D. L., GARZA-JUAREZ, A. J. & VAZQUEZ-RODRIGUEZ, J. A. 2016. Nutraceutical Properties of Bioactive Peptides in Common Bean (Phaseolus vulgaris L.). *Journal of Food Nutrition and Dietetics,* 2.

HERNÁNDEZ-LEDESMA, B. & HSIEH, C. (eds.) 2013. *Bioactive food peptides in health and disease,* Rijeka, Croatia: InTech.

HOSSAIN, S., AHMED, R., BHOWMICK, S., MAMUN, A. A. & HASHIMOTO, M. 2016. Proximate composition and fatty acid analysis of *Lablab purpureus* (L.) legume seed: implicates to both protein and essential fatty acid supplementation. *Springer Plus, 5*.

HOU, D., YOUSAF, L., XUE, Y., HU, J., WU, J., HU, X., FENG, N. & SHEN, Q. 2019. Mung Bean (Vigna radiata L.): Bioactive Polyphenols, Polysaccharides, Peptides, and Health Benefits. *Nutrients,* 11.

HSIEH, C., HERNÁNDEZ-LEDESMA, B. & LUMEN, B. O. D. 2020. Cancer Chemopreventive Potential of Seed Proteins and Peptides. *Nuts and Seeds in Health and Disease Prevention*. Elsevier Inc.

HUANG, Y., WANG, X., WANG, H., LIU, Y. & CHEN, Y. 2011. Studies on mechanism of action of anticancer peptides by modulation of hydrophobicity within a defined structural framework. *American Association for Cancer Research*.

IDOWU, A. O., FAMUWAGUN, A. A., FAGBEMI, T. N. & ALUKO, R. E. 2021. Antioxidant and enzyme-inhibitory properties of sesame seed protein fractions and their isolate and hydrolyzate. *Internation Journal of Food Properties* 24, 780-795.

JAGELSKA, E., BRAZDA, V., POSPISILOVA, S., VOJTESEK, B. & PALECEK, E. 2002. New ELISA technique for analysis of p53 protein/DNA binding properties. *Journal of Immunological Methods,* 267, 227– 235.

JAHANBANI, R., GHAFFARI, S. M., SALAMI, M., VAHDATI, K., SEPEHRI, H., SARVESTANI, N. N., SHEIBANI, N. & MOOSAVI-MOVAHEDI, A. A. 2016. Antioxidant and Anticancer Activities of Walnut (Juglans regia L.) Protein Hydrolysates Using Different Proteases. *Plant Foods for Human Nutrition,* 71, 402-409.

JAKUBCZYK, A., KARAS, M., RYBCZYNSKA-TKACZYK, K., ZIELINSKA, E. & ZIELINSKI, D. 2020. Current Trends of Bioactive Peptides—New Sources and Therapeutic Effect. *Food,* 9, 846.

JAN, R. & CHAUDHRY, G. 2019. Understanding Apoptosis and Apoptotic Pathways Targeted Cancer Therapeutics. *Advanced Pharmaceutical Bulletin,* 9, 205-218.

JOHNSHOPKINSMEDICINE. 2022. *Sarsomas* [Online]. Maryland, USA: Johns Hopkins Medicine. Available: https://www.hopkinsmedicine.org/health/conditions-and-diseases/sarcoma [Accessed 17-09-2022 2022].

KAMRAN, F. & REDDY, N. 2018. Bioactive peptides from legumes: Functional and nutraceutical potential. *Recent Advances in Food Science,* 1, 134-149.

KANNAN, A., HETTIARACHCHY, N. S., LAY, J. O. & LIYANAGE, R. 2010. Human cancer cell proliferation inhibition by a pentapeptide isolated and

characterized from rice bran. *Peptides,* 31, 1629 - 1634.

KARAMI, Z. & AKBARI-ADERGANI, B. 2019. Bioactive food derived peptides: a review on correlation between structure of bioactive peptides and their functional properties. *Journal of Food Science and Technology*.

KAUR, G. & DUFOUR, J. M. 2012. Cell lines. *Spermatogenesis,* 2, 1-5.

KHANGEBAM, B. & CHAKRABARTI, R. 2018. Viscera of *Labeo rohita*: A potential source of trypsin for industrial application. *Journal of Aquatic Food Product Technology,* 27.

KHARB, M., JAT, R. K. & GUPTA, A. 2012. A review on medicinal plants used as a source of anticancer agents. *International Journal of Drug Research and Technology,* 2, 177-183.

KORHONEN, H. & PIHLANTO, A. 2006. Bioactive peptides: Production and functionality. *International Dairy Journal,* 16, 945-960.

KWAN, Y. P., SAITO, T., IBRAHIM, D., AL-HASSAN, F. M. S., OON, C. E., CHEN, Y., JOTHY, S. L., KANWAR, J. R. & SASIDHARAN, S. 2016. Evaluation of the cytotoxicity, cell-cycle arrest, and apoptotic induction by *Euphorbia hirta* in MCF-7 breast cancer cells. *Pharmaceutical Biology,* 54, 1223-1236.

LEE, K., OH, Y., CHO, W. & MA, J. 2015. Antioxidant and Anti-Inflammatory Activity Determination of One Hundred Kinds of Pure Chemical Compounds Using Offline and Online Screening HPLC Assay. *Evidence-Based Complementary and Alternative Medicine*.

LETAI, A. 2017. Apoptosis and Cancer. *Annual Review of Cancer Biology,* 1, 275-294.

LI-CHAN, E. C. Y. 2015. Bioactive peptides and protein hydrolysates: research trends and challenges for application as nutraceuticals and functional food ingredients. *Current Opinion in Food Science,* 1.

LIANG, Y., LIU, J. & FENG, Z. 2013. The regulation of cellular metabolism by tumor suppressor p53. *Cell & Bioscience,* 3.

LIN, Y., MAURO, J. C. & KAUR, G. 2019. Bioactive Glasses for Cancer Therapy. *In:* KAUR, G. (ed.) *Biomedical, Therapeutic and Clinical Applications of Bioactive Glasses*. United Kingdom: Woodhead.

LIU, J., LIN, M., YU, J., LIU, B. & BAO, J. 2011. Targeting apoptotic and autophagic pathways for cancer therapeutics. *Cancer Letters,* 300, 105-114.

LOGUE, S. E., ELGENDY, M. & MARTIN, S. J. 2009. Expression, purification and use of recombinant annexin V for the detection of apoptotic cells. *Nature Protocols,* 4, 1383-1395.

LOPEZ-BARRIOS, L., GUTIERREZ-URIBE, J. A. & SERNA-SALDIVAR, S. O. 2014. Bioactive Peptides and Hydrolysates from Pulses and Their Potential Use as Functional Ingredients. *Journal of Food Science* 79, R273-R283.

LÓPEZ-CORTEZ, M. S., ROSALES-MARTÍNEZ, P., ARELLANO-CÁRDENAS, S. & CORNEJO-MAZÓN, M. 2016. Antioxidants Properties and Effect of Processing Methods on Bioactive Compounds of Legumes. *In:* GOYAL, A. K. (ed.) *Grain Legumes*. IntechOpen.

LUNA-VITAL, D. A., MOJICA, L., MEJÍA, E. G. D., MENDOZA, S. & LOARCA-PIÑA, G. 2015. Biological potential of protein hydrolysates and peptides from common bean (*Phaseolus vulgaris* L.): A review. *Food Research International,* 76.

MAASS, B. L., KNOX, M. R., VENKATESHA, S. C., ANGESSA, T. T., RAMME, S. & PENGELLY, B. C. 2010. Lablab purpureus—A Crop Lost for Africa? *Tropical Plant Biology,* 3, 123-135.

MAPHOSA, Y. & JIDEANI, V. A. 2017. *The Role of Legumes in Human Nutrition,* London, InTechOpen.

MARATHE, S. A., RAJALAKSHMI, V., JAMDAR, S. N. & SHARMA, A. 2011. Comparative study on antioxidant activity of different varieties of commonly consumed legumes in India. *Food and Chemical Toxicology,* 49, 2005-2012.

MARQUS, S., PIROGOVA, E. & PIVA, T. J. 2017. Evaluation of the use of therapeutic peptides for cancer treatment. *Journal of Biomedical Science,* 24.

MARTÍNEZ-LEO, E. E., A. M, M.-O., ACEVEDO-FERNÁNDEZ, J. J., MOO-PUCC, R. & SEGURA-CAMPOSA, M. R. 2019. Peptides from *Mucuna pruriens* L., with protection and antioxidant *in vitro* effect on HeLa cell line. *Journal of the Science of Food Agriculture,* 99, 4167-4173.

MATEMU, A., NAKAMURA, S. & KATAYAMA, S. 2021. Health Benefits of Antioxidative Peptides Derived from Legume Proteins with a High Amino Acid Score. *Antioxidants,* 10.

MCCONNELL, E. J., DEVAPATLA, B., YADDANAPUDI, K. & DAVIS, K. R. 2014. The soybean-derived peptide lunasin inhibits non-small cell lung cancer cell proliferation by suppressing phosphorylation of the retinoblastoma protein. *Oncotarget,* 6, 4649-4662.

MOHAMED, M. F., SAMIR, N., ALI, A., AHMED, N., ALI, Y., AREF, S., HOSSAM, O., MOHAMED, M. S., ABDELMONIEM, A. M. & ABDELHAMID, I. A. 2017. Apoptotic induction mediated p53 mechanism and Caspase-3 activity by novel promising cyanoacrylamide derivatives in breast carcinoma. *Bioorganic Chemistry,* 73, 43-52.

MONTOYA-RODRÍGUEZ, A., MILÁN-CARRILLO, J., REYES-MORENO, C. & MEJÍA, E. G. D. 2015. Characterization of peptides found in unprocessed and extruded amaranth (Amaranthus hypochondriacus) pepsin/pancreatin hydrolysates. *International Journal of Molecular Science,* 16, 8536 - 5884.

MORRIS, J. B. 2009. Morphological and Reproductive Characterization in Hyacinth Bean, *Lablab Purpureus* (L.) Sweet Germplasm with Clinically Proven Nutraceutical and Pharmaceutical Traits for Use as a Medicinal Food. *Journal of Dietary Supplements*, 263-279.

NAEEM, M., SHABBIR, A., ANSARI, A. A., AFTAB, T., KHAN, M. M. A. & UDDIN, M. 2020. Hyacinth bean (Lablab purpureus L.) – An underutilised crop with future potential. *Scientia Horticulturae,* 109551.

NAIKER, T. S., BAIJNATH, H., AMONSOU, E. O. & MELLEM, J. J. 2020a. The effect of soaking, steaming, and dehydration on the microstructure, physicochemical

properties and *in vitro* starch digestibility of flour produced from Lablab purpureus (L.) Sweet (hyacinth bean). *Journal of Food Processing and Preservation.*

NAIKER, T. S., BAIJNATH, H., AMONSOU, E. O. & MELLEM, J. J. 2020b. Effect of steaming and dehydration on the nutritional quality and functional properties of protein isolates produced from *Lablab purpureus* (L.) Sweet (hyacinth bean). *Journal of Food Processing and Preservation.*

NHS. 2022. *Non-Hodgkins Lymphoma* [Online]. UK: Crown. Available: https://www.nhs.uk/conditions/non-hodgkin-lymphoma/ [Accessed 18-09-2022 2022].

NIH. 2017. *National Cancer Institute* [Online]. [Accessed 20 December 2017 2017].

NOVIKOV, N. M., ZOLOTARYOVA, S. Y., GAUTREAU, A. M. & DENISOV, E. V. 2021. Mutational drivers of cancer cell migration and invasion. *British Journal of Cancer,* 124, 102-114.

OLAGUNJU, A. I., OMOBA, O. S., ENUJIUGHA, V. N., ALASHI, A. M. & ALUKO, R. E. 2018. Pigeon pea enzymatic protein hydrolysates and ultrafiltration peptide fractions as potential sources of antioxidant peptides: An *in vitro* study. *LWT - Food Science and Technology,* 97, 269-278.

ORONA-TAMAYO, D., VALVERDE, M. E. & LÓPEZ, O. P. 2018. Bioactive Peptides from Selected Latin American Food Crops – A Nutraceutical and Molecular Approach. *Critical Reviews in Food Science and Nutrition.*

PAULA, L. C. D., LEMES, A. C., NERI, H. F. D. S., GHEDINI, P. C., BATISTA, K. D. A. & FERNANDES, K. F. 2020. Antioxidant and antiperoxidative effect of polypeptides from common beans (*Phaseolus vulgaris*, cv BRS Pontal). *Brazilian Journal of Development,* 6, 50596-50580.

PAYNE, A. C., MAZZER, A., CLARKSON, G. J. J. & TAYLOR, G. 2013. Antioxidant assays – consistent findings from FRAP and ORAC reveal a negative impact of organic cultivation on antioxidant potential in spinach but not watercress or rocket leaves. *Food Science and Nutrition,* 1, 439-444.

PFEFFER, C. M. & SINGH, A. T. K. 2018. Apoptosis: A Target for Anticancer Therapy. *Internation Journal of Molecular Sciences,* 19.

QUINTAL-BOJÓRQUEZ, N. & SEGURA-CAMPOS, M. R. 2021. Bioactive Peptides as Therapeutic Adjuvants for Cancer. *Nutrition and Cancer,* 73, 1309-1321.

RAFFO, A. J., PERLMAN, H., CHEN, M., DAY, M. L., STREITMAN, J. S. & BUTTYAN, R. 1995. Overexpression of bcl-2 Protects Prostate Cancer Cells from Apoptosis *in vitro* and Confers Resistance to Androgen Depletion in Vivo1. *Cancer Research, 55*, 4438-4445.

RAMKISSON, S., DWARKA, D., VENTER, S. & MELLEM, J. J. 2020. *In vitro* anticancer and antioxidant potential of Amaranthus cruentus protein and its hydrolysates. *Food Science and Technology (Campinas)*.

RAO, S., CHINKWO, K. A., SANTHAKUMAR, A. B. & BLANCHARD, C. L. 2018. Inhibitory Effects of Pulse Bioactive Compounds on Cancer Development Pathways. *Diseases,* 6.

RAYAPROLU, S. J., HETTIARACHCHY, N. S., HORAX, R., KUMAR-PHILLIPS, G., LIYANAGE, R., LAY, J. & CHEN, P. 2017. Purification and characterization of a peptide from soybean with cancer cell proliferation inhibition. *Journal of Food Biochemistry,* 41.

REJHOVA, A., OPATTOVA, A., CUMOVA, A., SLÍVA, D. & VODICKA, P. 2018. Natural compounds and combination therapy in colorectal cancer treatment. *European Journal of Medicinal Chemistry,* 144, 582-594.

ROCHA, T. S., HERNANDEZ, L. M. R., CHANG, Y. K. & MEJÍA, E. G. 2014. Impact of germination and enzymatic hydrolysis of cowpea bean (Vigna unguiculata) on the generation of peptides capable of inhibiting dipeptidyl peptidase IV. *Food Research International,* 64, 799-809.

ROCHETTE, L., ZELLER, M., COTTIN, Y. & VERGELY, C. 2021. Antitumor Activity of Protons and Molecular Hydrogen: Underlying Mechanisms. *Cancers,* 13.

ROY, M., ULLAH, S., ALAM, M. & ISLAM, M. A. 2021. Evaluation of quality parameters and antioxidant properties of protein concentrates and hydrolysates of hyacinth bean (*Lablab purpureus*). *Legume Science,* e128.

RYE, C., WISE, R., JURUKOVSKI, V., DESAIX, J., CHOI, J. & AVISSAR, Y. 2016. *Biology* [Online]. Houston, Texas: OpenStax. Available: https://openstax.org/books/biology/pages/10-4-cancer-and-the-cell-cycle [Accessed 24 May 2022 2022].

SABBIONE, A. C., OGUTU, F. O., SCILINGO, A., ZHANG, M., AÑÓN, M. C. & MU, T. 2019. Antiproliferative Effect of Amaranth Proteins and Peptides on HT-29 Human Colon Tumor Cell Line. *Plant Food for Human Nutrition.*

SALAMPESSY, J., REDDY, N., PHILLIPS, M. & KAILASAPATHY, K. 2017. Isolation and characterization of nutraceutically potential ACEInhibitory peptides from leatherjacket (*Meuchenia* sp.) protein hydrolysates. *LWT - Food Science and Technology,* 80.

SÁNCHEZ-CHINO, X., JIMÉNEZ-MARTÍNEZ, C., DÁVILA-ORTIZ, G., ÁLVAREZ-GONZÁLEZ, I. & MADRIGAL-BUJAIDAR, E. 2015. Nutrient and Nonnutrient Components of Legumes, and Its Chemopreventive Activity: A Review. *Nutrition and Cancer,* 67, 401-410.

SARMADI, B. H. & ISMAIL, A. 2010. Antioxidative peptides from food proteins: A review. *Peptides,* 31, 1949-1956.

SAUNDERS, P. A., COOPER, J. A., ROODELL, M. M., SCHROEDER, D. A., BORCHERT, C. J., ISAACSON, A. L., SCHENDEL, M. J., GODFREY, K. G., CAHILL, D. R., WALZ, A. M., LOEGERING, R. T., GAYLORD, H., WOYNO, I. J., KALUYZHNY, A. E., KRZYZEK, R. A., MORTARI, F., TSANG, M. & ROFF, C. F. 2000. Quantification of Active Caspase 3 in Apoptotic Cells. *Analytical Biochemistry*, 114-124.

SBROGGIO, M., MONTILHA, M., FIGUEIREDO, V. G. D., GEORGETTI, S. & KUROZAWA, L. 2016. Influence of the degree of hydrolysis and type of enzyme on antioxidant activity of okara protein hydrolysates. *Food Science and Technology (Campinas),* 36, 375-381.

SEBASTIAN, R., JAYKAR, B. & GOMATHI, V. 2020. Current status of anticancer research in fabaceae family. *The Pharma Innovation Journal,* 9, 52-60.

SEKHON, J., GREWAL, S. K., SINGH, I. & KAU, J. 2017. Evaluation of nutritional quality and antioxidant potential of pigeonpea genotypes. *Journal of Food Science and Technology,* 54, 3598-3611.

SHARMA, P., KAUR, H., KEHINDE, B. A., CHHIKARA, N., SHARMA, D. & PANGHAL, A. 2020. Food-Derived Anticancer Peptides: A Review. *International Journal of Peptide Research and Therapeutics.*

SINGH, B. P., VIJ, S. & HATIB, S. 2014. Functional significance of bioactive peptides derived from soybean. *Peptides 54 (2014) 171–179,* 54, 171 - 179.

SINGH, P. 2011. *Antioxidant activity of food proteins and food protein hydrolysates.* Master of Science, McGill University.

SIPAHLI, S., DWARKA, D., AMONSOU, E. & MELLEM, J. 2022. *In vitro* antioxidant and apoptotic activity of Lablab purpureus (L.) Sweet isolate and hydrolysates. *Food Science and Technology (Campinas)*, 42.

SONKLIN, C., ALASHI, A. M., LAOHAKUNJIT, N. & ALUKO, R. E. 2021. Functional characterization of mung bean meal protein-derived antioxidant peptides. *Molecules,* 6, 1515.

SOSALAGERE, C., KEHINDE, B. A. & SHARMA, P. 2022. Isolation and functionalities of bioactive peptides from fruits and vegetables: A reviews. *Food Chemistry,* 366.

STENGLER, M. & ANDERSON, P. 2018. *Outside the box cancer therapies: Alternative therapies that treat and prevent cancer,* USA, Hay House.

SUBAGIO, A. 2006. Characterization of hyacinth bean (Lablab purpureus (L.) sweet) seeds from Indonesia and their protein isolate. *Food Chemistry* 95, 65 - 70.

SUBAGIO, A. & MORITA, N. 2008. Effects of protein isolate from hyacinth beans (*Lablab purpureus* (L.) Sweet) seeds on cake characteristics. *Food Science and Technology Research,* 14, 12-17.

SUNG, H., FERLAY, J., SIEGEL, R. L., LAVERSANNE, M., SOERJOMATARAM, I., JEMAL, A. & BRAY, F. 2021. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *A Cancer Journal for Clinicians*, 1-41.

SYNTHEGO. 2022a. *Everything You Need To Know About A549 Cells* [Online]. Synthego. Available: https://www.synthego.com/a549-cells#:~:text=A549%20cells%20are%20lung20carcinoma,to%20work%20with%20these%20cells. [Accessed 21-09-2022 2022].

SYNTHEGO. 2022b. *HEK293 Cells: Background, Applications, Protocols, and More* [Online]. Synthego. Available: https://www.synthego.com/hek293 [Accessed 21-09-2022 2022].

TACIAS-PASCACIO, V. G., MORELLON-STERLING, R., SIAR, E., TAVANO, O., BERENGUER-MURCIA, A. & FERNANDEZ-LAFUENTE, R. 2020. Use of Alcalase in the production of bioactive peptides: A review. *International Journal of Biological Macromolecules,* 165.

TAK, Y., KAUR, M., AMAROWICZ, R., BHATIA, S. & GAUTAM, C. 2021. Pulse Derived Bioactive Peptides as Novel Nutraceuticals: A Review. *International Journal of Peptide Research and Therapeutics,* 27, 207-2068.

TANG, C., WANG, X. & YANG, X. 2009. Enzymatic hydrolysis of hemp (Cannabis sativa L.) protein isolate by various proteases and antioxidant properties of the resulting hydrolysates. *Food Chemistry,* 114, 1484–1490.

TANIYA, M. S., RESHMA, M. V., SHANIMOL, P. S., KRISHNAN, G. & PRIYA, S. 2020. Bioactive peptides from amaranth seed protein hydrolysates induced apoptosis and antimigratory effects in breast cancer cells. *Food Bioscience,* 35.

THUMBRAIN, D., DWARKA, D., GERRANO, A. S. & MELLEM, J. J. 2020. Antioxidant and apoptotic potential of protein isolates derived from Vigna unguiculata (L.) Walp. *International Journal of Food Science and Technology, 55*, 2813-2823.

THUNDIMADATHIL, J. 2012. Cancer treatment using peptides: Current therapies and future prospects. *Journal of Amino Acids.*

TORRES-FUENTES, C., CONTRERAS, M., RECIO, I., ALAIZ, M. & VIOQUE, J. 2015. Identification and characterization of antioxidant peptides from chickpea protein hydrolysates. *Food Chemistry,* 180, 194-202.

UDENIGWE, C. C. & ALUKO, R. E. 2012. Food Protein-Derived Bioactive Peptides: Production, Processing, and Potential Health Benefits. *Journal of Food Science,* 71.

UMAYAPARVATHI, S., ARUMUGAM, M., MEENAKSHI, S., DRAGER, G., KIRSCHNING, A. & BALASUBRAMANIAN, T. 2014. Purification and Characterization of Antioxidant Peptides from Oyster (*Saccostre cucullata*) Hydrolysate and the Anticancer Activity of Hydrolysate on Human Colon Cancer Cell Lines. *International Journal of Peptide Research and Therapeutics,* 20, 231-234.

VAIJAYANTHI, P. V. & CHANDRAKANT, R. S. 2019. *Hyacinth Bean (Lablab purpureus L. Sweet): Genetics, Breeding and Genomics.,* Switzerland, Springer.

VERMA, A. K. & SINGH, S. 2020. Phytochemical analysis and *in vitro* cytostatic potential of ethnopharmacological important medicinal plants. *Toxicology Reports, 7*, 443-452.

WALI, A., MIJITI, Y., YANHUA, G., YILI, A., AISA, H. A. & KAWULI, A. 2021. Isolation and Identification of a Novel Antioxidant Peptide from Chickpea (Cicer arietinum L.)

Sprout Protein Hydrolysates. *International Journal of Peptide Research and Therapeutics,* 27, 219-227.

WANG, H., KHOR, T., SHU, L., SU, Z., FUENTES, F., LEE, J. & KONG, A. 2012. Plants Against Cancer: A Review on Natural Phytochemicals in Preventing and Treating Cancers and Their Druggability. *Anticancer Agents in Medicinal Chemistry,* 12, 1281-1305.

WHO. 2020. *International Agency for Research on Cancer* [Online]. Available: https://gco.iarc.fr/tomorrow/en/dataviz/isotype [Accessed 02 November 2020].

WONG, F., XIAO, J., WANG, S., EE, K. & CHAL, T. 2020. Advances on the antioxidant peptides from edible plant sources. *Trends in Food Science & Technology,* 99, 44-57.

XU, X., LAI, Y. & HUA, Z. 2019. Apoptosis and apoptotic body: disease message and therapeutic target potentials. *Bioscience Reports,* 39.

XUE, Z., WEN, H., ZHAI, L., YU, Y., LI, Y., YU, W., CHENG, A., WANG, C. & KOU, X. 2015. Antioxidant activity and anti-proliferative effect of a bioactive peptide from chickpea (*Cicer arietinum* L.). *Food Research International, 77*, 75 - 81.

YADA, R. Y. (ed.) 2004. *Proteins in food processing,* Cambridge, England: Woodhead Publishing Limited.

YAGHOUBZADEH, Z., GHADIKOLAII, F. P., KABOOSI, H., SAFARI, R. & FATTAHI, E. 2020. Antioxidant Activity and Anticancer Effect of Bioactive Peptides from Rainbow Trout (Oncorhynchus mykiss) Skin Hydrolysate. *International Journal of Peptide Research and Therapeutics* 26, 625-632.

YEO, J. & SHAHIDI, F. 2021. Bioactive peptides in health and disease: an overview. *Biologically Active Peptides*. Elsevier.

ZHANG, Q., TONG, X., LI, Y., WANG, H., WANG, Z., QI, B., SUI, X. & JIANG, L. 2019. Purification and Characterization of Antioxidant Peptides from Alcalase-Hydrolyzed Soybean (*Glycinemax* L.) Hydrolysate and Their Cytoprotective Effects in Human Intestinal Caco-2 Cells. *Journal of Agricultural Food Chemistry,* 67, 5772-5781.

ZIVNY, J., JR, P. K., PYTLIK, R. & ANDERA, L. 2010. The Role of Apoptosis in Cancer Development and Treatment: Focusing on the Development and Treatment of Hematologic Malignancies. *Current Pharmaceutical Design,* 16, 11-33.

ZUBAIR, H. & AHMAD, A. 2017. Cancer Metastasis: An Introduction. *In:* AHMAD, A. (ed.) *Introduction to Cancer Metastasis*. Academic Press.

Chapter 7: Appendices

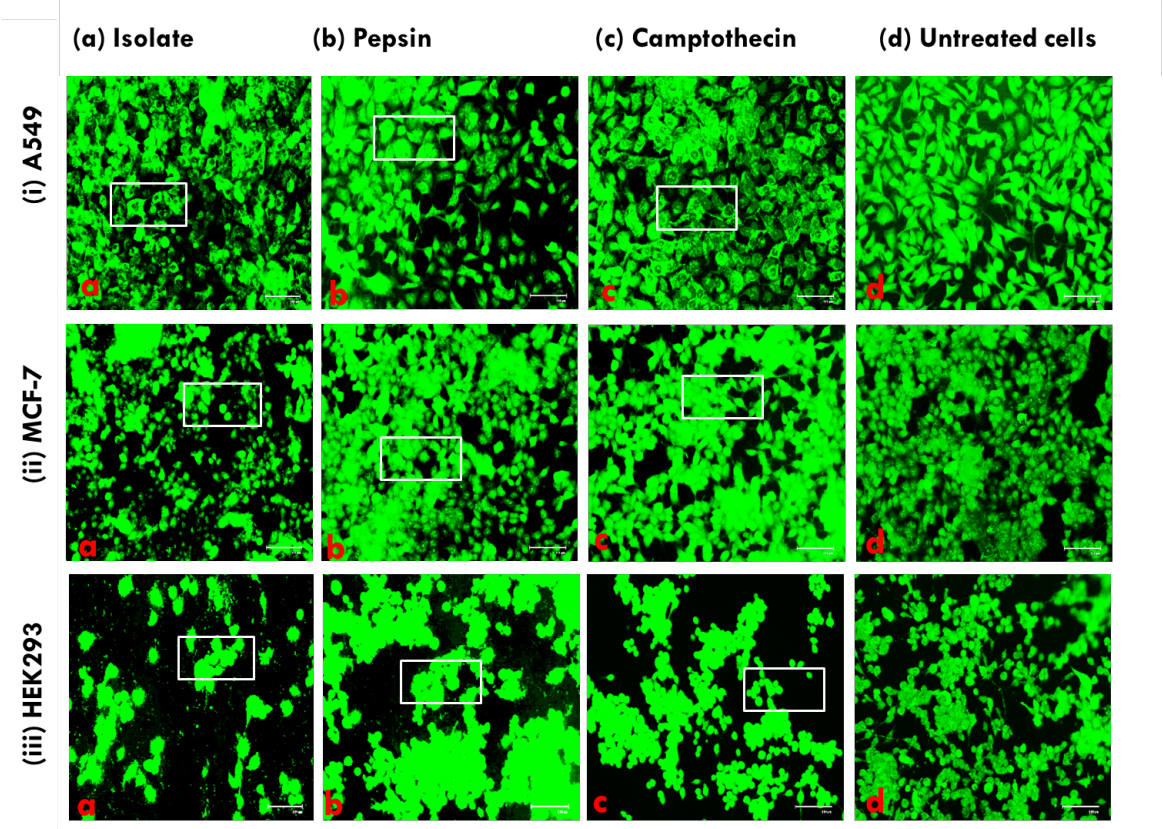

Figure 7.1: Observations of cell membranes by fluorescent microscopy of (i) A549, (ii) MCF-7 and HEK293 cells treated with (a) Isolate, (b) Pepsin hydrolysate; (c) Camptothecin and (d) Untreated cells at IC_{50} values determined by MTT assay. Observations were made at 10X under green light.

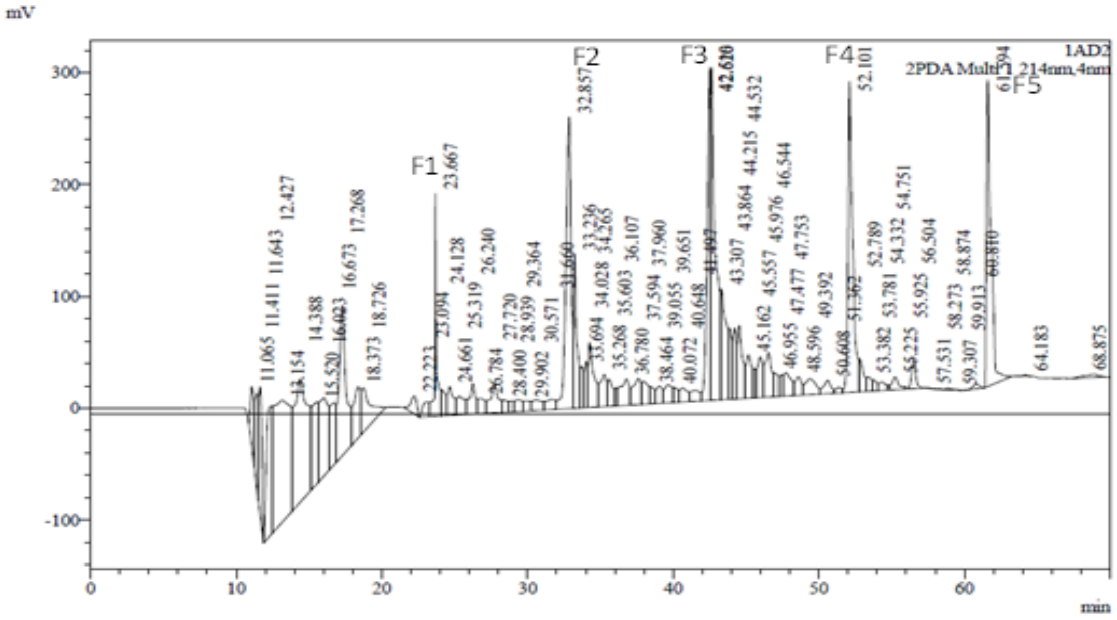

Figure 7.2: RP-HPLC chromatogram of the 3 kDA fraction derived from *L. purpureus* purified by ultrafiltration (F1 – Fraction 1, F2 – Fraction 2, F3 – Fraction 3, F4 – Fraction 4, F5 – Fraction 5)

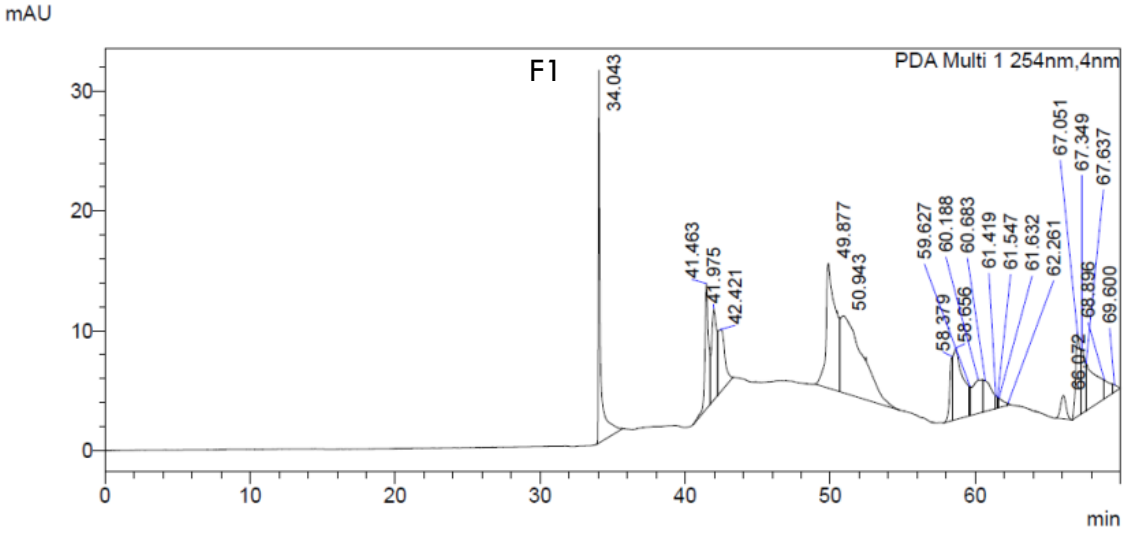

Figure 7.3: Fraction 1 (F1) derived from *L. purpureus* 3 kDa molecular weight fraction collected by RP-HPLC.

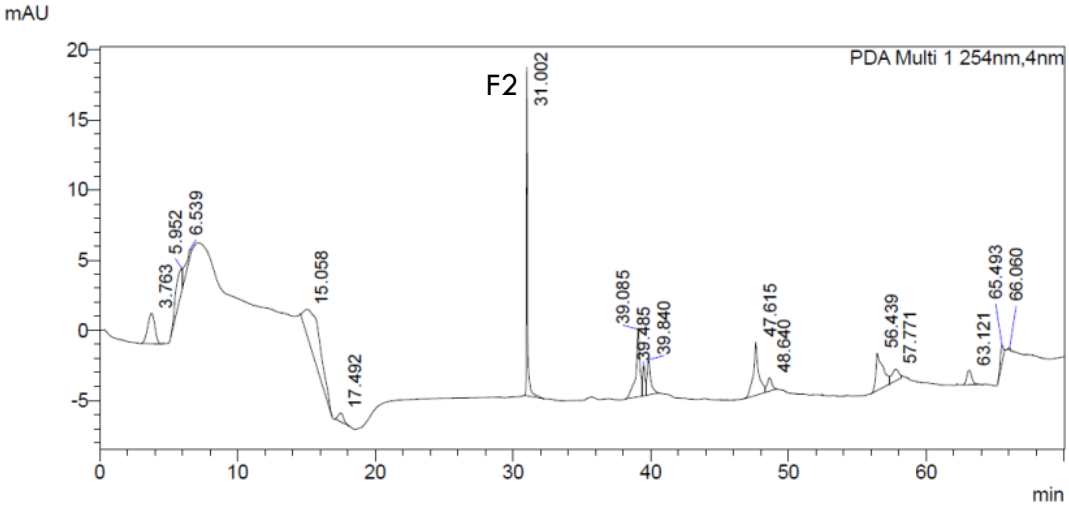

Figure 7.4: Fraction 2 (F2) derived from *L. purpureus* 3 kDa molecular weight fraction collected by RP-HPLC.

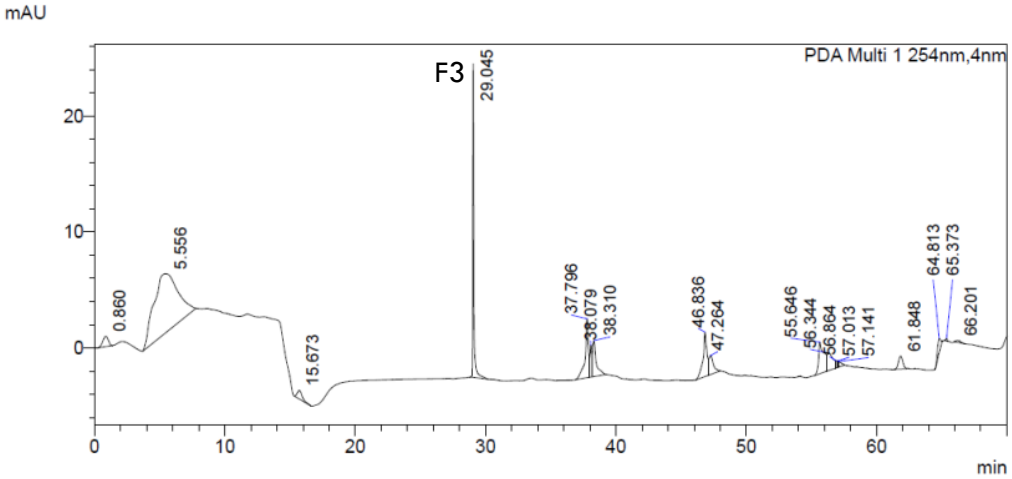

Figure 7.5: Fraction 3 (F3) derived from *L. purpureus* 3 kDa molecular weight fraction collected by RP-HPLC.

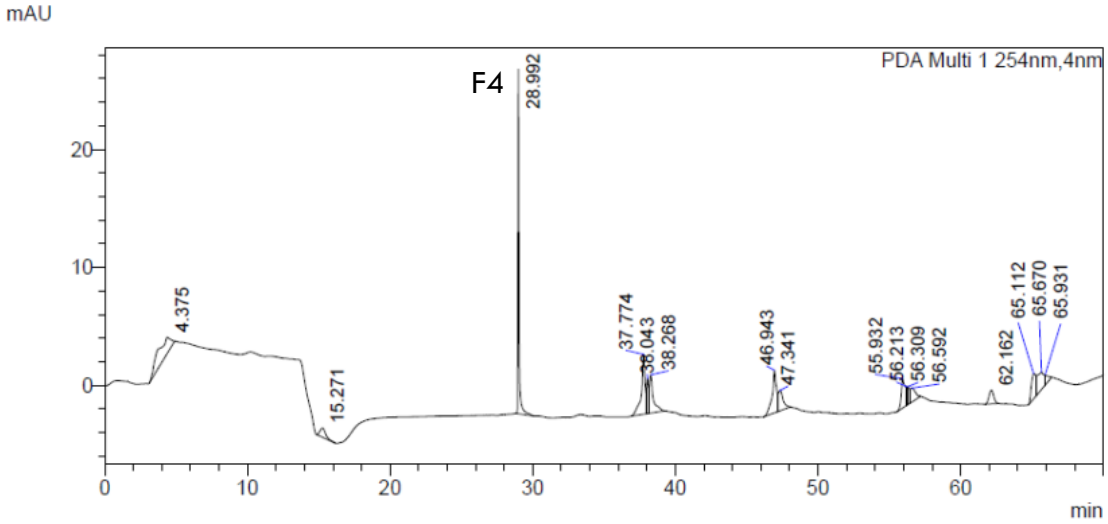

Figure 7.6: Fraction 4 (F4) derived from *L. purpureus* 3 kDa molecular weight fraction collected by RP-HPLC.

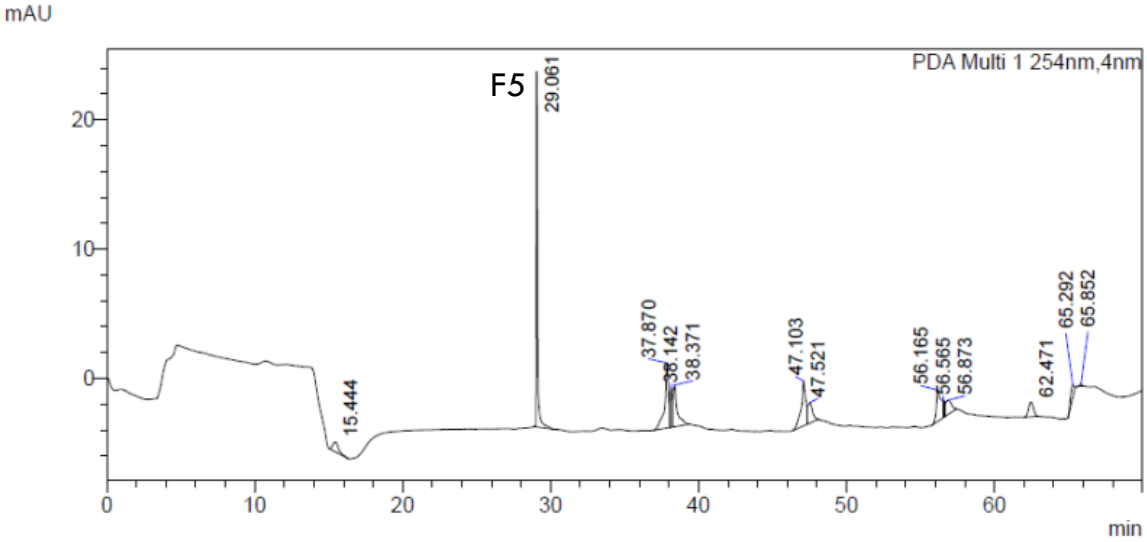

Figure 7.7: Fraction 5 (F5) derived from *L. purpureus* 3 kDa molecular weight fraction collected by RP-HPLC.

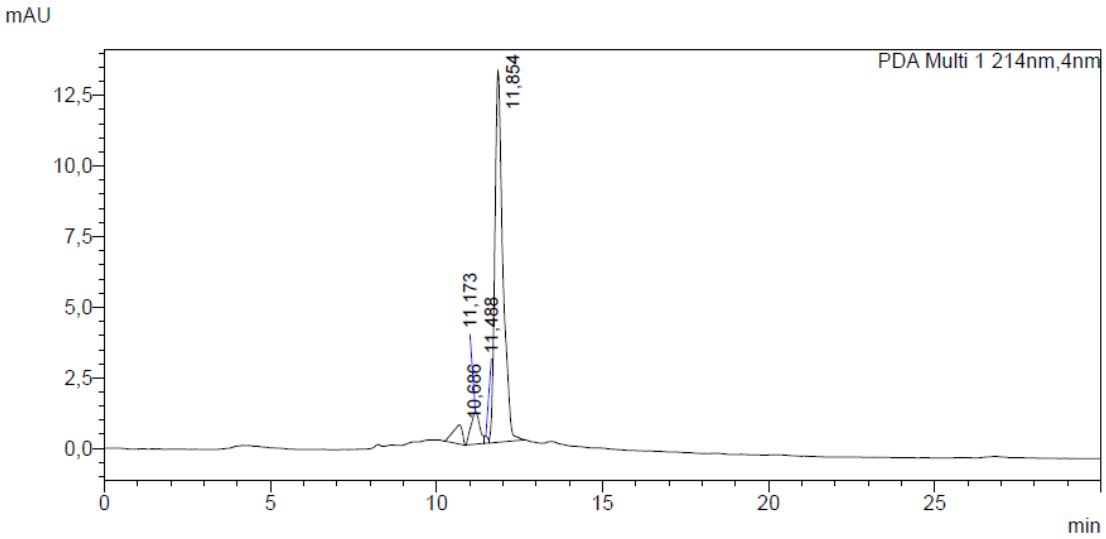

Figure 7.8: RP-LCMS Chromatogram of fraction 2 derived from *L. purpureus* 3 kDa molecular weight to confirm purity of the sample