Anticancer and Anti-Reactive Oxygen Species Activity of Bioactive Peptides Isolated from *Vigna unguiculata*

Submitted in complete fulfilment for the Degree of Master of Applied Sciences in Food Science and Technology in the Department of Biotechnology and Food Science, Faculty of Applied Sciences, Durban University of Technology, Durban, South Africa

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Reference Declaration

I, Sonaal Ramsookmohan (21508486) and Professor J.J. Mellem, in respect of the following dissertation entitled: Anticancer and anti-reactive oxygen species activity of bioactive peptides isolated from *Vigna unguiculata*

Declare that:

1. As of our knowledge:
    a. No other similar dissertation exists;
    b. The only similar dissertation(s) that exist(s) is/are referenced in my dissertation.

2. All references as detailed in the dissertation are complete in terms of all personal communication engaged in and published works consulted.

Signature of student	**03 April 2024** **Date**
Signature of promoter/ supervisor	**03 April 2024** **Date**
Signature of promoter/ supervisor	**03 April 2024** **Date**

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 Professor J.J. Mellem and Dr D. Dwarka.

Student's signature

Dedication

I would like to dedicate this work to my family and loved ones. Thank you for your unwavering support and encourage.

Acknowledgements

- My gratitude goes out to my family and loved ones for their constant encouragement, guidance and support.
- Professor J. Mellem and Dr Dwarka, firstly for allowing me to complete my studies under their supervision, which enabled me to work independently. I am also grateful for their guidance and support, without which, the completion of this project would not be possible.
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Research outputs

Publications

- Antioxidant and anticancer potential of hydrolysates isolated from *Vigna unguiculata*. *The Annals of the University Dunarea de Jos of Galati Fascicle VI – Food Technology*. 2024 [submitted]

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ABSTRACT

Cancer is a major cause of death globally and continues to escalate with current anticancer drugs associated with severe side effects and resistance driving the need for safer alternative therapeutics. Food proteins, from legumes, are a source of bioactive peptides and studies revealed that they are associated with various therapeutic properties. Cowpea (*Vigna unguiculata*) is an underutilized nutritious legume crop with promising potential due to its documented protein profile. Therefore, this study evaluated the *in vitro* anticancer effect of *V. unguiculata* peptides derived from alcalase and flavourzyme. Physicochemical properties such as water and oil absorption capacities, emulsifying properties, sub-unit composition, amino acid composition among others, were also assessed. Peptides were also evaluated for their antioxidant activity using superoxide radical scavenging, 1,1-diphenyl-2-picrylhydrazil (DPPH) and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assays as well as for their apoptotic potential using 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), reactive oxygen species (ROS) and caspase 3/7 assays on cancerous (Caco-2 and MCF-7) and healthy (C2C12) cell lines. From results obtained it was observed that the foam capacity for the peptides derived from alcalase and flavourzyme were 78.34 and 82.39%, respectively, which was noted to be significantly different. The physicochemical properties determined their potential application in food industries. Glutamic acid was the most abundant amino acid in all samples while methionine was noted to be the least concentrated amino acid in the flour and alcalase derived sample while proline was the least concentrated in the flavourzyme sample. Results from this study suggest that cowpea samples have antioxidant capabilities with enzymatic hydrolysis contributing to a higher capacity compared to that of raw flour ~~flour~~ samples. From the cowpea flour, the peptide sample derived from alcalase demonstrated the highest DPPH free radical scavenging activity (70.88-80.47%), followed by flavourzyme (67.27-75.84%), while the raw flour sample showed the lowest activity (24.28-66.17%). The ABTS scavenging capacity of the alcalase peptide was in the range of 35.26-85.92%. The MTT cytotoxicity assay revealed that the cowpea peptides and camptothecin showed different sensitivities on the MCF-7 cell lines. The IC_{50} values of flavourzyme peptide, alcalase peptide and camptothecin were 0.07, 0.09 and 0.07 µg/mL respectively.

Cell viability of the cowpea peptides and camptothecin (control) on the Caco-2 cells varied with the different concentrations. Alcalase and flavourzyme samples had IC_{50} values of 0.15 and 0.11 µg/mL respectively. The apoptotic potential of the peptides was further shown by the caspase 3/7 activity. From the results in this study, it can be ascertained that the cowpea peptides have potential as an anticancer therapeutic agent. Further research is necessary to determine mechanism of action and to conduct *in vivo* evaluations of these peptides using animal models.

1. INTRODUCTION

Cancer is a major cause of death globally with 116 391 new cases reported for 2020 in Southern Africa (Cancer Today, 2021). It is predicted that Sub-Saharan Africa could see an increase of 85% in the number of cancer cases by 2030 (Morhason-Bello et al., 2013), whilst the projected mortalities from cancer are estimated to reach over 13 million worldwide (WHO | Key statistics, 2020). Cancer is the abnormal growth and multiplication of cells in the body. In all types of cancer, abnormal cells begin to grow uncontrollably and spread into surrounding tissues (Chalamaiah et al., 2018). A successful anticancer drug should kill or incapacitate cancer cells without causing unnecessary damage to normal cells, which can be achieved through the induction of apoptosis in cancer cells (Sipahli et al., 2022). Therefore, there is a need for safer alternate therapeutics that are capable of terminating cancer cells without significantly harming healthy cells.

Poverty and lack of modern medicine results in 65-80% of the world's population to rely solely on plants for basic health care (WHO, 2008). There are anticancer drugs, but these are associated with severe side effects and resistance (Thumbrain et al., 2020). Epidemiological research has indicated that the regular intake of specific food items, such as certain fruits and vegetables, referred to as functional foods, is linked to a decreased likelihood of experiencing a range of chronic ailments, including but not limited to high blood pressure certain forms of cancer, obesity and diabetes. (Sabbione *et al*., 2019). Seeds of leguminous plants stand out as one of the most abundant plant reservoirs of proteins and amino acids essential for human nutrition (Thumbrain et al., 2020). Food proteins, from legumes, are a source of bioactive peptides and studies revealed that they are associated with various therapeutic properties (Garcia-Mora et al., 2015; Chan et al., 2016).

Cowpea (*Vigna unguiculata*) is an underutilized nutritious legume crop with promising potential. It stands out as an environmentally sustainable and climate-friendly crop which is indigenous to Africa. It serves as a significant provider of high-quality dietary protein as well as essential nutrients (Awika and Duodu 2017; Naiker et al., 2019).

Previous study by Thumbrain et al. (2020) demonstrated the effectiveness of cowpea protein isolates in inhibiting cell growth and triggering apoptosis in cancer cells. Additionally, the study revealed the protein isolate's role as a protective mediator in stressed non-cancerous cell lines, leading to a reversal in apoptotic activity. Therefore, this study focused on further investigating the anticancer activity of the peptides from cowpea and their activity on human colon adenocarcinoma (CACO-2), breast (MCF-7) and muscle (C2C12) cell lines to evaluate the mechanism of action. The initiation of apoptosis, a crucial mechanism, encompasses an energy-dependent cascade-driven process facilitated by particular proteases, notably caspases. Approaches aimed at surmounting tumour resistance to apoptotic pathways encompass the regulating caspase activity and reactive oxygen species (ROS) production.

2. LITERATURE REVIEW

2.1. Cancer Statistics

Throughout the world, the burden of cancer mortality and incidence is growing rapidly. An increasing trend in deaths and new cases from different cancers worldwide has be noted, particularly in low-and middle-income countries, over the past 20 years (Adeloye et al., 2016). In 2020 there were an estimate of 10 million deaths (9.9 million excluding non-melanoma skin cancer (NMSC), except basal cell carcinoma) and 19.3 million new cases (18.1 million excluding NMSC, except basal cell carcinoma) worldwide (Sung et al., 2021). According to Figure 2.1, lung cancer was the leading cause of cancer death followed by breast and colorectal cancer in 2020. In terms of most incidence, breast cancer was noted to be most prevalent, thereafter prostate and lung cancer in both sexes. The most commonly diagnosed cancer and cancer-associated mortality in women was breast cancer while colorectal cancer was shown to have the second most incidences together with lung cancer which resulted in the second most cancer deaths in women in 2020. In men, lung cancer resulted in the highest number of incidences and mortalities. Prostate cancer was the second most diagnosed whilst colorectal cancer was the second highest cause of death from cancer in 2020.

The prevalence of adverse effects resulting from cancer treatment is widespread, often extending beyond the treatment duration. These effects not only add to the overall burden of cancer but also contribute to the economic challenges associated with the disease (Schmitz et al., 2015). Chemotherapy-induced side effects are prevalent among cancer patients and can pose life-threatening risks. A study by Altun and Sonkaya, 2018 reported that the predominant side effects included nausea and vomiting (79.3%) and fatigue (74.7%). Additionally, frequently reported significant side effects comprised decreased appetite (65.5%), alterations in taste (60.9%), hair loss (60.0%), dry mouth (51.7%), and constipation (51.7%). More than 50% of the patients experienced each of these adverse effects.

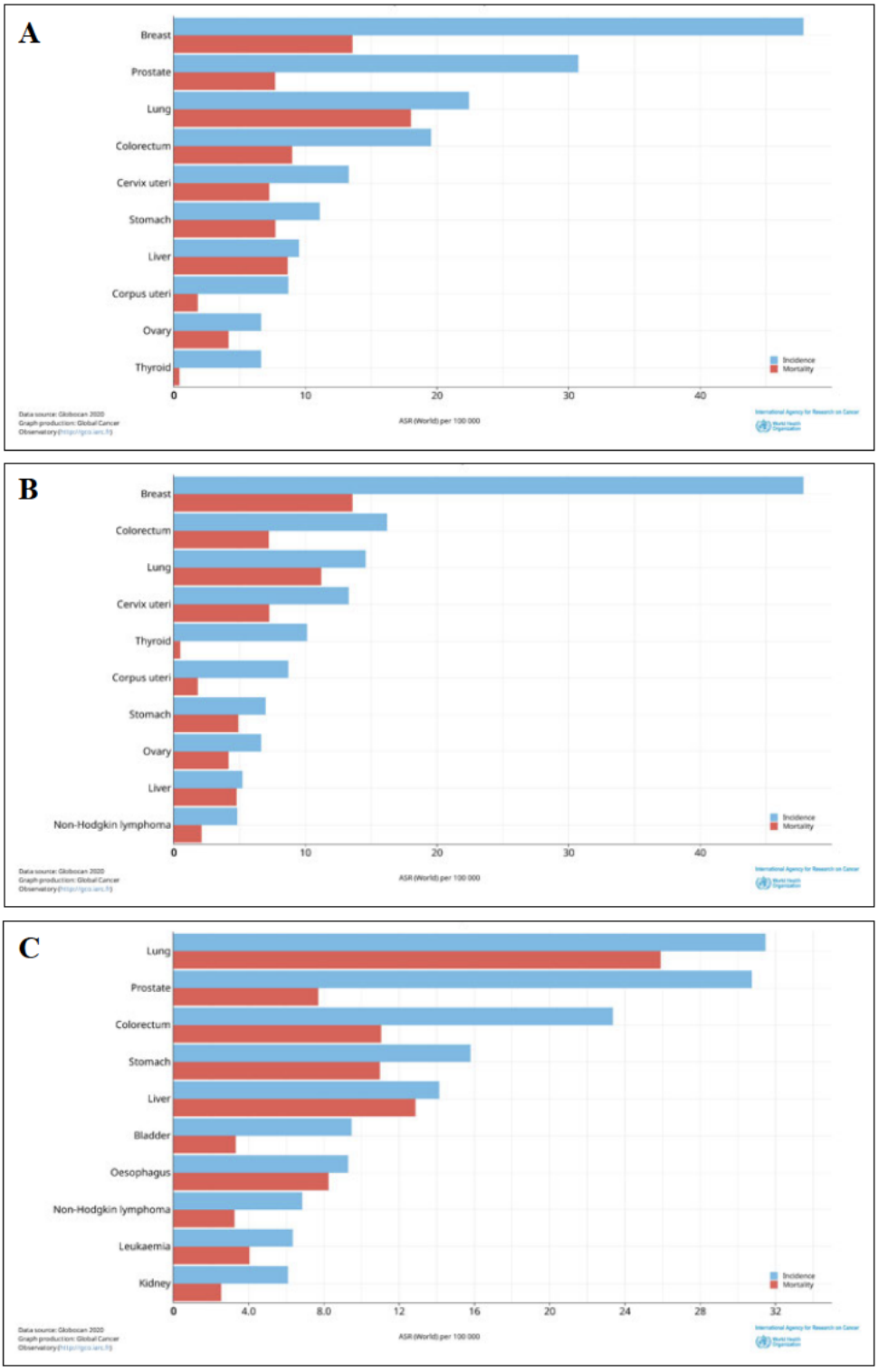

Figure 2.1: Estimated age-standardized incidence and mortality rates (world) in A - both sexes (excl. Nonmelanoma skin cancer), B - females and C - Males (blue bars = incidence, red bars = mortality) (GLOBOCAN, 2020).

2.2. Characteristics of Cancer: General Overview

Cancer is a name given to a collection of related diseases and refers to more than 270 different types of cancer diseases. Cancer can form virtually anywhere in the body where some cells divide uncontrollably and spread into surrounding tissues, in all types of cancer. In order to keep the human body healthy, normal cells grow and divide to produce new cells in a controlled manner. Once cells are damaged or become old, they die and are replaced with new cells. However, this process collapses once cancer develops. Cancer cells survive and grow- producing new, abnormal cells, instead of dying. These extra cells may form a growth called a tumour (Figure 2.2) (Mitra et al., 2018). Malignant growths, commonly referred to as cancerous tumours, possess the ability to metastasize to distant regions of the body through infiltration into either the bloodstream or the lymphatic system. Furthermore, they have the potential to inflict damage upon neighbouring tissues and organs. This aggressive proliferation of cancer cells is identified as a malignant tumour, which subsequently permits the invasion and deterioration of otherwise healthy tissues, including vital organs (Ferlay et al., 2021). The main characteristics of cancer are highlighted by genetic mutations and abnormalities. Genetic alterations impact immune response, cell proliferation and apoptosis, which have been showed to be involved in metastasis (Novikov et al., 2021).

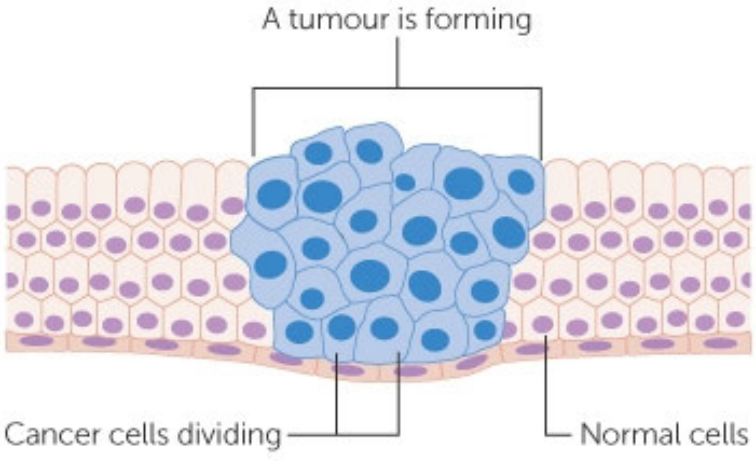

Figure 2.2: Normal cells versus cancer cells dividing and forming a tumour (Cancer Research UK, 2022).

2.2.1. Metastasis

The process of metastasis plays a pivotal role in the advancement of cancer and is a major cause of cancer-associated deaths. Metastasis refers to the spread of cancer. In the course of this mechanism, cancerous cells depart from their initial location and disseminate throughout the body, giving rise to secondary locations and eventual organ failure (Ganesh and Massagué, 2021; Novikov et al., 2021).

Metastasis can either occur by the tumour growing directly into the tissue surrounding it, the tumour cells travelling through the lymph system to other lymph nodes or by travelling through the bloodstream to other locations in the body (Lambert et al., 2017). The initial phase of the metastatic sequence is invasion. In this stage, cancer cells breach the nearby basement membrane and migrate into the adjacent tissue through the extracellular matrix (ECM) (Figure 2.3).

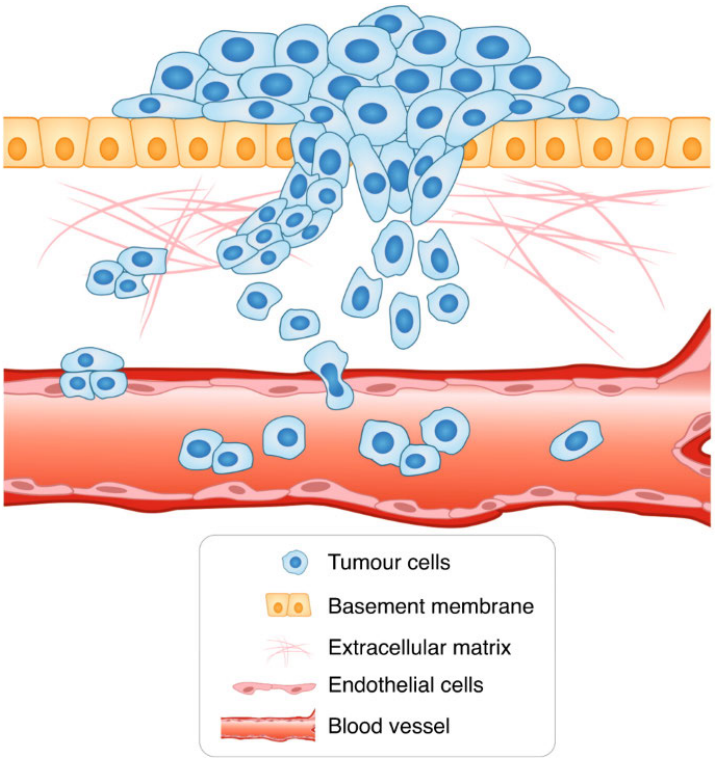

Figure 2.3: A model illustrating the invasion of cancer cell (Novikov et al., 2021).

2.2.2. Types of Cancer

Tumours, both malignant and benign, are classed according to the type of cell they arise from. Most cancers belong into one of the three main categories: sarcomas, carcinomas, and lymphomas or leukemias. Sarcomas are solid tumours of connective tissues such as bone, muscle, fibrous tissue, and cartilage, and are rare in humans. Carcinomas are more common, accounting for approximately 90% of cancer in humans and is the cancer of epithelial cells. Lymphomas and leukemias arise from cells of the immune system and from blood-forming cells, respectively, and includes approximately 8% of malignancies in humans. Tumours are further classified according to the type of cell involved and tissue of origin (such as breast or lung cancer). For instance, Erythroid leukemias arise from precursors of erythrocytes (red blood cells) and fibrosarcomas from fibroblasts (Cooper and Hausman, 2000).

2.2.3. Reactive Oxygen Species in Relation to Cancer

Reactive oxygen species (ROS) is a comprehensive designation encompassing unstable, reactive, and partially reduced oxygen compounds generated as by-products during routine metabolic activities. They serve as secondary messengers in cellular signalling, playing indispensable roles in numerous physiological processes within both normal and cancerous cells (Chio and Tuveson, 2017; Yang et al., 2018). Decades of exhaustive investigations conducted in the last fifty years have highlighted the significant involvement of ROS in cancer. While modest ROS levels can offer certain advantages, an excessive build-up can foster carcinogenesis. A distinguishing feature of cancer cells, setting them apart from their normal counterparts, is their capacity to generate heightened quantities of ROS and their heightened reliance on an antioxidant defence mechanism (Prasad et al., 2017).

In regular somatic cells, ~~reactive oxygen species~~ ROS are essential for various cellular processes, such as supporting immune defence mechanisms and serving as obligatory secondary messengers (Redza-Dutordoir and Averill-Bates, 2016). Reactive oxygen species can be generated through enzymatic or non-enzymatic means. Enzymatic mechanisms involve endothelial nitric oxide synthase (eNOS), NADPH oxidases (NOXs), lipoxygenase activity, xanthine oxidase, arachidonic acid metabolism, cyclooxygenase and enzymes within the cytochrome P450 family (Hart et al., 2015;

Gorrini et al., 2013). Meanwhile, the non-enzymatic route for ROS generation occurs through the mitochondrial respiratory chain. Consequently, the precise regulation of ROS/ redox equilibrium is of paramount importance for maintaining normal biological functions, including cell growth, senescence, cell survival, and the aging process (Kirtonia et al., 2020).

Environmental pollutants associated with cancer have been demonstrated to elevate the presence of ROS species, as exemplified by factors like UV exposure and smoking (Mouret et al., 2006). Additionally, since ROS are an inherent by-product of metabolic processes, the heightened metabolic activity that sustains increased proliferation in cancer cells leads to an augmented ROS production. The activation of several well-known oncogenes, such as BRCA1, Cmyc and Kras, triggers the generation of reactive oxygen species (Weinberg et al., 2010; Cao et al., 2007). Moreover, the onset of hypoxia in tumours, resulting from inadequate blood supply to the growing lesion, is another contributor to increased ROS levels (Azimi et al., 2017).

Disturbances in signaling pathways associated with tumorigenesis, such as alterations in integrin activation during cancer metastasis, are also correlated with heightened ROS production (Chiarugi et al., 2003). Collectively, these mechanisms lead to a significant escalation in ROS levels within cancer cells, but there remains substantial debate concerning the implications of ROS in tumorigenesis (Yang et al., 2018).

2.2.4. Role of Caspase- 3/7 in Cancer

Caspase-3 is a broadly distributed member of a preserved protein family, commonly acknowledged for its activated proteolytic functions in facilitating apoptosis. This process occurs in cells that respond to specific inducers of cell death, whether intrinsic or extrinsic in nature (Eskandari and Eaves, 2022). The apoptotic caspases can be classified into initiators (caspases-8, -9, and -10) and effectors (caspases-3, -6, and -7). Another set of caspases, namely caspases-1, -4, -5, and -12, form the group known as inflammatory caspases (Bibo-Verdugo and Salvesen, 2022). They mutually activate, triggering a series of biochemical events (Elmore, 2007). Following the activation of initiator caspases, executioner caspases come into play, cleaving various structural and regulatory proteins crucial for the dismantling of the cell.

Typically, an initiator caspase comprises of a prodomain of over 90 amino acids, while an effector caspase has a prodomain sequence of 20–30 residues. Caspases exist as inactive zymogens and require activation to exert their effects. The activation of caspase-9 initiates the activation of effector caspases (caspase-3 or caspase-7), characterized by internal cleavage that divides the small and large subunits. This process authenticates the activation of an initiator caspase. (Yadav et al., 2021).

Caspase-3 and caspase-7 serve pivotal roles as executioner enzymes, crucial for both apoptosis and the regular functioning of mammalian life. Initially, caspase-3 takes the form of an inactive, purely cytosolic homodimer referred to as procaspase-3, comprising a 277 amino acid protein. Upon receiving a signal from upstream, procaspase-3 experiences proteolytic cleavage (Adams and Cory, 2002). This process results in the generation of an N-terminal segment (175 amino acids) forming a large subunit p20 and a C-terminal segment (102 amino acids) forming a small subunit p12, with p20 further trimmed to yield a p17 subunit. Active caspase-3 comprises two p12 and two p17 subunits, where both p12 and p17 subunits contain the enzyme's active site non-covalently, incorporating an embedded catalytic cysteine (Cys). Complete activation involves both proteolytic processing and denitrosylation of Cys 163. In its active state, caspase-3 utilizes the Cys residue to cleave various substrates, such as poly(ADP-ribose) polymerase family member-16 (PARP-16), protein kinase C (PKC)-gamma and delta, procaspases 6, 7, 9, and beta-catenin (Chowdhury et al., 2008).

Caspase-3 plays a pivotal role in the programmed death of cells, by orchestrating the stages of cellular demise in a non-traumatic manner. Once activated, caspase-3 initiates the breakdown of proteins, ultimately leading to cell death. This enzyme plays a crucial role in cellular mechanisms and can be activated by any of the initiators, rendering it a primary target for anticancer therapy. As a frequently activated enzyme in cellular apoptosis, caspase-3 can be activated through pathways that depend on caspases or operate independently of mitochondrial cytochrome c release and caspase-9 function. In the apoptotic process, caspase-3 is essential for chromatin condensation and the fragmentation of deoxyribonucleic acid (DNA) in all examined cell types (Yadav et al., 2021).

In the realm of cancer treatment strategies, the activation of caspase-3-mediated apoptosis for inducing cytotoxicity has emerged as an intriguing technique. In the quest for innovative caspase-3 activators, several scientists and researchers have designed and documented various compounds capable of inducing caspase-3-mediated apoptosis, resulting in cytotoxic effects. These newly developed compounds exhibit potential in the advancement of anticancer drugs (Bhatiya et al., 2014; Vaidya et al., 2015, 2016).

2.2.5. Current Cancer Therapy

Despite the growing accessibility of conventional cancer treatment options, millions of patients continue to succumb to cancer-related fatalities annually (Torre et al., 2015). The metastasis of cancer cells, the recurrence of the disease, its inherent heterogeneity, and its resistance to chemotherapy and radiotherapy have rendered many traditional treatment approaches ineffective against a significant number of malignant tumours (Sun, 2015). Furthermore, cancer cells' ability to evade the immune response has also been cited as a factor contributing to treatment failure. Multiple shortcomings have been identified in conventional cancer therapy methods, often leading to unsatisfactory outcomes. Despite decades of research and advancements in the field of cancer treatment, conventional chemotherapy remains the predominant therapeutic approach for the majority of cancer cases. Nevertheless, chemotherapy operates by indiscriminately targeting rapidly dividing cells. Consequently, it cannot differentiate between normal, healthy proliferating cells and cancerous ones, rendering it incapable of addressing dormant or indolent cancerous conditions (Hilchie et al., 2011; Donnelly, 2004).

Chemotherapy is linked to a range of severe side effects, encompassing both immediate indicators of toxicity and later manifestations of chronic toxicity (Schirrmacher, 2017). These effects can vary in intensity, classified as ‘mild (grade 1), moderate (grade 2), severe (grade 3), or incapacitating or life-threatening (grade 4)’ according to the ~~World Health Organization~~ WHO classification. Immediate adverse reactions can manifest in various ways, affecting the hair, gastrointestinal tract, skin, kidneys, bone marrow and blood. Essentially, every organ in the body can be impacted, including vital organs like the heart, lungs, and brain. Notably, grade 3 and 4 neurotoxicity may lead to symptoms such as spasms, tingling sensations, lack of coordination, drowsiness, paralysis, and even coma.

Furthermore, the chronic repercussions of chemotherapy encompass issues like infertility, an increased risk of developing cancer, and the development of drug resistance (Schirrmacher, 2019). Numerous studies have documented that conventional chemotherapeutic drugs face challenges related to their non-selective distribution, resulting in suboptimal bioavailability, rapid elimination from the bloodstream, and relatively low solubility in bodily fluids (Cho et al., 2008; Han et al., 2013). The primary objective for any cancer treatment agent is to specifically target and destroy cancer cells while sparing the adjacent healthy cells and tissues, a goal that has not yet been fully realized (Yahya and Alqadhi, 2021).

2.3. Antioxidants in relation to cancer therapy

There are several factors behind the process of carcinogenesis. Multiple indications propose a close connection between oxidative stress and a varied range of illnesses, including cancer and cardiovascular disease (Zahra et al., 2021). The process of oxidation is a widespread and prevalent chemical reaction that impacts both living and non-living entities.

While oxygen is essential for the survival of aerobic organisms, it also gives rise to the production of free radicals (Haider et al., 2020). Free radicals are molecules characterized by an unpaired electron in their outer orbitals, making them highly reactive within the body. They exhibit reactivity by oxidizing (extracting an electron from) other atoms or, alternatively, by reducing (donating their electron to) other atoms (Adwas et al., 2019). A loss of equilibrium between free radicals and antioxidants results in the oxidative damage of proteins, fats, nucleic acids, and carbohydrates (Halliwell, 2007).

The initiation and progression of early stages in cancer are notably influenced by oxidative stress which is intrinsically associated to various stages of cancer. Cancer cells experience a significantly higher degree of oxidative stress compared to normal cells, attributable to oncogene-induced transformation, heightened metabolic activity, mitochondrial dysfunction etc. (Wall-Medrano and Olivas-Aguirre, 2020). Antioxidants are known to play a crucial role in protecting the body from the detrimental effects of free radical (Adwas et al., 2019).

An antioxidant is a molecule with the capacity to hinder or decelerate the oxidation of macromolecules and functions by diminishing or halting chain reactions, either by eliminating free radicals or impeding other oxidation processes through their own oxidation. Additionally, the undesired effects of ~~reactive oxygen species~~ ROS are controlled through the defensive cellular antioxidant system (Fuloria et al., 2021). The mechanisms employed by antioxidant defence include:

- Inhibition of the production of free radicals
- Scavenging of oxidants
- Conversion of toxic free radicals into less harmful substances
- Prevention of the generation of secondary toxic metabolites and inflammation mediators
- Interruption of the chain propagation of secondary oxidants
- Repair of damaged molecules
- Initiation and augmentation of the endogenous antioxidant defence system.

These defence mechanisms collectively work to safeguard the body from the impact of oxidative stress. Therefore, the appropriate utilization of antioxidants is believed to yield a beneficial impact on preventing cancer. Antioxidants are also employed in cancer treatment to mitigate the potential excessive toxicity of chemotherapy medications (Zinovkin et al., 2023).

2.3.1. Antioxidant Methods

Studies have concentrated on the process of oxidation involving free radicals and the comprehensive mechanism of action of antioxidants. This attention stems from the notable impact that neutral free radicals exert on the biological system (Gulcin, 2020). Numerous tests for antioxidants directly assess the transfer of H atoms or electrons from antioxidants to free radicals. The majority of these methods share similar principles and techniques. Among the frequently employed techniques are superoxide scavenging assay, 2,2-azinobis 3-ethylbenzthiazoline-6-sulfonic acid radicals (ABTS·+) and 1,1-diphenyl-2-picrylhydrazil (DPPH). Utilizing a stable free radical, DPPH, serves as a method to assess antioxidant activity similarly. The nitrogen atom's single electron in DPPH undergoes reduction to the corresponding hydrazine, achieved by accepting a hydrogen atom from antioxidants.

The DPPH· radical exhibits a notably stable and intense colour, leading to its widespread use. Once a solution of DPPH is combined with a substance capable of supplying a hydrogen atom, the violet color fades away, resulting in the reduced form of the DPPH radical (DPPH-H) (Yapıcı et al., 2021). The broader band is responsible for the intense violet colour of the DPPH· solution. The generation of hydrazine (DPPH-H) triggers the vanishing of the visible band, causing the solution's colour to shift from violet to pale yellow due to radical reduction through the transfer of hydrogen atoms from antioxidants, acting as hydrogen donors. DPPH maintains its stability as a free radical thanks to the delocalization of the spare electron across the entire molecule. Consequently, the DPPH radical does not undergo dimerization, a characteristic not shared by many other free radicals (Gulcin and Alwasel, 2023). The chemical structure of DPPH radical is depicted in Figure 2.4.

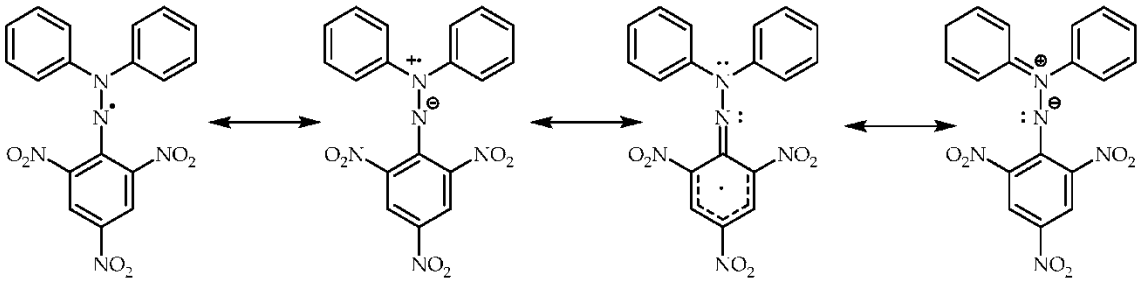

Figure 2.4: The chemical structures of 1,1-diphenyl-2-picrylhydrazil radical (DPPH·) (Gulcin and Alwasel, 2023).

The oxidation of ABTS by oxidants results in its radical cation, ABTS·+, characterized by intense coloration. The measurement of antioxidant capacity involves assessing the ability of test compounds to reduce the colour by directly interacting with the ABTS radical. Notably, ABTS·+ is effective for both lipophilic and hydrophilic compounds. These radicals are more reactive compared to DPPH radicals. The formation of the ABTS radical cation serves as the foundation for a spectrophotometric method used to quantify the overall antioxidant activity of substances (Gulcin and Alwasel, 2023). The effectiveness of the superoxide radical (O2.-) scavenging activity method depends on the ability of an antioxidant to neutralize the O2.- radical in a test tube.

This process involves the oxidation of NADH and the concurrent evaluation of the reduction of nitroblue tetrazolium (NBT) (Siddeeg et al., 2021). The mode of action of antioxidant reacting with free radicals is shown in Figure 2.5.

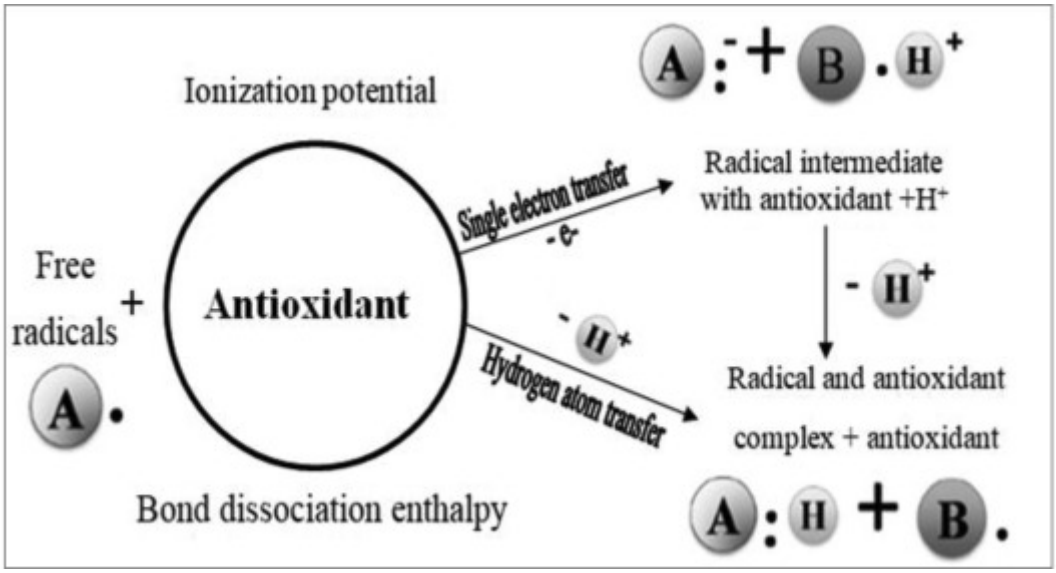

Figure 2.5: Mode of action of antioxidant reacting with free radicals (Siddeeg et al., 2021).

2.4. Natural compounds/Plants in cancer therapy

For years, traditional herbal medicine has been in practice. Over the past few decades, there has been a rising trend in the utilization of these herbal medicines for the treatment of numerous chronic diseases, owing to their remarkable pharmacological activities, economic feasibility, and lack of harmful effects (Rahman and Islam, 2013). Studies have shown that plants traditionally used in various cultures contain bioactive compounds. When administered in adequate doses, these compounds have positive effects on health (Garcia-Oliveira et al., 2021).

Plants contain active substances known as secondary metabolites, alkaloids, phenolics and flavonoids, terpenoids and glycosides, which play a crucial role in various essential biological processes (Dekebo, 2019). With over 50,000 secondary metabolites identified in the plant kingdom, their function can be perceived as either attracting or repelling, depending on the plant's needs (Dekebo, 2019; Tungmunnithum, et al., 2018).

The presence of these secondary metabolites benefits both plants and other living organisms with medicinal herbs reliant on secondary metabolites, which serve as antimicrobials, antioxidants, anticancer agents, and anti-inflammatories. There is significant interest within the scientific community in conducting pharmacological studies on plant compounds for their potential use as cancer suppressors. Additionally, plant products demonstrate synergy with chemotherapeutic drugs, reducing cellular resistance and enhancing their effectiveness (Alwahibi, 2020). Renowned for their beneficial impact on human health, antioxidants and other noteworthy bioactive components, including phenolic compounds, are employed in the treatment and prevention of diverse disorders (Tungmunnithum et al., 2018). These substances demonstrate antibacterial, anticancer, cardioprotective, anti-inflammatory properties and contribute to bolstering the immune system. Among these compounds, flavonoids represent the most extensive class of phenolic compounds present in nature (Al-Rimawi et al., 2022).

Typically, plant-derived anticancer compounds have been explored as potential options for developing new chemotherapeutics and improving the efficacy of conventional ones (Mao et al., 2020). However, these compounds come with several challenges, including low stability or solubility and difficulties in extraction from natural sources (Garcia-Oliveira et al., 2021).

2.5. Legumes as Anticancer Alternatives

Health and nutrition are known to be closely related and the ability of foods and their components have been shown to enhance the overall quality of life or reduce the risk of disease (Danquah and Agyei 2012). Legumes have been part of global diets since ancient eras, appreciated for their nutritional qualities. Nutritionally, legumes boast high protein and dietary fibre content, presenting an affordable and nutrient-rich food option. Additionally, they serve as a valuable source of vitamins and minerals (Silva et al., 2016).

~~Recent~~ Research has highlighted the potential health advantages of legumes that extend beyond fulfilling basic nutritional needs for humans (Barman et al., 2018; de Cedrón et al., 2018; Papandreou et al., 2019; Sathoff and Samac 2019; Zhang et al., 2018).

The presence of numerous bioactive compounds in legumes is linked to various health-promoting factors such as anti-obesity, anti-inflammatory, cardiovascular protective among others (Serventi and Dsouza, 2020). Studies have shown that a significant consumption of legumes is associated with a reduced risk of various cancer types (Li and Mao 2017; Zhu et al. 2015). As per the American Institute for Cancer Research (AICR), legumes encompass various compounds, namely saponins, lignans, polyphenolic phytochemicals and resistant starch, that may protect against cancer (Luna-Vital and de Mejía, 2018). Legumes are a good source of bioactive proteins with the seed proteins widely studied to produce peptides with a diversity of biological activities (González-Montoya et al., 2017). The peptides derived from legume proteins exhibit distinct physicochemical properties due to their amino acid composition. Consequently, they feature diverse mechanisms of action contributing to their potential in anticancer activities (Luna-Vital and de Mejía 2018).

2.5.1. Bioactive Peptides

Peptides are brief linear sequences of amino acids (AA), typically less than 50 AA in length, frequently stabilized by disulphide bonds. They are generated through rational approaches, displaying heightened specificity in binding and modulating a targeted protein interaction (Marqus et al., 2017). Bioactive peptides can be generated by various biotechnological processes such as fermentation and enzymatic hydrolysis. Bioactive peptides offer a notably promising approach to address a variety of diseases, including cancer. Chemo-preventive agents are anticipated to be safe, cost-effective, and readily available. Peptides meets this criteria and are ideal drug candidates due to their low production cost, are regarded as safer alternatives to synthetic compounds because they are naturally present in the typical human diet, having a wide range of acceptance and availability, a relatively high tissue penetration and can be easily modified (Li et al., 2014; Hilchie et al., 2019; Kurrikoff et al., 2019).

Bioactive peptides provide unique benefits in contrast to other chemotherapeutic agents, exhibiting high affinity and tissue penetration, precise target specificity, and minimal toxicity (Bhutia et al., 2019). As indicated by Cicero et al. (2017), bioactive peptides derived from plant, animal, and marine sources demonstrate selective cytotoxic activity against various cancers both *in vitro* and *in vivo*.

Nevertheless, many of these studies lack toxicity screening in non-cancerous cell lines (Thumbrain et al., 2020). Bioactive peptides exert anti-tumour activity via several key mechanisms:

a) The initiation of apoptosis, a process dependent on energy and mediated through specific proteases or caspases, can be achieved through various strategies. Overcoming tumour resistance to apoptotic pathways involves activating pro-apoptotic receptors, reinstating p53 activity, modulating caspases, and inhibiting proteasomes (Burz et al., 2009)
b) Halting the intermediate formation of tumors can be achieved by regulating cellular mechanisms linked to cell proliferation and survival, or by influencing biosynthetic pathways that govern cell growth (Kornienko et al., 2013);
c) Modulating the function of the immune system involves enhancing the expression of tumour-associated antigens (antigenicity) in cancer cells, prompting tumour cells to release danger signals that activate immune responses (immunogenicity), or heightening the susceptibility of tumour cells to be recognized and eliminated by the immune system (Díaz-Gómez et al., 2017; Zitvogel et al., 2013).

A study by Ortiz-Martinez et al. (2017) reported that maize peptides resulted in apoptotic induction on HepG2 cells. Previous studies by Hsieh et al. (2018) and Wan et al. (2017) have reported that soybean peptides exerted anticancer activities on colon, lung and breast cancer. Vital et al. (2014) revealed that peptides in common bean fractions inhibited human colorectal cancer cells. Previous research has indicated that a peptide fraction obtained from a soybean line high in oleic acid demonstrates anti-proliferative effects on various cancer cell lines, including blood (CCRF-CEM), colon (Caco-2 and HCT-116), and liver (HepG-2) cells. A singular, purified soy peptide demonstrated ≥80% inhibition of human colon and blood cancer cells. The purification of the peptide fraction yielded a potent single peptide consisting of 158 amino acid residues with the highest anti-proliferative activity, making it suitable for testing against other prominent cancer cells (Rayaprolu et al., 2017). The anticancer impact of maize peptides has been substantiated through *in vitro* models. HepG2 cells exhibited heightened anticancer activity upon exposure to maize peptides derived from the enzymatic hydrolysis of proteins extracted from corn gluten meal (Li et al., 2013).

In the study, corn peptides (CPs) were evaluated for anti-tumour activity in H22-tumor bearing mice with treatment of animals with these peptides resulting in an inhibition of tumour growth. Ortiz-Martinez et al. (2017) reported significant anti-cancer effects of maize peptides in HepG2 cells. The peptide fractions isolated from albumin alcalase hydrolysates from maize exhibited greater strength than those from proteins derived from the same maize source. Treating HepG2 cells with the peptide fraction from various maize varieties led to an average 4-fold increase in apoptotic induction rates. These findings indicate an anti-proliferative effect of peptide fractions from both varieties, potentially linked to the reduction in the expression of antiapoptotic factors (Díaz-Gómez et al., 2017). The multifunctional nature of peptides allows them to function not only as agents with inherent anti-cancer properties but also to stabilize drugs, improve the cellular uptake of proteins or drugs, enable precise targeting of cancer cells using therapeutic or imaging agents, and even act as components in cancer vaccines (Whitfield and Soucek, 2019).

2.6. Cowpea (*Vigna unguiculata*)

Cowpea is a food legume belonging to the Fabacea family (Figure 2.6, 2.7). All cultivated cowpeas are categorized under the species *Vigna*, which is divided into four cultivar groups: *textilis*, *biflora, unguiculata and sesquipedalis*. The physiological factors such as colour, size, yield, maturity time and taste can be used for differentiation of cultivars (Jayathilake et al., 2018; Oyewale and Bamaiyi 2013). Cowpea is indigenous to Africa for over six thousand years and is found extensively in areas characterized by temperate and tropical climates due to its drought and heat tolerance, which makes it a climate-change and environment friendly crop (Naiker *et al*., 2019; Awika and Duodu 2017; Jaichand *et al*., 2020). It is the most widely produced pulse grain behind common dry bean and chickpea (Awika and Duodu 2017; ~~FAO, 2014).~~

Cowpea is cultivated across Africa, Southeast Asia, Latin America and Southern United States but West Africa holds the predominant share, contributing over 87% to global production and utilization of cowpea, making it the world's largest producer and consumer of this crop (Jayathilake *et al*., 2018; ~~FAO, 2014~~). This legume is of vital importance to the livelihoods of millions of people in Africa as it has been incorporated into human diets and utilized as animal feed for forage.

The dry grain can be consumed fried, steamed or boiled according to different preparations, for human consumption. Likewise, fresh pods, fresh seeds and young leaves have been consumed in certain regions of the world (Carvalho et al., 2017). Cowpea is commonly known as "poor man's meat" owing to its high-quality protein, substantial nutritional value, and widespread consumption, particularly in developing nations, catering to the dietary needs of millions (Naiker et al., 2019; Carvalho et al., 2017). The dry grain has a protein content in the range of proteins 23–32%, and it is rich in essential amino acids such as tryptophan (68 $mg.g^{-1}$ N) and lysine (427 $mg.g^{-1}$ N) (Carvalho et al., 2017).

Apart from nutritional benefits, cowpea is also considered as a source of significant bioactive compounds that could contribute to various health benefits for humans. Polyphenols and peptides are important groups of bioactive compounds found in cowpea, which exert health promoting properties (Awika and Duodu 2017). Epidemiological evidence suggests that including cowpea in the diet provides protective effects against the onset of various chronic conditions, including diabetes (Barnes et al., 2015), various types of cancer (Khalid and Elharadallou, 2013), gastrointestinal disorders (Trehan et al., 2015), and more (Jayathilake et al., 2018).

Figure 2.6: Cowpea seeds (Dreamstime.com, 2022).

Figure 2.7: Cowpea flowering (Crop gene bank, 2022).

2.6.1. Nutritional Component of Cowpea

In terms of nutrition, cowpea is comparably similar to other legumes, featuring a relatively low-fat content and a high concentration of total protein. Cowpea is acknowledged as a nutrient-dense food with low energy density. On a dry basis, an average cowpea grain comprises approximately 23-32% protein, 50-60% carbohydrates, and around 1% fat (Jayathilake et al., 2018). A study by Naiker et al. (2019) demonstrated the chemical composition of five cowpea cultivars as shown in Table 2.1. The protein found in cowpea is abundant in lysine, surpassing the levels found in cereal grains. This makes cowpea a natural complement to cereals in the creation of balanced meals. As a result, cowpea has gained recognition as a legume with high-quality protein, constituting a remarkable source of various health-promoting components such as phenolic compounds and bioactive peptides etc. (Jayathilake et al., 2018).

Table 2.1: Chemical composition for five cultivars of cowpea flour under respective parameters [Adapted from Naiker et al., 2019]

Cultivar	Protein (%)	Fat (%)	Total starch (%)	Ash (%)	Moisture (%)	Fiber (%)	Amylose (%)
Glenda	25.64±0.49	1.12±0.05	51.33±0.68	3.36±0.05	8.25±0.20	10.30±0.36	19.15±0.42
Veg Cowpea 2	25.69±0.45	1.50±0.16	50.99±0.36	3.64±0.02	7.90±0.19	10.28±0.42	16.72±0.73
Veg Cowpea 3	26.33±0.49	1.94±0.03	51.08±0.67	3.44±0.42	7.85±0.25	9.36±0.30	16.96±0.42
Makathini	24.30±0.54	1.21±0.09	51.17±0.74	3.11±0.09	7.35±0.17	12.86±0.28	17.45±0.00
Embu Buff	24.75±0.53	1.19±0.07	51.12±0.75	2.71±0.11	9.29±0.19	10.94±0.46	17.20±0.42

2.6.2. Cowpea Proteins and Bioactive Peptides

Research indicates that cowpea possesses a distinctive and intricate protein profile, encompassing globulins, albumins, glutelins, and a singular prolamin (one protein band). The predominant component of cowpea protein is the globulin fraction (50-70%), further categorized into two fractions: 11S (legumin) and 7S (vicilin/β-vignina) (Jayathilake et al., 2018).

Albumins and globulins are recognized as the primary storage proteins in cowpea (Tchiagam et al., 2011). Albumin fractions in seeds exhibit variability, ranging between 8.2-11.9%. They are classified as enzymatic and metabolic proteins, such as lipoxygenase, protease inhibitors, and lectins (Gupta et al., 2010). Following in significance are glutelins, constituting 14.4-15.6% of the total proteins (Gupta et al., 2010). Prolamins, the least abundant storage protein type in cowpea, range from 2.3-5.0%, characterized by high proline and glutamine contents (Tchiagam et al., 2011; Jayathilake et al., 2018). The unique characteristics and quality of the protein within a food group are principally determined by its amino acid composition and the physiological utilization of specific amino acids during digestion and absorption (Elharadallou et al., 2015; Jayathilake et al., 2018).

In addition to the importance of essential amino acids, there is a growing focus on protein-derived fragments referred to as peptides, which have potential implications in preventing or treating chronic metabolic disorders. These natural peptides are produced through enzymatic hydrolysis or fermentation and are known to have favourable effects within the body. The ability of these peptides to create beneficial physiological conditions for proper bodily functions classifies them as 'bioactive.'

Peptides, typically consisting of 3 to 20 amino acid residues, are produced through enzymatic proteolysis of diverse animal and plant proteins. Additionally, recent *in vitro* investigations have demonstrated that peptides derived from cowpea exhibit antioxidant properties and contribute to the prevention of cancer (Cicero et al., 2017; Domínguez-Perles et al., 2016). A study by Tian et al. (2013) revealed that protein inhibited proliferation of MBL2 lymphoma and L1210 leukaemia cells.

In a study by Thumbrain et al. (2020) it was also demonstrated that the protein isolates of cowpea successfully inhibited cell growth and induced apoptosis in cancerous cells. It was shown that the protein isolate acts as a protecting mediator in stressed non-cancerous cell line by causing a reversal in apoptotic activity.

2.7. Enzymatic Hydrolysis

Various physical and chemical treatments, including the enzymatic catalysis of food proteins, are utilized to produce bioactive peptides. Nonetheless, enzymatic hydrolysis has proven to be the most effective approach for obtaining bioactive peptides from protein sources (Kannan et al., 2008; Kannan et al., 2010; Kannan et al., 2012; Korhonen and Pihlanto, 2006; Rayaprolu et al., 2017). Enzymatic hydrolysis is typically carried out under carefully controlled conditions (temperature, pH, enzyme activity and substrate concentration). In this proteolysis process, susceptible peptide bonds are cleaved, releasing bioactive peptides. Various enzymes can hydrolyse proteins under their optimal conditions, and multiple proteolytic enzymes can be employed simultaneously or sequentially to hydrolyse the protein and produce peptides with short sequences if their optimum hydrolysis conditions align (Ulug et al., 2021).

Controlled enzymatic hydrolysis can yield peptides with bioactivity, including an anti-proliferative effect against cancer cell lines. Research has demonstrated that enzyme catalysis generates peptides from diverse food sources that function as physiological biomolecules (Gibbs et al., 2004; Kannan et al., 2010; Li et al., 2014; Ohba et al., 2004; Rayaprolu et al., 2017). The preference for enzymatic hydrolysis in producing bioactive peptides stems from its non-impact on nutritive value, short reaction time, ease of scalability, and predictability. Furthermore, numerous studies have indicated that enzymatic hydrolysis yields peptides and hydrolysates exhibiting antioxidant and antitumor properties in Hela cells (He at al., 2013; He et al. 2020).

2.8. Physicochemical Characterization of Peptides

Besides their bioactivities, protein peptides derived from food exhibit diverse physicochemical properties such as lipid binding, emulsification, solubility and foaming influenced by their length, composition and sequence (Cho et al., 2014; Pokora et al., 2013). Consequently, these peptides from food sources hold great potential as ingredients for the development of functional foods (Chalamaiah et al., 2012; Chalamaiah et al., 2018).

2.8.1. Water and oil absorption capacity

Water absorption capacity (WAC) and oil absorption capacity (OAC) reflects the interaction of protein with water or oil in food system. It is the entrapment or the ability to hold water or oil against gravity (Oyeyinka et al., 2016). There are numerous parameters such as shape, conformational characteristics as well as size that play a role in determining the water absorption capacity (Malomo et al., 2014). Water absorption capacity is also impacted by the physicochemical environment such as ionic strength, presence of lipids and solubility with regards to hydrophilic amino groups which are the primary sites of protein-water interaction as well as the balance between the polar and non-polar amino acids of the protein molecules (Malomo et al., 2014). The low hydrophilic properties may result in a low water absorption capacity (Nosenko et al., 2014).

The oil absorption capacity is the ability of oil to bind the hydrophobic portion of proteins. It relates to emulsifying capacity which a functional property that involves hydrophobicity. The oil absorption capacity is determined by the source and species of the protein (Malomo and Aluko, 2015). The protein surfaces with more hydrophobic sites absorb more oil and surfaces with more hydrophilic sites absorb more water. The oil absorption capacity is an important factor that will determine the use of proteins as potential ingredients (Malomo and Aluko, 2015).

2.8.2. Foaming properties

Foams are two phase systems that consists of a dispersed phase (gas) and a continuous phase (liquid) (Tan et al., 2011). Foams are formed through the unfolding and absorption of the protein at the air water interface, as well as film formation around the air bubble that prevents them from collapsing. Structural disorder, metastability and protein molecular flexibility characterizes good foams, thus once foams are formed, they are thermodynamically stable over time (Ptaszek, 2013). Foaming properties include foaming capacity and stability. The foam capacity of a protein refers to its capability to create foam under specific conditions, including temperature, pH, and salt concentration. Foaming stability, on the other hand, measures how effectively the protein can maintain the volume of the foam over time (Stone et al., 2015). Foaming properties of protein can be influenced by various factors such thermal treatment, protein concentration, mechanical shear, pH, the behaviour of the interface and the interaction of protein with other food ingredients (Stone et al*.,* 2015). Low foam stability is achieved at elevated pH levels because of the heightened net charge, leading to weakened protein-protein interactions. This, in turn, diminishes the protein's capacity to create robust interfacial films at the air-water interface (Tan et al*.,* 2011).

2.8.3. Emulsifying properties

Emulsifying capacity is the ability of proteins that allow the mixing of two molecules that do not normally mix (water and oil) by acting as an intermediary agent. This is an important property because interactions between water and oil is not possible in food systems, which is why there is a requirement for a surface-active agent (Malomo and Aluko 2015). The speed at which protein permeates the interface and its ability to change shape under the impact of surface tension for instance, surface denaturation determines the suitability of the flour sample as an emulsifier (Barac et al*.,* 2015). The stability of the emulsions and enhanced formed-oil-water-phases of most formulated foods depend on the interaction between the hydrophilic portion with water and the interaction of the hydrophobic portion with oil (Tang, 2017). A well-developed surface hydrophobicity and relatively stable conformation, good water solubility, a balance between the non-polar, charged and polar amino acids and relatively low molecular weight are qualities proteins should have to form emulsions (Barac et al., 2015).

The emulsifying properties of proteins are often expressed as emulsion capacity and stability (Boye et al., 2010). The emulsifying capacity gauges the quantity of oil that can be emulsified per unit of protein, while emulsifying stability assesses the emulsion's resistance to structural changes over a specified duration (Boye et al., 2010).

2.8.4. Protein Solubility

The solubility in an aqueous solution determines the functionality of proteins in food applications as well as food processing (Oyeyinka et al., 2016). Protein solubility is the equilibrium between the hydrophobic and hydrophilic interactions.Parameters such as pH, ionic strength, temperature and food matrix medium influences protein solubility. Another critical, contributing factor is the protein's isoelectric point. The isoelectric point is the point where proteins have no net charge. The solubility is the lowest near and at the isoelectric point (Mundi and Aluko 2012). This is due to the amphoteric nature of the protein which results in precipitation at the pH correlating to the isoelectric point, thus leading to minimum protein solubility (Malomo and Aluko 2015). Furthermore, the solubility is influenced by the type of source of the plant protein, thereby affecting the functional properties of the final peptides (Barac et al., 2015). Other than the isoelectric point, the pH of the environment plays an important role in determining protein solubility (Oyeyinka et al., 2016).

2.8.5. Sub-unit Composition

The SDS-PAGE method is used to study protein-based variation among different organisms. It is used to detect several types of protein sub-units of different organisms (Jiang et al., 2016). The proteins are separated in an electric field through a solid polymer called a gel matrix, smaller proteins migrate faster because of less friction from the gel matrix. The rate of migration through the gel matrix is also impacted by protein's structure and charge (Ruo.mbl.co.jp, 2019). The SDS-PAGE profile of cowpea protein isolates as demonstrated by Gómez et al. (2021) is shown in Figure 2.8 below. The cowpea isolates exhibited polypeptides within the molecular weight range of 42-80 kDa for the vicilin fraction and 94, 33, 25, and 20 kDa for the albumin fraction. In the presence of reducing conditions, polypeptides of 80 kDa (arrow 1 and 3) and 42 kDa (arrow 2 and 4) were not observed in I8 and I10.

Instead, new bands were identified, corresponding to a polypeptide of 29 kDa (arrow 5 and 7) and 19 kDa (Fig. 4A, arrow 6 and 8). These results suggest the existence of disulfide bonds in proteins of higher molecular weight.

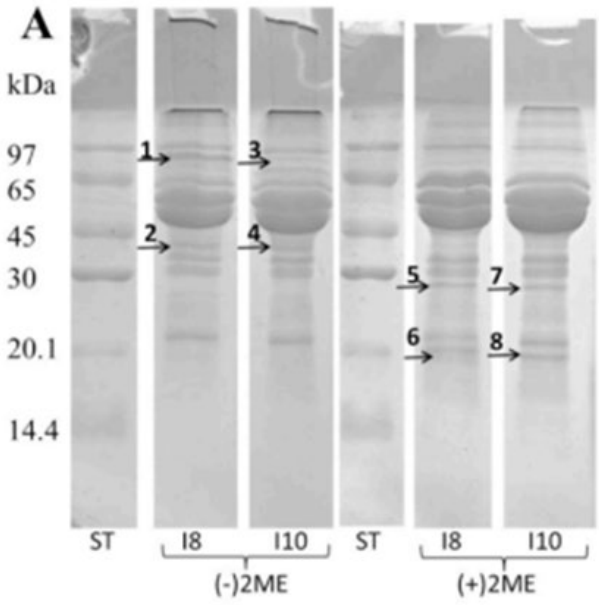

Figure 2.8: Electrophoresis profile of cowpea protein isolates under non-reducing [(−)2ME] and reducing [(+)2ME] conditions (Gómez et al., 2021).

2.8.6. Amino Acid Content

The building blocks of protein are said to be amino acids. Amino acid profiles serve as indicators of the nutritional qualities and functionalities of proteins. The content of essential and nonessential amino acids is a key parameter that offers crucial nutritional information regarding the protein quality of legumes (Keskin et al., 2022). Amino acids can be classified as dispensable, indispensable and conditionally indispensable during the biosynthesis process, based on their availability and biological activity (Diaz et al., 2015). Humans and organisms cannot synthesize essential or indispensable amino acids and their absence hampers the synthesis of protein-based enzymes and tissues. Chronic essential amino acid deficiency has been reported to severely affect organs of the body and systems including brain function of children and infants, immunodeficiency etc (Meyers et al., 2006).

Non-amino acid sources of nitrogen synthesize dispensable amino acid and they are usually found in foods such as wheat and rice. Sometimes amino acids are considered limiting due to the inability of dispensable amino acids to meet the requirements for human beings. (Diaz et al., 2015). Usually, animal source protein contains all the essential amino acids in suitable amounts while plant proteins are incomplete and contain inadequate amounts of essential amino acids. Lysine was reported as the first-limiting amino acid in wheat and maize flour by scientist Thomas Osborne (Osborne and Mendel, 1914). In addition, oat, barley and rice were reported to have a very low lysine content (Buckner et al., 1915). In contrast, White et al., (1955) noted that lysine is not limiting in quinoa. Teka et al. (2020) reported eighteen amino acids were found in cowpea varieties. Among the eighteen amino acids identified, nine were categorized as essential amino acids (EAA), while the remaining nine were non-essential amino acids (NEAA). Among the essential amino acids (EAAs), leucine exhibited the highest abundance, followed by lysine, valine, tryptophan, and histidine. while, methionine was the least abundant. Glutamic acid and glutamine were the most abundant non-essential amino acids.

2.9. Antioxidant Activity of Legumes

Legumes are also known as a source of natural antioxidants and their antioxidant capacity depends on the biological variety of the plant, and is observed over broad ranges (Amarowicz and Pegg, 2008; Matemu et al., 2021). The increasing focus on natural antioxidants has spurred extensive investigation into bio-functional peptides derived from a diverse range of legume-based food items and their by-products. These peptides were initially dormant within the protein structure but can be produced through digestion in the gastrointestinal tract as well as non-gastrointestinal processes, including the use of commercial proteases and fermentation.

Over the past few decades, natural antioxidants sourced from proteins of legume origin have gained significant attention as safer alternatives to synthetic antioxidants. These alternatives aim to restore the oxidant–antioxidant balance, consequently diminishing the risk of diseases associated with oxidative stress (Lourenço et al., 2019). Peptides derived from legumes might demonstrate antioxidant activity by specific amino acids functioning as metal-chelating and hydrogen-/electron-donating agents.

These interactions with free radicals can lead to the termination of radical chain reactions or prevent their formation (Torres-Fuentes et al., 2015; Aderinola and Duodu, 2022). Foxtail millet protein (prolamin) was hydrolysed by Ji et al. (2019) using alcalase and trypsin, and the antioxidant activities were assessed through DPPH and ORAC assays. The hydrolysate obtained with alcalase exhibited superior antioxidative activities in both assays compared to the trypsin-derived hydrolysate. Nadzri et al. (2021) produced chickpea hydrolysates using alcalase and papain, reported that the chickpea hydrolysates from alcalase exhibited a higher antioxidant activity on DPPH as compared to the chickpea hydrolysate from papain. Islam et al. (2022) evaluated the antioxidant activity of soybean hydrolysates derived from alclase and protamex by conducting the ABTS assay. The results indicated that the alcalase hydrolysates had the highest ABTS radical scavenging activity (70.21%) compared to the protamex hydrolysate (52.31%).

3. AIM AND OBJECTIVES

3.1. Research Problem and Aim

Current therapeutic anticancer drugs are expensive and associated with severe side effects and resistance, therefore the need for cost efficient alternates with lower risks. Therefore, the aim of this study was to investigate the anticancer and anti-reactive oxygen species activity of peptides derived from *Vigna unguiculata* to inhibit cancer cells. The reason for this approach is that food proteins, from legumes, are a source of bioactive peptides and studies revealed that they are associated with various therapeutic properties

3.2. Objectives

- To produce peptides from alcalase and flavourzyme and evaluate their physicochemical properties (water & oil absorption capacity, emulsifying & foaming properties, sub-unit composition, solubility and amino acid composition).
- To determine the antioxidant activities of peptides from cowpea by conducting DPPH, ABTS and superoxide radical scavenging methods.
- To determine the anti-proliferative activity of cowpea peptides human colon adenocarcinoma (CACO-2), breast (MCF-7) and muscle (C2C12) cell lines using the MTT assay.
- To determine the reactive oxygen species and anticancer potential of cowpea peptides using the cellular ROS assay and caspase-3/7 glo detection assay.

4. METHODOLOGY

4.1. Sample Preparation

Cowpea samples were obtained from the Agricultural Research Council-Vegetable, Industrial and Medicinal Plants (ARC-VIMP), Pretoria, South Africa. Seeds were inspected, cleaned, and soaked for 24 h in distilled water (5% m/v). Dehulled cowpea seeds were then dried in a drying oven (Memmert, South Africa) at 30°C overnight and milled through a 180 µM sieve, into flour. The flour was passed through a 180 µm sieve and defatted with ~~n~~-hexane overnight (1:3 m/v) (Naiker et al., 2019).

4.1.1. Preparation of protein isolate

The protein isolate was prepared through salt extraction-dialysis, following the method ~~as per~~ of Mohan and Mellem (2020). Defatted flour was combined with 0.1 M sodium phosphate buffer (pH 8.00) containing 6.4% potassium chloride (KCl) at a ratio of 1:10 (w/v) and agitated at 500 rpm for 24 h at room temperature. Subsequently, the mixture was centrifuged (Eppendorf 5810 R) at 4 500 × *g* for 20 min at 4°C. The resulting supernatant was collected and subjected to dialysis against Milli-Q™ water at 4°C, with the water refreshed three times daily for a duration of 72 h. After dialysis, the extract was centrifuged at 5 000 × *g* for 15 min, freeze-dried, and stored at −30°C.

4.1.2. Preparation of peptides by enzymatic hydrolysis

Peptides were prepared following the procedure outlined by Sipahli et al. (2021). In summary, two proteases, namely Flavourzyme (Sigma Aldrich: ≥500 U/g) at pH 7 and 50°C, and Alcalase (Sigma Aldrich: ≥0.75 U/mL) at pH 8 and 50°C, were employed. Protein isolates were dispersed in distilled water (1:20 w/v), and enzymes were added at 5% of the substrate ratio on a dry basis for all reactions. Each substrate underwent a 4 h incubation, with regular pH adjustments and monitoring. Subsequently, the substrate was heated to 100°C using a water-bath, for 10 min, cooled to 37°C, centrifuged at 10 000 × *g* for 30 min, and the supernatant freeze-dried.

4.2. Physicochemical characterization

4.2.1. Water and oil absorption capacities

Peptide sample (2.50 g) was added to 20 mL distilled water and sunflower oil, respectively. The suspensions were centrifuged for 10 min at 3000 × *g*. The water and oil released after centrifugation was weighed and expressed as water absorption capacity (WAC) and oil absorption capacity (OAC) (%), respectively (Naiker et al., 2019).

4.2.2. Emulsifying properties

The method of Hamid et al. (2015) was used to determine emulsifying properties. A 1% suspension was added to 5 mL sunflower oil and thereafter homogenized. The emulsions were centrifuged for 5 min at 1 100 x *g* and the following formulas used to calculate the emulsion capacity and stability:

$$\text{Emulsion capacity (\%)} = \frac{\text{Height of emulsified layer (mL)}}{\text{Total height of tube contents (mL)}} \times 100 \quad \text{.....[1]}$$

$$\text{Emulsion stability (\%)} = \frac{\text{Height of emulsified layer after heating (mL)}}{\text{Height of layer before heating (mL)}} \times 100 \quad \text{.....[2]}$$

4.2.3. Foaming properties

The method outlined by Hamid *et al*. (2015) was used to evaluate foaming properties. Peptide suspensions (2%) were blended for 5 min and the foam capacity determined from the following formula:

$$\text{Foam capacity (\%)} = \frac{\text{vol. after shear (mL)} - \text{vol. before shear (mL)}}{\text{vol: before shear (mL)}} \times 100 \quad \text{.....[3]}$$

Foam stability was determined after the contents were allowed to stand for 1 h at room temperature and the foam stability evaluated using the following formula:

$$\text{Foam stability (\%)} = \frac{\text{vol. of foam (mL) at 60 min}}{\text{Initial foam (mL) vol.}} \times 100 \quad \text{.....[4]}$$

4.2.4. Protein solubility

The protein solubility was determined according to Ramsookmohan et al. (2020) with the solubility of peptides determined at pH values of 2, 4, 6, 8 and 10. A 1% peptide suspension was created using distilled water and stirred overnight. The suspensions were centrifuged at 1 100 × *g* for 10 min. Subsequently, 15 mL of the supernatant was distributed into five centrifuge tubes, and the pH individually adjusted to 2, 4, 6, 8, and 10. Following this, the mixture was incubated at room temperature for 1 h and then centrifuged at 5 000× *g* for 15 min with Bradford used to determine protein concentration (Bradford, 1976).

4.2.5. Sub-unit composition by SDS-PAGE

The SDS–PAGE of cowpea peptides was done using Invitrogen (2010) under reducing conditions. NovexTM Bolt Bis-Tris gels were used with the Invitrogen Mini Gel tank under a constant voltage (200 V).

4.2.6. Amino acid analysis

Amino acid analysis was carried out by Central Analytical Facilities, University of Stellenbosch using a Waters Acquity Ultra Performance Liquid Chromatography (UPLC) equipped with a photodiode array (PDA) detector. One microliter of sample/ standard solution was injected into the mobile phase, conveying the derivatized amino acids onto a Waters UltraTag C18 column (2.1 × 50 mm × 1.7 µm) held at 60°C with instrument control and data acquisition were performed using MassLynx software.

4.3.Antioxidant Potential

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

The DPPH radical scavenging assay was performed following the procedure outlined by Sipahli et al. (2022) with slight adjustments. Samples were dissolved in distilled water containing 1% Triton X-100 at concentrations ranging from 1 to 5 mg/mL. A mixture of 100 µL of a DPPH solution (100 µM) and 100 µL of the sample was prepared and placed in a 96-well plate, followed by a 30 min incubation period in the dark. The absorbance was then measured at 517 nm.

Glutathione served as the positive control, while the blank consisted of distilled water and DPPH. The calculation of scavenging activity was performed using the following formula:

$$Radical\ scavenging\ activity = \frac{Absorbance\ (blank) - Absorbance\ (sample)}{Absorbance\ (blank)} \times 100 \quad \ldots\ldots\ldots\ldots [5]$$

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

The ABTS radical scavenging assay was conducted by the method outlined by Sipahli et al. (2022). A mixture of 7 mM ABTS and 2.45 mM potassium persulfate in a 1:1 (v/v) ratio was gently agitated for 16-20 h in a dark environment. The ABTS stock solution was subsequently diluted with a 10 mM potassium phosphate buffer at a pH of 7.4 until the absorbance at 734 nm reached the desired level of 0.70±0.02. Following this, 100 µL of the ABTS reagent was combined with 100 µL of the sample (at concentrations ranging from 1 to 5 mg/mL) in a 96-well plate, and the mixture incubated at 30°C for 4 min and the absorbance measured at 734 nm. Glutathione served as the positive control, and the blank consisted of buffer solution. The determination of scavenging capacity was carried out using the subsequent formula:

$$Scavenging\ activity = \frac{Absorbance\ (blank) - Absorbance\ (sample)}{Absorbance\ (blank)} \times 100 \quad \ldots\ldots\ldots\ldots\ldots\ldots [6]$$

4.3.3. Superoxide radical scavenging assay

The superoxide radical scavenging activity was assessed using method by He et al. (2013) with modifications. Samples and standards (1-5 mg/mL) were prepared in 0.1 M NaOH. In brief, 80 µL of 50 mM Tris-HCl (pH 8.3) with 1 mM EDTA was added to 80 µL of the sample. Subsequently, 40 µL of 1.5 mM pyrogallol prepared in 10 mM HCl was introduced, and the absorbance was measured at 420 nm over a 4 min period. Glutathione served as the positive control, and 50 mM Tris-HCl the blank with the following formula used to determine scavenging capacity:

$$Superoxide\ radical\ scavenging\ capacity\ (\%) = \frac{\Delta\ Abs\ (control) - \Delta\ Abs\ (sample)}{\Delta\ Abs\ (control)} \times 100 \quad \ldots [7]$$

4.4.Anti-proliferative Potential

4.4.1. Cell culture

Human muscle (C2C12), breast cancer (MCF-7), and human colon cancer (Caco-2) cell lines were provided by the Department of Human Physiology at the University of KwaZulu-Natal (Westville campus). These cells were cultured at 37°C in a humidified incubator with a 5% CO_2 atmosphere, using Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics [10,000 U/mL penicillin and 10,000 U/mL streptomycin sulfate (pen/strep)]. Given the potential for antibiotics to impact cellular phenotype and morphology, they were used in a reduced concentration of 1% pen/strep for this study. Cell cultures were grown until they reached approximately 80% confluence, with regular media replacement as required. Upon reaching confluence, the cells were trypsinized and sub-cultured.

4.4.2. Cell viability

The cytotoxicity of the peptides was assessed using the 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, following the method by Dwarka et al. (2017) with minor modifications. Cells were seeded at a density of $1x10^2$ cells/mL, with 50 µL of DMEM, in a 96-well flat-bottom plate and incubated for 24 h at 37°C under a 5% CO_2 atmosphere. Subsequently, the cells were exposed to 50 µL of peptides, which were prepared in 5% dimethyl sulfoxide (DMSO) at concentrations ranging from 1 000 to 7.8 µg/mL and incubated for another 24 h. Camptothecin was employed as the positive control. To assess cell viability, a 20 µL aliquot of MTT solution (5 mg/mL) prepared in phosphate-buffered saline (PBS) was added to the cells and incubated for 4 h at 37°C and 100 µL of DMSO added to dissolve the formazan salt that forms.

The absorbance was measured at 570 nm using a microplate spectrophotometer (Multiscan Go, Thermo Scientific) and the percentage cell viability determined using the following formula:

$$\%\ Cell\ viability = \frac{Absorbance\ of\ treated\ cells}{Absorbance\ of\ untreated\ cells} \times 100 \quad \text{.....[8]}$$

4.4.3. Reactive oxygen species activity

The cellular ROS Assay kit (ab113851, DCFDA Cellular ROS Detection Assay Kit, Abcam) was used to detect the production of intracellular ROS. The assay was conducted as per manufacturer's protocol. Briefly, the respective cells were seeded in 96-well plates. The following day, the media was removed and 1 x buffer was added. The buffer was ~~the~~ removed and cells were stained with diluted 2′,7′-dichlorofluorescin diacetate (DCFDA) solution and thereafter incubated for 45 min at 37°C in darkness. Then, after removing the solution, the buffer was used to rinse the cells. The cells were treated with respective peptides, using their IC_{50} values. The plate was measured at 485 nm excitation and 535 nm emission on a Biotek Fluorescence plate reader.

4.4.4. Caspase- 3/7

The Caspase-Glo 3/7 kit (Promega Corporation, 2019,) was employed to determine the caspase 3/7 activity. The assay was executed following the manufacturer's protocol. Cells were initially seeded at a density of $1x10^2$ cells/mL and incubated for 24 h. Subsequently, cells were treated with peptides using their IC_{50} values for each respective cell line. For the reaction, 100 µL of Caspase-Glo reagent was added and incubated for 1 h. The blank consisted of 5% DMSO and Dulbecco's modified Eagle's medium (DMEM) and the positive control was camptothecin. Subsequent analysis was performed using a fluorescence spectrophotometer (GloMax)

4.5. Statistical Analysis

Results are expressed as the mean for replicate data (n=3) and evaluated by analysis of variance (ANOVA) using Tukey post-test, performed using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego California USA, 2007).

5. RESULTS AND DISCUSSION

Water absorption capacity (WAC) refers to the ability of a product to interact with water in an environment where water is limiting or insufficient (Omoba et al., 2018). In this study the WAC was 2.01 and 2.41 g/g, respectively for the Alcalase and Flavourzyme samples with no significant difference; however, there was a significant difference ($p < 0.05$) between the flour and the peptides samples (Table 5.1). Similar findings were found in a study by Xu et al. (2021) who reported WAC for lentil isolate and chickpea protein isolate from Alcalase to be in the range of 2.1-2.5 g/g. Peptides obtained from enzymatic hydrolysis using Alcalase and Flavourzyme demonstrated a higher WAC than raw cowpea flour 1.29 g/g. This may be attributed to the significant ability of the peptides to unfold, swell and dissociate, which exposes additional binding sites, whereas with the raw flour, other components such as carbohydrates are present which may hinder the process (Kaur and Singh, 2007). Samples exhibiting a superior water absorption capacity can be appropriate to use in preparing complementary foods (Obi and Okoye, 2017).

Oil absorption capacity (OAC) refers to proteins non-polar side chains binding to the oil (Du *et al*., 2018). The OAC of peptides from Alcalase (1.68 g/g) and Flavourzyme (1.99 g/g) were higher than the raw flour sample (0.84). The oil absorption capacity of the flour corresponds to those previously reported in a study by Naiker et al. (2019) for cowpea flours (0.71-0.84 g/g). The presence of non-polar side chains that bind the hydrocarbon chains of oils, may be the reason for the higher oil absorption capacity observed in the peptides (Sathe et al., 1982; Kaur and Singh, 2007). The OAC of the samples observed in this study were noted to be lower than those reported by Tontul et al. (2018) (3.15-3.65 g/g) for chickpea protein isolates. The enhanced protein aggregation and disulphide group formation could have been responsible for the decreased OAC of the peptides as compared to that of the chickpea isolates (Mune and Sogi, 2015).

The emulsion capacity of flour was 31.65% which was significantly different ($p < 0.05$) to the peptides derived from alcalase and flavourzyme, which were noted to be 39.98 and 39.87% respectively, which was not significantly different.

The emulsion stability was noted to be significantly different which was 82.83% for alcalase sample, 85.78% for the flavourzyme and 83.64% for the flour sample however; the emulsion stability of the flour sample was not significantly different to the peptides from alcalase.

Protein specificity or other factors such as solubility may be responsible for the variation in emulsifying properties (Shevkani et al., 2015; Naiker et al., 2019). It has also been noted that the presence of hydrophobic sites on the surface of the protein molecule plays a significant role for the adsorption of protein at the water-oil interface during the formation of the emulsion (Tan et al., 2014; Karaca et al.,2011). The foaming capacity is influenced by the unfolding of the protein structure owing to its dispersion at the surface tension of air and water, while foam stability is due to a cohesive dense film that forms around the air bubble (Hassan et al., 2019; Khan et al., 2011). The foam capacity and stability for the flour was 75.70 and 85.47%, respectively. The peptides derived from alcalase exhibited a foam capacity of 78.34% and the foam stability was 91.24%. The flavourzyme derived peptides exhibited a foam capacity of 82.39% and foam stability of 87.18%. A significant difference was noted for both the foaming capacity and stability. The foaming properties in this study were noted to be lower than that reported for pea protein (133.3-263.3%) (Stone et al., 2015). The lower foaming properties of the peptides may be representative of a slow formation of film at the air-water interfaces together with a lower film visco-elasticity (Wani et al., 2015; Iyenagbe et al., 2017). Unhydrolyzed proteins and large peptides may result in an inhibitory effect on the foaming properties by steric hindrance at the interface of the foam and/or hydrophobic interaction (Tsumura et al., 2005).

Table 5.1: Functional properties of cowpea peptides using alcalase and flavourzyme

Sample	WAC (g/g)	OAC (g/g)	Emulsion capacity (%)	Emulsion stability (%)	Foam capacity (%)	Foam stability (%)
Control	1.29 ± 0.03^{b}	0.84 ± 0.07^{b}	31.65 ± 3.62^{a}	83.64 ± 4.68^{a}	75.70 ± 2.60^{ab}	85.47 ± 4.74^{ab}
Alcalase	2.01 ± 0.01^{a}	1.68 ± 0.05^{a}	39.98 ± 5.13^{a}	82.83 ± 4.6^{a}	78.34 ± 2.84^{a}	91.24 ± 2.04^{a}
Flavourzyme	2.41 ± 0.03^{a}	1.99 ± 0.07^{a}	39.87 ± 4.74^{a}	85.78 ± 4.3^{b}	82.39 ± 3.88^{b}	87.18 ± 1.83^{b}

Values reported are means±standard deviation of triplicate determinations. Mean values with different superscript letters within the same column are significantly different ($p < 0.05$).

The protein solubility of the cowpea flour and peptides at different pH values are shown in Figure 5.1. The cowpea samples show a typical U-shaped protein solubility profile, having the lowest solubility near pH 4 and higher solubility in both the acidic and alkaline region. The protein solubility of cowpea flour, Alcalase peptide and Flavourzyme peptide was in the range of 58-70% at pH 2.0 that decreased to 12-27% at pH 4. The isoelectric point of the cowpea samples resulted in minimum solubility at pH 4 as the peptides have an extremely low net charge or a net charge of zero (Tan et al., 2014) with an increase in the protein solubility observed from pH 6-10.

Cowpea peptides produced from Alcalase had a lower protein solubility than those produced from Flavourzyme and flour at pH 2 but resulted in an increase in solubility from pH 7-10. For pH values below or above the isoelectric pH of a protein indicates that there is a net charge on the protein surface which induced the protein-solvent interaction thus resulting in a higher solubility. The increased solubility of these peptides can be attributed to their reduced molecular size and the newly exposed ionisable amino and carboxyl groups, which contribute to an enhancement in the peptides' hydrophilic properties (Periago et al., 1998). Similar protein solubility profiles were reported in studies by Tsumura et al. (2005) and Shevkani et al. (2015) for soy protein hydrolysates and cowpea protein isolates, respectively.

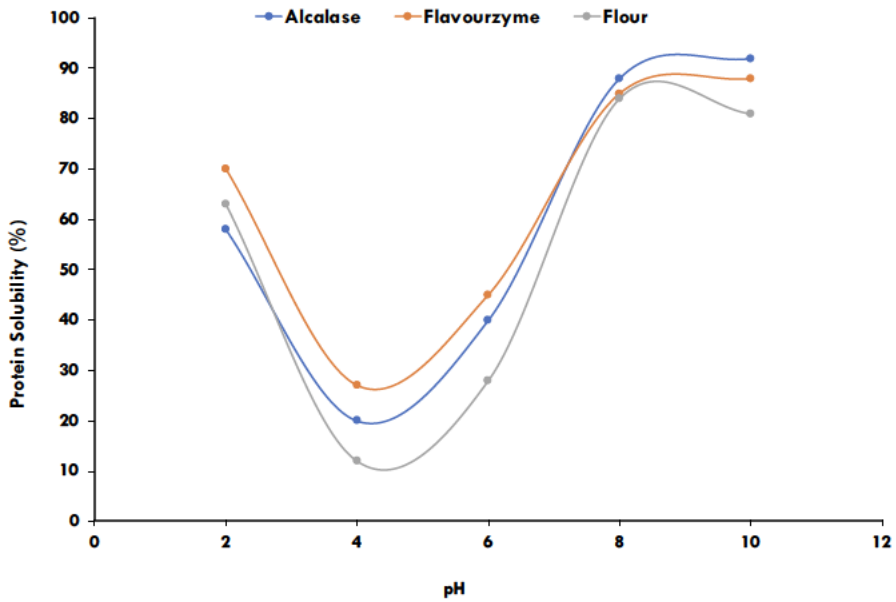

Figure 5.1: Protein solubility curve for cowpea flour and peptides from alcalase and flavourzyme.

Figure 5.2 illustrates the electrophoretic profiles of the cowpea peptides under reducing and non-reducing conditions. The cowpea peptides were characterized by polypeptide subunits between 200 and 6.5 kDa, with main polypeptides of molecular mass between 67-45 and 35-21 kDa. Some of the major bands suggest that the 7S globulin (vignin) fraction was the major component of cowpea hydrolysates with similar observations reported by Mune Mune (2015) for cowpea hydrolysates. Hydrolysis with alcalase altered the protein molecular weight distribution pattern and exhibited a reduction in the intensity of bands, especially the major bands around 67 kDa. This indicates that the respective cowpea peptide subunits are most susceptible to enzymatic hydrolysis (Ghribi et al., 2015; Xu et al., 2019). The differences in the band profiles between the flavourzyme and alcalase peptides show associated enzyme specificity for available catalytic sites. According to previous studies, aggregation of peptides is a consequence of the digestion with glutamyl endoprotease enzymes (Xia et al., 2012; Creusot and Gruppen, 2007; Gómez et al., 2021). It has been reported that Alcalase performs subtilism and exhibits glutamyl endoprotease activity, which releases peptides with glutamic acid at the C-terminal and hydrophobic patches in the sequence, contributing to aggregation (Spellman et al., 2005; Gómez et al., 2021).

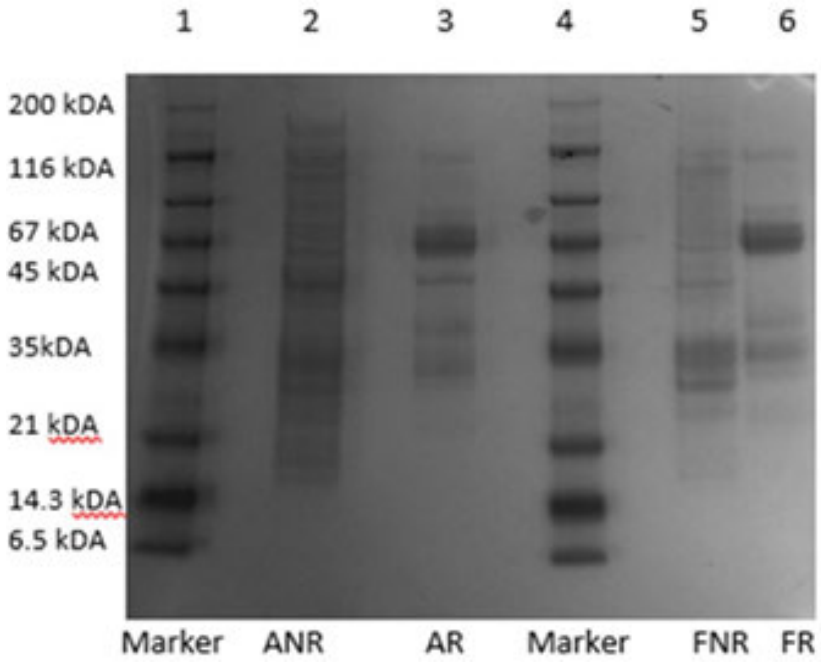

Figure 5.2: SDS-PAGE of cowpea peptides under non-reducing and reducing conditions [ANR - alcalase non-reduced, AR - alcalase reduced, FNR - flavourzyme non-reduced, FR - flavourzyme reduced].

The amino acid composition of cowpea flour and peptides derived from alcalase and flavourzyme is displayed in Table 5.2. Amino acids are the building blocks of protein and there are 20 that occur naturally and assist with metabolism. Amino acids are categorized into essential and non-essential types. The acquisition of essential amino acids (EAAs) primarily occurs through the consumption of dietary proteins, while non-essential amino acids can be synthesized internally by humans. The nine EAAs crucial for human health include lysine (Lys), isoleucine (Ile), tryptophan (Trp), valine (Val), histidine (His), leucine (Leu), threonine (Thr), phenylalanine (Phe) and methionine (Met). Among these, leucine, isoleucine, and valine are categorized as branched-chain amino acids (BCAAs). Comprising approximately 40% of the body's total amino acid requirement, BCAAs have garnered significant attention in the last decade due to their ability to enhance protein synthesis and influence metabolism. Recent findings have demonstrated that EAAs function not only as protein building blocks but also as signalling molecules that regulate various biological processes (Xiao and Guo, 2022).

It was observed that the cowpea flour and peptides derived from alcalase and flavourzyme comprised of the essential amino acids (flour: 9.97, alcalase peptide: 23.04 and flavourzyme peptide: 24.35 g/100 g) and non-essential amino acids (flour: 12.89, alcalase peptide: 27.25 and flavourzyme peptide: 31.91 g/100 g) which makes cowpea a complete protein or high-quality protein (Sharma and Saini, 2022). Glutamic acid was the most abundant amino acid in the flour (4.53 g/100 g), alcalase peptide (9.81 g/100 g) and flavourzyme peptide (10.95 g/100 g) was found to be the most abundant amino acid, Aspartic acid was the second most abundant amino acid, 2.86 and 6.79 g/100 in the cowpea flour and flavourzyme derived peptide respectively, while leucine was the second most abundant amino acid in the alcalase derived peptide (4.87 g/100 g). Lysine was the most ~~the~~ concentrated essential amino acid in the cowpea flour (2.43 g/100 g), while leucine was noted to be the most concentrated essential amino acid in the alcalase (4.87 g/100 g) and flavourzyme (5.18 g/100 g) peptides respectively. Methionine was noted to be the least concentrated amino acid in the flour and alcalase peptide with values of 0.39 and 1.21 g/100 g respectively, while proline was the least concentrated in the flavourzyme peptide (1.01 g/100 g). A study by Siddiqi et al. (2020) also reported glutamic acid to be most abundant in wheat.

Essential amino acids, alternatively termed indispensable amino acids, are amino acids that cannot be synthesized by humans and other vertebrates from metabolic intermediates. These amino acids need to be provided through an external diet as the human body lacks the metabolic pathways necessary for their synthesis (Hou and Wu, 2018). Non-essential amino acids, also referred to as dispensable amino acids, are those that can be omitted from a diet. The human body has the capability to synthesize these amino acids by relying solely on the essential amino acids (Lopez and Mohiuddin, 2020). In this study, the major non-essential amino acid in all the cowpea samples was noted to be glutamic acid. The total essential amino acid values of 9.97, 23.04 and 24.35g/100 were obtained for the flour, alcalase and flavourzyme samples respectively.

Amino acids can be categorized based on the extent of interaction of their side chains with water. Amino acids with aliphatic side chains (Pro, Ile, Met Ala, Val and Leu) and aromatic side chains (Tyr, Phe and Trp) are considered hydrophobic, resulting in limited solubility in water. On the other hand, polar (hydrophilic) amino acids are highly soluble in water and fall into either the charged category (Arg, Asp, Glu, His, and Lys) or the uncharged category (Ser, Thr, Asn, Gln, and Cys) (Damodaran, 2008). The acidic amino acids were greater than the basic amino acids in all cowpea samples indicating that the protein is mainly acidic in nature. The Fischer's ratio is a metric used to assess the biological activity of proteins, indicating the ratio between branched-chain amino acids and aromatic amino acids. In this study, the Fischer's ratio of the flavourzyme peptide was <1.8, while the cowpea flour and alcalase peptide were >1.8.

A study by Mohan and Mellem, (2020) reported the amino acid composition of lablab protein isolates derived from salt extraction, isoelectric precipitation, and micellization precipitation and revealed that leucine (7.44-9.26 g/100 g) was the predominant essential amino acid, while glutamic acid (15.27-23.05 g/100 g) was the most abundant non-essential amino acid. The aromatic amino acids of protein isolates ranged from 8.95 to 10.17 g/100 g, along with branched-chain amino acids ranging from 14.91-18.78 g/100 g. In this study, some of the amino acids may have undergone some degree of loss during hydrolysis (Darragh et al., 1996) with the variation in amino acid composition observed in the alcalase and flavourzyme peptides potentially attributed to the difference in specificity of the two proteases.

Table 5.2: Amino acid profile of cowpea flour and peptides derived from alcalase and flavourzyme (g/100g)

Amino Acid (AA)		Flour	Alcalase	Flavourzyme	Reference*
Essential Amino Acid (EAA)	**Histidine**	0.70	1.66	1.89	2.40
	Threonine	0.89	1.85	1.90	5.10
	Lysine	2.43	3.55	3.89	6.30
	Methionine	0.39	1.21	1.20	3.20
	Valine	1.28	3.35	3.18	7.60
	Isoleucine	1.08	2.58	2.74	5.60
	Leucine	2.03	4.87	5.18	8.30
	Phenylalanine	1.17	3.97	4.37	5.10
	Subtotal	9.97	23.04	24.35	43.60
Nonessential Amino Acid (NAA)	**Arginine**	1.64	2.08	3.43	-
	Serine	1.23	1.72	3.09	-
	Glycine	0.96	2.19	2.05	-
	Aspartic Acid	2.86	4.42	6.79	-
	Glutamic Acid	4.53	9.81	10.95	-
	Alanine	1.07	2.69	2.22	-
	Proline	0.03	2.36	1.01	-
	Tyrosine	0.57	1.98	2.37	-
	Subtotal	12.89	27.25	31.91	-
BCAA (LIe+Leu+Val)		4.39	10.8	11.1	-
Aromatic AA (Phe+Tyr)		1.74	5.95	6.74	-
Fischer's ratio (BCAA/AAA)		2.52	1.82	1.65	-
Basic Amino Acids		4.77	7.29	9.21	-
Acidic Amino Acids		7.39	14.23	17.74	-

**Egg was used as the reference* (*FAO/WHO/UNU, 1985)*

DPPH is a nitrogen-centred free radical compound known for its stability. It finds widespread use in assessing the antioxidant capacity of peptides (Karadag et al., 2009). This stability is attributed to its resonance stability and the unique blocking of benzene rings (Biswas et al., 2010). When DPPH encounters a substance capable of donating a proton (H+), this radical is effectively scavenged. Consequently, the rich purple hue associated with DPPH shifts to a pale yellow colour. This colour transformation is a result of the DPPH radical accepting an electron, thereby transforming into a stable diamagnetic molecule, leading to a reduction in absorbance (Liu et al., 2016; Krishnappa et al., 2014).

The DPPH radical-scavenging activity is depicted in Figure 5.3. The DPPH activity of the flour and peptides changed in a concentration-dependent manner with the concentration ranging from 0-5 mg/mL. The cowpea protein flour and peptides showed linear correlations between scavenging ability and concentration. As seen in figure 11, the control – glutathione exhibited the best activity (74.55-85.4%). From the cowpea samples, the peptide derived from alcalase demonstrated the highest DPPH radical-scavenging activity (70.88-80.47%), followed by the flavourzyme sample (67.27-75.84%), while the flour sample showed the lowest activity (24.28-66.17%). The flour sample was significantly different to the alcalase peptide, Flavourzyme peptide and glutathione sample, but there was no significant difference observed between glutathione, alcalase peptide and flavourzyme peptide. The IC_{50} is a commonly employed parameter for quantifying the efficiency of antiradical properties, with a low IC50 value indicating a robust scavenging activity. The best IC_{50} for peptides from alcalase, flavourzyme, flour sample and glutathione were 1.47, 2.67, 4.79 and 0.95 mg/mL, respectively. A previous study by Chen et al. (2017) also showed a similar trend with the DPPH scavenging capacity of proteins increasing in a dose-dependent manner. However, in a study by Sipahli et al. (2022) it was reported that the lablab isolate (1.81 mg/mL) exhibited better activity than the hydrolysates (2.76, 3.81, 4.47 mg/mL) which differed to the findings from this study. However, Sipahli et al. 2022 reported that the DPPH radical scavenging activity showed that the control had the lowest IC_{50}, which corresponds to this study. Making direct comparisons between various studies proves challenging due to the ~~to the~~ different variables and difference in experimental conditions.

The results in this study indicates that the hydrolysis of the flour improved the free radical scavenging activity and antioxidant ability of cowpea. The enzymatic hydrolysis contributed to the higher antioxidant capacity of the peptide samples as compared to the flour sample as proteolysis process cleaves susceptible peptide bonds and releases bioactive peptides (Ulug et al., 2021). The results suggest that the scavenging activity of the cowpea samples reveal that they were able to scavenge the DPPH radical effectively.

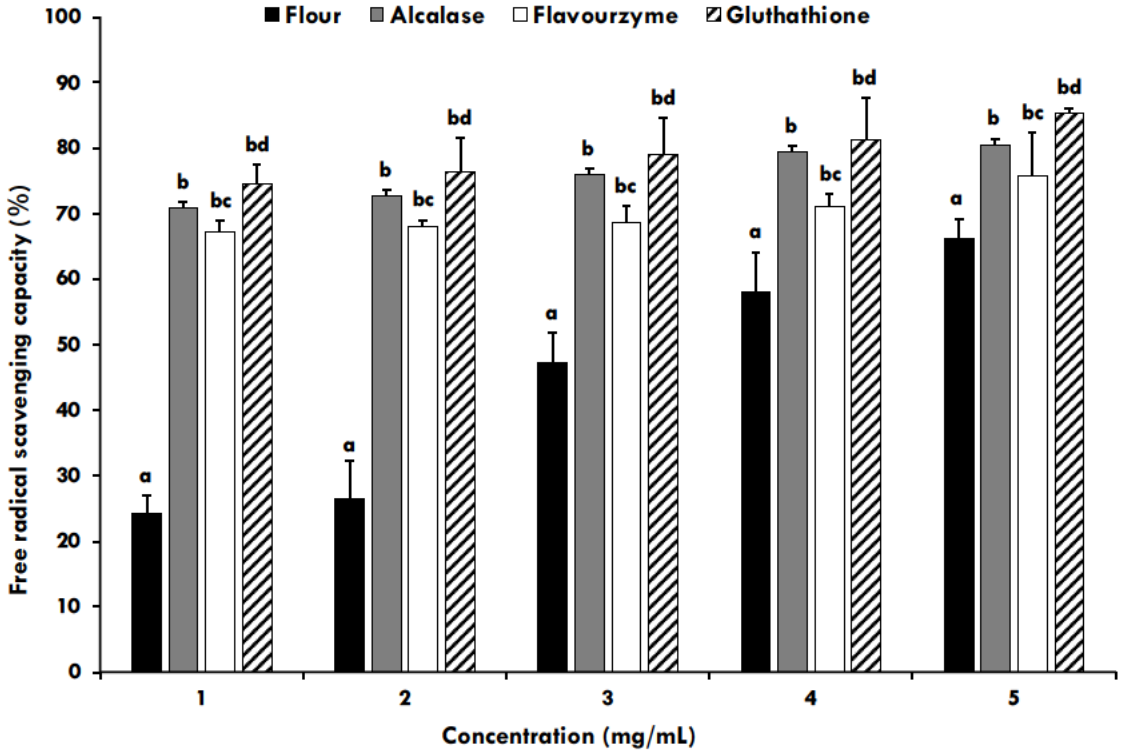

Figure 5.3: DPPH radical scavenging capacity of cowpea flour, peptides and control (glutathione). Data represents mean±SD (n= 3). Values with different superscript letters are significantly different ($p<0.05$).

The ABTS assay evaluates an antioxidant's capacity to stabilize the ABTS radical cation (ABTS ·+) through an electron transfer process. The formation of the green-blue chromophore ABTS ·+ occurs as a result of the reaction between ABTS and potassium persulfate ($K_2S_2O_8$) with the extent of colour change directly proportionate to the degree of inhibition of ABTS ·+ (Benkhaira et al., 2022; Kokina et al., 2019). ~~This proves to~~ This may be more sensitive when assessing the antioxidative capabilities of protein hydrolysate samples, as it can gauge their capacities at low inhibitory concentrations (Liu et al., 2016).

ABTS radical scavenging properties of cowpea samples are shown in Figure 5.4. The cowpea flour and peptides showed an increasing trend with the ABTS scavenging capacity increasing with increasing concentrations, as also seen in the DPPH scavenging assay, with the scavenging capacity reaching a plateau around the concentration 4 mg/mL. The scavenging capacity percentage were in the range of 18.38-48.76, 35.26-85.92, 30.78-83.14 and 66.69-80.25% for flour, alcalase, flavourzyme and glutathione samples, respectively at all concentrations. The flour sample was significantly different to the glutathione sample but not significantly different to the alcalase and flavourzyme peptides. The lowest IC_{50} values for the various samples are as follows: flour: 4.36; glutathione: 3.81; flavourzyme peptide: 1.33; alcalase peptides:1.27 mg/mL. The alcalase peptide was the most active in scavenging ABTS, greater than glutathione, the positive control. The ABTS scavenging ability of the peptides was higher than lablab hydrolysates (Sipahli et al., 2022). However, Liu et al. (2022) reported superior scavenging capacity of mung bean hydrolysates. These results are in accordance with those reported by Thumbrain et al. (2020) for cowpea isolates.

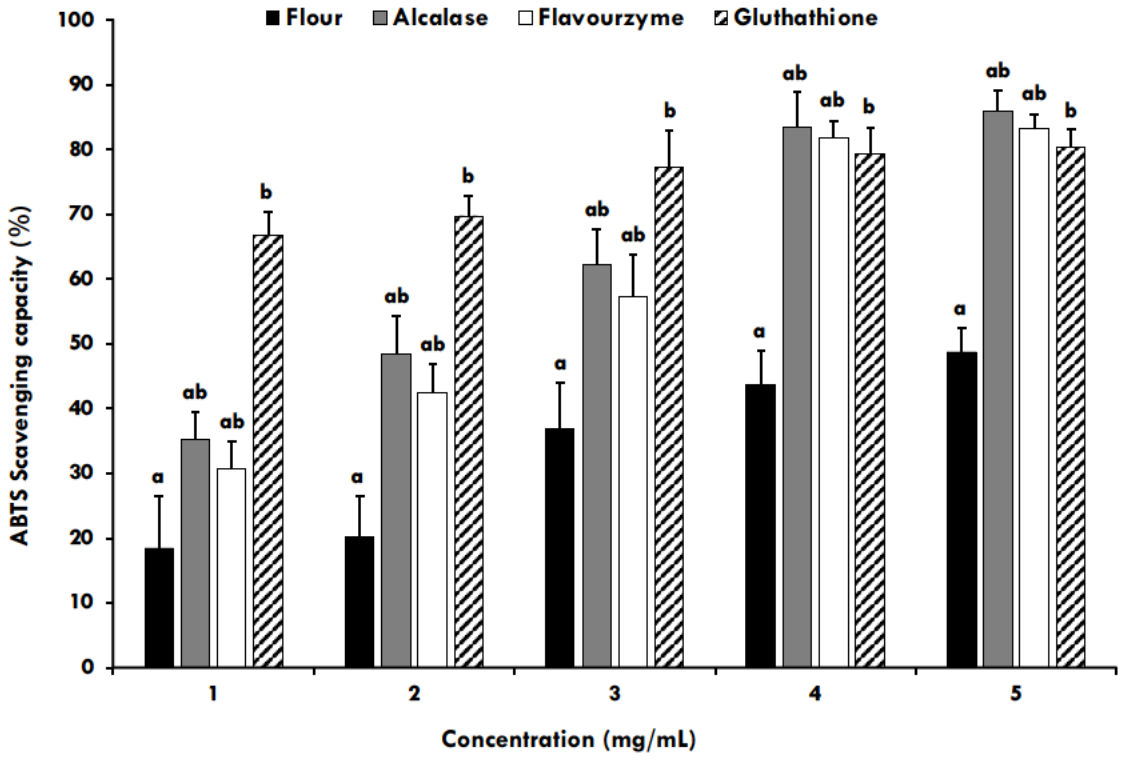

Figure 5.4: ABTS radical scavenging capacity of cowpea flour, peptides and control (glutathione). Data represents mean±SD (n= 3). Values with different superscript letters are significantly different ($p<0.05$).

To further confirm the antioxidant activity, the superoxide radical scavenging activities of cowpea samples were determined at the same concentration as the DPPH and ABTS assays, and is illustrated in Figure 5.5. The scavenging activity of all samples increased from concentration 1-3 mg/mL with glutathione exhibiting the highest capacity. From concentration 4-5 mg/mL the scavenging capacity of glutathione plateaued. At concentration 5 mg/mL alcalase peptide showed the highest scavenging ability (85.59%) followed by flavourzyme (83.13%), glutathione (82.25%), and the flour sample demonstrated the lowest scavenging capacity (32.10%). The best IC_{50} value for alcalase, flavourzyme, glutathione and flour were 1.51, 2.58, 2.63, 6.18 mg/mL. The flour sample was significantly different to the alcalase peptide, flavourzyme peptide and glutathione samples, but no significant difference was noted between the alcalase peptide, flavourzyme peptide and glutathione sample. The lower IC_{50} of the peptides compared to the flour and positive control indicate that enzymatic hydrolysis was beneficial for improving the superoxide radical scavenging activity of cowpea samples. A study by Liu et al. (2022) reported mung bean protein hydrolysates fractions to be in the range of 1.00-2.43 mg/mL.

Results from this study suggest that the cowpea samples have antioxidant capabilities with enzymatic hydrolysis contributed to the higher antioxidant capacity of the peptide samples as compared to the raw flour sample. The variation in the radical scavenging capacity of cowpea samples may be attributed to the various peptide compositions formed during the process of hydrolysis which are known to contribute to the radical-scavenging capabilities of the resulting peptides (Cumby et al., 2008). Furthermore, the presence of free amino acids within the peptides are another significant factor affecting their scavenging capacity. This may be attributed to the hydroxyl groups of amino acids possessing the ability to convert free radicals into more stable compounds through their hydrogen donation activity (Farvin et al., 2014; Wong et al., 2020). Previous studies have reported that a decrease in the tyrosine (Tyr) content within a sequence may lead to a reduction in oxidation resistance (Davalos et al., 2004). Concurrently, alternative studies have also proposed that tripeptides containing tyrosine (Tyr) or tryptophan (Trp) in the C-terminal position exhibit robust free radical scavenging activity (Wang et al., 2016). Additionally, factors such as the degree of hydrolysis (DH), choice of protease, molecular weight (MW) profile and solubility are intricately linked to the antioxidant properties of protein peptides (Jun et al., 2004).

While hydrolysed proteins exhibit noteworthy antioxidant properties, the precise influence of the amino acids within their composition on their capacity to hinder lipid peroxidation remains a subject that lacks comprehensive understanding (Guidea et al., 2020). Existing research highlights particular amino acids may be key determinants of peptide antioxidant activity (Dash and Ghosh, 2017). Gaining insights into the connection between the amino acid composition of peptides and their antioxidant effectiveness could pave the way for the development of a novel category of safe, multifunctional antioxidants with broad applicability in the realms of food and medicine. In this context, amino acids demonstrate their distinctiveness compared to other antioxidants by potentially serving as multifunctional agents capable of impeding various oxidative pathways. The interplay between amino acids and peptides is at the forefront of driving multifunctional research within this domain (Guidea et al., 2020).

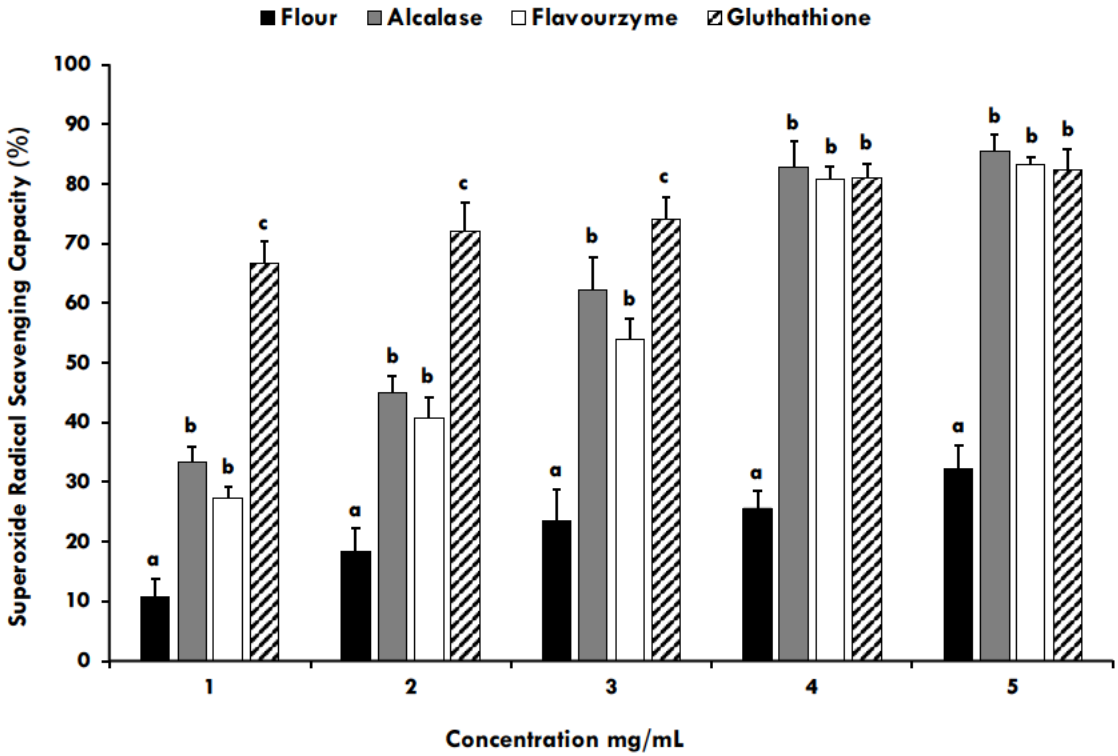

Figure 5.5: Superoxide radical scavenging capacity of cowpea flour, peptides and control (glutathione). Data represents mean±SD (n= 3). Values with different superscript letters are significantly different ($p<0.05$).

The MTT assay was used to evaluate the cytotoxicity of the cowpea peptides on cancerous cell lines (Caco-2 and MCF-7) and healthy cell lines (C2C12) to determine the anticancer potential of peptide fractions. The peptides should be toxic to the cancer cell lines while being non- toxic to the healthy cell line, in order for the peptides to be considered a good anticancer agent. The MTT assay determines the amount of cell activity by reducing the yellow colour and the formation of insoluble formazan crystals by living cells, which shows mitochondrial function (Akbarmehr et al., 2023). The dead cells lose the ability to reduce tetrazolium salts into coloured formazan products. Viable cells with active metabolism convert MTT into a purple-coloured formazan product. Thus, the intensity of the coloured product is directly proportional to the number of viable cells present in the culture (Kamiloglu et al., 2020).

The cell viability of the Caco-2 cell line is shown in Figure 5.6 A. The cell viability of the cowpea peptides and camptothecin (control) on the Caco-2 cells varied with the different concentrations, with alcalase peptide being in the range of 57.88-106.96%, flavourzyme peptide: 48.55-96% and camptothecin (control): 45.90-103.57%.

Camptothecin had the lowest viability for most concentrations. From the peptide samples, flavourzyme exhibited the lowest cell viability. At concentration 7.81 µg/mL, camptothecin had the highest viability (103.57%) whilst flavourzyme peptide had the lowest viability (96%). It was observed that the lowest viability for peptides and camptothecin was at 1000 µg/mL. Camptothecin exhibited the lowest viability (45.90%), followed by flavourzyme peptide (48.55%), with alcalase peptide having the highest cell viability (51.88%). No significant differences ($p < 0.05$) was observed between the samples. Camptothecin had the lowest IC_{50} of 0.07 µg/mL. Alcalase peptide and flavourzyme peptide had IC_{50} values of 0.15 and 0.11 µg/mL respectively.

Figure 5.6 B illustrates the cell viability of MCF-7 cells. The cowpea peptides and camptothecin showed different sensitivities on the MCF-7 cell lines. Camptothecin had the lowest viability for most concentrations. Alcalase peptide demonstrated the lowest cell viability (99.08%) at concentration 15.625 µg/mL. Flavourzyme peptide had the lowest viability (138.13, 62.51%) at concentrations 7.81 and 1000 µg/mL respectively.

The lowest cell viability was observed at 1000 µg/mL. Flavourzyme peptide demonstrated the lowest viability (62.51%), followed by alcalase peptide (64.20%) whilst, camptothecin, the chemo-preventative control, had the highest cell viability (68.50%). There was no significant difference ($p < 0.05$) between the samples. The IC_{50} values of flavourzyme peptide, alcalase peptide and camptothecin were 0.07, 0.09 and 0.07 µg/mL respectively.

The cell viability of the healthy muscle cell line is demonstrated in Figure 5.6 C. The cell viability for camptothecin, alcalase peptide and flavourzyme peptide were 45.33- 93.64, 50.34-95.66 and 48.82-117.91%. The flavourzyme peptide had the highest cell viability for most concentrations, while camptothecin showed the lowest viability. The highest cell viability (117.91%) was observed at concentration 250 µg/mL for the flavourzyme peptide. A significant different was observed between cells treated with flavourzyme and camptothecin. The alcalase peptide had the highest IC_{50} (0.23 µg/mL), followed by flavourzyme peptide (0.19 µg/mL) and camptothecin had the lowest IC_{50} 0.10 µg/mL).

From results it was observed that the cowpea peptides had an anti-proliferative impact on the cancer cells. From the cowpea peptides, flavourzyme exhibited the best activity as it had the lowest cell viability, at certain concentrations, on cancer cells whilst also exhibiting a high cell viability on healthy cells thereby not greatly effecting the normal cells. Sipahli et al. (2022) reported the effect of *L. purpureus* isolates and hydrolysates on the proliferation A459 and MCF-7 and normal cell line HEK293. It was observed that the proteolytic process improved the cytotoxicity of *L. purpureus*, with the pepsin hydrolysate, being most effective on the A549 cell line with an IC_{50} of 119.60 µg/mL, which was lower than that of the control, camptothecin. A similar observation was reported by Akbarmehr et al. (2023) who investigated the cytotoxic activities of corn pollen protein hydrolysates. The results showed that the hydrolyzed sample reduced the viability of cancer cells in a concentration-dependent manner compared to the primary protein. The lowest cell viability for the hydrolysate was 32% at a concentration of 5 mg/mL. Anticancer activity of *Amaranthus cruentus* hydrolysates and chia (*Salvia hispanica*) were proven (Ramkisson et al., 2020; Quintal-Bojórquez et al., 2021).

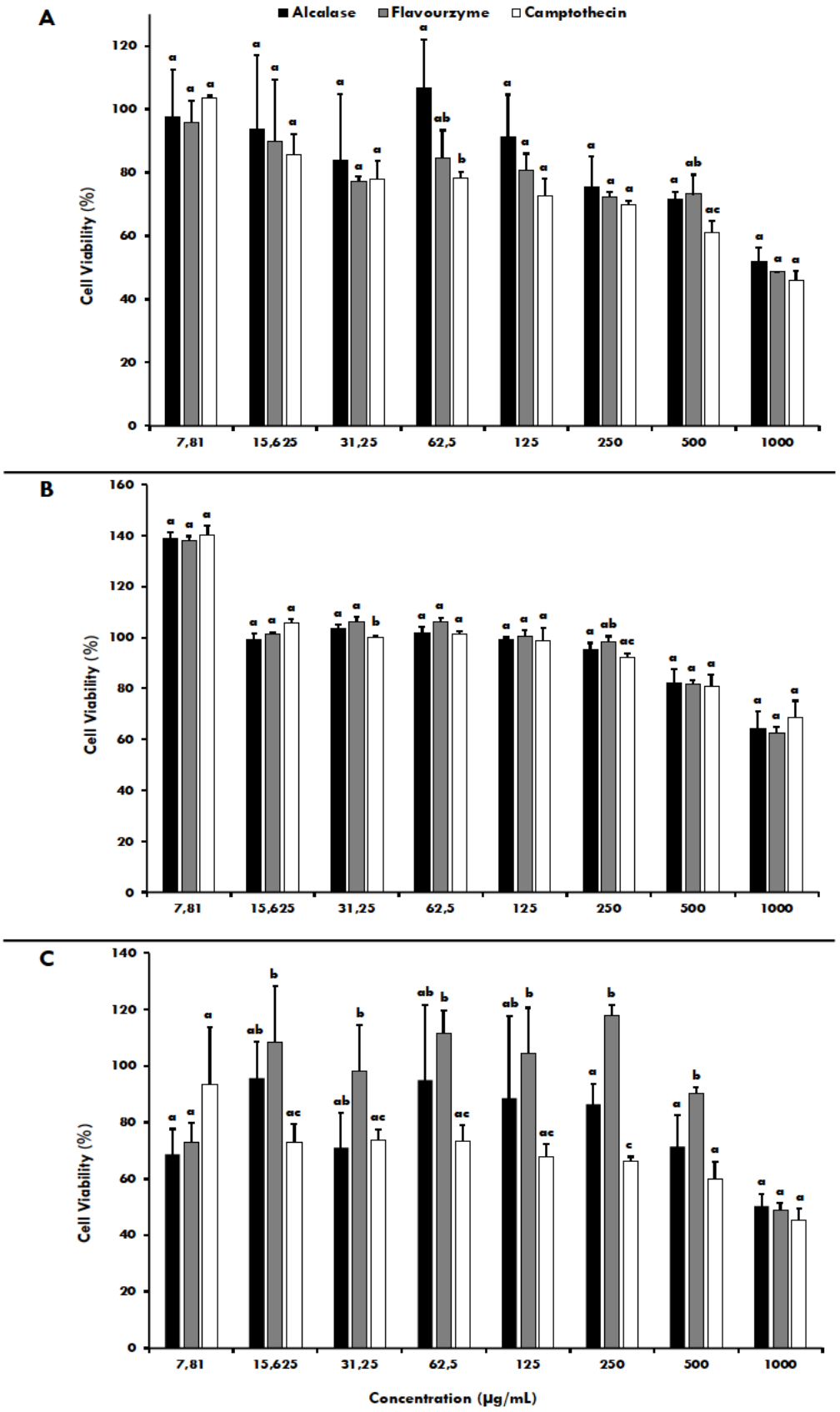

Figure 5.6: Cell viability of cowpea peptides (Alcalase and Flavourzyme) and control (camptothecin) against Caco-2 (A), MCF-7 (B) and C2C12 (C) cell lines. Data represents mean±SD (n= 3). Values with different superscript letters are significantly different ($p<0.05$).

~~Reactive oxygen species~~ ROS are small molecules typically known for their high activity and short lives. These ROS are naturally generated as by-products during oxidative phosphorylation within mitochondria, facilitating metabolic energy production. Furthermore, various external factors such as ultraviolet (UV) radiation, inflammatory responses, chemical substances, and natural compounds possess the capability to generate pro-oxidant compounds such as ROS (Badroon et al., 2020). Reactive oxygen species (ROS) have been linked to DNA mutations, aging, and cell death. Moderate levels of ROS can be advantageous for regular cells, as they stimulate proliferation pathways. However, elevated ROS levels pose risks to both healthy and tumour cells, leading to cell death either by apoptosis or necrosis (Nguyen et al., 2020; Taiwo et al., 2020). Certain anticancer medications employed in chemotherapy, such as doxorubicin, taxanes, vinca alkaloids, and antimetabolites, trigger heightened oxidative stress, ultimately causing the demise of cancer cells (Barrera, 2012).

The elevated levels of ROS could initiate both extrinsic and intrinsic apoptotic pathways. In the extrinsic pathway, ROS activate death receptors on the cell membrane, initiating caspase-8, which in turn directly activates caspases-3/-6/-7, ultimately leading to the initiation of apoptosis (Badroon et al., 2020). Conversely, ROS have the ability to trigger intrinsic pathways, leading to the release of cytochrome C from mitochondria and the initiation of apoptosis via the mitochondrial pathway. Damage to mitochondrial DNA results in the disturbance of respiratory chain function, leading to the decline of mitochondrial membrane potential and the disruption of ATP synthesis (Redza-Dutordoir and Averill-Bates, 2016). Excessive production of ROS may be linked to disruptions in cell homeostasis, mitochondrial impairment, and apoptosis (Xie et al., 2018). Activation of apoptosis pathways through the mitochondrial pathway is considered a crucial step in the process of apoptosis (Nguyen et al., 2020). As shown in Figure 5.7 the cells treated with alcalase peptide, flavourzyme peptide and camptothecin resulted in elevated ROS levels. The camptothecin treated samples exhibited the highest fold-change followed by flavourzyme and alcalase samples, on the cancer cell lines as well as the healthy cell line (C2C12). However, the ROS levels of the C2C12 cells were noted to be lower for the alcalase (0.93), flavourzyme (1.12) and camptothecin (1.52) compared to Caco-2 (Alcalase:1.60, flavourzyme: 2.19 and camptothecin: 3.21) and MCF-7 (alcalase: 1.85, flavourzyme: 2.77 and camptothecin: 3.4).

The increased ROS levels in the Caco-2 and MCF-7 cell lines may have resulted in mitochondrial dysfunction and subsequently, apoptosis as explained above. The increased ROS levels in the cancer cell lines may have played a role in the decreased cell viability observed in figures 14 & 15. Previous studies by Bagheri et al. (2018) and Zhang et al. (2011) showed that the ethanolic extract of *Brucea javanica* and seed oil of ~~*Brucea javanica*~~ B. *javanica* exhibited increased ROS levels on cancer cells and induced apoptosis.

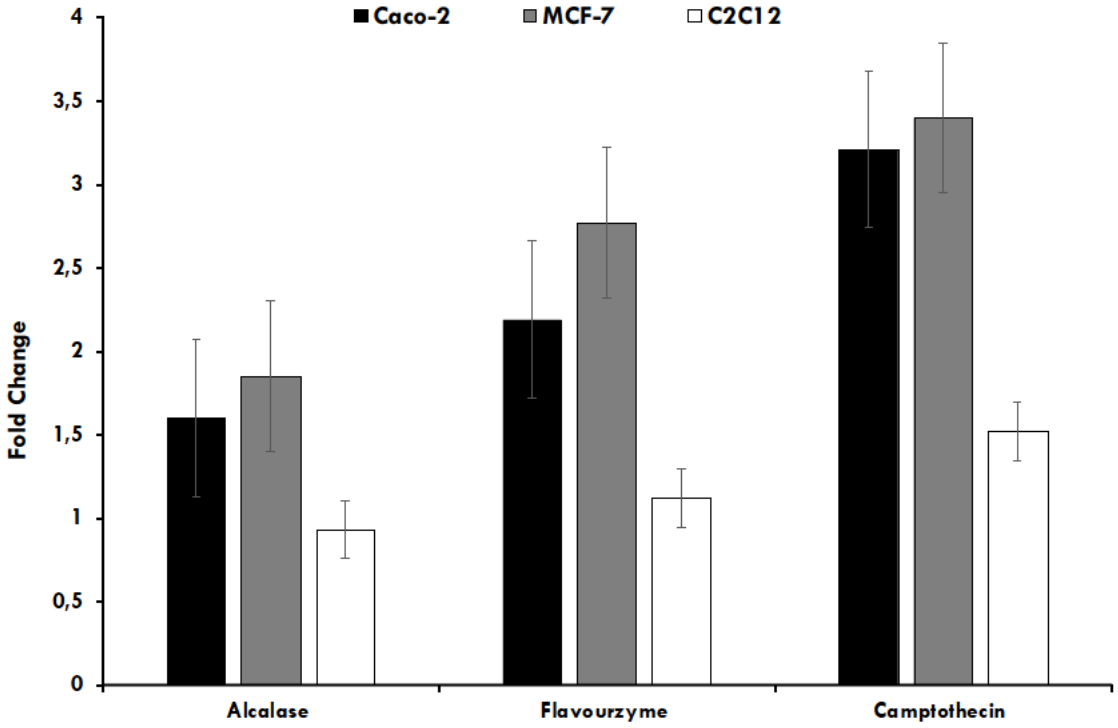

Figure 5.7: Reactive oxygen species (ROS) activity of cells treated with cowpea peptides and control (camptothecin) at IC_{50} values obtained by MTT assay, across the respective cell lines. Data represents mean±SD (n= 3).

Caspases are part of a class of cysteine proteases that exist in cells as inactive zymogens. They play a role in catalytic activation during the initiation of apoptosis. Caspases can be categorized into three types: inflammatory caspases (such as caspase 1, 4, 5, 11, and 12), initiator caspases (such as caspase 2, 8, 9, and 10) and executioner caspases (including caspase 3, 6, and 7) (Theofilas et al., 2021). Caspase-3 and caspase-7 serve as pivotal executor enzymes essential for the process of apoptosis.

Caspase-3 is responsible for initiating the phases of cellular demise in a non-traumatic fashion Once activated, caspase-3 initiates the degradation of proteins, ultimately resulting in cell death. Caspase-3 holds a paramount role in cellular mechanisms and can be activated by any of the initiators, making it a primary target for anticancer therapy (Yadav et al., (2021). Caspase-3 plays a crucial role in apoptotic chromatin condensation and the fragmentation of deoxyribonucleic acid (DNA) in all examined cell types. Additionally, it is necessary for the disassembly of the cell and the formation of apoptotic bodies (Porter and Jänicke, 1999).

The caspase- 3/7 reagent induces cell lysis, leading to caspase cleavage of the DEVD substrate and the subsequent production of luminescence. The luminescent output directly correlates with the level of caspase activity present in the sample (Payne et al., 2013). Caspase 3/7 activation for this study is illustrated in Figure 5.8. The caspase activity was higher in the Caco-2 cells (alcalase: 207013, flavourzyme: 124800 and camptothecin: 55898 FU) and MCF-7 cells (alcalase: 12326, flavourzyme: 11196 and camptothecin: 10966 FU) as compared to the healthy cell line, C2C12 (alcalase: 10342, flavourzyme: 4076 and camptothecin: 6510 FU). The alcalase peptide treated cells were not significantly different to the flavourzyme peptide cells and to the camptothecin treated cells for the Caco-2 cell line. For the MCF-7 cell line, there was no significant difference between the cells treated with alcalase peptide, flavourzyme peptide and camptothecin. A significant difference was noted between the cells treated with alacalse peptide and cells treated with the flavourzyme peptide, however no significant difference was shown between the cells treated with camptothecin, alcalase peptide in the healthy cell line (C2C12). The cell viability is inversely proportional to fluorescence units (FU) (Sipahli et al., 2022; Butterick et al., 2014) so the increased caspase activity may have contributed to the decreased cell viability of the cancer cell lines observed in figures 14 & 15. These results indicate that the alcalase and flavourzyme peptides induced apoptosis in the cancerous cell lines via caspase-dependent pathways.

Previous study by Sabbione et al. (2019) reported that the fluorescence intensity of colon tumour cells treated with amaranth proteins were similar to the control. A study by Gupta and Bhagyawant, (2021) showed that the chickpea peptide exhibited increased caspase activities in a dose dependent manner.

Ishikawa (human endometrial adenocarcinoma) cells treated with 5, 50, 100, 250 and 500 µg/ml of chickpea peptide resulted in the activation of caspases-3 by 1.3, 1.7, 2.5, 3.5 and 4.8 fold, compared to the positive and negative control, respectively. The findings by Mazumder et al. (2023) proved that lupin extracts showed apoptotic activity by increasing caspases-3/7 cleavage in colorectal carcinoma cells.

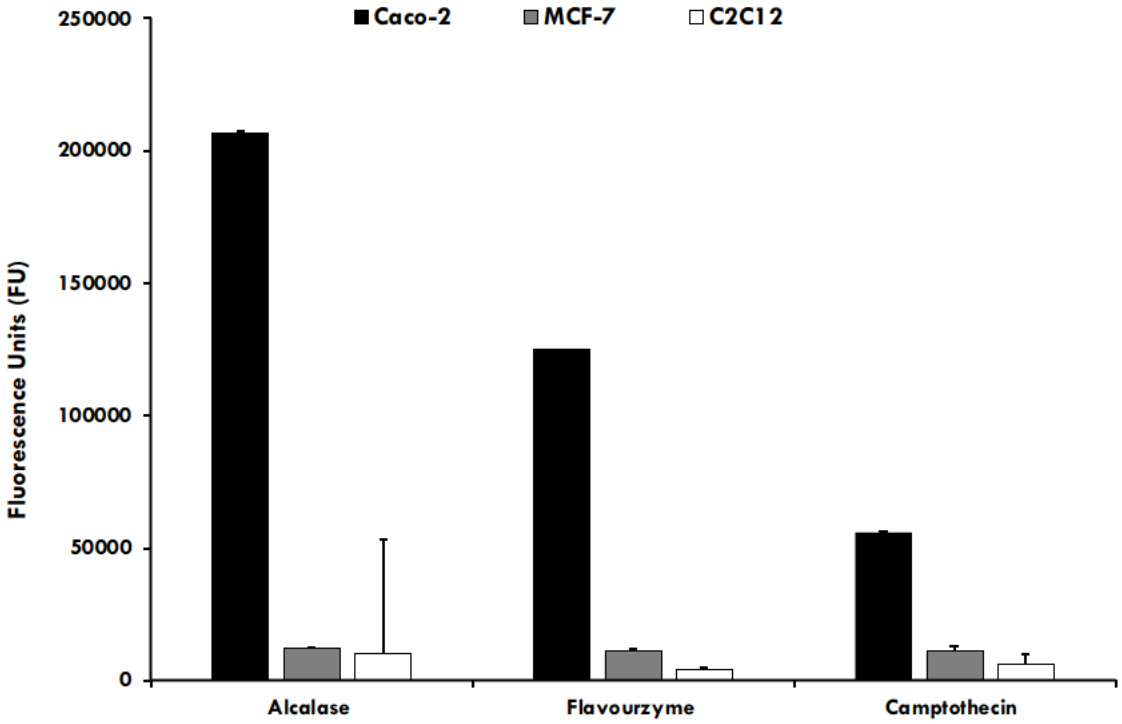

Figure 5.8: Caspase 3/7 activity measured as fluorescence units of respective cells treated with cowpea peptides and at IC50 values obtained by MTT assay, across the respective cell lines. Data represents mean ± SD (n= 3)

6. CONCLUSION

This study demonstrated that *Vigna unguiculata* (cowpea) peptides presents a practical application for producing value-added legume-based ingredients for potential food applications from a processing perspective. This could increase the value of cowpea by expanding its use and contributes to the legume grain sector but it also exhibits antioxidant activity as well as potential use of the cowpea peptides as an anticancer therapeutic agent.

Enzymatic hydrolysis was shown to improve the antioxidant and potential anticancer properties of the peptides when compared to the raw cowpea flour. From the peptide samples, flavourzyme exhibited the best cytotoxicity on the cancer cell lines (Caco-2 and MCF-7), having IC_{50} values of 0.11 µg/mL and 0.07µg/mL respectively. The luminescent output showed caspase 3/7 activity and was found to be higher in the Caco-2 cells and MCF-7 cells as compared to the healthy cell line, C2C12, which further established apoptotic activity. This work supports the idea of using *Vigna unguiculata* peptides as a potential functional food with health-promoting benefits.

It is important to note that further research is necessary to delve into the structural characteristics, explore additional health advantages, assess the impacts of alternative enzymes, determine specific peptides responsible and the possible mechanism of action as well as to conduct *in vivo* evaluations of these peptides using animal models.

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