Characterisation and antiproliferative effect of okra seed protein isolate on human cancer cell lines

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Reference Declaration

I, Miss Letisha Maistry - 21854516 and Prof JJ Mellem do hereby declare that in respect of the following dissertation – Title: Characterisation and antiproliferative effect of okra seed protein isolate on human cancer cell lines

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Authors Declaration

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

Student's signature

“Matha Pitha Guru Deivam”

translating to

“Mother father teacher God”

Mother is the first God as she brings you into this life. To my mum, thank you for your guidance and blessings. Your love, support, and strength made this journey possible.

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Publications Outputs

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Abstract

The increasing interest in plant-derived bioactive compounds has led to significant focus on okra seed protein isolate (OSPI) due to its notable nutritional and therapeutic properties. This research explores the structural and molecular characteristics of OSPI, aiming to assess its potential application as a nutraceutical in cancer therapy. A comprehensive amino acid profile showed that OSPI is rich in glutamic acid (14.46 g/100g), aspartic acid (7.56 g/100g), and arginine (9.42 g/100g), with a high proportion of hydrophobic (31.39 g/100g), and branched-chain amino acids (14.24 g/100g), known to support immune modulation and antitumour activity. Solubility analysis demonstrated peak protein solubility (91.86%) at pH 9, which is a critical factor affecting its functional efficacy. Molecular weight profiling indicated the presence of 7S and 11S globulin polypeptides, which are associated with encapsulation efficiency in nutraceutical development. Spectroscopic analysis, including FTIR and XRD, established the presence of α-helices and β-sheet structures, further supporting the protein's conformational stability and amorphous nature, promoting bioavailability in pharmaceutical formulations. Particle size denoted monomodal distributions with the smallest size depicted at 0.144±0.10 µm, highlighting its suitability in drug delivery systems. The cytotoxic and antiproliferative effect of OSPI was evaluated using human cancer cell lines. In lung carcinoma cells (A549), OSPI reduced viability to 37.73% at 15 µg/mL and demonstrated a lower IC_{50} (21.80 µg/mL) than the standard chemotherapeutic agent camptothecin (524.85 µg/mL). Defatted okra flour (DOF) and OSPI significantly upregulated p53 levels with apoptotic induction against A549, as presented by Annexin V-binding. In hepatocellular carcinoma cells (Hep-G2), OSPI activated caspase-3/7, -8, and -9 at levels comparable to camptothecin, suggesting the initiation of both intrinsic and extrinsic apoptotic pathways. Furthermore, okra flour (OF) promoted reactive oxygen species (ROS) generation more effectively than camptothecin in A549 and Hep-G2 cells, reinforcing its potential oxidative stress-mediated apoptotic effects. These findings collectively underscore OSPI as a potential candidate for pharmaceutical and nutraceutical applications, particularly in cancer therapy. Its structural stability, solubility, and bioactive profile support its utilisation in the development of plant-based therapeutic interventions.

Chapter 1: Introduction

The surge in world population, estimated to approach 9 billion by 2040, is intensifying the burden on global food and healthcare systems (Galan, 2025). This demographic increase places immense pressure on food systems, healthcare resources, and environmental sustainability, necessitating innovative approaches to ensure long-term human well-being. One area that has drawn attention is the exploration of alternative protein sources that not only meet dietary requirements but also offer therapeutic value in addressing chronic diseases, particularly cancer. Proteins are fundamental biomolecules, serving as structural and functional components of nearly every cellular process. Beyond their nutritional role, proteins are integral to metabolic signalling, enzyme catalysis, immune responses, and tissue repair (Kumar et al., 2022). Proteins obtained from legumes, seeds, nuts, and vegetables have emerged as promising alternatives to conventional animal-derived sources due to their sustainability, affordability, and potential to act as therapeutic agents (Kumar et al., 2022). Plant-derived proteins serve as essential nutrients and offer promising pharmacological benefits, particularly in managing non-communicable diseases including diabetes, cardiovascular conditions, and various cancers (Shahnaz et al., 2024). The amino acid composition, hydrophobicity, molecular size, charge, and surface reactivity are directly linked to health-promoting properties of plant proteins (Sim et al., 2021). These properties influence digestibility and bioavailability but also contribute to their functionality in cellular interactions. Plant proteins enriched with essential amino acids and antioxidant properties have shown promise in modulating immune responses, regulating apoptosis, and hindering the propagation of cancer cells (Shahnaz et al., 2024), aligning with the global shift towards green and biocompatible medicine.

Cancer remains one of the primary sources of fatalities globally, with a growing burden in both developed and developing countries. The International Agency for Research on Cancer (IARC) reported 20 million new cases in 2022, with projections of 35 million by 2050 (Bray et al., 2024). Factors including geographic location, genetic predisposition, environmental exposure, and socioeconomic disparities influence the incidence and mortality of cancer (Rahman et al., 2018). In South Africa, the burden of cancer is substantial, with breast cancer being the most frequently diagnosed, accounting for 22.6% of all cancers and 16% of cancer-related mortalities among females (Ayeni et al., 2023). The high prevalence and mortality rates accentuate the urgency for effective prevention and treatment approaches. While existing treatments, such as chemotherapy, radiation therapy, surgery, and immunotherapy, have advanced significantly, it is often allied with adverse side effects, such as immunosuppression, gastrointestinal distress, and organ toxicity (Avilés-Gaxiola et al., 2020). Moreover, the development of resistance to conventional treatments highlights the need for novel, less invasive, and more selective treatments.

This has resulted in natural proteins and peptides emerging as promising alternatives due to their ability to induce apoptosis in malignant cells, modulate oxidative stress, and selectively engage with tumour markers. Proteins with high hydrophobicity and negative charge profile are particularly effective in traversing cancer cell membranes, increasing cytotoxicity while being non-invasive to normal tissues (Chalamaiah et al., 2018). These unique properties bring about possibilities for protein-based anticancer therapies derived from natural plant sources. One underexplored plant with notable therapeutic potential is *Abelmoschus esculentus*, commonly known as okra. Indigenous to African countries and generally harvested in tropical and subtropical regions, okra has been utilised for nutritional and medicinal purposes across numerous cultures (Muimba-Kankolongo, 2018). Traditionally, okra has been employed in the treatment of ailments such as dysentery and gonorrhea, and is rich in vitamins, minerals, and bioactive compounds, including polysaccharides, polyphenols, and proteins (Dantas et al., 2021). Numerous findings have highlighted that okra extracts possess antioxidant, anti-inflammatory, hypolipidemic, and antitumour properties (Elkhalifa et al., 2021).

Particularly noteworthy is the protein content of okra seeds, which has been reported to reach 35% in crude form and over 90% in purified isolates (Nnamezie et al., 2021). These proteins are rich in key amino acids such as lysine and tryptophan (Elkhalifa et al., 2021), as well as hydrophobic and aromatic residues that may enhance bioactivity (Ofori et al., 2020). While okra polysaccharides and flavonoids have been widely studied for their therapeutic potential, the anticancer properties of okra seed protein isolate (OSPI) remain underexplored. Given the mounting global cancer burden, the drawbacks of conventional remedies, and the advantages of plant-based interventions, this study aims to characterise okra seed protein isolate and assess its antiproliferative effect on human cancer cell lines. Therefore, by evaluating its structural composition, bioactivity, and mechanism of action in human cancer cell lines, this research seeks to contribute to the development of novel, biocompatible therapeutic alternatives that are accessible, affordable, and align with sustainable healthcare innovations. The specific objectives for this study are outlined as follows:

1. To characterise the chemical and structural composition of okra seed protein isolate by assessment of the amino acid profile, molecular weight, Fourier transform infrared spectroscopy (FTIR), x-ray diffraction (XRD), scanning electron microscopy (SEM), and zeta potential.
2. To determine the antiproliferative activity and induction of apoptosis of okra seed protein isolate using the MTT assay and annexin V-PE detection kit on human cancer cell lines.
3. To determine the effect of okra seed protein isolate on key enzymes involved in the mechanism of cancer using caspase-3/7, caspase-8, caspase-9, and p53 expression assays on human cancer cell lines.
4. To determine the reactive oxygen species (ROS) inhibition of okra seed protein isolate on human cancer cell lines using cellular ROS.

Chapter 2: Literature review

2.1 Prevalence of cancer

Cancer remains one of the leading causes of mortality and morbidity worldwide, affecting all populations regardless of geography, ethnicity, or socioeconomic status. According to Sung et al. (2021), approximately 19.3 million new cancer cases and 10 million deaths ensued globally, with the number expected to increase to 28.4 million by 2040 due to aging populations, urbanisation, and lifestyle factors. The burden is shifting increasingly toward low income and middle-income countries, where more than 70% of cancer deaths now occur primarily due to limited access to preventative services, early detection, and effective treatment (W.H.O., 2025). In these regions, cancers such as breast, cervical, liver, and stomach are the most prevalent, whereas high-income countries report increased incidence of prostate, colorectal, and lung cancers. Lifestyle factors such as tobacco use, alcohol intake, and poor diet account for a significant proportion of preventable cancers (Kocarnik et al., 2022). Moreover, infectious agents, including human papillomavirus (HPV), hepatitis B and C viruses (HBV/HCV), and *Helicobacter pylori,* are linked to nearly 15% of all cancer cases globally, particularly in less developed regions (de Martel et al., 2020).

2.2 Global cancer statistics

According to the International Agency for Research on Cancer (IARC), there were 20 million new cancer cases and 9.7 million deaths worldwide in 2022 (Bray et al., 2024). The global burden of cancer is anticipated to increase by 77% to approximately 35.3 million new cases by 2025 (Bray et al., 2024). The regions most affected include Asia (49.3% of cases), followed by Europe (22.8%) and North America (13.3%) (Sung et al., 2021) with the global projected cancer incidence and mortality outlined in Table 2.1.

Table 2.1. Global cancer incidence and mortality projections (2020-2025)

Year	New Cases (million)	Deaths (million)	Reference
2020	19.3	10.0	(Sung et al., 2021)
2021	19.7	10.2	-
2022	20.0	10.4	(Bray et al., 2024)
2023	20.3	10.6	(IRAC, 2024)
2024	20.6	10.8	(IRAC, 2024)
2025	20.9	11.1	(IRAC, 2024)

2.2.1 Most common cancers globally

As of 2022, the five most prevalent cancers globally, based on incidence, are lung, breast, colorectal, prostate, and stomach cancers (Figure 2.1). Lung cancer leads with approximately 2.5 million new cases, accounting for 12.4% of all cancer diagnoses, and is also the primary cause of cancer-related deaths, with about 1.8 million fatalities (18.7% of total cancer deaths) (Bray et al., 2024). Breast cancer follows closely with 2.3 million new cases (11.6%) and 670,000 deaths (6.9%) (Bray et al., 2024). Colorectal cancer ranks third, with 1.9 million new cases (9.6%) and 900,000 deaths (9.3%) (Bray et al., 2024). Prostate cancer is fourth in incidence, recording 1.5 million new cases (7.3%) (Bray et al., 2024), while stomach cancer is fifth, with 970,000 new cases (4.9%) and 660,000 deaths (6.8%) (Bray et al., 2024). These statistics underscore the significant global burden of these cancers and emphasize the necessity for targeted preclusion, early detection, and effective therapy strategies worldwide.

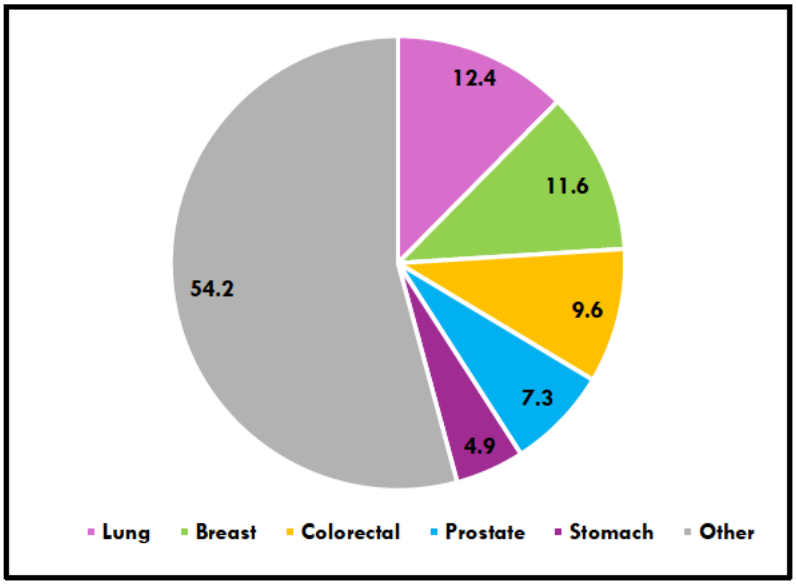

Figure 2.1. Global distribution of the top 5 cancers (2022).

2.2.2 Top 5 cancers in South Africa (2022)

As of the latest data from the National Cancer Registry for 2022, the five most commonly diagnosed cancers in South Africa are breast, prostate, cervical, lung, and colorectal (Table 2.2). Socioeconomic, genetic, and infectious disease-related factors influence South Africa's cancer landscape. In 2022, 111 321 new cases occurred in South Africa, of which 64 547 resulted in death.

Table 2.2. Top 5 most common cancers in South Africa (2022) with an estimated annual incidence and key risk factors

Cancer	Annual incidence (2022)	Key risk factors	Reference
Breast	14 712	Genetics, obesity, hormonal factors	(Bray et al., 2024)
Prostate	12 903	Age, ethnicity (higher in Black men), family history	(Bray et al., 2024)
Cervical	10 532	Persistent HPV infection, inadequate screening, HIV co-infection	(Bray et al., 2024)
Lung	9446	Tobacco, smoking, occupational exposures (e.g., asbestos), HIV-related immunosuppression	(Bray et al., 2024)
Colorectal	7338	Diet high in processed meats, low fibre ingestion, sedentary lifestyle, obesity	(Bray et al., 2024)

2.3 Physiology of cancer

Cancer arises from the conversion of normal cells into abnormal cells through a multistep progression known as carcinogenesis. This transformation involves genetic mutations and epigenetic alterations that disrupt normal cellular mechanisms, leading to uncontrolled cell proliferation and tumour formation. Central to this concept are the hallmarks of cancer (Figure 2.2) as revised by Hanahan (2022). Tumours can be categorised into two major types based on their behaviour and pathology viz. benign or malignant.

2.3.1 Benign tumours

Benign tumours are non-cancerous growths (Table 2.3) characterised by well-differentiated cells that closely resemble their regular counterparts. These tumours grow slowly, remain localised, and do not invade surrounding tissues or metastasize to distant sites (Figure 2.3) (Patel, 2020). While generally not life-threatening, benign tumours can result in complications if they compress adjacent structures or secrete hormones (Patel, 2020).

2.3.2 Malignant tumours

In contrast, malignant tumours are cancerous (Table 2.3) and are composed of poorly differentiated cells that exhibit uncontrolled growth, invasiveness, and the potential to metastasize (Patel, 2020). These tumours can infiltrate neighbouring tissues (Figure 2.3) and proliferate to distant organs through the bloodstream or lymphatic system (Patel, 2020). Malignant cells often display genetic instability, leading to further mutations that promote tumour progression and resistance to therapy.

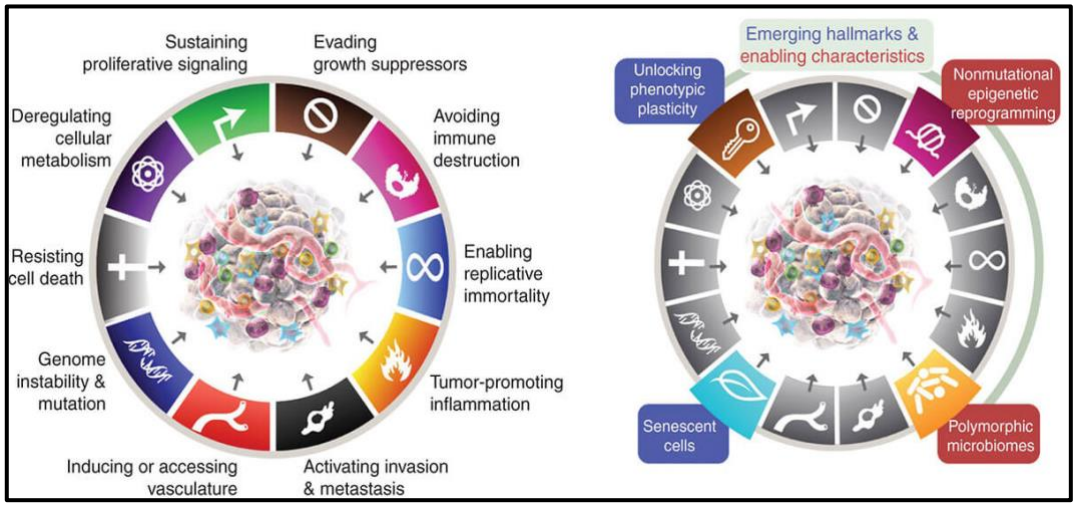

Figure 2.2. Hallmarks of cancer (Hanahan, 2022).

Table 2.3. The primary difference between benign and malignant tumours

Benign	Malignant	Reference
Non-cancerous	Cancerous	(Whelan, 2024a)
Gradual growth	Rapid growth	(Whelan, 2024a)
Non-fatal	Fatal	(Whelan, 2024a)
Unable to penetrate & attach adjacent tissue	Penetrate and attach to adjacent tissue	(Whelan, 2024a)
Typically do not return once removed	Can return once removed	(Whelan, 2024a)
Symmetrical	Asymmetrical	(Whelan, 2024a)
Encapsulated	Unencapsulated	(Whelan, 2024a)

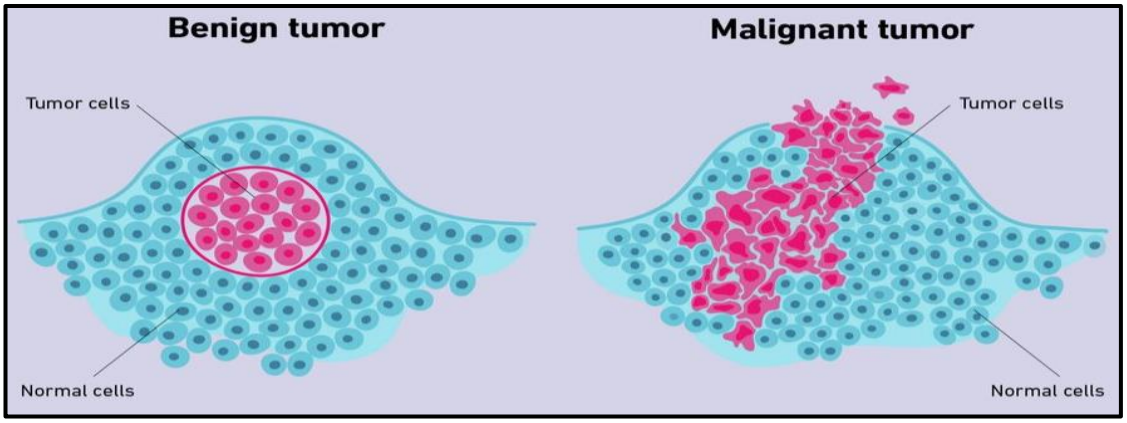

Figure 2.3. Morphological differences of benign and malignant tumours (Whelan, 2024b).

2.3.3. Molecular mechanisms and tumour microenvironment

The progression of benign tumour formation to malignant tumours comprises multiple molecular changes, including the activation of oncogenes, deactivation of tumour suppressor genes, and modifications in signalling pathways that regulate cell growth and apoptosis (Zhang et al., 2024a).

The tumour microenvironment, consisting of stromal cells, blood vessels, and extracellular matrix components, plays a vital role in supporting tumour growth and facilitating metastasis (Binnewies et al., 2018). Interactions between cancer cells and the microenvironment can promote angiogenesis, immune evasion, and resistance to therapy (Binnewies et al., 2018). Thus, understanding the physiological differences between benign and malignant tumours is critical for precise diagnosis, prognosis, and the development of effective treatment strategies.

2.4 Classification of different types of cancer

2.4.1 Carcinomas

Carcinomas are the most common type of cancer, accounting for approximately 80-90% of all cancer cases (Whelan, 2024b). They originate from epithelial cells, which line the internal and external surfaces of the body, including the skin, glands, and organs such as the lungs, liver, breast, and gastrointestinal tract. These cells are constantly renewing, making them particularly susceptible to genetic mutations over time. Carcinomas can further be subdivided based on their epithelial origin (Table 2.4). For instance, adenocarcinomas arise from glandular tissues (e.g., breast, prostate, and colon), while squamous cell carcinomas develop from the flat epithelial cells lining the skin and mucous membranes (Hanahan, 2022). Due to their high incidence, carcinomas are the most extensively studied type of cancer, with established screening protocols and numerous targeted therapies now available.

2.4.2 Sarcomas

Sarcomas are relatively rare and arise from mesenchymal cells, which form the connective tissues of the body, including bones, muscles, fat, cartilage, and blood vessels (Whelan, 2024b). Sarcomas can occur in any part of the body; therefore, they are a heterogeneous group with over 50 subtypes. Two primary categories include osteosarcomas (originating in bone) and soft tissue sarcomas (affecting fat, muscle, and fibrous tissue) (Table 2.4). Unlike carcinomas, which typically present later in life, sarcomas are more common in children and young adults. These tumours are often aggressive, with a high tendency for local invasion and metastasis via the bloodstream rather than lymphatic propagation. Treatment typically includes surgical resection combined with chemotherapy or radiation therapy, but outcomes remain poor for high-grade metastatic sarcomas (Sung et al., 2021).

2.4.3 Blastomas

Blastomas are a group of rare, aggressive cancers that primarily affect children and originate from precursor or immature cells known as "blasts" (Whelan, 2024b), which resemble embryonic tissue. These tumours typically arise in organs during early development. They are named based on the tissue or organ of origin, such as neuroblastoma (nervous tissue), nephroblastoma or Wilms tumour (kidney), hepatoblastoma (liver), and retinoblastoma (eye) (Table 2.4) (Whelan, 2024b). Blastomas stem from embryonic cells; thus, they often exhibit rapid growth and can metastasize quickly if not diagnosed early. Genetic mutations, especially those affecting tumour suppressor genes such as RB1 in the retinoblastoma or WT1 in the nephroblastoma, play an essential role in their development (Joyce et al., 2018). Diagnosis usually involves imaging, histopathology, and molecular analysis, while treatment typically comprises a combination of surgery, chemotherapy, and radiation therapy (Board, 2024).

2.4.4 Leukaemia

Leukaemias are cancers of the blood and bone marrow, characterised by the uncontrolled proliferation of abnormal white blood cells (Bispo et al., 2020). Unlike solid tumours, leukaemia does not form discrete masses but advances throughout the bloodstream and impairs normal haematopoiesis. It is classified into four main types based on the cell line affected (myeloid or lymphoid) and the accelerated progression (acute or chronic) (Table 2.4): acute lymphoblastic leukaemia (ALL), acute myeloid leukaemia (AML), chronic lymphocytic leukaemia (CLL), and chronic myeloid leukaemia (CML). Leukaemias are among the most common cancers in children, particularly ALL, but they can also occur in adults, especially AML and CLL. Symptoms often include fatigue, frequent infections, and unexplained bruising due to bone marrow failure. Treatment involves chemotherapy, targeted agents, and in some cases, bone marrow transplantation (Kocarnik et al., 2022).

2.4.5. Lymphomas

Lymphomas are cancers that originate in the lymphatic system, specifically malignant neoplasms in lymphocytes (Lewis et al., 2020), a type of white blood cell critical to the immune response. These cancers typically present as solid tumours in lymph nodes, spleen, or other lymphatic tissues. Lymphomas are primarily divided into Hodgkin lymphoma (HL) and non-Hodgkin lymphoma (NHL) (Table 2.4) (Lewis et al., 2020). The presence of Reed-Sternberg cells characterises HL and generally has a more favourable prognosis with standard therapies. NHL comprises a diverse group of malignancies that vary greatly in behaviour, prognosis, and response to treatment.

These include aggressive forms such as diffuse large B-cell lymphoma (DLBCL) and more indolent types such as follicular lymphoma. Immunotherapy, including monoclonal antibodies (e.g., rituximab), has revolutionised lymphoma treatment, particularly for B-cell subtypes (Binnewies et al., 2018).

2.4.6 Melanomas

Melanomas are malignant tumours that develop from melanocytes (Table 2.4), the pigment-producing cells primarily located in the skin but also present in the eyes (uveal melanoma) and mucosal surfaces. Although melanoma accounts for a smaller percentage of skin cancers, it is liable for the majority of skin cancer-related fatalities due to its elevated metastatic potential. Risk factors such as excessive exposure to ultraviolet (UV) radiation, genetic predisposition, and the presence of atypical moles. Melanoma often presents as asymmetrical pigmented lesions with irregular borders and colour variations. Early detection is critical, as localised melanoma can be effectively treated with surgical excision. Advanced cases may require immunotherapy or targeted therapy (e.g., BRAF inhibitors) to manage metastatic disease (Hanahan, 2022).

2.4.7 Central nervous system (CNS) cancers

Central nervous system (CNS) cancers impact the brain (Table 2.4) and spinal cord and include a diverse range of tumours, with glioblastoma multiforme (GBM) being one of the most aggressive and fatal. These tumours can originate from glial cells, such as astrocytes (astrocytomas), oligodendrocytes (oligodendrogliomas), or ependymal cells (ependymomas). CNS is particularly challenging due to the sensitive location, limited regenerative capacity and neural tissue, and the protective blood-brain barrier, which restricts drug delivery. Symptoms of CNS cancers depend on the tumour's location and may include headaches, seizures, cognitive impairment, and motor dysfunction. Treatment typically comprises a combination of surgery, radiation therapy, and chemotherapy; however, the prognosis remains poor for high-grade tumours, especially GBM, which has a median survival of less than 15 months (Dienstmann et al., 2017).

Table 2.4. Overview of types of cancer

Classification	Example	Origin site	Incidence 2022 (%)	Reference
Carcinomas	Adenocarcinoma	Lung	12.4	(Whelan, 2024a, Bray et al., 2024)
	Squamous cell	Oesophagus (Mucous membranes - organs)	2.6	
Sarcomas	Osteosarcoma	Bone	-	(Whelan, 2024a, Bray et al., 2024)
	Kaposi sarcoma	Lining of blood & lymph vessels	0.2	
Blastomas	Retinoblastoma	Retina in the back of the eye	-	(Whelan, 2024a, Bray et al., 2024)
	Hepatoblastoma	Liver - right lobe	4.3	
Leukaemia	Lymphoid/ myeloid	Lymphoid cells (white blood cells) or myeloid cells (immature white blood cells)	2.4	(Karunarathna et al., 2024, Bray et al., 2024)
	Acute or chronic	Fast growing (immature blood cells) or slowing growing (mature blood cells)		
Lymphomas	Hodgkin	Lymphatic system - Reed Sternberg cells	0.4	(Lewis et al., 2020, Bray et al., 2024)
	Non-Hodgkin	Lymphatic system - No reed Sternberg cells	2.8	
Melanomas	Melanocytes	Skin	1.7	(Gajaria et al., 2021, Bray et al., 2024)
		Mucous membranes (Stomach)	4.9	
Central Nervous System Cancers	Glioblastoma	Spine/brain	-	(Nabors et al., 2020, Bray et al., 2024)
	Medulloblastoma	Brain (cerebellum)	1.6	

2.5 Overview of traditional cancer treatments

Chemotherapy, radiation, surgery, targeted therapy, and immunotherapy (Table 2.5) are the cornerstone modalities in cancer treatment, each with distinct mechanisms and applications. Chemotherapy employs cytotoxic drugs that non-specifically target rapid division of cells, leading to side effects due to damage to normal proliferating tissues (Zeien et al., 2022). Radiation therapy utilises ionizing radiation to induce DNA damage in cancer cells, often used pre- or post-operatively to reduce tumour burden or prevent recurrence (Rocha et al., 2022). Surgical intervention remains essential for localised tumours, offering the potential for complete tumour excision and diagnostic assessment through biopsy (Alieva et al., 2018). Targeted therapy is a more precise approach, representing specific molecular pathways such as tyrosine kinases or hormone receptors involved in tumour progression, reducing systemic toxicity compared to chemotherapy (Min and Lee, 2022). Immunotherapy, including checkpoint inhibitors and CAR-T cells, has modernised oncology by augmenting the immune system's ability to distinguish and eliminate tumour cells, particularly in melanoma and lung cancer (Garg et al., 2024).

Table 2.5. Summary of traditional cancer therapies

Therapy	Mechanism	Side Effects	Reference
Chemotherapy	DNA damage, mitosis inhibition	Nausea, fatigue	(Zeien et al., 2022)
Radiation	DNA strand breaks	Skin burns, fibrosis, secondary cancers	(Rocha et al., 2022)
Surgery	Tumor excision	Pain, infection, organ dysfunction	(Alieva et al., 2018)
Targeted	Inhibits specific receptors	Rash, hypertension, liver issues	(Min and Lee, 2022)
Immunotherapy	Enhances immune response	Inflammation, autoimmune-like effects	(Garg et al., 2024)

2.5.1 Chemotherapy mechanisms and effects

Chemotherapy is considered to arrest the growth and spread of cancer by targeting rapidly dividing cells (Amjad et al., 2023), thereby preventing tumour invasion and metastasis. However, due to its adverse effects on healthy proliferating cells, significant side effects often occur. Traditional chemotherapeutic agents typically disrupt the synthesis of DNA, RNA, proteins, or their function in malignant cells, leading to cell death either directly or through the activation of apoptosis (Amjad et al., 2023). Chemotherapeutics cannot distinguish cancer cells from other proliferating cells. They also damage healthy tissues (e.g., bone marrow, gut lining, hair follicles), leading to the typical toxic side effects of chemotherapy (Amjad et al., 2023). Moreover, each dose of cytotoxic drugs typically kills a fraction of tumour cells, not all of them (Palmer et al., 2019). As a result, numerous cycles of chemotherapy are required to reduce tumour burden over time (Amjad et al., 2023).

2.5.1.1 Combination chemotherapy

Combination chemotherapy can be utilised to improve tumour control and prevent resistance by the use of multiple drugs with different mechanisms or cell-cycle targets. Combination regimens attack cancer cells "from multiple sides" and kill both actively dividing and more dormant tumour cells, making it difficult for resistant clones to emerge (Amjad et al., 2023). In practice, combination therapy is standard in many protocols; for example, curative combinations for testicular cancer or lymphoma involve 3-5 drugs (e.g., R-CHOP) due to their non-overlapping actions. The use of medications with distinct targets and toxicity profiles allows near maximal dosing for each agent while lowering the chance that any single mutation confers cross-resistance (Amjad et al., 2023). This multi-agent approach typically produces better response rates and more prolonger remissions than single-drug therapy (Amjad et al., 2023).

2.5.1.2 Clinical significance of chemotherapy

Chemotherapy can be administered at different stages of cancer care. Neoadjuvant therapy (preoperative chemotherapy) is used to shrink tumours before surgery, while adjuvant therapy (postoperative chemotherapy) removes microscopic residual disease. Adjuvant chemotherapy is the standard of care for many common cancers, especially breast, lung, colorectal, and ovarian cancer, because it significantly improves long-term survival (Amjad et al., 2023).

Chemotherapy is often combined with radiation therapy, "chemoradiation", either to increase tumour shrinkage before surgery or as a curative treatment itself for certain cancers such as head, neck, lung, and anal cancers (Amjad et al., 2023). Moreover, chemotherapy remains the primary systemic treatment for metastatic disease, with regimens tailored by tumour type and patient factors.

2.5.1.3 Key principles guiding chemotherapy

Oncologists follow several foundational principles when designing chemotherapy regimens. The fractional kill hypothesis suggests that each dose of chemotherapy kills a constant fraction of tumour cells, not a constant number. For example, a drug might kill 99% of cells per dose; the surviving 1% may repopulate. Thus, even very effective drugs require multiple cycles to eradicate the tumour (Palmer et al., 2019). The dose-response linearity theory proposes that the neoplastic tumours are directly proportional to the dose of the agent and its efficacy. This underlies strategies like dose-dense or intensive chemotherapy (Amjad et al., 2023). The third principle, the Goldie-Coldman hypothesis, suggests that tumours develop resistance through random mutations, emphasising the need for combination therapy. Therefore, early and multi-drug treatment minimises the chance that any clone is resistant to all agents (Amjad et al., 2023). There is a vast array of chemotherapeutic agents that can be categorised by their mechanism of action (Table 2.6).

Table 2.6. Classification of chemotherapeutic agents according to their mechanism of action

Chemotherapeutic agent	Example	Drug	Mechanism of action	Side effects	Reference
Alkylating Agents	Nitrogen mustard	Cyclophosphamid, bendamustine	Generate a reactive alkyl group (R-CH_2^+) that binds to nucleophilic sites on proteins and nucleic acids, disrupting essential cellular processes which interferes with DNA replication and transcription, hindering cell division and function	Nausea, vomiting, infertility, neurotoxicity, pulmonary fibrosis	(Amjad et al., 2023)
	Nitrosoureas	Carmustine, lomustine			
	Platinum analogs	Carboplatin, cisplatin			
Antimetabolites	Cytidine analogs	Azacitidine, decitabine	DNA methyltransferase inhibition	Myelosuppression-decreased production of blood cells in bone marrow	(Amjad et al., 2023)
	Folate antagonists	Methotrexate, pemetrexed	Folate reduction		
	Purine analogs	Cladribine, clofarabine	Mimic the structure of guanine and function as deceptive metabolites within the cell		
Antimicrotubular Agents	Topoisomerase II inhibitors	Doxorubicin, daunorubicin	RNA & DNA synthesis inhibition	Myelosuppression, mucositis	(Amjad et al., 2023)
	Topoisomerase I inhibitors	Irinotecan, topotecan	Inhibition release of Topoisomerase I from DNA break site	Dose-limiting diarrhoea, neutropenia	
	Taxanes	Paclitaxel, docetaxel	Block the formation of microtubules during M phase	Hypersensitivity reactions, myelosuppression, peripheral neuropathy	

2.5.2 Radiation

Radiation therapy is a broadly used cancer therapy that engages high-energy radiation to irradicate cancer cells or inhibit their growth (Rocha et al., 2022). The primary mechanism involves damaging the DNA within cancer cells, either directly or indirectly through the generation of free radicals (Rocha et al., 2022). This damage impairs the cell's ability to replicate and ultimately leads to cell death. Radiation can be delivered externally using machines such as linear accelerators or internally through radioactive substances positioned adjacent or within the tumour (brachytherapy) (Maani and Maani, 2022). The treatment is typically localised, allowing for targeted destruction of cancerous tissue while sparing as much surrounding healthy tissue as possible. Advances in technology, such as intensity-modulated radiation therapy (IMRT) and image-guided radiation therapy (IGRT), have significantly enhanced accuracy and reduced side effects (Grégoire et al., 2020). Radiation therapy may be used alone or in conjunction with other treatments including surgery, chemotherapy, after surgery (adjuvant), or as palliative care to relieve symptoms in advanced cancer cases (Rocha et al., 2022). Despite its effectiveness, radiation therapy may lead to side effects depending on the treatment site, including fatigue, skin irritation, and localised tissue damage.

2.5.3 Surgery

Surgery is one of the oldest and most effective methods for treating cancer, especially when the disease is detected at an early stage. It involves the physical removal of the tumour and, in some cases, surrounding tissues or lymph nodes to prevent the spread of cancer. Surgical intervention can serve multiple purposes. It may be curative, diagnostic (via biopsy), preventative (removal of precancerous lesions), or palliative to alleviate symptoms in advanced cancer cases. The success of surgical treatments is contingent on factors such as tumour size, location, stage, and the patient's overall health (Religioni et al., 2021). In many cancers, especially those confined to a single area, surgery remains the primary treatment option. It is often combined with other therapies like chemotherapy or radiation, either before surgery to shrink a tumour or after surgery to remove residual cancer cells. Modern surgical techniques, including minimally invasive procedures such as laparoscopy, have improved recovery times, reduced complications, and minimised damage to healthy tissues.

2.5.4 Targeted therapy

Targeted therapy is a category of treatment that utilises drugs to precisely target cancer cells, offering more effective results with less side effects compared to traditional chemotherapy (Min and Lee, 2022). Conversely chemotherapy, which affects all actively proliferating cells, targeted therapy aims to interfere with specific molecules and pathways in cancer cell growth, proliferation, and survival (Min and Lee, 2022). It has revolutionised cancer treatment by utilising agents like monoclonal antibodies (mAbs) and small kinase inhibitors (SMKIs) that precisely target molecular pathways involved in cancer cell growth and survival.

2.5.4.1 Monoclonal antibodies

Monoclonal antibodies target extracellular ligands, membrane receptors, and membrane-bound proteins, functioning via ligand-binding barriers, ligand-receptor interaction neutralisation, or target molecule internalisation (Zahavi and Weiner, 2020). For example, trastuzumab targets HER2 in breast cancer, while cetuximab and panitumumab target EGFR in colorectal cancer (Zahavi and Weiner, 2020). These mAbs can also enhance host antitumour immunity by creating a bridge between tumour and immune cells, leading to antibody-dependent cellular cytotoxicity (ADCC) (Kersh et al., 2018).

2.5.4.2 Small molecule kinase inhibitors

Small molecule kinase inhibitors primarily suppress protein kinases, vital for cancer cell transformation and survival, by blocking their enzymatic activity (Min and Lee, 2022). These inhibitors bind to the ATP-binding pocket of kinases, with different types (types I, II, III, IV, V, VI) employing varied mechanisms to suppress kinase activity (Lu et al., 2020). An example includes imatinib, which targets BCR-ABL in chronic myelogenous leukaemia, and gefitinib, an EGFR inhibitor used in non-small cell lung cancer (NSCLC) (Min and Lee, 2022).

2.5.4.3 Resistance mechanisms

A noteworthy challenge in targeted therapy is the emergence of drug resistance (Figure 2.4). Primary resistance occurs due to intrinsic cellular, genetic, or epigenetic modifications, while acquired resistance develops over time due to various molecular and cellular changes (Min and Lee, 2022). Mechanisms include mutations in kinase domains, activation of compensatory signalling pathways, and interactions with the tumour microenvironment (Min and Lee, 2022). Overcoming resistance requires strategies such as developing inhibitors that target resistance mutations, combining therapies, and indirectly manipulating cellular targets (Meng et al., 2021).

While targeted therapies have led to remarkable survival benefits in some cancers, they are effective for patients with targetable driver mutations or abnormalities. Side effects can still occur due to cross-reactivity with normal cells, and drug resistance remains a significant challenge (Min and Lee, 2022). Despite these limitations, targeted therapy represents a fundamental shift toward precision medicine in cancer treatment.

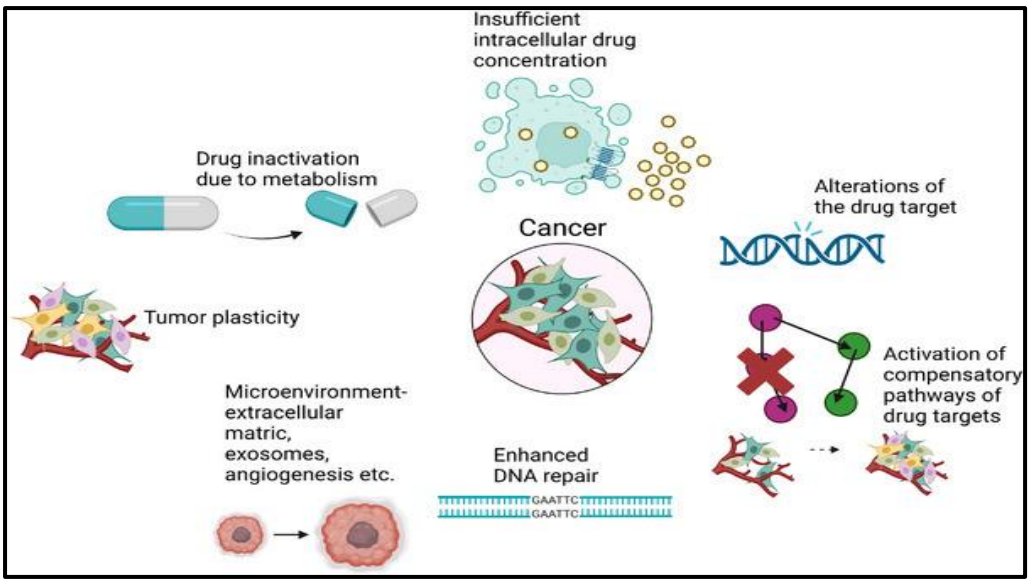

Figure 2.4. Key mechanisms of cancer resistance to therapy (Lei et al., 2023).

2.5.5 Immunotherapy

Immunotherapy is a form of cancer therapy that employs the body's immune system to recognise and kill cancer cells (Garg et al., 2024). Unlike conventional therapies that directly target tumours, immunotherapy improves or reestablishes the immune system's natural ability to fight cancer. This approach can offer long-lasting protection and is particularly effective in certain cancers, such as melanoma, lung, and kidney cancers. There are several types of immunotherapy, including immune checkpoint inhibitors (ICIs), which block proteins like PD-1, PD-L1, or CTLA-4 that cancer cells use to weaken the immune response, allowing cancer cells to be destroyed (Garg et al., 2024). Another form is CAR-T cell, where a patient's T cells are genetically altered to target cancer cells better (Garg et al., 2024). Other methods include cancer vaccines and immune-stimulating agents, which boost the overall activity of the immune system (Garg et al., 2024). While it offers the potential for durable responses, not all patients respond similarly, and side effects such as inflammation and autoimmune-like reactions can arise.

2.6 Apoptosis

Apoptosis is a central process that facilitates the systematic elimination of unnecessary, damaged, or malfunctioning cells, playing a vital role in maintaining cellular balance and removing potentially pre-cancerous cells (Leary et al., 2024). This form of programmed cell death is particularly significant in cancer treatment, as many therapeutic strategies are designed to trigger the death of tumour cells (Leary et al., 2024). Apoptosis transpires via two primary pathways, specifically the intrinsic and extrinsic routes (Figure 2.5), both of which ultimately lead to the activation of caspases, which are the key enzymes responsible for implementing cell death (Van Opdenbosch and Lamkanfi, 2019).

2.6.1 Intrinsic pathway

The intrinsic apoptotic pathway is prompted by internal cellular stress. It is primarily focused on the mitochondria, which store crucial proteins such as cytochrome c and the second mitochondria-derived activator of caspases (SMAC) (Kist and Vucic, 2021). The release of these proteins occurs through mitochondrial outer membrane permeabilization (MOMP), a process regulated by a balance between pro-apoptotic and anti-apoptotic members of the B-cell lymphoma 2 (BCL-2) protein family (Leary et al., 2024). This disruption favours MOMP, leading to the release of cytochrome c and SMAC into the cytosol, ultimately resulting in the activation of caspases (Kist and Vucic, 2021) (Figure 2.5).

2.6.2 Extrinsic pathway

Conversely, the extrinsic apoptotic pathway is initiated at the surface of the cell by death receptors belonging to the tumour necrosis factor (TNF) receptor superfamily. A caspase cascade is triggered that results in programmed cell death due to the activation of these receptors by external death ligands. A key point of crosstalk between the two pathways involves caspase-8 and BH3-interacting domain death agonist (BID). Caspase-8 cleaves BID, converting it into an active form that promotes mitochondrial permeabilization by activating BAX/BAK, thereby linking the extrinsic signals to the intrinsic mitochondrial pathway (Leary et al., 2024) (Figure 2.5).

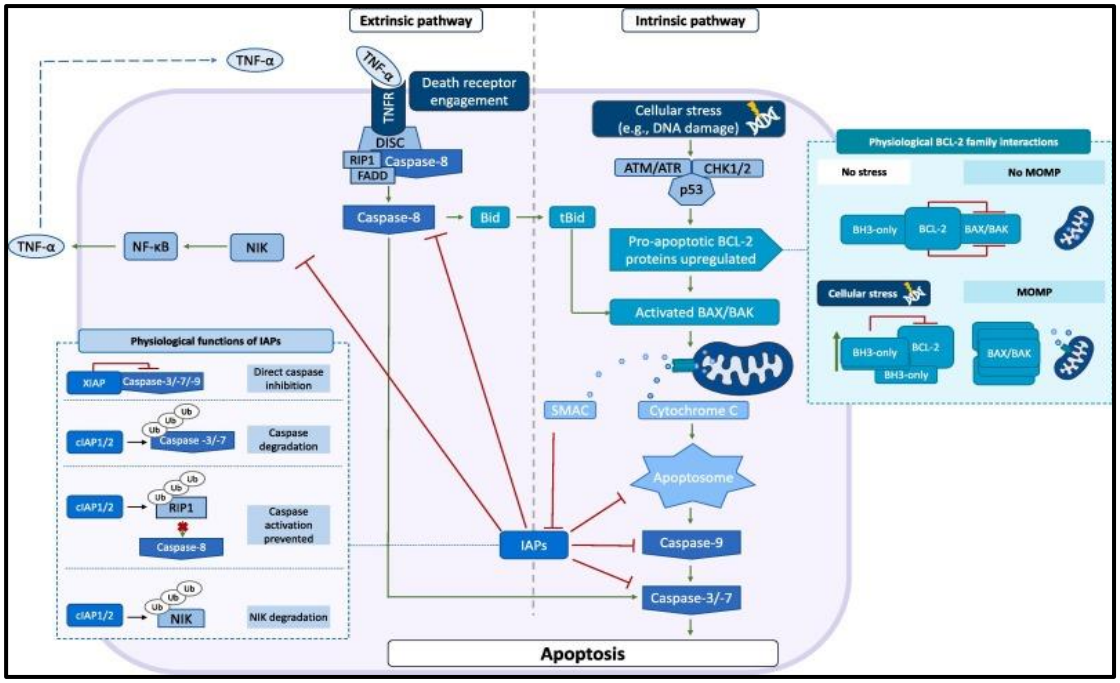

Figure 2.5. Summary of the main components involved in intrinsic and extrinsic apoptotic signalling pathways (Leary et al., 2024).

2.6.3 Apoptosis in cancer

A hallmark of cancer includes the evasion of apoptosis; thus, tumour cells frequently alter apoptotic components. For instance, overexpressing Bcl-2/Bcl-xL, mutating p53, or downregulating caspases. This dysregulation not only promotes tumour survival but also contributes to drug resistance (Mustafa et al., 2024). The modulation of apoptotic pathways holds immense potential in cancer treatment, where promoting apoptosis in malignant cells could lead to tumour reversion (Mustafa et al., 2024).

2.7 Caspase

Caspases are a family of cysteine proteases that play a crucial role as regulators in programmed cell death, with distinct subgroups executing particular roles. There are initiator caspases such as caspase 8 and 9, which begin apoptotic signalling, while the executioner caspases, including caspases 3 and 7, dismantle the cell's structural and regulatory components (Sahoo et al., 2023). Moreover, there are indications that caspases play roles in non-apoptotic processes, such as proliferation, differentiation, and migration, which are often observed in cancer, where dysregulated caspase activity can aggravate malignancy or chemotherapeutic resistance (Ghorbani et al., 2024).

2.7.1 Caspase-3 and caspase-7

Caspase-3 and caspase-7 are the principal executioner caspases in apoptosis (Mustafa et al., 2024). These cysteine proteases exist as inactive zymogens and are cleaved into active subunits by initiator caspases (8 and 9) in response to death signalling (Mustafa et al., 2024). Structurally similar, caspase-3 and caspase-7 recognise the same tetrapeptide motifs in substrates and carry out the execution phase of apoptosis (Julien and Wells, 2017). Once activated, they cleave over one hundred cellular proteins, including PARP, ROCK1, and lamin A, to induce DNA fragmentation, membrane blebbing, and the formation of apoptotic bodies (Mustafa et al., 2024). Furthermore, caspase-3 and caspase-7 play decisive roles in triggering irreversible cell death, as their function is stringently controlled by internal cellular signals and inhibitor of apoptosis proteins (IAPs) (Sahoo et al., 2023) to prevent inadvertent cell elimination. Mechanistically, caspase-3/7 is activated via both intrinsic and extrinsic apoptotic pathways (Figure 2.5). Intrinsic apoptosis occurs via the activation of cytochrome c which activates caspase-9, further triggering the cleavage of procaspase-3/7 (Mohammad et al., 2021). The activation of caspase-8 indirectly activates caspase-3/7 through mitochondrial amplification, leading to extrinsic apoptosis. Moreover, numerous studies emphasize the central role caspase-3 plays in apoptosis as it cleaves DNA fragmentation factor (DFF-45), inhibiting caspase-activated DNase (ICAD), thus causing DNA laddering (Sahoo et al., 2023). Caspase-7 overlaps with caspase-3; however, it also cleaves distinct substrates such as cytokeratins during apoptosis (Lamkanfi and Kanneganti, 2010).

2.7.2 Caspase-3/7 activation and plant proteins

Plant proteins and peptides are known to activate caspase-3/7 in cancer cells. A prime example is Lunasin from soybean, which exhibited activation of caspases -8 and -9, which led to caspase-3 activation in leukaemia and breast cancer cells (Kaufman-Szymczyk et al., 2023). According to Deesrisak et al. (2021), a peptide (IM-7) from sesame seeds upregulated CASP3 gene expression and induced apoptosis in leukaemia cells. Bioactive peptides from pulses such as chickpea and mung bean have been reported to promote apoptosis. A previous study by Ghadiri et al. (2024) noted that pea and legume-derived peptides induced cell cycle arrest and apoptosis in tumours. These findings suggest plant-derived proteins and peptides may exert anticancer effects by engaging executioner caspases. Overall, triggering caspase-3/7 is a common mode by which plant proteins inhibit antitumour effects (Mazalovska and Kouokam, 2020).

2.8 Caspase 8

Caspase-8 is a key initiator of the extrinsic apoptotic pathway. It consists of long pro-domains (DED) motifs that are activated by the death-inducing signalling complex (DISC) (Zhang et al., 2024b). Upon ligand binding, death receptors (DR), such as TNF, and adaptor proteins, including FADD, bring procaspase-8 molecules into proximity with DISC, thereby causing autoproteolytic activation (Tummers and Green, 2017). Active caspase-8 thereafter directly cleaves and activates caspase-3/7, driving apoptosis (Mustafa et al., 2024). Additionally, caspase-8 cleaves the BH3-only protein BID to truncated BID (tBID), linking extrinsic signals to mitochondrial permeabilization (Huang et al., 2016).

2.8.1 Caspase 8 and plant proteins

Plant-derived proteins have previously shown activation of caspase-8. According to Mazalovska and Kouokam (2020), lectins, a carbohydrate-binding protein, can trigger extrinsic apoptosis in tumour cells. Moreover, *polygonatum cyrtonema* lectin (PCL) induced apoptosis in fibrosarcoma cells via caspase-8/-3 cascade (Zhang et al., 2010). The soybean peptide lunsain also showed activation of caspase-8 in apoptosis initiation against breast cancer and leukaemia (Kaufman-Szymczyk et al., 2023). Furthermore, calotroposid A isolated from C*alotropis gigantea* induced apoptosis in colon cancer due to the expression of caspase-8 (Mutiah et al., 2018).

2.9 Caspase 9

Caspase-9 is a principal initiator of intrinsic apoptosis via the mitochondrial pathway. In healthy cells, procaspase-9 is present in the cytosol in an inactive form. The release of mitochondria in conjunction with cytochrome c and dATP induces its binding with Apaf-1, thus promoting the formation of the apoptosome, a heptameric wheel that recruits multiple procaspase-9 molecules (Mustafa et al., 2024). Caspase-9 zymogens within the apoptosome cleave each other into active enzymes. The activated caspase-9 further prompts executioner procaspases 3 and 7 to begin the death phase (Mustafa et al., 2024). As a result, caspase-9 serves as a key player converting mitochondrial stress signals into apoptosis.

2.9.1 Caspase 9 in plant proteins

The mitochondrial outer membrane permeabilization (MOMP) induced in Hep-G2 and HeLa cells by isoflavone prunetin led to the release of cytochrome c, triggering the activation of caspase-9, thus highlighting the intrinsic apoptotic pathway (Jeong et al., 2024).

In a study by Dia and de Mejia (2011), lunasin from soybeans increases Bax and clusterin, while reducing Bcl-2, leading to cytochrome c release and caspase-9 activation, indicating caspase-9 plays a role in apoptosis of colon cancer cells. Moreover, the upregulation of caspase-9 was observed in HCT116 cells (colon cancer) following treatment with *Pulicaria crispa*, indicative of apoptosis (Nabil et al., 2025). In HeLa cells, Joseph et al. (2024) determined that mitochondrial apoptosis was triggered due to the significant induction of caspase-9 by a triterpenoid from *Aphanamixis polystachya*.

2.10 Tumour suppressor protein: p53

The p53 protein was first identified in 1979 during studies of SV40 viral oncogenesis. A ~53 kDa cellular protein bound to the SV40 T antigen was discovered by Linzer and Levine (1979), resulting in the name p53 (Song et al., 2024). Early studies using transforming viruses misled researchers to classify p53 as a proto-oncogene; however, by 1989, it became clear that p53 suppressed transformation. Subsequent studies redefined p53 as a tumour suppressor, and its gene TP53 found on chromosome 17p13.1 was found to encode 393-amino-acid proteins (Wang et al., 2023). The p53 protein was thereafter recognised as "guardian of the genome" that limits the proliferation of cells under stress, halting the cell cycle or triggering apoptosis (Capaccia et al., 2022).

2.10.1 Structure and function of p53

Human p53 is a multidomain transcription factor of 393 amino acid residues (Wang et al., 2023). It contains an N-terminal transactivation domain (TAD) (residues 1-61), proline-rich domains (64-92), a central DNA-binding domain (DBD) (96-292), a tetramerization domain (324-356), and a C-terminal regulatory region (364-393) (Wang et al., 2023). Physiologically, p53 remains inactive and unstable through interaction with the E3 ubiquitin ligase MDM2, which binds the TAD and marks p53 degradation (Wang et al., 2023). The induction of stresses such as DNA damage or hypoxia (Figure 2.6) results in p53 being rapidly modified post-translationally to block the binding of MDM2 (Fischer and Sammons, 2024). For instance, kinases, including ATM (Figure 2.6) and ATR, phosphorylate the N-terminal region of p53, which weakens its association with MDM2, thus increasing the p53 protein levels in cells (Wang et al., 2023). Moreover, acetylation of the C-terminal lysines by co-activators (CBP/p300) further stabilises and enhances the DNA-binding affinity of p53 (Figure 2.6) (Wang et al., 2023). As a result, p53 levels increase, allowing it to serve as a sequence-specific transcription factor. According to Song et al. (2024), as a transcription factor, p53 binds specific DNA response elements and activates hundreds of target genes involved in cell cycle arrest, DNA repair, apoptosis, senescence, and other antitumour functions (Song et al., 2024).

Targets include *CDKN1A/p21* (cell cycle inhibitor) and pro-apoptotic genes such as *BAX*, *PUMA*, and *NOXA* (Figure 2.6) (Zilu et al., 2022). Through the effectors, wild type p53 enforces cell cycle checkpoints and eliminates or arrests cells with irreparable damage. In addition to its transcriptional role, p53 has non-traditional roles in processes such as DNA repair and apoptosis, through direct interactions with DNA repair proteins, which are essential in maintaining genomic stability (Guo et al., 2024).

2.10.2 Wild-type and mutant roles of p53

In normal cells, p53 acts as a tumour suppressor, halting the cell cycle to allow DNA repair or inducing apoptosis if the damage is severe (Song et al., 2024). Mutations in the TP53 gene underscore this barrier to tumorigenesis and are the most common genetic alterations in human cancers (Song et al., 2024). According to Guo et al. (2024), over 50% of tumours carry a TP53 mutation, these mutant forms typically lose their ability to bind specifically to the DNA sequences of key target genes such as *CDKN1A/p21* and BAX, impairing the ability to trigger cell cycle arrest or initiate apoptosis efficiently (Guo et al., 2024). Moreover, mutant p53 often interacts abnormally with other transcription factors, such as p63 or chromatin regulators, reprogramming gene expression to promote proliferation, metastasis, and therapy resistance (Song et al., 2024).In contrast, wild-type p53 tumours typically produce better outcomes as they trigger chemotherapy or radiotherapy-induced cell death, whereas mutant p53 cancers tend to be more aggressive and resistant (Song et al., 2024). For instance, cancers with functional p53 respond better to DNA-damaging treatments, since p53 can drive apoptosis in damaged cells (Song et al., 2024). Furthermore, this has prompted the reactivation of p53 in tumours; however, mutant p53's altered structure often lacks DNA-binding or tetramerization, making drug targeting difficult (Wang et al., 2023).

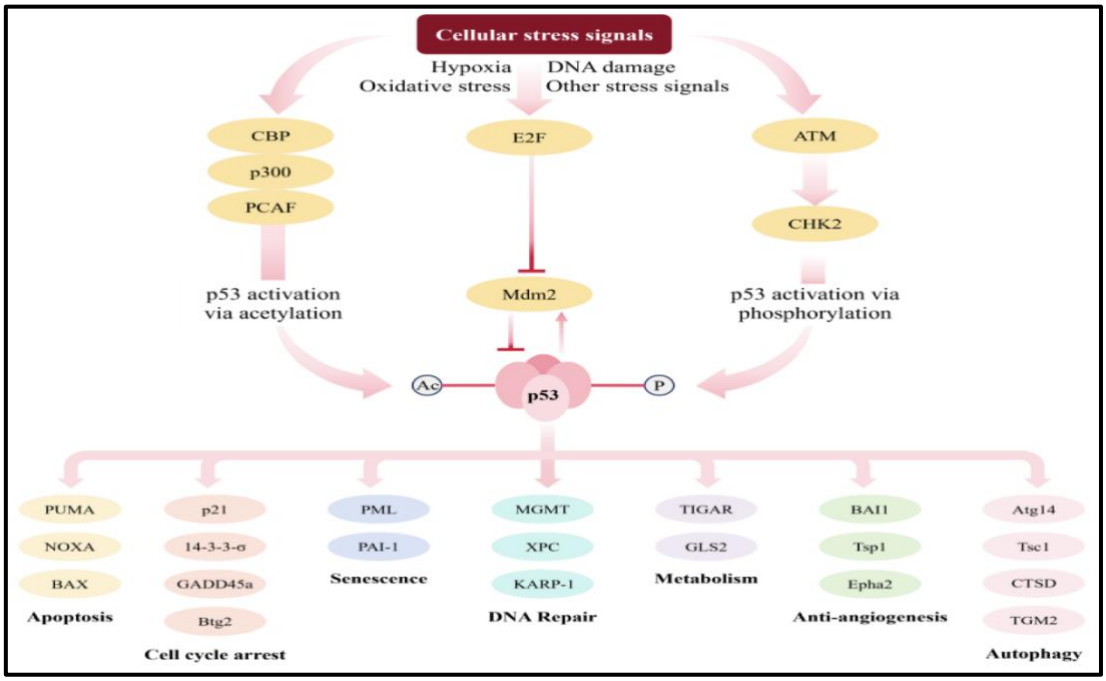

Figure 2.6. Cellular stress signals and function of p53 (Peng et al., 2024).

2.11 Reactive oxygen species (ROS)

Reactive oxygen species (ROS) are highly reactive molecules that contain oxygen and include both free radicals, such as superoxide anion (O_2^-), as well as non-radical species, including hydrogen peroxide (H_2O_2) (Biswa Mohan et al., 2021). These compounds are primarily generated as natural by-products of cellular metabolism, especially during mitochondrial oxidative phosphorylation (Vikas et al., 2020). While ROS are essential for numerous physiological functions such as cell signalling, immune defence, and maintaining homeostasis, an imbalance or excessive accumulation can lead to harmful cellular effects, particularly in cancer. Reactive oxygen species can be classified based on their site of generation as endogenous or exogenous, each with distinct sources, functions, and implications for cellular health and disease. Endogenous ROS are generated within the cell and arise from mitochondrial electron transport activity, oxidases, peroxisomal metabolism, endoplasmic reticulum (ER) stress, and the activity of immune cells such as phagocytes (Zhang and Wong, 2021). They are primarily produced as a result of metabolic activities and intracellular signalling. Exogenous ROS are produced outside the cell, often released into the extracellular environment by immune cells or through oxidative enzymes on the plasma membrane. Exogenous sources, including environmental pollutants, cigarette smoke, alcohol consumption, and ultraviolet (UV) radiation, can also induce ROS production or introduce reactive species into cells (Hajime et al., 2021).

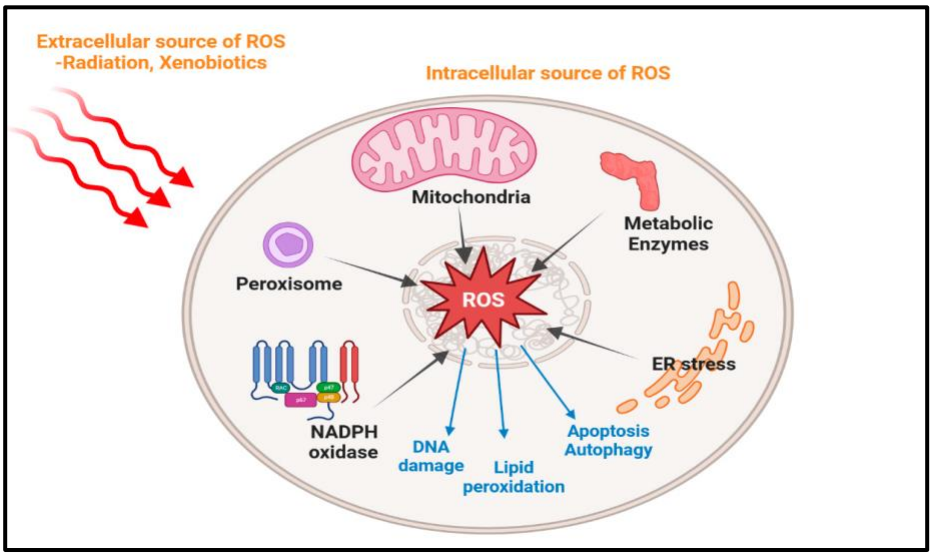

Figure 2.7. Sources of endogenous and exogenous reactive oxygen species (Hong et al., 2024).

2.11.1 The role of ROS in non-cancerous cells

In healthy cells, ROS levels are regulated to maintain a balance known as redox homeostasis between their production and neutralisation (Shah and Rogoff, 2021). This balance is primarily sustained through antioxidant molecules such as glutathione and NADPH (Shah and Rogoff, 2021). At normal levels, ROS serve important signalling roles, acting as secondary messengers in processes such as cell growth, differentiation, immune responses, and tissue repair (Shah and Rogoff, 2021). However, when ROS levels become increased, they can cause oxidative stress, damaging cellular components such as DNA, and proteins which can ultimately lead to programmed cell death (apoptosis) or cellular aging (senescence) (Shah and Rogoff, 2021). To prevent this, cells employ a range of antioxidant defences, including enzymes like superoxide dismutase, as well as systems involving peroxiredoxin and thioredoxin (Shah and Rogoff, 2021). The expression of antioxidant proteins such as NQO1 is also regulated in response to oxidative stress, further supporting redox regulation (Shah and Rogoff, 2021).

2.11.2 The role of ROS in cancerous cells

Reactive oxygen species play a dual and complex role in cancer, influencing both cell survival and death depending on their concentration (Ju et al., 2024). At low to moderate concentrations, ROS promote cancer cell proliferation, migration, and drug resistance (Ju et al., 2024). They act as signalling molecules that activate pathways such as NRF2, NF-κB, and PI3K/Akt, which enhance cell growth and survival (Ju et al., 2024). Conversely, when ROS levels are high, they induce oxidative stress, leading to damage to essential biomolecules such as proteins, nucleic acids, lipids, and organelles, ultimately resulting in cell death (Ju et al., 2024).

Several cell death pathways are allied with elevated levels of ROS, including apoptosis, autophagy, ferroptosis, and endoplasmic reticulum (ER) stress-induced apoptosis (Ju et al., 2024, Li et al., 2022). In apoptosis, ROS can activate p53 and JNK, stimulating pro-apoptotic Bcl-2 proteins, leading to cytochrome c release and caspase activation (Ju et al., 2024). Ferroptosis, another cell death mechanism, relies on the oxidation of membrane phospholipids containing polyunsaturated fatty acids (PUFAs) and is enhanced by high levels of ROS (Endale et al., 2023). Autophagy, while generally a survival mechanism, can also lead to cell death under excessive ROS conditions by targeting specific cellular components for degradation (Singh et al., 2024). ER stress, induced by ROS-mediated accumulation of misfolded proteins, can activate the unfolded protein response (UPR) and, if unresolved, trigger apoptosis (Ju et al., 2024).

Cancer cells adapt to manage ROS levels by upregulating antioxidant enzymes such as SOD, CAT, PRDX, and GPX and activating signalling pathways like NRF2 to enhance cellular resilience against oxidative stress (Ju et al., 2024). Thus, understanding the circumstances in which ROS promote or inhibit cancer development is crucial for emerging therapies that decrease ROS to avert cancer development or elate ROS to induce cancer cell death (Ju et al., 2024).

2.11.3 Mechanisms of ROS generation in cancer cells

2.11.3.1 Mitochondrial dysfunction

Mitochondria, the primary source of energy for cancer cells, produce ROS during oxidative phosphorylation (Romesberg and Van Houten, 2022). Elevated ROS levels can bring about mitochondrial DNA damage and dysfunction, further increasing ROS production (Romesberg and Van Houten, 2022).

2.11.3.2 Tumour microenvironment

The tumour microenvironment consists of both cancerous and various normal cell types (Zhao et al., 2023). Among these, cancer-associated fibroblasts (a type of stromal cell) release hydrogen peroxide (H_2O_2), contributing to extracellular oxidative stress that facilitates tumour progression and invasion (Chan et al., 2018).

2.11.3.3 Environmental and metabolic factors

Exposure to carcinogens, chronic inflammation, radiation, and high metabolic demands further exacerbate ROS levels in cancer cells (Shah and Rogoff, 2021).

2.11.3.4 Protein-protein interactions

ROS can induce or disrupt disulphide bonds in proteins, thereby modulating protein-protein interactions involved in cell adhesion, transcription, regulation, and apoptosis (Iliadis and Papanikolaou, 2024). For instance, a previous study elucidated that ROS can impair E-cadherin/β-catenin binding in colorectal cancer, promoting epithelial-mesenchymal transition (EMT) and metastasis (Trejo-Solis et al., 2021).

2.11.4 Pathways of ROS involved in cancer

Reactive oxygen species are embedded in the molecular network that regulates cancer development, progression, and resistance to therapy. These reactive molecules influence several signalling pathways that regulate key cellular processes including proliferation, metabolism, and metastasis. In cancer, ROS act as both modulators and messengers, affecting various molecular cascades either by promoting or suppressing tumour growth depending on their concentration.

2.11.4.1 PI3K/AKT pathway

The phosphoinositide 3-kinase (PI3K)/AKT pathway (Figure 2.8) is crucial for promoting cell survival, growth, and metabolic activity (Trinh et al., 2024). This pathway can be activated by ROS through inhibition of the tumour suppressor phosphatase and tensin homolog (PTEN) (Trinh et al., 2024), which negatively regulates PI3K. Oxidation of cysteine residues in PTEN leads to its inactivation, thereby enhancing PI3K signalling and promoting uncontrolled cell proliferation (Trinh et al., 2024). Additionally, ROS can modulate AKT phosphorylation, further enhancing cell survival signals in tumour cells (Trinh et al., 2024).

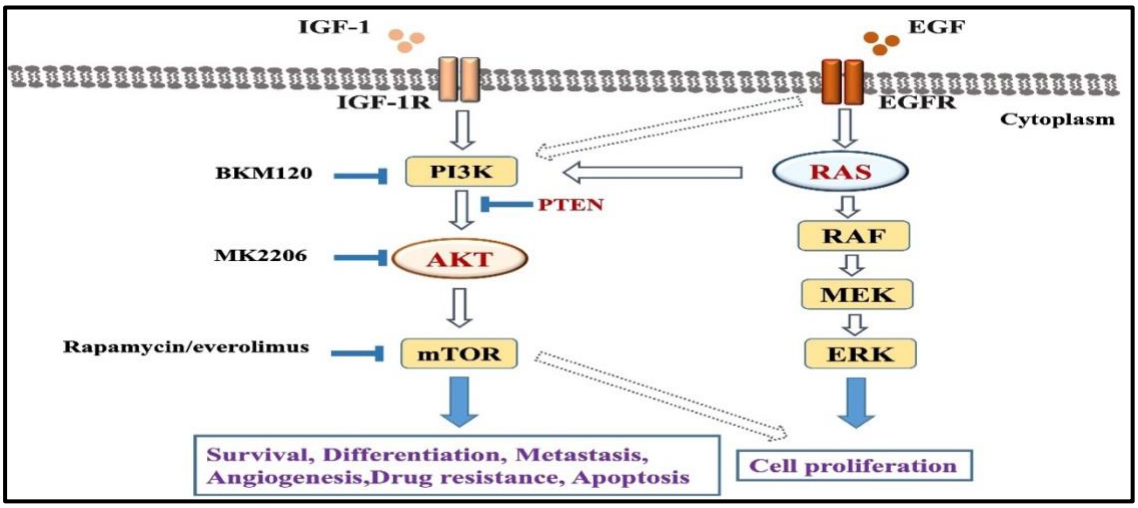

Figure 2.8. Overview of the PI3K/AKT pathway (Zhong et al., 2023).

2.11.4.2 NF-κB pathway

Nuclear factor-kappa B (NF-κB) is a transcription factor that regulates immune responses, inflammation, and cell survival (Hong et al., 2024). The NF-κB pathway (Figure 2.9) is activated by ROS, thus stimulating the degradation of its inhibitor, IκB, through the activation of IκB kinase (IKK) (Hong et al., 2024). Once released, NF-κB translocates to the nucleus and promotes the expression of anti-apoptotic and pro-inflammatory genes (Hong et al., 2024). This contributes to tumour cell survival, immune evasion, and resistance to chemotherapy.

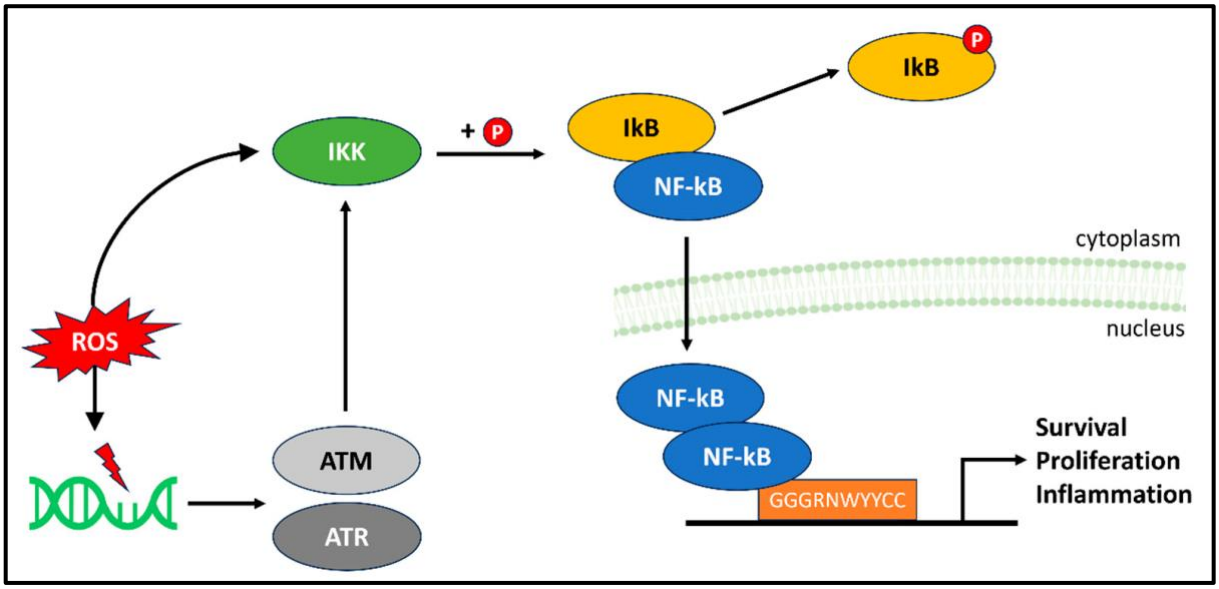

Figure 2.9. Overview of the NF-κB pathway (Hong et al., 2024).

2.11.4.3 p53 pathway

The tumour suppressor p53 is responsible for DNA repair, cell cycle arrest, and apoptosis in response to cellular stress. Reactive oxygen species can both activate and inhibit p53 (Figure 2.10). Low to moderate ROS levels can trigger p53 activation through phosphorylation by stress kinases, leading to apoptosis or senescence. However, excessive ROS may oxidize cysteine residues within the DNA-binding domain of p53, impairing its transcriptional activity. Mutant or dysfunctional p53 in numerous cancers contributes to the inability of cells to undergo ROS-induced apoptosis.

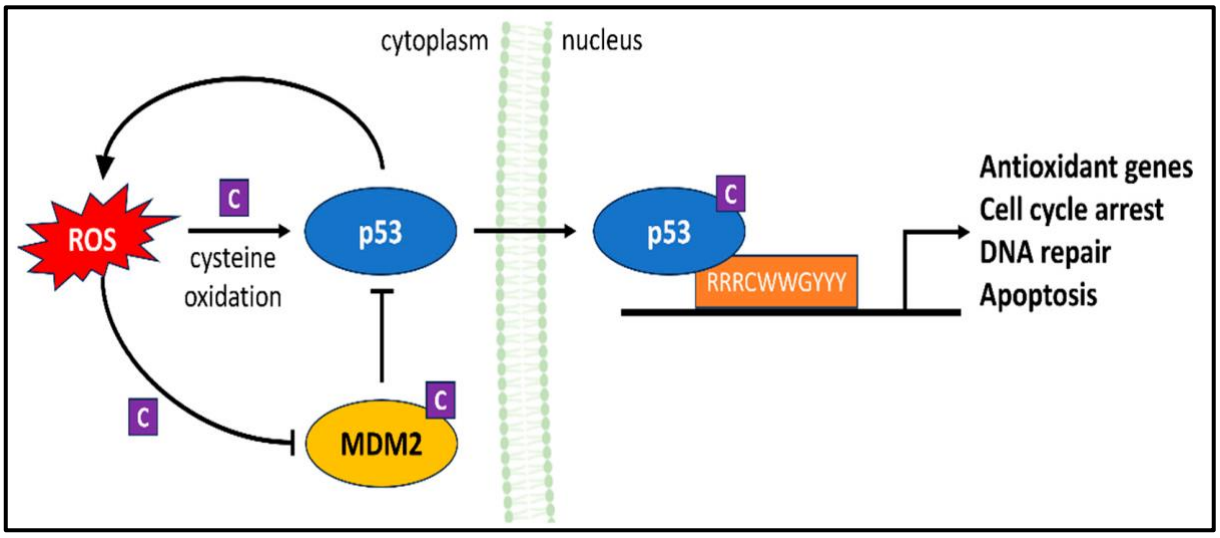

Figure 2.10. Overview of the p53 ROS-mediated pathway (Hong et al., 2024).

2.11.4.4 Bcl-2 and mitochondrial apoptosis pathway

The mitochondrial or intrinsic apoptotic pathway is influenced by ROS as it alters the balance between pro-apoptotic and anti-apoptotic members of the Bcl-2 family. For instance, high ROS can promote conformational changes and oligomerization of Bax and Bak, leading to mitochondrial membrane permeabilization, cytochrome c release, and caspase activation (Figure 2.11) (Qian et al., 2022). Conversely, ROS can modify and impair anti-apoptotic proteins such as Bcl-2 and Mcl-1, thereby shifting the cellular balance toward apoptosis (Qian et al., 2022).

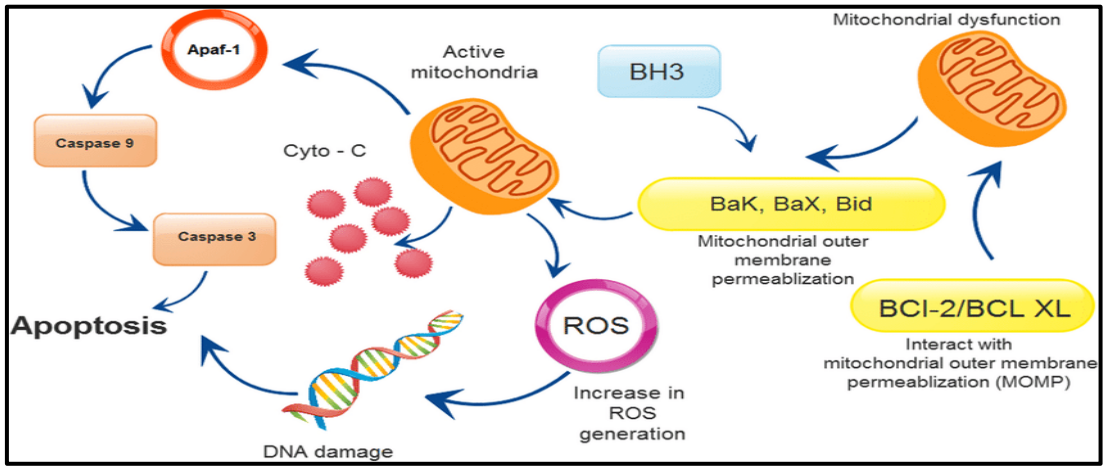

Figure 2.11. Influence of ROS on Bcl-2 and mitochondrial apoptotic pathways (Rofeal and Abdelmalek, 2021).

2.12 Amino acids in cancer therapy

Amino acids play a crucial role in cancer development and progression by fueling biosynthetic processes, maintaining redox balance, and supporting rapid proliferation (Xiang and Hong-Sheng, 2023). Tumour cells often exhibit altered amino acid metabolism, particularly an increased uptake of glutamine, serine, and methionine, to meet their energetic and anabolic requirements (Lieu et al., 2020). These metabolic adaptations sustain tumour growth in addition to influencing epigenetic regulation and resistance to therapy. For instance, glutamine acts as a carbon and nitrogen source for nucleotide amino acid synthesis, while methionine metabolism affects methylation patterns involved in oncogene expression (Xiang and Hong-Sheng, 2023). Thus, targeting amino acid components is emerging as a potential therapeutic approach in numerous cancers.

2.12.1 Targeting amino acid metabolism

Cancer cells often display an increased dependency on specific amino acids for their rapid proliferation and survival (Xiang and Hong-Sheng, 2023), which can be exploited therapeutically. One approach involves inhibiting key enzymes involved in amino acid metabolism. For instance, inhibitors of glutaminase (GLS), particularly CB-839, have indicated potential in treating multiple cancers, such as ovarian (Shen et al., 2020), often as combination therapy (Xiang and Hong-Sheng, 2023). Moreover, targeting the serine synthesis pathway (SSP) with inhibitors such as NCT503 has demonstrated synergistic effects on glioblastoma in chemotherapy (Jin et al., 2022). The inhibition of pyrroline-5-carboxylate reductase 1 (PYCR1) has also shown efficacy in multiple myeloma in combination with bortezomib (Jin et al., 2022). In addition, focusing on amino acid deprivation, where targeting specific amino acids essential for cancer cell growth can impede their proliferation (Xiang and Hong-Sheng, 2023). In various leukaemia treatments, L-asparaginase (ASNase) is utlised to break down the asparagine produced by the cancer cells; the depletion of this amino acid could inhibit their growth as the cells cannot synthesize it adequately (Chiu et al., 2020). Furthermore, dietary modifications, such as methionine restriction, have also been explored to induce energy loss in cancer cells and enhance the efficacy of other treatments, including immunotherapy (Morehead et al., 2023). Moreover, understanding the link between amino acid metabolism, redox balance, and epigenetic regulation provides additional therapeutic avenues (Xiang and Hong-Sheng, 2023). The disruption of these interconnected pathways could result in impairment of cancer cell survival and promote cell death (Xiang and Hong-Sheng, 2023). The reprogramming of amino acid metabolism in cancer cells presents a significant opportunity for developing novel and targeted therapeutic interventions (Faubert et al., 2020).

2.13 Plant protein isolates

Plant proteins and their hydrolysates have emerged as promising anticancer agents due to their bioactive properties and their ability to modulate key cellular pathways, induce apoptosis, and inhibit tumour proliferation. In contrast, conventional chemotherapeutics, often exhibit systemic toxicity (Avilés-Gaxiola et al., 2020), while plant-derived proteins offer high biocompatibility, low toxicity, and selective targeting of cancer cells, rendering them attractive candidates for nutraceutical and pharmaceutical applications (Bhutia et al., 2019). Several studies have demonstrated the efficacy of plant protein isolates against different cancer cell lines emphasizing its therapeutic potential in targeting multiple cancer types through mechanisms such as caspase activation and ROS generation (Table 2.7). Collectively, these properties position plant proteins as multifunctional agents capable of complementing or replacing conventional therapies, paving the way for innovative, plant-based anticancer approaches.

Table 2.7. Studies on the role of plant protein isolates and hydrolysates in cancer therapy

Plant protein	Type of cancer	Mechanism of action	Reference
Quinoa protein hydrolysate (trypsin)	Colon (Caco-2 cells)	Induced apoptosis via caspase-3 pathway cell cycle arrest	(Fan et al., 2022)
Rice bean protein hydrolysate (pepsin and pancreatin)	Cervical (SiHa) & ovarian (SKOV-3)	Triggered mitochondrial apoptosis, ↑ ROS	(González-Cruz et al., 2025)
Soy protein hydrolysates (bromelain & thermolysin)	Oral squamous carcinoma (HSC-3)	Cell cycle arrest, caspase-3 activation, & mitochondrial depolarization	(Hsieh et al., 2022)
Amaranth cruentus protein hydrolysates (alcalase, trypsin, & pepsin)	Breast (MCF-7)	Apoptotic induction	(Ramkisson et al., 2020)
Cowpea (*Vigna unguiculata*) protein isolate	Lung carcinoma (A549)	Apoptotic induction	(Thumbrain et al., 2020)
Legume protein hydrolysates (proteases)	Colorectal (T84, HCT15, & SW480)	Antitumour activity, ↓ cell viability and inhibition of tumour migration	(Fuel et al., 2022)
Walnut protein hydrolysate (proteases)	Cervical (HeLa), liver carcinoma (HepG2), & breast (MCF-7)	Triggered apoptosis and autophagy pathways	(Ma et al., 2015)
Mung bean protein hydrolysate (papain)	Liver (HepG2)	Antiproliferative activity & apoptotic induction	(Ghadiri et al., 2024)
Sesame seed protein hydrolysate (pepsin)	Leukaemia	Activated apoptosis and autophagy by ↑ caspase-3, ↓ Bcl-2	(Deesrisak et al., 2021)
Rapeseed protein isolate	Breast	↑ p53, ↑ Bax, & ↓ Bcl-2	(Zhang et al., 2025a)

↑ - increased; ↓ - decreased

2.14 Okra (*Abelmoschus esculentus*)

Okra (*Abelmoschus esculentus*), is a extensively recognised and versatile crop commonly referred to as "Lady's finger" (Elkhalifa et al., 2021) (Figure 2.12). Okra is considered to have originated near Ethiopia, where historical evidence suggests it was extensively cultivated by the Egyptians as early as the 12th century BCE, gradually expanding across the Middle East and North Africa (Elkhalifa et al., 2021). Moreover, botanical hypotheses propose a dual origin theory for okra species, *A. esculentus* is believed to have originated in North India, while *A. ficulneus* likely emerged in Ethiopia (Swamy, 2023). Today, okra is cultivated in warm tropical and subtropical climates annually on a commercial scale across Africa, Asia, and the Americas (Islam, 2019), where it serves not only as a food source but also holds potential in traditional medicine and industrial applications due to its diverse phytochemical composition.

Figure 2.12. Okra (*Abelmoschus esculentus*)

2.14.1 Okra as a nutraceutical

Okra has gained traction due to its abundance of bioactive compounds supporting human health. The varying components of okra have been utilised in traditional treatment for gastrointestinal, inflammatory, and metabolic ailments (Dantas et al., 2021). Previous research has denoted the bioactivity of okra, for instance, okra mucilage and extracts have been shown to lower blood glucose, cholesterol, modulate immunity, and exhibit antioxidant and anti-inflammatory effects (Table 2.8) (Elkhalifa et al., 2021).

Table 2.8. The health benefits of okra components and phytochemicals

Okra component	Health benefit	Mode of action	Reference
Dietary fibre & mucilage	Mitigates and prevents constipation	Binds toxins and lubricates intestines, acting as a natural evacuant	(Das et al., 2019)
Mucilage	Ulcer relief	Okra is alkaline which neutralises acid & coats the digestive tract increasing recovery of peptic ulcers	(Das et al., 2019)
Polysaccharides	Antidiabetic	↓ glucose levels and body weight	(Fan et al., 2014)
Flavonoids & polyphenols (okra seeds)	Antifatigue	↓ BLA & BUN, combats fatigue by ↑ liver glycogen reserves	(Xia et al., 2015)
Lectin	Anticancer	↓ proliferation of MCF-7 cells	(Monte et al., 2014b)
Flowers	Anticancer	Inhibition of colorectal cancer	(Elkhalifa et al., 2021)
Seed extract	Anticancer	↑ cytotoxicity in MCF-7 cells	(Chaemsawang et al., 2019)
Pulp	Anticancer	Oxidative stress and mitochondrial apoptosis in Jurkat T-cells	(Monte et al., 2014b)
Pectin	Anticancer	Induction of apoptosis in melanoma cells (B16F10) by Gal-3 interaction	(Vayssade et al., 2010)

↑ - increased; ↓ - decreased

The diverse health benefits of okra components, ranging from antidiabetic and antioxidant activity to antiproliferative properties highlighted in Table 2.8, collectively underscore its potential as a nutraceutical, while positioning okra as more than a traditional vegetable. Its ability to modulate physiological processes through mechanisms such as oxidative stress reduction, apoptotic induction, and immune enhancement reflects the growing interest in utilising plant-based interventions in mitigating chronic diseases. These attributes validates its pharmacological applications and further emphasizes evaluation of specific okra components, particularly its seed protein, which remain underexplored as a natural anticancer therapeutic agent.

Chapter 3: Chemical and structural characterization of okra (*Abelmoschus esculentus)* seed protein isolate as a potential therapeutic agent

Abstract

Okra seed protein isolate (OSPI) has garnered increasing interest due to its bioactive potential and nutritional value. This research focused on analyzing the structural characteristics and molecular composition of OSPI to evaluate its potential therapeutic applications. The amino acid profile revealed a diverse range of amino acids, with leucine (6.56 g/100 g), glutamic acid (14.46 g/100 g), and arginine (9.42 g/100 g) identified as the most abundant. Hydrophobic amino acids (31.39 g/100 g) along with branched-chain amino acids (14.24 g/100 g) further underscore their bioactivity, particularly in immune modulation and cancer therapy. Protein solubility analysis indicated optimal solubility (91.86%) at pH 9, a key factor influencing bioavailability. The molecular weight profile identified polypeptide bands corresponding to 2S albumins, known for their antimicrobial and immunomodulatory properties. FTIR and XRD analyses highlighted the presence of α-helical and β-sheet structures, contributing to OSPI's functional stability. Additionally, OSPI exhibited an amorphous nature, which is beneficial for pharmaceutical applications due to enhanced bioavailability. Particle size analysis demonstrated a monomodal distribution, with OSPI displaying the smallest size (0.14±0.10 μm), indicative of improved drug delivery potential. Collectively, these findings emphasize OSPI's therapeutic potential, particularly in disease prevention and treatment, supporting its potential application in pharmaceutical and nutraceutical industries.

Keywords: Okra; protein isolate; amino acid; hydrophobicity; therapeutic; disease

3.1 Introduction

Proteins are fundamental macronutrients that serve as building blocks of life, contributing significantly to the integrity and biological processes of organisms across humans, plants, and animals (Kumar et al., 2022). They regulate physiological pH, act as signalling molecules and hormones, primarily constitute enzymes that catalyse metabolic pathways, support the immune system, serve as bioactive agents, and are integral to disease prevention (Kumar et al., 2022). Moreover, the functionality of proteins is affected by multiple intrinsic and extrinsic elements, including amino acid composition, protein structure, molecular size, extraction parameters, pH, and surface characteristics such as solubility (Li et al., 2020). Proteins are macromolecules consisting of a linear sequence of amino acids linked by peptide bonds, influencing protein quality (Sim et al., 2021). Plant-based proteins, in particular, exhibit distinct amino acid profiles, each contributing unique characteristics and potential health benefits. Plant-derived protein sources are becoming increasingly popular due to their environmental sustainability and cost-efficacy (Kumar et al., 2022).

The growing interest in plant proteins extends to their therapeutic potential (Tan et al., 2023). Plant proteins have numerous health benefits, particularly in cancer treatments, where they demonstrate excellent tissue penetration, target selectivity, and minimal toxicity owing to their amino acid composition (Bhutia et al., 2019). These proteins provide the essential amino acids our bodies need to repair tissues and synthesize enzymes, hormones, and other vital molecules (Tomar et al., 2021). Beyond their structural functions, plant proteins are instrumental in reducing the risk of numerous health conditions, such as heart disease, diabetes, and cancer (Ansori, 2021, Shahnaz et al., 2024). The bioactivity of amino acids and peptides, influenced by factors such as hydrophobicity and molecular charge, plays a fundamental role in determining their efficacy in targeting specific diseases (Peighambardoust et al., 2021).

Plant proteins are derived from various sources, such as legumes, pulses, vegetables, nuts, and oilseeds, such as chickpeas, bambara groundnuts, and soybeans. According to Bonciu (2020), certain vegetables can also serve as natural therapeutic agents capable of treating a range of diseases, such as diabetes and hypertension. *Abelmoschus esculentus*, commonly known as okra or lady's finger, is a well-known angiosperm producing edible seed pods. Native to Africa but now grown commercially worldwide, okra thrives in warm temperate climates across tropical and subtropical regions of Africa, Asia, and America (Ofori et al., 2020). As a member of the *Malvaceae* family, okra has long been valued for its nutritional content and medicinal potential (Ofori et al., 2020).

Historically, okra has served multiple purposes, including acting as a cooling agent, appetizer, astringent, and aphrodisiac (Elkhalifa et al., 2021). Furthermore, it has been reported that, traditionally, okra is used to treat chronic dysentery and gonorrhea (Esmaeilzadeh et al., 2020). Okra seeds are rich in bioactive compounds, including proteins, and provide a wealth of fundamental vitamins and minerals including vitamins C and potassium, vital for preserving wellness and immune function (Liu et al., 2021). It has also proven effective in alleviating chronic illnesses such as diabetes (Liu et al., 2021). Pharmacologically, okra demonstrates antioxidant and anti-inflammatory properties, with polysaccharides, polyphenols, and flavonoids exhibiting both antioxidant and antifatigue activities *in vivo* and *in vitro* (Liu et al., 2021). These compounds have increased the scavenging rates of free radicals, superoxide, and DPPH radicals and enhanced swimming endurance in test subjects (rat model) (Gao et al., 2018, Xia et al., 2015). Okra seeds, aqueous extracts, and polysaccharides have also shown *in vitro* antidiabetic potential, decreasing α-glucosidase and α-amylase activities (Yuan et al., 2019).

Okra polysaccharides have also been shown to demonstrate hypolipidemic activities, reducing cholesterol absorption and binding to bile acids (Kahlon et al., 2007), while methanol extracts of okra seeds display antibacterial and antifungal properties (Petropoulos et al., 2017). Additionally, ethanol extracts of okra and quercetin have shown *in vitro* immunomodulatory activity by decreasing reactive oxygen species and reducing microglial activation (Mairuae et al., 2017), along with okra polysaccharides having exhibited antitumor activity, enhancing phagocytosis in RAW264.7 cells (Zheng et al., 2014). Lectins from okra seeds have been reported to increase the expression of key genes such as P21, caspase 3, and caspase 9 involved in apoptosis (Monte et al., 2014a). While extensive research has explored the role of okra polysaccharides, polyphenols, and flavonoids in mitigating chronic diseases, the therapeutic potential of the okra seed protein isolate has yet to be discerned, despite it being a rich source of bioactive proteins.

3.2 Materials and methods

3.2.1 Preparation of okra seed protein isolate

The okra seeds [ARC-okra24], sourced from the Agricultural Research Council (ARC)-VIMP, were milled to produce okra flour (OF). The resultant flour was sieved (250 μm) and defatted using hexane (1:5 w/v). The defatted okra flour (DOF) was subsequently utilized in the preparation of okra seed protein isolate (OSPI) with minor modifications, according to Nnamezie et al. (2021). The DOF was mixed with distilled water (dH_2O) (1:15 w/v), stirred for 10 min and the pH adjusted to 10 using 1 M sodium hydroxide (NaOH).

The resultant slurry was stirred for 2 h, followed by centrifugation (10 000 x g, 30 min). The supernatant was adjusted to pH 4.5 using 1 M hydrochloric acid (HCL) and centrifuged (10,000 x g, 30 min) with the pellet obtained resuspended in dH_2O and adjusted to pH 7 using 1 M NaOH. The sample was stored at -80°C for 24 h and thereafter lyophilized.

3.2.2 Protein

Protein content and yield

The protein content of OSPI was determined using the Bradford protein assay, whereas the crude protein content of DOF was determined via the Kjeldahl method, applying a conversion factor of N × 5.30 (Bradford, 1976). The protein yield was then calculated using the following equation:

$$Protein\ yield = \frac{Protein\ content\ of\ isolate\ (\%) - Protein\ content\ of\ defatted\ flour(\%)}{Protein\ content\ of\ isolate\ (\%)} \times 100 \quad \text{.....(1)}$$

Protein solubility

Protein solubility was evaluated according to Chen et al. (2017) with minor modifications. In brief, 10 mg of the sample was dissolved in 10 mL of dH_2O, and the pH adjusted between 3-9 using 0.01 M NaOH or 0.01 M HCl. The suspension was vortexed (2 min, room temp), and centrifuged (8 000 x g, 15 min). The protein content in the supernatant was quantified using the Bradford method (Bradford, 1976), and protein solubility was calculated using the following equation:

$$Protein\ solubility\ (\%) = \frac{Total\ amount\ of\ protein\ in\ supernatant}{Total\ amount\ of\ protein\ in\ the\ sample} \times 100 \quad \text{.....(2)}$$

3.2.3 Chemical and structural composition

Amino acid composition

The amino acid profiles of the samples were analyzed in accordance with Wheat et al. (2008), using a Waters Acquity Ultra Performance Liquid Chromatograph (UPLC). Amino acids were quantified either as free amino acids or after protein hydrolysis. Free amino acids were analyzed by injecting 1 µL of the sample into the mobile phase, which carried the derivatized amino acids onto a Waters UltraTag C18 column (2.1 x 50 mm x 1.7 µm) kept at 55°C for 10 minutes. Protein hydrolysis was carried out through acid digestion with 6 N HCl (110°C, 18 h). Chromatographic analysis of the amino acids was conducted using MassLynx software for instrument control and data acquisition.

Gel electrophoresis

Gel electrophoresis was used to determine the molecular weights of OSPI and OF under reducing and non-reducing conditions following the manufacturer's protocol. Briefly, 20 mL of Bolt™ MES SDS running buffer (20X) was diluted with 380 mL deionized water and poured into the SureLock Tandem Midi Gel Tank. An Invitrogen™ precast gel (4-12%) was placed in the tank. To prepare the samples, 10 mg of each was disseminated in 1 mL of phosphate-buffered saline (pH 7.4). Then each sample (10 µL) was combined with 10 µL of Bolt™ LDS sample buffer (4X), and the volume adjusted to 40 µL. For reducing conditions, 4 µL of Bolt™ reducing agent (10X) was added. The samples were heated (70°C, 10 min), and 15 µL pipetted into each well. The Spectra™ Multicolor Broad Range Protein Ladder (10-260 kDa) was used as the molecular weight standard.

Infrared spectra analysis

The secondary structure of the samples was examined using an Agilent Cary 630 Fourier Transform Infrared Spectrophotometer (FTIR) with a diamond-attenuated total reflectance (ATR) module, based on the method outlined by Madurapperumage et al. (2022). A resolution of 8 cm^{-1} across a range of 4 000 to 650 cm^{-1}, with 32 cycles of data collection.

X-ray diffraction (XRD)

The XRD analysis of the samples was carried out at the Council for Scientific and Industrial Research (CSIR) using a Bruker AXS D8 Advance powder X-ray diffractometer. The diffractometer was set to a tube current of 40 mA and a 45 kV generator voltage, following the modified method of Sahni et al. (2020), with modifications. A fixed divergence slit (0.38 mm) and a copper anode were utilized.

Particle size (PS) and zeta potential (ZP)

The particle size and zeta potential were determined using a Litesizer DLS 500 (Anton Paar) fitted with Kalliope software. Before evaluation, the samples (1 mg/mL) were hydrated in dH_2O for 1 h. The analysis was conducted at 25°C with a solvent refractive index of 1.33 (Alabi et al., 2023).

Microstructure analysis

Transmission electron microscopy (TEM) was used to analyze the microstructure (JEOL 2100 HRTEM, Japan) of OSPI at the Electron Microscopy Unit [University of KwaZulu Natal (UDW Campus)] as described by Du et al. (2023).

3.2.4 Statistical analysis

The analyses were conducted in triplicate, and the results are presented as mean ± standard deviation. Statistical significance for values with $p<0.0001$ was established by means of a two-way analysis of variance (ANOVA), performed with GraphPad Prism 10 version 10.4.1 (627) for Windows (GraphPad Software, San Diego, CA, USA).

3.3 Results and discussion

Amino acids (AAs) are the fundamental building blocks of life, playing a crucial role in maintaining optimal bodily function. They serve as precursors to produce secondary metabolic molecules with significant biological importance (Ling et al., 2023). Amino acids can be classified as essential, which the body requires but cannot synthesize; non-essential, which the body can produce; and conditional, which are not crucial unless during illness (Ahmed et al., 2021, Akbay et al., 2024). Table 3.1 depicts the overall amino acid composition of the flours is less than that of the protein isolate, likely due to the higher purity of the OSPI. Moreover, the protein isolate amino acid content is higher than the Food and Agriculture Organization (FAO) recommendation, indicating its potential as a valuable nutritional source. Leucine, known for enhancing muscle protein synthesis, is the principal essential amino acid in the samples, with OSPI showing the highest content at 6.56 g/100g. This is slightly higher than a previous study's okra protein isolate and hydrolysate levels (Ijarotimi et al., 2023). Moreover, among the non-essential amino acids, glutamic acid (14.46 g/100g), which may include glutamine and arginine (9.42 g/100g), was prominent in OSPI, aligning with the results found by Ijarotimi et al. (2023) and Nnamezie et al. (2021). Glutamine and arginine are also conditional amino acids essential in regulating immune response, neurotransmission, improving endothelial function, and preventing cardiovascular disease (Chen et al., 2024). In addition, Dai et al. (2017) elucidated that arginine could penetrate and disrupt cell membranes, leading to cytotoxic effects in cancer cells, further emphasizing the therapeutic potential of OSPI. Furthermore, aspartic acid, which may include asparagine, was notably present at 7.56 g/100g. Yamaguchi et al. (2016) also attributed antiproliferative activity in tumor cells to a combination of aspartic and glutamic acids. These findings highlight the potential of OSPI in disease prevention, particularly in cancer therapy.

OSPI also exhibited a significantly high content of hydrophobic amino acids (HAAs), totaling 31.39 g/100g, consistent with the findings of Ijarotimi et al. (2023) for okra protein isolate and hydrolysate. Chalamaiah et al. (2018) noted that food-derived HAAs could facilitate immunomodulatory activity and enhance the specificity of target cells for potential anticancer therapeutics, further underscoring the therapeutic potential of OSPI.

Moreover, proteins with higher levels of branched-chain amino acids (BCAAs) compared to aromatic amino acids (AAAs) are beneficial for health, with applications in antitumor activity and liver damage repair (Wang et al., 2024). OSPI exhibited a BCAA content of 14.24 g/100g, almost double that of the AAA content, emphasizing its pharmaceutical potential. The presence of these significant amino acids, combined with the high HAA content, suggests the bioactive potential of OSPI for therapeutic applications.

Table 3.1. Amino acid profile of okra seed flour (OF), defatted okra flour (DOF), and okra seed protein isolate (OSPI) (g/100g)

	Amino acid	OF	DOF	OSPI	FAO recommendation[a]
Essential AA	**Histidine**	0.73	1.05	2.16	1.9
	Threonine	1.14	1.62	2.52	1.4
	Lysine	2.35	3.24	4.45	-
	Methionine	0.74	1.01	3.06	-
	Valine	1.82	2.67	4.59	3.5
	Isoleucine	1.06	1.61	3.09	2.8
	Leucine	2.47	3.69	6.56	6.6
	Phenylalanine	1.48	2.31	4.12	-
	Total Essential AA	11.79	17.2	30.55	-
Non-essential AA	**Serine**	1.64	2.36	4.05	-
	Arginine	3.59	5.24	9.42	-
	Glycine	1.77	2.57	3.63	-
	Aspartic acid	3.83	4.81	7.56	-
	Glutamic acid	6.35	8.43	14.46	-
	Alanine	1.56	2.18	3.48	-
	Proline	1.34	1.98	3.11	-
	Tyrosine	1.12	1.58	3.38	-
	Total Non-essential AA	21.20	29.15	49.09	-
Total amino acids		32.99	46.35	79.64	-
Phenylalanine + Tyrosine (AAA)		2.60	3.89	7.50	6.3
Methionine (SCAA)		0.74	1.01	3.06	2.5
Isoleucine +Leucine + Valine (BCAA)		5.35	7.97	14.24	-
Fischer's ratio (BCAA/AAA)		2.06	2.05	1.90	-
Basic		6.67	9.53	16.03	-
Acidic		10.18	13.24	22.02	-
Hydrophobic		11.59	17.03	31.39	-
Hydrophilic		2.78	3.98	6.57	-

OF: okra flour, DOF: defatted okra flour, OSPI: okra seed protein isolate, AAA: aromatic amino acids, SCAA: sulphur containing amino acids, BCAA: branched amino acids, Basic: lysine; arginine; histidine, Acidic: aspartic

and glutamic acid, Hydrophobic: alanine, valine, isoleucine, leucine, tyrosine, phenylalanine, proline, methionine, Hydrophilic: serine, threonine[a] (FAO/WHO/UNU, 1985)

The molecular weight of proteins is crucial in determining their bioavailability and bioactivity (Ijarotimi et al., 2023), as the size of the bioactive peptides within proteins influences their bioavailability (Patil et al., 2022). Under non-reducing conditions, the samples exhibited three prominent high-intensity polypeptide bands (Figure 3.1) around 55, 64, and 75 kDa (lanes 1 & 2). The 55 and 64 kDa bands correspond to vicilin (7S globulin) subunits, which are predominantly found in legume proteins and have been observed in protein isolates from bambara groundnut and hyacinth beans (Alabi et al., 2023, Naiker et al., 2020). The protein band around 75 kDa represents convicilin, the α-subunit of vicilin (Alabi et al., 2023). The low molecular weight bands for the samples (lanes 1 & 2) between 10-20 kDa, which are associated with basic legumin (11S globulins), were observed under non-reducing conditions. The polypeptide band at 20 kDa corresponds to studies conducted by Naiker et al. (2020) and Alabi et al. (2023) for hyacinth bean and bambara groundnut protein isolates, respectively. Moreover, nanoparticles derived from legumin and vicilin proteins of fava beans have shown promise as encapsulation materials for nutraceutical compounds, demonstrating biocompatibility with CaCo-2 cells and efficient nutrient delivery (Fang et al., 2024). This highlights the potential of legumin and vicilin as effective drug delivery carriers, further supporting the therapeutic potential of OSPI, as both proteins are abundant in OSPI and serve as rich sources of bioactive peptides.

However, Antonia et al. (2019) inferred that protein bands <20 kDa correspond to 2S albumins. 2S albumins are low-molecular-weight proteins characterized by a high concentration of glutamine, arginine, and asparagine (Souza, 2020); the predominant amino acids present in OSPI are associated with numerous health benefits, as previously stated. Additionally, 2S albumins exhibit numerous biological advantages, such as being an antibacterial agent that can limit and prevent pathogenic bacteria's progression in humans (Laxminarayan et al., 2013). In a study by Maria-Neto et al. (2011), 2S albumins derived from sesame seeds demonstrated strong inhibitory effects against *Klebsiella* species, a gram-negative bacterium responsible for pneumonia and other infectious diseases. The biological activities exhibited by 2S albumins suggest significant potential for development as antibiotic agents (Souza, 2020). Therefore, the presence of 2S albumins in OSPI underscores its potential in therapeutics. Under reducing conditions (lanes 3 & 4) revealed fewer polypeptide bands with a downward shift, which were observed with a band at 14 kDa being absent. This suggests the presence of disulfide bonds in the non-reduced state of the samples (Naiker et al., 2020). A polypeptide band at 48 kDa is observed in both OF and OSPI under reducing

conditions, corresponding to the β-conglycinin subunit present in soybean protein isolates (González-Montoya et al., 2018).

This subunit has demonstrated a decrease cholesterol levels and influence obesity, highlighting the therapeutic potential of OSPI (Hu et al., 2023). Moreover, the protein isolate denotes a band of 9 kDa under reducing conditions. In a previous study conducted by González-Montoya et al. (2018), protein fractions between 5-10 and >10 kDa from germinated soybean protein exhibited high potency in inhibiting the proliferation of CaCo-2 cells. Furthermore, soybean peptides with molecular weights >10 kDa effectively inhibited breast and cervical cancer growth (Marcela et al., 2016). The protein bands at 9, 10, and >10 kDa in OSPI highlight its potential for application as a pharmaceutical agent. Additionally, no significant differences can be observed between OF and OSPI under both conditions.

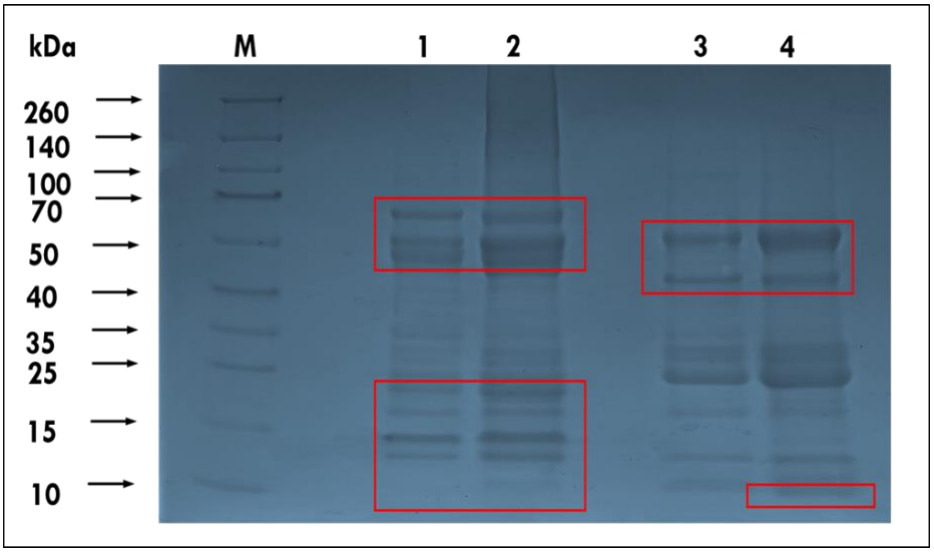

Figure 3.1. Molecular weight profile of okra samples. [Lane 1: okra seed flour (OF) & Lane 3: okra seed protein isolate (OSPI) (Non-reducing), Lane 2: okra seed flour (OF) & Lane 4: okra seed protein isolate (OSPI) (Reducing), M: marker.]

The solubility of proteins aids in determining their functionality and can be impacted by pH and amino acid composition (Li et al., 2020). Poor solubility can affect the bioavailability of proteins and its therapeutic efficiency. As depicted in Figure 3.2, both flours showed no significant differences within the pH range of 3-9. Overall, the protein solubility of the flours increased at each pH, exhibiting the highest solubility in the alkaline region, except DOF at pH 9 (74.52%). The protein isolate exhibits a similar trend to the flours, showing the highest solubility (91.86%) among the samples at pH 9. The protein isolate demonstrates a similar pattern to the flours, having the highest solubility (91.86%) amongst the samples at pH 9. Additionally, OSPI was shown to be the least soluble at pH 5 near the isoelectric point. This is likely due to protein precipitation caused by

the absence of a net charge on the molecules and can also indicate variations in the surface structure of proteins (Tan et al., 2023, Chen et al., 2020).

Moreover, comparable findings were discerned for sunflower protein isolate, which was insoluble between pH 4-5.5 and solubility gradually increased to pH 7 (Ivanova et al., 2013). A study on four soybean cultivars was comparable to the results achieved for OSPI exhibiting minimum solubility around pH 5 (Chen et al., 2020). According to Yagasaki et al. (1997) and Chen et al. (2020) the low solubility of proteins near the isoelectric point could indicate β-conglycinin found in soybean protein. Moreover, as established in the molecular weight profile, β-conglycinin was identified in OSPI with potential health benefits. Thus, the low solubility of OSPI near pH 5 corresponding to the β-conglycinin subunit further emphasizes its therapeutic potential. The amino acid composition of samples differ, therefore displaying varying degrees of solubility as the different charged groups (polar and non-polar) affect the solubility of the samples (Mir et al., 2019), as indicated by the deviation in results. The high solubility of OSPI suggests increased bioavailability for its application as a potential therapeutic agent. This potential is underscored by a study conducted by Zhang et al. (2017) in which a widely used chemotherapeutic agent, Docetaxel (DTX), was combined with soy protein isolate (SPI). The results indicated that the inclusion of SPI notably improved the solubility of DTX within a pH range of 7 to 10 (Zhang et al., 2017). Furthermore, the DTX-SPI suspension indicated a systolic delivery, which, in turn, supplemented the cell cytotoxicity against A549 cells (lung cancer). This study not only validates the high solubility of OSPI but also highlights its ability as a delivery and pharmaceutical agent against diseases such as cancer, particularly given its high solubility at an alkaline pH.

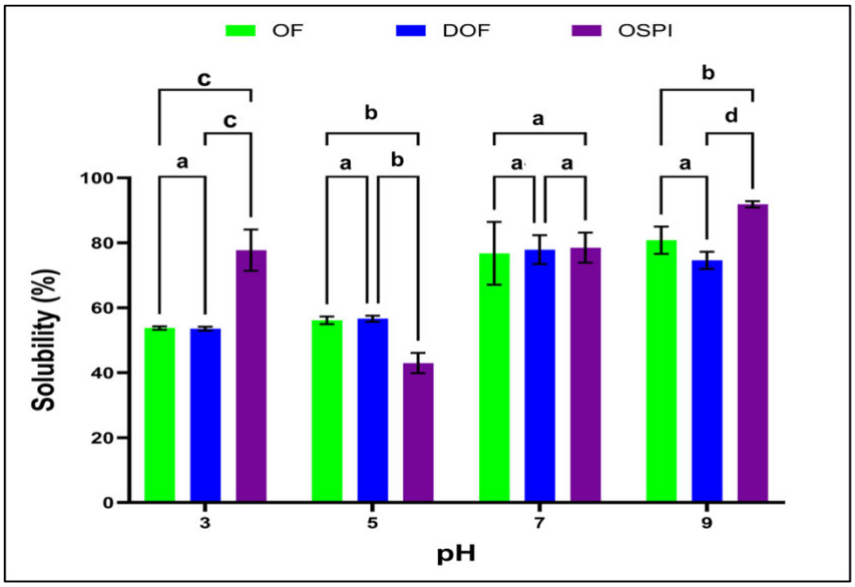

Figure 3.2. Effect of pH on the solubility of okra flour (OF), defatted flour (DOF), and okra seed protein isolate (OSPI). Data denotes mean±standard deviation (n = 3). Values with the same superscript letters

are not significantly different (p<0.0001).

The FTIR spectra (Figure 3.3) depicts no significant shifts in wavenumbers among the samples. Protein-containing samples display nine distinct spectral lines within the absorption range of approximately 200 to 3300 cm^{-1} (Sadat and Joye, 2020). These bands result from various oscillatory modes of the amide groups present in peptide bonds and are classified as amides A, B, and I-VII. Various characteristic peaks related to proteins were observed. The peak at 1636 cm^{-1} for the flours and protein isolate indicates amide I, corresponding to results achieved by Antonia et al. (2019) for quinoa protein. The amide I band is primarily associated with the stretching vibrations of C=O bonds in the peptide backbone and is widely recognized as the most sensitive and commonly used band for analyzing protein secondary structures (Sadat and Joye, 2020). Moreover, the proportion of each protein secondary structure can be determined by examining the area under the peaks in the original spectra, which are ascribed to specific structural components (Sadat and Joye, 2020). The amide I peak is positioned between 1628-1642 cm^{-1} on the infrared spectrum, which is typically attributed to β-sheet structures (Krimm and Bandekar, 1986, Sadat and Joye, 2020).

Amide II is usually denoted in the region of ~1500 to ~1550 cm^{-1} as described by Antonia et al. (2019) and Madurapperumage et al. (2022), respectively. Thus, the bands found at 1543 cm^{-1} in the flours and 1524 cm^{-1} in the protein isolate link to results of quinoa protein (Antonia et al., 2019) and in pulse proteins such as chickpeas (Madurapperumage et al., 2022). An estimated peak fitting proposes that the flours are positioned within the range of 1540-1545 cm^{-1}, suggesting an association to α-helical structures, while the protein isolate is located between 1520-1525 cm^{-1} correlating to β-sheet secondary configurations (Aboul-eneın et al., 2014, Sadat and Joye, 2020). Additionally, identifying protein secondary structure bands is challenged further by the absorption of amino acid side chains within the protein (Sadat and Joye, 2020). This absorption overlaps with the amide I and II bands, accounting for approximately 10 to 20% of the total absorption in these regions for globular proteins (Kong and Yu, 2007, Sadat and Joye, 2020). Furthermore, Sadat and Joye (2020) elucidated that aromatic amino acid side chains also influence the spectrum in the amide I and II regions; in particular, amides I and II lie closest to arginine at 1633 cm^{-1} on the spectra and are associated with C=N symmetric stretching. As previously mentioned, arginine was one of the primary amino acids in OSPI, and it had numerous associated therapeutic benefits. Thus, identifying amide bands further emphasizes the potential of OSPI as a therapeutic agent. A peak at 1234 cm^{-1} was noted in OF and at 1230 cm^{-1} in DOF and OSPI. These band frequencies allude to amide III, similar to quinoa protein at ~1200 cm^{-1} (Antonia et al., 2019).

Moreover, Madurapperumage et al. (2022) showed that lentil protein amide III laid between 1232-1470 cm^{-1} representing C-H, CH2, and CH3 bends of sulphur-containing amino acids (SAA). C-H, CH2, and CH3 stretches were identified as lentil proteins in all samples at 2922 cm^{-1}. These stretches were observed between 2826-2995 cm^{-1}, signifying SAA (Madurapperumage et al., 2022). Whilst the peak at 2922 cm^{-1} reflected in all the samples, OF showed a long, narrow peak, whereas the peak in DOF appeared shorter and more stretched, and OSPI emerged flatter and more expansive. In addition, a broad peak attributed to amide A was observed in all samples. OF was depicted at 3276 cm^{-1}, and a slight shift in wavenumber to 3273 cm^{-1} denoted DOF and OSPI. These results correlate with quinoa protein (amide A: 3270-3310 cm^{-1}) (Antonia et al., 2019).

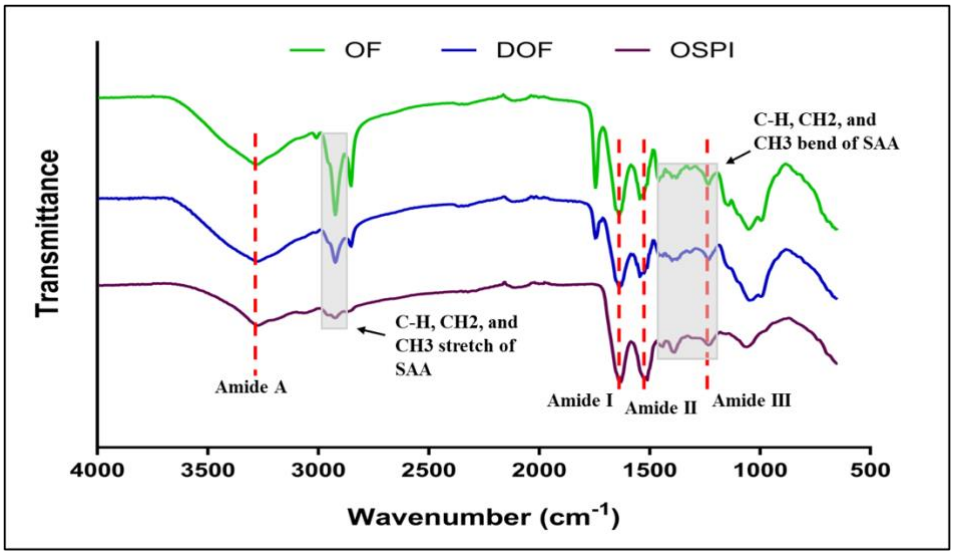

Figure 3.3. FTIR spectra of okra seed flour (OF), defatted okra flour (DOF), and okra seed protein isolate (OSPI).

The size of a crystal influences the diffraction angle (2θ) and intensity (Sahni et al., 2020). Moreover, these factors are crucial for understanding potential conformational changes within protein structures and their interactions with other molecules (Sahni et al., 2020). Smaller crystals produce lower diffraction intensities and broader diffraction angles (Sahni et al., 2020). The diffraction peak around 7° indicates crystalline region I, while the peak shoulder near 2θ = 19° represents crystalline region II (Figure 3.4) (Sahni et al., 2020). These peaks are comparable to alfalfa and sunflower protein isolates in which the broad band around 2θ = 20° was ascribed to the amorphous nature of the protein (Sahni et al., 2020, Malik et al., 2016). Thus, suggesting that the peak at 2θ=19° in OSPI could indicate the amorphous nature of OSPI. According to Aitipamula and Vangala (2017) proteins with amorphous structures benefit therapeutics as they offer a higher dissolution rate and enhanced bioavailability for drug development, suggesting that OSPI could serve as a promising pharmaceutical agent.

Additionally, the differences in peak intensity may result from the varying interactions between protein molecules and polyphenols (Sahni et al., 2020). The prominent diffraction peaks in both regions I and II correspond to the reflections of 7S (α-helix) and 11S (β-sheet) globulins, respectively, within the polypeptide chain structure, as identified by gel electrophoresis (Jhan et al., 2021). Furthermore, OSPI displayed a minor peak around 81°, similar to soybean protein isolate, which has previously shown an affinity for vitamin D encapsulation (Khan et al., 2020), further highlighting the therapeutic potential of OSPI. Additionally, the minor peak shows low intensity and a broad angle, which alludes to OSPI being smaller in size.

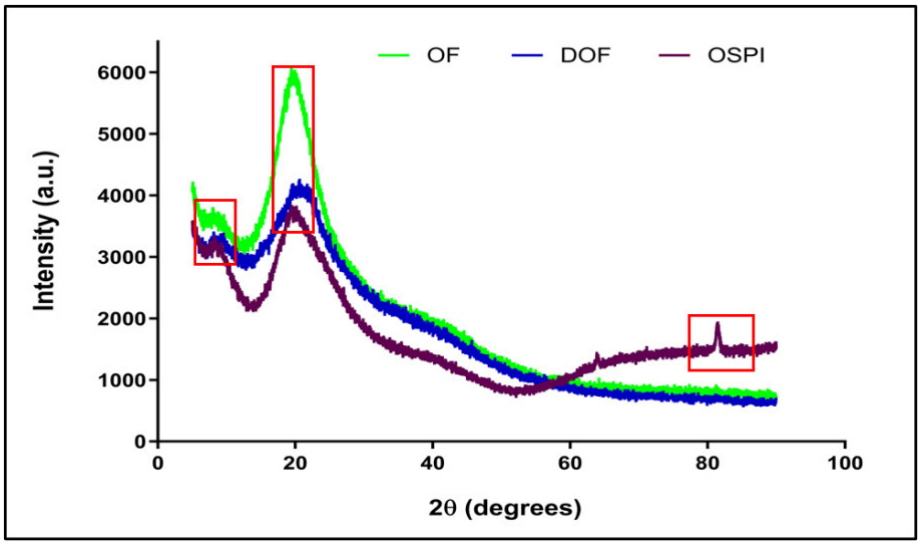

Figure 3.4. X-ray diffractogram of okra seed flour (OF), defatted okra flour (DOF), and okra seed protein isolate (OSPI).

The polydispersity index (PDI) indicates particle size distribution and uniformity (Hoseini et al., 2023). The PDI values of 0.10-0.25 denote a narrow distribution of particles, while a broad distribution is indicated by values greater than 0.50 (Hoseini et al., 2023). OF and OSPI were shown to have PDI values of 2.20 and 2.70, respectively, aligning to a wide distribution. DOF showed a PDI value of 0.22, corresponding to a narrower distribution, suggesting a monodispersity of particles. Moreover, (Danaei et al., 2018) highlighted that particle size and PDI could impact drug delivery, cellular uptake, and encapsulation efficiency, directly affecting therapeutic outcomes. As shown in Figure 3.5, the samples depicted a monomodal distribution. OSPI appeared the smallest at 0.14±0.10 µm, compared to OF and DOF, which reflected at 0.43±0.20 and 1.37±0.10 µm, respectively. The small size of OSPI suggests its potential as a long-circulating carrier, ideal for drug delivery to tumors, while OF and DOF are better suited for transdermal and intravenous administration, respectively (Danaei et al., 2018). The applications mentioned above emphasize the pharmacological potential of OSPI.

The zeta potential, which represents the surface charge of a protein, is a vital factor in its stability, functionality, and interactions within biological systems. Understanding this property for therapeutic applications is essential, as it influences protein behavior, including solubility, aggregation tendency, and interactions with cells or tissues (Németh et al., 2022). A zeta potential above ±30 mV is typically considered stable (Thakur et al., 2020). As illustrated in Figure 3.5, OSPI exhibited the highest stability with a zeta potential of −29.70±2.60 mV, compared to OF and DOF, which had zeta potentials of −27.50±1.10 and −25.00±0.60 mV, respectively. The zeta potential of OSPI was similar to that of soybean protein, as reported by Chen et al. (2020). Moreover, You et al. (2021) denoted exosome-like nanovesicles derived from plants, with surface charges of −14.80 and −15.20 mV, proved effective as encapsulation materials for therapeutic drug delivery to human cells. This suggests that OSPI, with its higher stability, could be efficiently utilized in drug delivery applications.

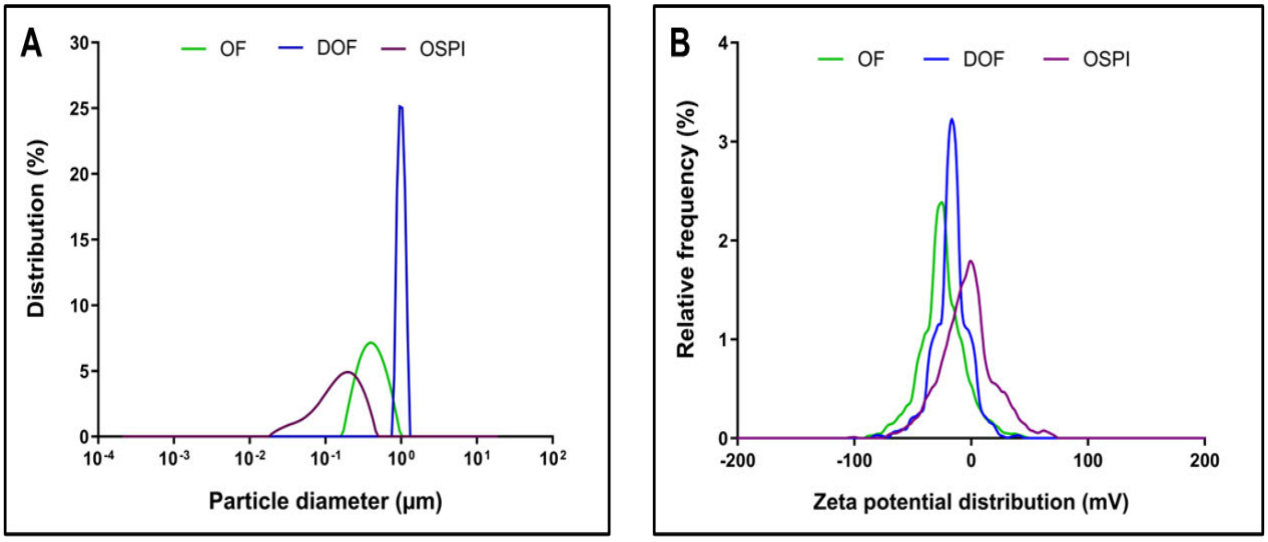

Figure 3.5. Particle size (A) and Zeta potential (B) of okra seed flour (OF), defatted okra flour (DOF), and okra seed protein isolate (OSPI).

Transmission electron microscopy (TEM) can be used to identify and characterize the microstructure of proteins (Sung et al., 2015). Figure 3.6 depicts the TEM of the flours and protein isolate particles. OF and DOF had median sizes of 12.20 and 9.90 nm, respectively, while OSPI showed an average size of 17.20 nm. The hydrodynamic diameter obtained from particle size analysis showed an inverse correlation with the TEM analysis, which revealed smaller sizes. The smaller sizes observed by TEM could be attributed to differences in sample state during analysis (Du et al., 2023), as the particle size samples are hydrated, potentially increasing the average particle size. The particle sizes suggest that these samples could be suitable for drug delivery via the reticuloendothelial system, allowing for deposition in various organs throughout the body (Danaei et al., 2018).

Furthermore, the particles displayed a near-spherical shape with a smooth surface, which are key characteristics contributing to effective drug delivery owing to OSPI potential in therapeutics (Priscila Laiz et al., 2023).

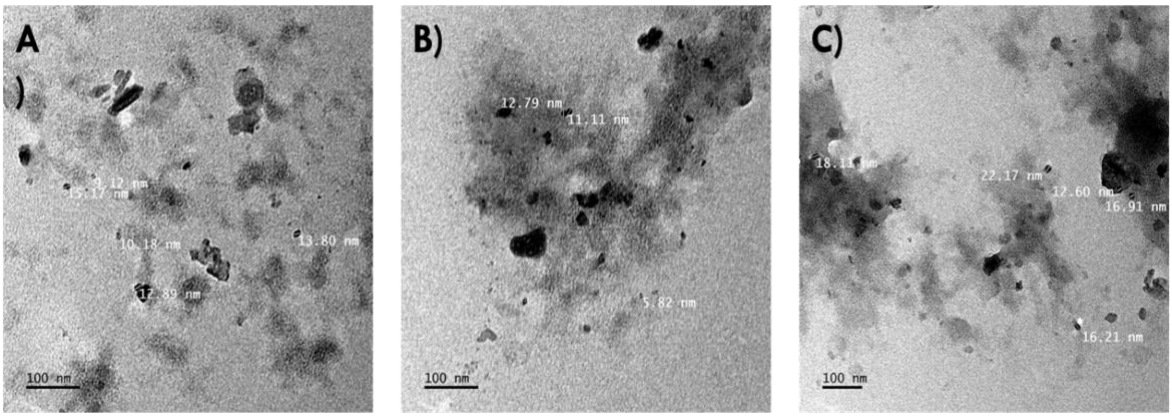

Figure 3.6. TEM micrographs of (A) okra seed flour (OF), (B) defatted okra flour (DOF), (C) and okra seed protein isolate (OSPI).

3.4 Limitations and future perspectives

Although okra seed protein isolate (OSPI) exhibits promising therapeutic potential, there are limitations to the research. This research fundamentally focuses on *in vitro* analyses, which may not fully replicate *in vivo* conditions, hypothetically limiting the direct clinical applicability of the results. Additionally, factors such as protein solubility, bioavailability, and stability under physiological conditions require further investigation to ensure effective therapeutic delivery. The presence of hydrophobic amino acids and low molecular weight peptides suggest potential bioactivity; however, their specific mechanisms of action and pharmacokinetics remain to be elucidated. Future research should explore *in vitro* and *in vivo* validation using cell culture and animal models to confirm the therapeutic efficacy of OSPI. Furthermore, exploring the synergistic effects of OSPI with other bioactive compounds for enhanced solubility and bioavailability, possibly through nanocarrier systems, could improve its pharmaceutical applications.

3.5 Conclusion

This study highlights the therapeutic potential of okra seed protein isolate (OSPI), emphasizing its rich amino acid composition, structural properties, and bioactive potential. OSPI exhibited an essential amino acid profile higher than FAO recommendations, with notable leucine, glutamic acid, arginine, and hydrophobic amino acid content. These amino acids contribute to immune regulation, neurotransmission, cardiovascular health, and anticancer activity, reinforcing OSPI's potential for disease prevention and treatment. The study also confirmed OSPI's high solubility, particularly in alkaline conditions, which enhances its bioavailability and functional properties. Electrophoresis revealed the presence of 7S and 11S globulins, along with 2S albumins, known for their antibacterial and immunomodulatory properties. Additionally, FTIR analysis confirmed the presence of key amide bands associated with secondary protein structures. At the same time XRD data indicated an amorphous nature beneficial for pharmaceutical applications due to increased dissolution and bioavailability. The small particle size and monodispersity of OSPI suggest its suitability for drug delivery systems, while its polydispersity index (PDI) indicates potential for encapsulation efficiency. These findings collectively underscore OSPI's potential as a functional protein with applications in therapeutic formulations, particularly in cancer treatment, antimicrobial agents, and nutraceuticals. Further studies on its bioactivity in complex biological systems could pave the way for its integration into the pharmaceutical and functional food industries.

Chapter 4: Antiproliferative effect of okra seed protein isolate on Hep-G2 and A549 cancer cell lines

Abstract

Cancer is a non-communicable disease characterised by the uncontrolled proliferation of abnormal cells and is one of the leading causes of mortality worldwide. According to the International Agency for Research on Cancer (IARC), approximately 20 million new cancer cases were recorded in 2022, with projections indicating that global incidence could increase to 35 million by the year 2050. In the present study, the antiproliferative effect of protein isolated from okra seeds were evaluated on Hep-G2 and A549 cancer cell lines. Okra seed protein isolate (OSPI) exhibited cytotoxic effects on A549 with cell viability of 37.73% at 15.62 µg/mL. The protein isolate (21.80 µg/mL) and defatted okra flour (DOF) (168.37 µg/mL) denoted lower IC_{50} values than camptothecin (524.85 µg/mL), suggesting that lower doses could potentially inhibit A549 proliferation compared to camptothecin a known chemopreventative agent. Okra flour (OF) denoted the highest apoptotic induction in A549 cells through Annexin V-binding. OSPI expressed similar levels of caspase 3/7 in Hep-G2 cells as camptothecin, indicating that OSPI could induce apoptosis in Hep-G2 cells. OSPI demonstrated efficiency in activating caspase 8 and 9 in Hep-G2, further highlighting the capability of OSPI as a potential therapeutic agent. A549 cells, known to display wild type p53, showed significant levels of p53, particularly when treated with DOF (34.68 µg/mL) and OSPI (36.06 µg/mL), suggesting the potential to induce apoptosis. Okra flour expressed moderate levels of ROS induction in A549 cells, higher than that of camptothecin. These results highlight the therapeutic potential of OSPI in disease treatment, underscoring its potential in both the pharmaceutical and nutraceutical industries.

Key words: Okra, protein, apoptosis, caspase-3/7, caspase-8, caspase-9, p53

4.1 Introduction

Cancer comprises a diverse group of complex diseases characterised by the unregulated proliferation and division of abnormal cells within the body (Jan, 2019). It remains a precarious international health concern and ranks among the leading causes of mortality worldwide. According to the International Agency for Research on Cancer (IARC), there were an estimated 20 million new cases reported in 2022 (Bray et al., 2024). The global cancer incidence is expected to increase substantially, with projections estimating around 35 million cases by 2050 (Bray et al., 2024). The prevalence of cancer varies widely and is predisposed by factors such as geographic region, socioeconomic status, and access to healthcare. The occurrence of certain types of cancers is more common in specific populations, influenced by genetic, environmental, and lifestyle factors, including alcohol use (Rahman et al., 2018). Common types of cancer include breast cancer, lung cancer, colorectal cancer, and prostate cancer (Ferlay et al., 2021). The incidence of cancer often increases as societies age and lifestyles evolve, making it a critical issue that requires further research culminating in prevention strategies, early detection, and innovative treatments.

Conventional cancer treatments include surgery, chemotherapy, and radiation therapy. While these therapies have made substantial strides in advancing survival rates and quality of life for many patients, they are not without disadvantages. Conventional treatments often result in a range of side effects, including nausea, fatigue, hair loss, and weakened immune function due to toxicity (Avilés-Gaxiola et al., 2020). These therapies are administered to destroy cancerous cells; however, due to their non-specific delivery, they often lead to damage of healthy cells (Sharma et al., 2021). Moreover, these treatments may not always provide a complete cure, resulting in a recurrence or development of resistance to treatment over time (Lei et al., 2017). Thus prompting a need for alternative treatment approaches that are less toxic to healthy cells and more cost-effective (Shoombuatong et al., 2018).

The utilisation of plant proteins has gained traction in recent years. Plant proteins possess several significant benefits compared to traditional treatments, including excellent tissue penetration and affinity, strong target selectivity, and minimal toxicity due to their primary composition being amino acids (Bhutia et al., 2019). Proteins derived from food or food components contain amino acids such as alanine and leucine, which are considered to possess hydrophobic properties (Chalamaiah et al., 2018). The characteristics of amino acids and peptides, such as their hydrophobicity and molecular charge, influence their bioactivity against specific diseases (Ahmed et al., 2021).

The advantage linked to hydrophobic amino acids is associated with their ability to enhance the interaction between the external membrane of malignant cells and therapeutic proteins or peptides, increasing cytotoxic efficacy and cell selectivity (Chalamaiah et al., 2018). Furthermore, Bonciu (2020) stated that selected vegetables can be utilised as natural therapeutic agents aiding in the treatment of several diseases. Okra, commonly known as "lady's finger" or "gumbo", is a multipurpose, nutrient-rich crop with seeds containing linoleic acid, and is particularly high in minerals such as calcium (Elkhalifa et al., 2021, Muimba-Kankolongo, 2018). When compared to legumes, okra has a comparatively high protein content, with previous studies having reported values as high as 35% crude protein concentrated in the seeds (Nnamezie et al., 2021).

Okra seeds can be beneficial due to their unique combination of seed protein rich in tryptophan, lysine, and sulphur-containing amino acids, deeming it the "perfect villagers" vegetable. A protein content of 90.24% isolated from okra seeds, with an amino acid composition rich in hydrophobic and aromatic amino acids at 30.46 and 9.96%, respectively, was previously highlighted in a study by Nnamezie et al. (2021). Amino acids influence the anticancer potential of proteins and peptides, as the hydrophobic amino acids are soluble across cancer cell membranes due to their intercellular interaction. In contrast, aromatic amino acids support penetration of cancerous cells (Avilés-Gaxiola et al., 2020).

Furthermore, okra extracts and their bioactive components, including polysaccharides and lectins, have demonstrated notable antitumour properties by enhancing macrophage activity, promoting tumour apoptosis, inhibiting tumour proliferation, and exerting immunomodulatory and pro-apoptotic effects in various cancer models (Liu et al., 2021). For instance, lectin from okra seed meal significantly reduced cell growth of breast cancer (MCF-7) cells, achieving 63% inhibition, and promoted upregulation of pro-apoptotic genes (*caspase-3*, *caspase-9*, and *p21*) with approximately 72% of cell death occurring predominantly through apoptosis (Monte et al., 2014b). In addition, polysaccharides found in okra flower stimulated the phagocytic activity of RAW264.7 cells, inducing the secretion of cytokines such as nitric oxide and tumour necrosis factor-alpha (TNF-α) by activating the NF-κ B pathway (Zheng et al., 2014). Despite these promising findings, studies specifically investigating okra seed protein isolate remain scarce. This research addresses this gap by evaluating the antiproliferative and apoptotic effects of OSPI on human cancer cell lines (A549 and Hep-G2), thereby advancing the current understanding of okra seed protein as potential natural therapeutic agents and situating OSPI within the broader context of plant-based antiproliferative strategies.

4.2 Materials and methods

4.2.1 Cell culture

The human embryonic kidney cell line (HEK293) served as the healthy cell model, while cancer cell lines included human liver hepatocellular carcinoma (Hep-G2) and lung adenocarcinoma (A549) cells obtained from the University of Technology (UKZN) in the disciplines of Pharmaceutics, Biomedical Science, and Biochemistry. Cells were maintained at 37°C with a relative humidity (RH) of 95% and a carbon dioxide (CO_2) level of 5%.

4.2.2 Cell viability

The cell viability of okra flour (OF), defatted okra flour (DOF), and okra seed protein isolate (OSPI) was determined according to Ramkisson et al. (2020) using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay with minor modifications. The cells (1×10^5 cells/mL) were subjected to 50 µL of each sample, and a serial dilution was performed (1000-7.8 µg/mL) and incubated for 24 h. An aliquot of 20 µL MTT solution (5mg/mL) was then added to the cells, followed by a 4-h incubation period at 37°C. Thereafter, 100 µL of dimethyl sulfoxide (DMSO) was added per well. The absorbance was measured at 570 nm using the Multiscan Go microplate spectrophotometer (ThermoFisher Scientific, USA), and cell viability calculated using the following equation:

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

4.2.3 Apoptosis

The apoptotic pathway of the samples was determined using the RealTime-Glo™ Annexin V Apoptosis kit as per the manufacturer's procedure (Promega Corporation). The IC_{50} values determined by the MTT assay were carried over. An aliquot of 100 µL of cells (1×10^5 cells/mL) was plated and left to adhere overnight. Following this, 50 µL of the sample (IC_{50} value) was added and incubated for 24 h. Thereafter, 50 µL of the 2X detection reagent was introduced into each well, and the relative luminescence units (RLU) were read after one hour of incubation using the Multiscan Go microplate spectrophotometer (ThermoFisher Scientific, USA).

4.2.4 Caspase 3/7, 8 and 9

The Caspase-Glo 3/7, 8, and 9 kits (Promega Corporation) were used to assess the activity of the above caspase enzymes, according to the manufacturer's procedure. A 100 µL cell suspension (1×10^5 cells/mL) was pipetted and incubated for 24 h. The cells were treated with the sample (50 µL) and incubated for 24 h. Caspase-Glo 3/7, 8, and 9 solutions (50 µL) were added to each well,

and the RLU was read after 30 min of incubation using the Multiscan Go microplate spectrophotometer (ThermoFisher Scientific, USA).

4.2.5 Human p53

Cells were seeded (1×10^5 cells/mL) and left to attach overnight. Thereafter, 50 µL of the sample was added, followed by a further 24-h incubation. Subsequently, an extraction buffer was used to lyse the treated cells, following analysis using the Human p53 ELISA kit (Catalogue number BMS256) obtained from ThermoFisher Scientific. The absorbance was read at 450 nm using the Multiscan Go microplate spectrophotometer (ThermoFisher Scientific, USA).

4.2.6 Reactive oxygen species (ROS)

The reactive oxygen species potential of the samples was determined using the ROS-Glo™ H2O2 kit as per the manufacturer's protocol. A 100 µL cell suspension (10 000 cells/well) was pipetted. After attachment, the cells were treated with 50 µL of the sample followed by a 24-h incubation. Thereafter, the hydrogen peroxide (H_2O_2) substrate was added to the cells 6 h before the 24-h completion time. Subsequently, ROS-Glo solution (100 µL) was added to each well and incubated for 20 min. The RLU was read using the Multiscan Go microplate spectrophotometer (ThermoFisher Scientific, USA).

4.2.7 Statistical analysis

All analyses were performed in triplicate, and the data are expressed as mean±standard deviation. Statistical significance ($p<0.0001$) was determined using a two-way analysis of variance (ANOVA), carried out with GraphPad Prism version 10.4.1 (627) for Windows (GraphPad software, San Diego, CA, USA).

4.3 Results and discussion

4.3.1 Cell viability

The flour samples, protein isolate, and camptothecin showed moderate toxicity on the cell viability of HEK293, as presented in Figure 4.1A. Camptothecin, the control depicted cell viability ranging from 34.41-67.72% between 7.81-1000 µg/mL. Moreover, a lower cell viability was observed for camptothecin against A549 cells (Figure 4.1B) with an optimum toxicity of 26.76% at 125

µg/mL. The protein isolate exhibited cytotoxic effects against A549 cells, reducing cell viability to 37.73% at a concentration of 15.62 µg/mL. The IC_{50} values for camptothecin, OSPI, and DOF were 524.85, 21.80, and 168.37 µg/mL, respectively. The protein isolate and defatted flour denoted lower IC_{50} values than camptothecin, suggesting that these could better inhibit the proliferation of A549 cells at a lower dosage in comparison to a known chemopreventative agent.

A similar result was obtained by Ramkisson et al. (2020), where a trypsin hydrolysate from *Amaranthus cruentus* showed a lower IC_{50} value than camptothecin in A549. Furthermore, Sipahli et al. (2021) established that a pepsin hydrolysate derived from *Lablab purpureus* presented an IC_{50} value of 119.60 µg/mL for A549, indicating that OSPI better hinders the proliferation of A549 cells. Additionally, OSPI displayed greater cytotoxicity compared to protein isolates from soybean, black soybean, adzuki bean, and mung bean with IC_{50} values of 431.00, 327.90, 720.40, and 505.10 µg/mL, respectively, for ovarian cancer (Chen et al., 2017). Black soybean protein isolated inhibited 69% of ovarian cancer cells at 600 µg/mL, whereas OSPI inhibited 62.27% at a lower concentration (15.62 µg/mL), suggesting greater therapeutic potential of A549 cells.

The cell viability of camptothecin and OSPI for Hep-G2 ranged from 53.78-84.48% and 80.70-98.96% respectively (Figure 4.1C). The flour (OF) and defatted flour (DOF) showed similar viability between 68.37 and 98.05%. The IC_{50} values for OF, DOF, and OSPI were 234.79, 86.11, and 157.66 µg/mL, respectively, while camptothecin was significantly higher at 551.05 µg/mL. The defatted flour displayed a lower IC_{50} value than black soybean protein at 88.9 µg/mL against SMMC-7721 cells (hepatocellular carcinoma) (Chen et al., 2017), indicating DOF to be slightly more effective as a potential therapeutic agent. In addition, adzuki and mung bean proteins indicated higher IC_{50} values (391.00 µg/mL and 323.60 µg/mL) for SMMC-7721 cells (Chen et al., 2017) compared to OSPI, further highlighting OSPI's therapeutic properties.

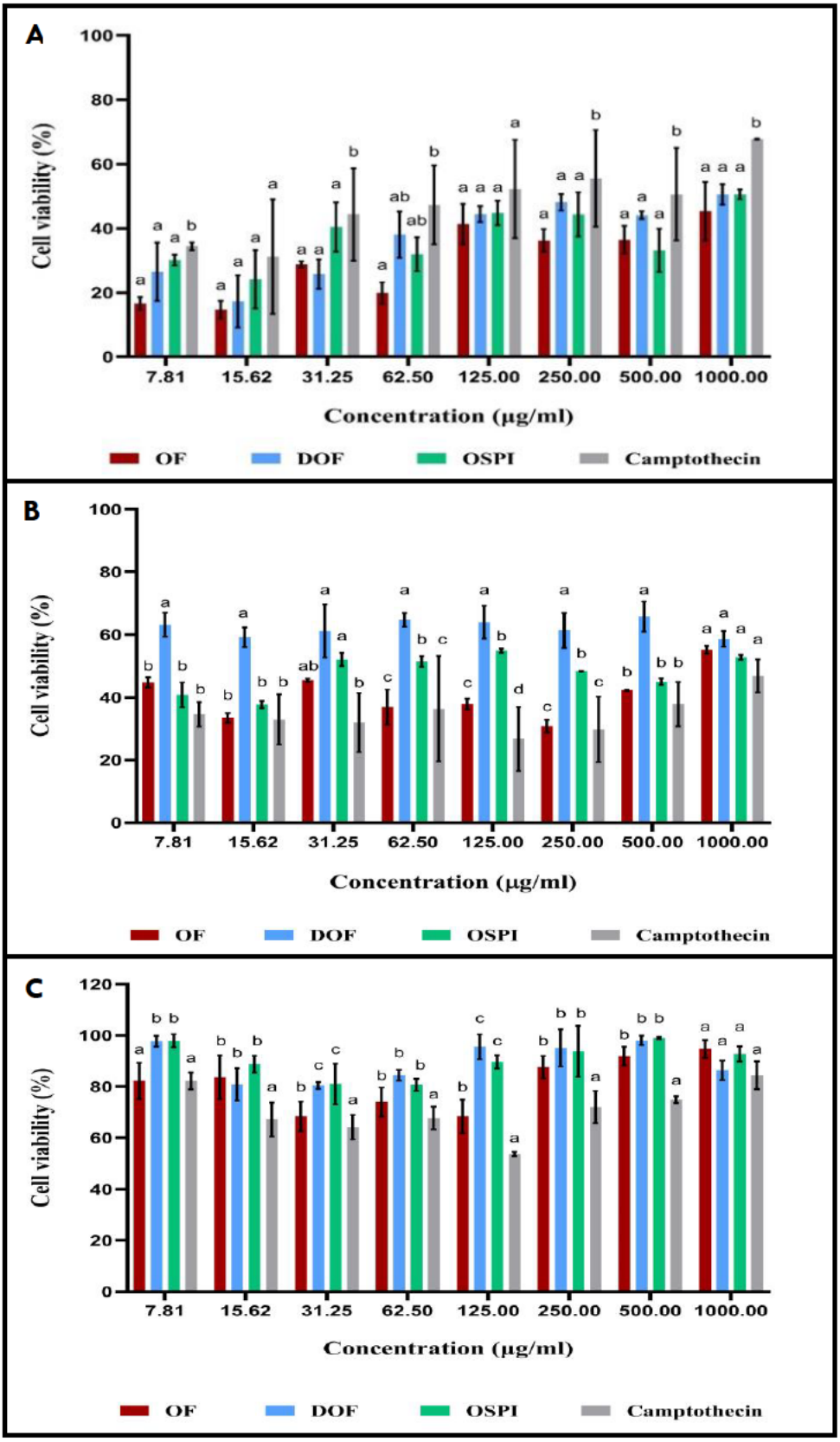

Figure 4.1: The effect of okra flour (OF), defatted okra flour (DOF), okra seed protein isolate (OSPI), and camptothecin at varying concentrations (7.81-1000 µg/mL) on the cell viability of [A - HEK293, B - A549, and C - Hep-G2]. Data denotes mean±SD. Values with different superscript letter indicate statistically significance ($p<0.05$).

4.3.2 Apoptotic induction

The apoptotic induction in HEK293, A549, and Hep-G2 cells was assessed using Annexin V-binding, which quantifies the externalisation of phosphatidylserine (PS) on the outer cell membrane as an indicator for apoptosis (Taniya et al., 2020, Birge et al., 2016). Apoptotic induction of camptothecin occurred in A549 cells with moderate and low induction in HEK293 and Hep-G2, respectively (Figure 4.2). A previous study obtained similar results for camptothecin, which predominantly showed apoptosis in A549 cells (Thumbrain et al., 2020). The protein isolate depicted elevated RLU levels for HEK293 in comparison to the control camptothecin (Figure 4.2), which was previously observed for *Embu buff* protein derived from *Vigna unguiculata (L.) Walp* expressing an increase in HEK293 cells undergoing apoptosis (Thumbrain et al., 2020). This could be attributed to elevated concentrations of the protein isolate, indicating a threshold-dependent effect (Soong et al., 2025). Furthermore, OF denoted the highest apoptotic induction compared to DOF and OSPI in A549, whereas in Hep-G2 cells, all samples exhibited reduced apoptosis compared to camptothecin. This trend was identified in MCF-7 cells treated with *Embu buff* protein (Thumbrain et al., 2020). Moreover, Fuel et al. (2022) alluded that legume flours induced higher apoptosis compared to their protein hydrolysates in colorectal cancer due to antioxidant-polyphenol synergy, correlating to the results of okra flour against A549. Camptothecin induced the highest apoptotic response in A549 and Hep-G2 cells, while OSPI triggered a moderate but selective pro-apoptotic effect in cancer cells with minimal induction in HEK293, suggesting cancer-targeting potential.

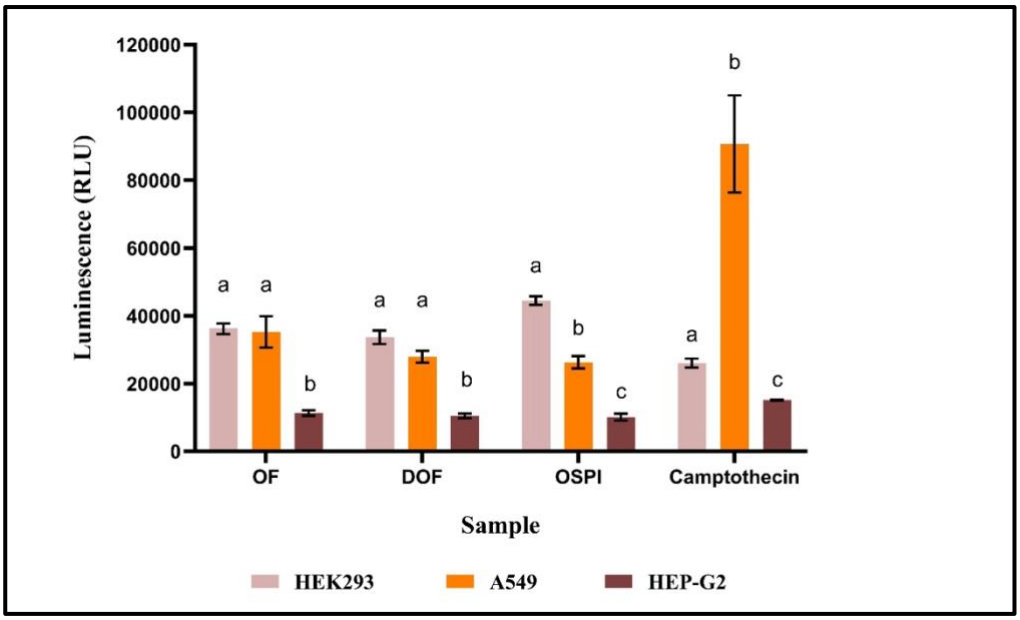

Figure 4.2. Annexin V-based luminescence assay indicating apoptotic induction in HEK293, A549, and Hep-G2 cells following treatment with okra flour (OF), defatted okra flour (DOF), okra seed protein isolate (OSPI), and the chemotherapeutic agent camptothecin. Data denotes mean±SD. Values with different superscript letter indicate statistically significance ($p<0.05$) of each sample mean compared across cell lines within the same row.

4.3.3 Caspase activity

The activation of caspases is a key event that marks the onset of apoptosis (Thumbrain et al., 2020). Caspases 3 and 7 are known as executioner caspases (Mustafa et al., 2024), due to their role in inducing the final phase of apoptosis (Boice and Bouchier-Hayes, 2020). They cleave several structural and regulatory proteins, leading to the loss of cellular function and the distinct morphological changes associated with apoptotic cell death (Boice and Bouchier-Hayes, 2020). As shown in Figure 4.3A, camptothecin generated a high level of caspase 3/7 in Hep-G2 cells, followed by OSPI. This indicates that OSPI could induce apoptosis in Hep-G2 cells similar to camptothecin. In a study by Alzamami et al. (2023), increased caspase 3/7 activity in Hep-G2 cells by compound 5 was attributed to the ability of the compound to promote apoptosis by induction of these executioner proteins. Furthermore, OSPI expressed low levels of caspase 3/7 in HEK293 cells alongside A549 cells, suggesting it is nontoxic to healthy cells and possesses a non-apoptotic effect on A549 cells. Conversely, findings by Ramkisson et al. (2020) and Thumbrain et al. (2020) utilising *Amaranth cruentus* and *Embu buff* protein isolates, respectively, denoted elevated levels of caspase 3/7 in HEK293, indicating the nontoxicity of OSPI. The flour samples OF and DOF exhibit similar levels of caspase 3/7 activation in Hep-G2, whereas they demonstrate low activation in HEK293 alongside OSPI. Additionally, OF exhibited caspase 3/7 activation in A549 cells compared to camptothecin, suggesting it could be utilised to generate apoptosis. Mazumder et al. (2023) established that lupin seed flour triggered apoptosis in colorectal cancer cells by induction of caspase 3/7. The ability of OSPI and OF to stimulate caspase 3/7 in Hep-G2 and A549 cells refers to its antiproliferative activity in therapeutics. Initiator proteases such as caspase 8 and 9 are essential in triggering apoptotic signalling pathways (Mustafa et al., 2024). Caspase 9 especially plays a role in intrinsic apoptosis of mitochondrial signalling pathways, which are activated when cytochrome c is released (Lee et al., 2020, Ren et al., 2024). Moreover, once caspase 9 is initiated, it directly cleaves the activation of caspases 3 and 7 (Brentnall et al., 2013). In this study camptothecin expressed high levels of caspase 9 in Hep-G2 cells (Figure 4.3C), which correlates with the elevated activation of caspase 3/7. Similarly, caspase 8 levels in Hep-G2 cells for camptothecin (Figure 4.3B) were comparable to those of caspase 9. The notable intensities of caspase 8 and 9 induced by camptothecin suggest its effectiveness as an anticancer agent. The protein isolate demonstrated efficiency in activating both caspase 8 and 9 in Hep-G2 alongside OF. Similar results were found in a study by Mutiah et al. (2018) where the activation of caspase 8 by a glycoside terpenoid in *Calotropis gigantea* led to apoptosis in colon cancer cells through the extrinsic pathway. The cleavage of caspase 8 by a compound derived from *Ficus carica* in breast and ovarian cancer cells activated apoptosis (Al-Jadaan, 2020).

Soy and cowpea peptides have been found to induce apoptosis in breast cancer due to G0/G1 arrest via the activation of caspase 9, suggesting OSPI could be beneficial as a therapeutic agent (Zhang et al., 2025b). In MCF-7 cells, aviculin, a ligand-based compound from *Lespedeza cuneata*, initiated apoptosis through mitochondrial signalling, by activation of caspase 9, further highlighting the capability of OSPI. The combination of caspase 3, 8, and 9 activations by OSPI in Hep-G2 cells suggest that it could be effective in intrinsic and extrinsic apoptosis, as observed by Ren et al. (2024) for Hep-G2 cells treated with soy protein isolate.

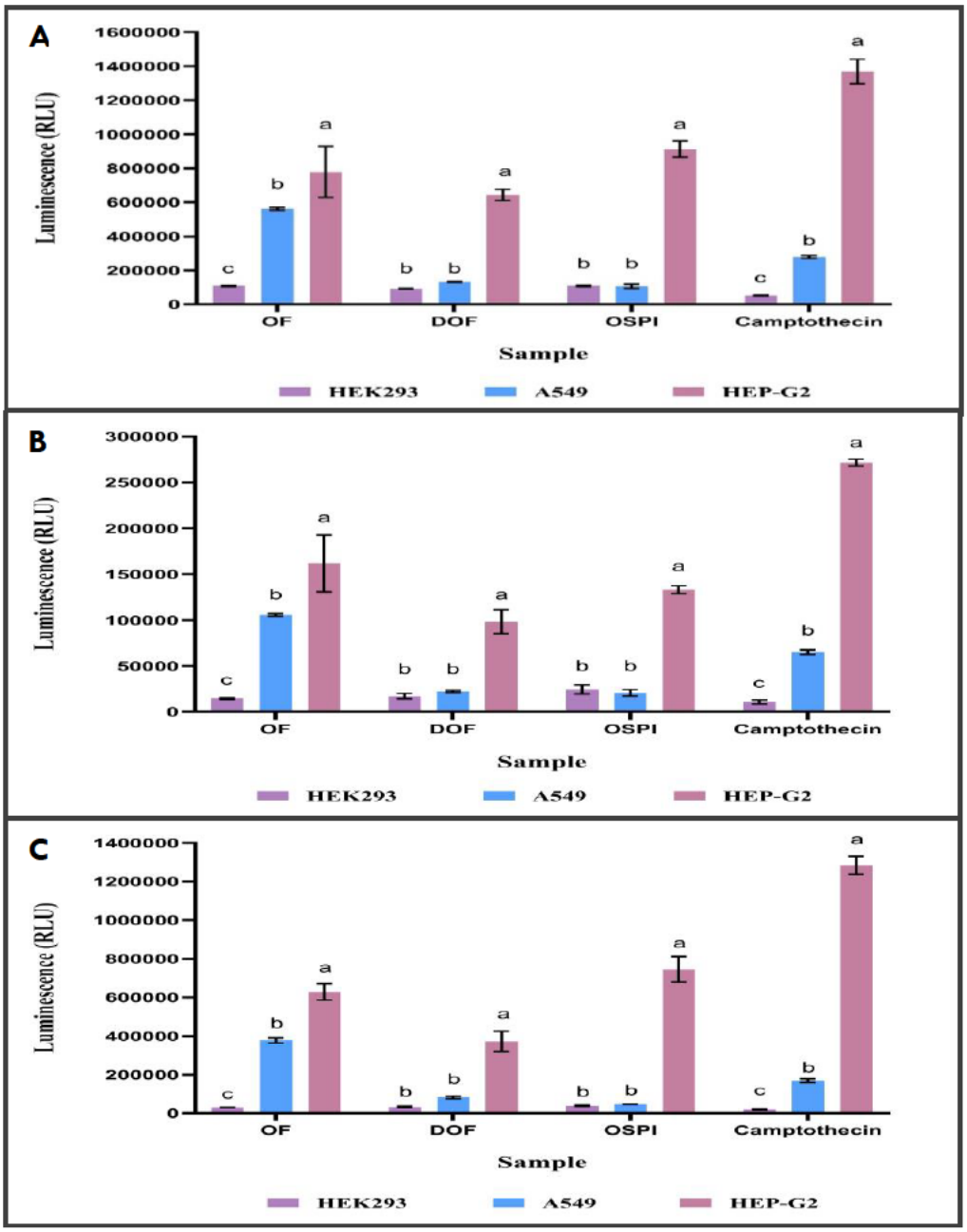

Figure 4.3. Caspase activity showing the activation of (A) caspase 3/7, (B) caspase 8, and (C) caspase 9 in HEK293, A549, and Hep-G2 cells treated with okra flour (OF), defatted okra flour (DOF), okra seed protein isolate (OSPI), and camptothecin. Data denotes mean±SD. Values with different superscript letter indicate statistically significance ($p<0.05$).

4.3.4 Human p53

As a transcription factor, p53 binds to specific DNA response elements. It regulates the expression of numerous target genes that play critical roles in cell cycle arrest, apoptosis, senescence, and other tumour-suppressive processes (Song et al., 2024). In the non-tumorigenic HEK293 cells, which retain functional p53, elevated p53 protein levels (87.90-98.14 µg/mL) were expressed for all sample treatments, including camptothecin (78.46 µg/mL) (Table 4.1), suggesting a response to cellular stress. This does not necessarily link to apoptosis, as p53 can induce temporary growth arrest or the initiation of DNA repair (Green and Kroemer, 2009). In contrast, A549 cells, known to display wild-type p53, showed significant levels of p53, particularly when treated with DOF (34.68 µg/mL) and OSPI (36.06 µg/mL) (Table 4.1). These findings are indicative of potential apoptotic or cell cycle arrest signalling, comparable to a study of plant-derived fenugreek extract demonstrating upregulation of p53, thus promoting apoptosis in MCF-7 cells (Alrumaihi et al., 2021). Camptothecin denoted the highest p53 induction (158.15 µg/mL) in A549 cells, consistent with its established mechanism of activating p53 through the inhibition of topoisomerase I (García et al., 2014). In Hep-G2 cells, all samples expressed low levels of p53, suggesting that apoptosis in Hep-G2 is not activated via p53. Relative luminescence units (RLU) indicate caspase activation as a measure of apoptotic induction. Camptothecin significantly activated all caspase pathways in Hep-G2 cells, confirming its role as an inducer of intrinsic and extrinsic apoptosis.

Table 4.1. p53 protein levels in human cancer cell lines treated with okra flour (OF), defatted okra flour (DOF), okra seed protein isolate (OSPI), and camptothecin

Sample	HEK-293 (µg/mL)	A549 (µg/mL)	Hep-G2 (µg/mL)
OF	87.90±0.11^{a}	9.16± 0.08^{a}	0.09±0.07^{a}
DOF	98.14±0.11^{b}	34.69±0.01^{b}	0.06±0.01^{a}
OSPI	94.46±0.08^{c}	36.07±0.01^{c}	0.06±0.00^{a}
Camptothecin	78.46±0.22^{d}	158.16±0.13^{d}	0.06±0.00^{a}

Data denotes mean±SD. Values with different superscript letter indicate statistically significance ($p<0.05$) of each sample mean compared across cell lines within the same row.

4.3.5 Reactive oxygen species

In both normal and malignant cells, reactive oxygen species (ROS) function as secondary messengers in cellular signalling and are key to multiple biological processes (Biswa Mohan et al., 2021). As presented in Figure 4.4, HEK293 exhibited increased levels of ROS, particularly following treatment with OF and OSPI. The ROS generation surpassed that observed with camptothecin, a well-established oxidative stress inducer. This indicates that the samples may induce oxidative stress in non-cancerous cells due to broad or non-targeted redox interactions (Sies and Jones, 2020). Moreover, this may be due to cellular proliferation and adaptive responses to oxidative stress, as various ROS species are known to modulate key functions such as signal transduction, metabolism, and gene expression (Biswa Mohan et al., 2021, Hong et al., 2024). Okra flour expressed moderate levels of ROS induction in A549 cells, higher than DOF, OSPI, and camptothecin, suggesting OF possesses greater ROS-mediated apoptotic induction. In Hep-G2 cells, all samples denoted low levels of ROS, including camptothecin. According to Gorrini et al. (2013) and Trachootham et al. (2009), internal oxidative stress prompts many cancer cells to adapt, thereby stimulating their innate antioxidant defence system, which results in a lower production of ROS. In a previous study on A549 and Hep-G2 cells, the activation of the Nrf2 pathway enhanced the production of the antioxidant glutathione, contributing to their defence against oxidative stress (Zhang et al., 2018), which could have resulted in the low ROS generation in this study. OF depicted elevated ROS generation in A549 cells, while OSPI moderately elevated ROS in cancer cells.

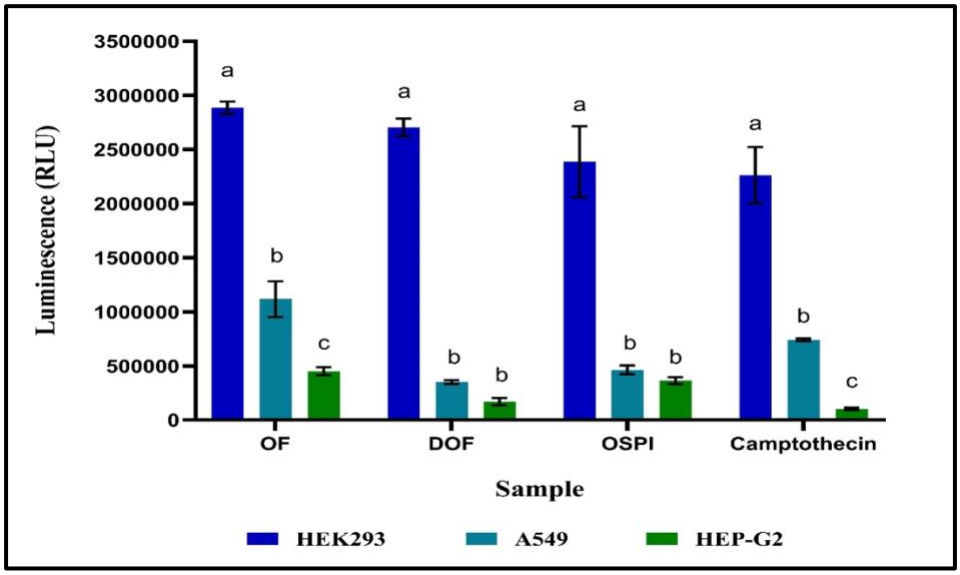

Figure 4.4. Reactive oxygen species (ROS) levels in HEK293, A549, and Hep-G2 cells following treatment with okra flour (OF), defatted okra flour (DOF), okra seed protein isolate (OSPI), and camptothecin. Data denotes mean±SD. Values with different superscript letter indicate statistically significance ($p<0.05$).

4.3.6 Conclusion

This study demonstrated the antiproliferative activity and pro-apoptotic properties of okra seed protein isolate (OSPI) against A549 and Hep-G2 human cancer cell lines. The protein isolate reduced the cell viability of A549 cells at relatively low concentrations (15.62 µg/mL) and further exhibited superior efficacy in comparison to camptothecin. Apoptotic induction via the activation of caspases -3/7, -8, and -9 enhanced expressions of p53, further supporting the role of OSPI in potentially targeting cancer cell survival pathways. The moderate generation of reactive oxygen species (ROS) and increased Annexin V-binding were indicative of the involvement of oxidative stress-mediated apoptosis. Overall, these outcomes propose that OSPI holds value as a potential natural therapeutic agent, with promising applications in both pharmaceutical and nutraceutical development.

Chapter 5: General discussion

Plant-derived proteins have long been recognised for their nutritional value; however, increasing attention is being placed on their bioactive and therapeutic properties, particularly in the realm of chronic diseases such as cancer. These proteins in native, isolated, or hydrolysed forms contain diverse amino acid profiles and structural motifs that may confer immunomodulatory, antiproliferative, and apoptotic effects. There are numerous plant sources of bioactive proteins; among these, okra seed protein isolate (OSPI) has emerged as a potential contender due to its rich composition of essential and functional amino acids, favourable solubility characteristics, and structural properties that enhance its biological interactions. This study explored the bioactive potential of OSPI in both biochemical and cellular frameworks, with a focus on its application in targeting human cancer cells such as A549 (lung carcinoma) and Hep-G2 (hepatocellular liver carcinoma).

The amino acid profile of OSPI denoted a rich concentration of arginine, leucine, glutamic acid, and aspartic acid. Furthermore, a high hydrophobic and branched-chain amino acid (BCAA) composition was observed. These amino acids are involved in immune regulation, metabolic signalling, and cytotoxic activities, indicating OSPI to be ideal for both nutritional and therapeutic interventions. The high hydrophobic amino acids (HAA) (31.39 g/100g) and BCAA (14.24 g/100g) support its capacity for targeted antiproliferative activity, as previously demonstrated in studies on legume based proteins (Ramkisson et al., 2020, Chen et al., 2017). The protein isolate displayed a high solubility (91.86%) at pH 9 and was least soluble at pH 5 near the isoelectric point. The low solubility of proteins near the isoelectric point could be indicative of β-conglycinin as found in soy protein (Chen et al., 2020). In addition, the high solubility is a relevant characteristic in drug delivery and absorption, as enhanced solubility improves bioavailability, a crucial factor in therapeutic applications (Maistry et al., 2025).

Electrophoretic analysis of OSPI showed prominent polypeptide bands corresponding to vicilin (7S), convicilin, and legumin (11S), structural proteins common in legumes. Notably, low molecular weight bands (<20 kDa) were identified, likely representing 2S albumins, which are associated with immunomodulatory effects (Souza, 2020). These albumins are characterised by a high concentration of glutamine, asparagine, and arginine (Souza, 2020), which have previously been highlighted as the predominant amino acids present in OSPI. Moreover, a polypeptide band at 48 kDa indicated the presence of β-conglycinin as previously noted by the solubility analysis, further indicating the therapeutic potential of OSPI.

These structural proteins, in conjunction with the amorphous nature of OSPI observed through X-ray diffraction (XRD), suggest enhanced compatibility in pharmaceutical formulations. The presence of α-helices and β-sheet structures identified by Fourier Transform Infrared Spectroscopy (FTIR) further confirms OSPI's structural stability, supporting its suitability for therapeutic and functional food applications.

The biological efficacy of OSPI was demonstrated through *in vitro* cancer cell assays, particularly against A549 and Hep-G2 cells. The cell viability of A549 showed a significant reduction (37.73%) when treated with OSPI at a concentration of 15.62 µg/mL, with an IC_{50} of 21.80 µg/mL, markedly lower than that of the standard chemotherapeutic agent camptothecin (524.85 µg/mL). The cytotoxic effect demonstrated by OSPI surpassed that of several plant proteins, including black soybean, adzuki, and mung bean (Chen et al., 2017), highlighting the potency of OSPI at low concentrations. In addition, defatted okra flour (DOF) displayed an IC_{50} of 86.11 µg/mL in Hep-G2 cells, suggesting both DOF and OSPI may serve as cost-effective and accessible therapeutic agents.

Apoptotic induction, as determined by Annexin-V binding, confirmed OSPI's ability to stimulate programmed cell death in cancer cells. The highest apoptotic induction was observed in A549 cells by okra flour (OF), likely due to the synergistic effects of polyphenols and antioxidant compounds in the whole flour (Fuel et al., 2022). In contrast, OSPI induced apoptosis in Hep-G2 cells, as evidenced by elevated caspase 3/7 activity, which mirrors apoptotic responses triggered by camptothecin and other established chemotherapeutics (Boice and Bouchier-Hayes, 2020, Alzamami et al., 2023). The ability of both OF and OSPI to selectively activate executioner caspases in cancer cells while not being highly toxic to healthy cells (HEK293) suggests a favourable therapeutic index, enhancing their relevance in drug development. Initiator caspases -8 and -9 were significantly activated in OSPI-treated Hep-G2 cells, confirming the involvement of both extrinsic and intrinsic apoptotic pathways. This dual pathway activation enhances therapeutic versatility, particularly in tumours that may develop resistance to single-pathway agents (McDonald et al., 2025). These findings are supported by previous studies where soy protein and fenugreek extract activated similar apoptotic signalling through caspase cascades and p53-dependent mechanisms (Alrumaihi et al., 2021, Ren et al., 2024) .

The p53 tumour suppressor protein, a central regulator of apoptosis and cell cycle arrest, was notably upregulated in A549 cells treated with OSPI and DOF. This upregulation aligns with previous findings linking plant extracts to p53 activation and cancer suppression (Alrumaihi et al., 2021).

While p53 levels were elevated in non-cancerous cells, these increases may reflect stress response mechanisms unrelated to apoptosis (Green and Kroemer, 2009). The moderate increase of p53 in cancer cells suggests that OSPI may modulate p53-dependent apoptosis, especially in cancer cells that retain wild-type p53, such as A549.

Reactive oxygen species (ROS) analysis further supported OSPI's therapeutic potential. The flour and protein isolate denoted substantial ROS generation in A549 cells, surpassing even camptothecin in some cases, suggesting ROS-mediated apoptotic pathways could be involved. In contrast, ROS levels were low in Hep-G2 cells, possibly due to the activation of the Nrf2 antioxidant defence pathway, as reported in liver cancer cell models (Zhang et al., 2018). These differential ROS responses underscore the specificity of OSPI activity, suggesting it may be optimised for certain types of cancer.

Additionally, particle characterisation exhibited OSPI to possess a small size (0.14±0.10 µm) and a high negative zeta potential (-29.7 mV), indicative of favourable colloidal stability and drug delivery potential. The near-spherical morphology and monomodal particle distribution further support OSPI's use as a carrier in pharmaceutical applications, especially for targeted cancer therapy (Danaei et al., 2018, You et al., 2021).

Conclusion and recommendations

This study provides a comprehensive application for the structural integrity, bioactivity, and therapeutic promise of okra seed protein isolate. Its molecular composition and solubility profile, alongside its cytotoxicity and apoptotic effects on cancer cells, highlight OSPI as a natural, multifunctional bioactive compound with broad potential in cancer therapy, drug delivery, and functional nutrition. Its dual apoptotic pathway activation, selectivity for cancer cells, and biocompatible encapsulation features reinforce its candidacy for further research in the pharmaceutical and nutraceutical industries. However, additional research is required to establish enhanced *in vitro* and *in vivo* validation, synergistic formulations, and targeted delivery systems to harness OSPI's therapeutic potential fully.

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