EXPLORATION OF THE REGULATION OF IMMUNOMODULATORY GENES BY *COMMELINA BENGHALENSIS* AQUEOUS EXTRACT IN BREAST CANCER CELLS

By

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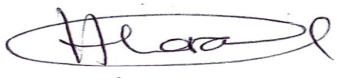
Declaration

I, Hellen Laura Mnisi, hereby, declare that the work submitted in this dissertation to the University of Limpopo for the degree of Master of Science in Biochemistry has not been previously submitted by me at this or any other University. This is my own work by design and in execution, and where use of others work was utilised, it has been duly acknowledged.

Signature : 

Date : *31 October 2024*

Dedication

I dedicate this work to My Lord Jesus Christ and Saviour for his continuous strength, love, peace and grace.

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I would like to kindly extend my gratitude and appreciation to the following:

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Oral and Poster Presentations

Oral Presentation

- Mnisi HL, Mbita Z., and Morafo V. (2022). Investigating the regulation of the immunomodulatory genes in *Commelina benghalensis*-treated breast cancer cells. 13th Faculty of Science and Agriculture Postgraduate Research Day, Bolivia Lodge, Polokwane, South Africa.

Poster Presentations

- Mnisi HL, Laka K and Mbita Z (2021). The effect of cobalt chloride on breast cancer MDA-MB 231 cell line. PACRI Inaugural International Cancer Meeting, Future Africa, University of Pretoria, South Africa.
- Mnisi HL, Mbita Z, Morafo V (2024). Investigating the effects of *Commelina Benghalensis* extracts in Breast Cancer cells. SASBMB Congress 2024, University of Venda.

Abbreviations

A	
APCs	Antigen-Presenting Cells
AIC	Aluminium Chloride
B	
BRCA1	breast cancer 1
BRCA2	breast cancer 2
BCR	B cell Receptor
BPA	Bisphenol A
C	
Cb	*Commelina benghalensis*
CD40/CD40L	Cluster of differentiation 40/ Cluster of differentiation 40 Ligand
CTLA-4	Cytotoxic T-Lymphocyte-Associated protein 4
CEF	Chloroform/Ethyl Acetate/Formic Acid
D	
DCIS	Ductal Carcinoma in Situ
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DMEM	Dulbecco's Modified Eagle Medium
DPPH	2, 2-Diphenyl-1-picrylhydrazyl
E	
EMW	Ethyl Acetate/Methanol/Water
EGCG	Epigallocatechin gallate
EMT	Epithelial-Mesenchymal Transition
ER	Oestrogen Receptor-Positive
F	
FBS	Foetal bovine serum
FC	Folin-Ciocalteu
FRAP	Ferric ion reducing power

G	
GPX3	Glutathione peroxidase 3
H	
HDAC	Histone deacetylase
HER2	Human Epidermal Growth Factor Receptor 2
I	
IDC	Invasive Ductal Carcinoma
IBC	Inflammatory Breast Cancer
ILC	Invasive Lobular Carcinoma
L	
LCMS	Liquid Chromatography-Mass Spectrometry
M	
MTT	3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide
MHC	Major Histocompatibility Complex
N	
NK	Natural Killer
NA_2CO3	Sodium Carbonate
$NaNO_2$	Sodium Nitrite
NaOH	Sodium Hydroxide
NO	Nitric Oxide
P	
PD-L1	Programmed Death-Ligand
PTEN	Phosphatase and TENsin
PBS	Phosphate Buffered Saline
PSN	Penicillin, Streptomycin, and Neomycin
Q	
QE/g	quercetin acid equivalent/gram
ROS	Reactive Oxygen Species
S	
SEM	Standard Error Mean

T

TAE/g	Tannic Acid Equivalents/gram
TEA	Toluene/Ethanol/Ammonia
TNBC	Triple-Negative Breast Cancer
TLC	Thin Layer Chromatography
TNF	Tumour Necrosis Factor
TNF-a	Tumour necrosis factor -alpha
TCRs	T cell receptors
TPC	Total Phenolic Content
TTC	Total Tannin Content
TFC	Total Flavonoid Content
TCR	T Cell Receptor
TREG	Regulatory T cell

U

UV/VIS	Ultraviolet/Visible

W

WHO	World Health Organization

Abstract

Cancer is a noteworthy global health issue, and according to predictions by the World Health Organization, the number of new cases was projected to increase to approximately 29-37 million by 2040, worldwide (WHO, 2020, Sung *et al*., 2021). Female breast cancer has been shown to be the leading cause of global cancer incidence in 2020, with reported 2.3 million new cases, representing 11.7% of all cancer cases (Reyes-Monasterio *et al*., 2022). Treatments such as surgery, radiotherapy, and chemotherapy are used to counteract breast cancer; however, these treatment approaches are now ineffective, which has led to the shift of interest towards the use of medicinal plants such as *Commelina benghalensis (Cb)*, which has been traditionally used in folk medicine to treat various ailments, including inflammation-related conditions. Studies reveal that the plant contains bioactive compounds, such as flavonoids, tannins and phenolics, which possess anti-inflammatory, antioxidant, and antitumor properties (Islam *et al*., 2018; Islam *et al*., 2017; Islam *et al*., 2016). Thus, the aim of the study was to determine the antioxidant and anticancer properties of *Cb* aqueous extract in breast cancer cells.

Breast cancer cell lines, MDA-MB 231 and MCF-7, were cultured and maintained in appropriate growth media. The *Cb* was extracted using only water, and this was done to mimic what the traditional healers do, when they prepare medicinal plants concoctions for medicinal purposes. The profiling of the bioactive compounds was done using Thin Layer Chromatography (TLC), phytochemical screening, and the Liquid Chromatography-Mass Spectrometry (LC-MS). The total amount of phenols, flavonoids and tannin in the *Cb* aq-extract was quantified using the Folin-Ciocalteu Colorimetric Assay. Additionally, the antioxidant activity was done using 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) and free radical antioxidant power (FRAP) assays. Furthermore, the cytotoxicity effect of *Cb* aqueous extract was determined against the breast cancer using 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay. The Muse® Cell Count and Viability assay was done to confirm the MTT results. Morphological changes were observed using light microscopy and the images were captured using computer connected to microscope. Moreover, Annexin V assay was conducted to investigate the potential cell death that may be induced by the *Cb* leaf-aq extract.

The TLC plates demonstrated that the *Cb* root, leaf and stem-aq extract have bioactive molecules. The quantitative phytochemical analysis using Folin-Ciocalteu Colorimetric Assays, showed that the *Cb* aq extracts had high phenolic, flavonoid and low tannin contents Folin-Ciocalteu Colorimetric Assays. Moreover, the *Cb* aq-extracts demonstrated antioxidant properties, and this was mostly observed in the leaf and the root parts of the plant. The LC-MS results of *Cb* leaf-aq extract demonstrated the presence of bioactive compounds that have been previously shown to exhibit anticancer cancer immunomodulatory activities. In this study, three compounds with immunomodulatory effects; namely, Abietic acid, Vorinostat and Lauramide, were identified. These compounds regulate a number of cytokine genes, and these include TGF-*b*1, IGF1R, IFNγ, IL-2, IL-4, IL-10, IL-13. Additionally, the *Cb* leaf-aq significantly (**P < 0.01, ***P <0.001 and ****P <0.0001) reduced the viability of MDA-MB 231 cells after 24- and 48-hours treatment with 500 and 1000µg/ml compared to other parts of the plant. Furthermore, the *Cb* leaf aqueous extract significantly (****P <0.0001) induced apoptosis of MDA-MB-231 cells after their treatment with the IC_{50} concentration (750µg/mL). These findings collectively support the conclusion that the phytocompounds identified in *Cb* have anticancer properties and a potential to modulate immunological responses in breast cancer cells.

1. Introduction

Cancer is a group of diseases characterised by the uncontrolled growth and spread of abnormal cells, which have the ability to invade and destroy normal tissues, potentially affecting various organs and systems (Rahman *et al*., 2022). Female breast cancer has been shown to be the leading cause of global cancer incidence in 2020, with an estimated 2.3 million new cases, representing 11.7% of all cancer cases (Reyes-Monasterio *et al*., 2022). Normally, cells in the body grow, divide, and die in a controlled, programmed manner and forming new cells needed; however, in cancer, this process is disrupted; and thus, cells continue to grow and divide uncontrollably, leading to the formation of tumours or cell masses within tissues (Pena-Romero and Orenes-Pinero*,* 2022). The tumours can interfere with the normal functioning of tissues organs and, in some instances, spread to other parts of the body through a process called metastasis (Saini *et al*., 2020).

There are many different types of cancers, each with its own characteristics, behaviour, and treatment options, one of which is breast cancer (Mertz *et al*., 2022). Breast cancer remains a significant global health burden, affecting millions of women, worldwide, and representing a leading cause of cancer-related deaths (WHO, 2023). According to the World Health Organization (WHO, 2017), breast cancer accounts for approximately 25% of all cancer cases in women, globally. Despite significant advancements in breast cancer research and treatment, the complexity and heterogeneity of this disease pose ongoing challenging (Cardoso *et al*., 2022).

The conventional treatment approaches for breast cancer have shown efficacy in many cases; however, some patients experience treatment resistance or encounter severe side effects that negatively impact their quality of life (Lai, 2023). Further, metastatic breast cancer remains challenging to manage, and is associated with a lower survival rate (Lim and Hortobagyi, 2016). As a result, there is a growing interest in exploring alternative and complementary approaches to effectively combat breast cancer (Bhetia and Mokashi, 2023). Among these approaches, immunotherapy has emerged as a promising strategy (Donatova *et al*., 2022). Immunotherapy harnesses the body‘s immune system to recognise and attract cancer cells, selectively (Jeanbart *et al.,* 2015). This approach has revolutionised cancer treatment in various malignancies, including melanoma, lung cancer and bladder cancer (Bielcikova, *et al*., 2022).

In recent years, immunotherapy has shown encouraging results in breast cancer treatment, particularly in subtypes with high levels of infiltration by immune cells, such as Triple-Negative Breast Cancer (TNBC) [Gupta *et al*., 2022]. Immune checkpoint inhibitors, monoclonal antibodies that block certain proteins on cancer cells and immune cells, have shown potentials in enhancing the immune response against breast cancer cells (Kumar *et al*., 2021). However, despite these advances, not all breast cancer patients respond to immunotherapy, highlighting the need to explore additional anticancer

immunomodulatory agents, especially from medicinal plants that can complement existing treatment strategies (Patel *et al*., 2020).

1.1. *Commelina benghalensis*

Natural compounds derived from plants, like *Commenlina benghalensis (Cb)*, also known as Wandering Jew, or Khotsoana in South Sotho, have gained attention for their potential anticancer properties (Gupta *et al*., 2022 and Kumar *et al*., 2021). *Cb*, a widely distributed weed, has been traditionally used in folk medicine to treat various ailments, including inflammation-related conditions. Studies have revealed that the plant contained a diverse array of bioactive compounds, such as flavonoids, tannins and phenolics, which possess anti-inflammatory, antioxidant, and anti-tumour properties (Islam *et al*., 2018, Islam *et al*., 2017 and Islam *et al*., 2016). If *Cb* has potent antioxidant activity and demonstrates anti-cancer activities against breast cancer cells, it may also offer a complementary therapeutic approach to enhance the immune response against tumour cells, potentially improving treatment outcomes and patient survival rates.

1.2. *Phytochemicals and immunomodulation*

Phytochemicals are bioactive compounds found in plants that can have various effects on human health (Thakur *et al*., 2020). Some phytochemicals have been studied for their potentials to modulate the immune system, where Immunomodulation provides the ability to enhance or suppress immune responses as needed to maintain immune balance and function (Behl *et al*., 2021). Phytochemicals with potential immunomodulatory effects include flavonoids, alkaloids, terpenes, polysaccharides, and other compounds (Ali Reza *et al*., 2023). These compounds may influence immune cells, cytokines (cell signalling molecules) and other components of the immune response (Hussain *et al*., 2023).

1.3. *Problem Statement*

Cancer remains one of the leading causes of death worldwide, and one of the problematic cancers amongst women, is breast cancer (Siegal *et al*., 2015). A recent study on cancer statistics has shown that breast cancer has exceeded lung cancer as the leading cancer, with 2.3 million new cases reported, worldwide (Sung *et al*., 2021). The increasing numbers can be attributed to the lack of effective anticancer drugs and late diagnosis. The current treatment strategies, which include chemotherapy, radiotherapy, and surgery, have several side effects, lack specificity and efficacy (Gavamukulya *et al*., 2014). New treatment strategies that are safe with fewer or no side effects are needed, and one of which could be the use of medicinal plants. Thus, medicinal plants are gaining popularity in cancer research as potential sources of innovative anticancer therapeutics (Wangchuk, 2018). Additionally, a number of anticancer agents have been reported to modulate immune responses, which have been shown to increase their anticancer therapeutic effects (Petroni *et al*., 2021). One of the medicinal plants that has shown

potential anticancer activities is *Cb*; however, its anticancer mechanism of action remains less understood (Shakya, 2016).

1.4. *Aim of the study*

The aim of the study was to determine the potential bioactive compounds from *Commelina benghalensis* aqueous extract that can regulate immunomodulatory genes implicate in anticancer activities.

1.5. Objectives of the study

The objectives of the study were to:

i. profile the bioactive compounds within the *C. benghalensis* aqueous extract using the thin layer chromatography (TLC),
ii. qualitatively characterise the bioactive compounds using phytochemical screening,
iii. quantitatively evaluate the total phenolic, flavonoid and tannic content in the *C. benghalensis* aqueous extract,
iv. quantitatively assess potential antioxidant activity of *C. benghalensis* aqueous extract using both 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and Ferric ion reducing power (FRAP) assays,
v. culture and maintain MDA-MB 231 and MCF-7 breast cancer cell lines,
vi. determine the cytotoxicity of *C. benghalensis* aqueous extract against breast cancer cells MDA-MB 231 and MCF-7 using both the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) to determine the IC_{50} of the *C. benghalensis* aqueous extract,
vii. confirm the IC_{50} of *C. benghalensis* aqueous extract using the Muse cell count and viability assay
viii. analyse the effect of the *C. benghalensis* aqueous extract on the morphological changes on breast cancer cells using light microscopy,
ix. determine the molecular mechanisms of *C. benghalensis* aqueous extract on the human breast cancer cells by analysing cell cycle arrest and apoptosis, and
x. identify phytochemicals with immunomodulatory effects using Liquid Chromatography–Mass Spectrometry (LC-MS).

1.6. *Organisation of the thesis*

This dissertation is structured into five chapters; namely, the introduction (Chapter one), literature review (Chapter two), methodology (Chapter three), results (Chapter four), discussion and conclusion (Chapter five), and list of references. The summary of each chapter is written below:

1.7.1. Summary of Chapter one

This chapter elicits the background on cancer, specifically, breast cancer, as one of the problematic diseases mostly effecting women, worldwide. This chapter also introduces natural compounds, which are derived from plants, such as *Commelina benghalensis (Cb)*, which have gained attention due to their potential anticancer and antioxidant properties that can offer complementary therapeutic approach to enhance the immune response against tumour cells; potentially, improving treatment outcomes and patient survival rates. The chapter also includes the aims and the objectives of the study.

1.7.2. Summary of Chapter two

This chapter provides a review on the causes, symptoms, types and treatments of breast cancer, leading to the understanding of how medicinal plants or natural compounds containing active biomolecules may impact breast cancer; for an example, flavonoids found in different plants have antioxidant, anti-inflammatory and anti-cancer effects.

1.7.3. Summary of Chapter three

This chapter covers all the materials, methodologies and all the procedures followed and performed to accomplish the aim and the objectives of this study. These methods include Thin Layer Chromatography (TLC), Liquid Chromatography-Mass spectrometry (LC-MS), phytochemical analysis of the bioactive compounds present in *Commelina benghalensis* Cell viability assays, morphological analysis and Annexin V assay for apoptosis analysis.

1.7.4. Summary of Chapter four

This chapter covers all the outcomes that were observed and analysed to address the objectives of the study.

1.7.5. Summary of Chapter five

This chapter provides the discussion, interpretation, analysis and explanation of the results that were obtained in chapter four conclusion of the research project.

2. Introduction

Breast cancer remains a prevalent and serious health concern, demanding continued research for novel therapeutic strategies. One promising avenue lies in harnessing the body's immune system to combat cancer cells. This literature review delves into anticancer and immunomodulatory potential of *Commelina benghalensis (Cb)*, a medicinal plant traditionally used in various culture, specifically focusing on its effect on breast cancer cells.

2.1. *2.1. Cancer*

Cancer is a group of diseases characterised by the uncontrolled growth and spread of abnormal cells in the body (Bhole *et al*., 2021). These abnormal cells can form tumours and invade nearby tissues, impairing the normal functioning of organs and systems (Pan and Winkler, 2022). Cancer can occur in virtually any part of the body and has a significant impact on health and mortality, worldwide (Arnold *et al*., 2020). The most common types of cancer, worldwide, include breast, lung, colorectal, prostate, stomach, and liver cancers and vary in incidence based on factors such as geography, genetics, and lifestyle (Hamdi *et al*., 2021).

2.2. *2.2 Breast Cancer*

Breast cancer is a complex disease that arises from the abnormal growth and behaviour of cells within the breast tissue being one of the most prevalent cancers, worldwide, and predominantly affects women, although men can also develop breast cancer, albeit at a lower incidence rate (Francies *et al*., 2020). The disease originates in the breast, a part of the body primarily composed of glandular tissue responsible for producing milk, and various supportive structures (Ciesla *et al*., 2020). Breast cancer begins when certain cells within the breast tissue undergo genetic mutations or changes that disrupt their normal regulation, these mutated cells begin to divide and multiply at an accelerated rate, leading to the formation of a mass or lump called a tumour (Singh and Ali. 2023). This uncontrolled proliferation of cells is a hallmark of cancer. As the abnormal cells continue to grow, they can accumulate to form a tumour which can be classified as benign or malignant (Boutry *et al*., 2022). Benign tumours are non-cancerous and typically do not spread to other parts of the body and on the other hand, malignant tumours are cancerous and have the potential to invade nearby tissues and spread to distant sites through the bloodstream or lymphatic system (Allugunti., 2022). Cancer cells from the primary tumour can break away and enter either the bloodstream or the lymphatic vessels, which are part of the body's immune system and these circulating cancer cells, known as metastatic cells, Breast cancer can manifest in various ways, hormones like oestrogen and progesterone can play a significant role in the growth of certain types of breast cancer,

leading to the development of hormone receptor-positive tumours (Satpathi *et al*., 2023) Early detection is crucial for improving breast cancer outcomes.

2.3.1. Causes of breast cancer

Breast cancer is a complex disease resulting from a combination of genetic, environmental, hormonal, and lifestyle factors. Genetic mutations such as BRCA1 and BRCA2, are associated with an increased risk of breast cancer (Mavaddat *et al*., 2010). Mutations in these genes impair the body's ability to suppress tumour formation and repair damaged DNA. Reproductive history impacts breast cancer risk such as late age at first childbirth, nulliparity (never giving birth), and shorter periods of breastfeeding have been linked to an increased risk, potentially due to hormonal fluctuations (Mitrunen and Hirvonen, 2003). Exposure to ionising radiation, especially during early life or certain medical procedures, can damage DNA and increase the likelihood of genetic mutations that initiate breast cancer. Genetic predisposition, hormonal influences, environmental exposures, reproductive history, lifestyle choices, radiation exposure, and hormone replacement therapy all contribute to the development of breast cancer (Mukherjee *et al*., 2013 and Manouchehri *et al*., 2022).

2.3.2. Symptoms of breast cancer

A palpable lump or mass in the breast is one of the most common early signs of breast cancer, the lump may feel different from surrounding tissue and should be evaluated by a healthcare professional (Marini *et al*., 2022 and Schaper *et al*., 2020) Unexplained changes in the size, shape, or contour of the breast could indicate the presence of a tumour and such changes may be visible or noticeable upon touch (Sousa *et al.,* 2022). In some individuals, persistent redness, warmth, or unusual colour changes on the breast may be an indication of underlying issues. Also, changes in the appearance of the nipple, such as inversion (turning inward), scaling, or crusting, warrant medical attention (Mitchell and Johnson, 2022). Awareness of the above-mentioned symptoms is crucial for the early detection and prompt management of breast cancer. While the signs and symptoms may not necessarily indicate breast cancer, however, they should not be ignored. Thus, regular self-examinations, clinical breast exams, and mammograms are essential and encouraged for timely breast cancer diagnosis and treatment.

2.3.3. Types of breast cancer

Ductal Carcinoma *in Situ* (DCIS) is a non-invasive type of breast cancer where abnormal cells are confined to the milk ducts and have not spread into surrounding tissues (Bassey *et al*., 2022). Invasive Ductal Carcinoma (IDC) is the most common type of invasive breast cancer which originates in the milk ducts and invades nearby breast tissues. It may present as a lump, skin changes, or other symptoms (Charishma *et al*., 2020). Invasive Lobular Carcinoma (ILC) begins in the milk-producing glands (lobules) and can spread to nearby tissues (Offiah *et al*., 2023). Inflammatory Breast Cancer (IBC) is a rare but

aggressive form of breast cancer which often causes the breast to become red, swollen, and tender. These changes are due to cancer cells blocking lymph vessels in the skin (Irakleidis and Tan, 2019). Accurate diagnosis, subtype identification, and personalised treatment approaches are essential for optimizing patient outcomes.

2.3.4. Breast cancer treatment

Lumpectomy, also known as breast-conserving surgery, is a procedure that involves the removal the “lump” tumour while preserving the surrounding healthy tissue (Puls *et al*, 2023). Mastectomy on the other hand involves the complete removal of the breast tissue. Radiation Therapy, involves the focused use of high-energy rays to target and destroy cancer cells (Alati *et al*, 2023). Chemotherapy involves the use of drugs to kill or slow the growth of cancer cells and it is administered systemically, affecting the entire body (Seynhaeve *et al*, 2020). Hormone Therapy targets hormone receptor-positive (HR+) breast cancers by blocking the effects of oestrogen or progesterone (Bai *et al*., 2021). Hormone therapy is commonly used for HR+ cancers and may involve medications like tamoxifen or aromatase inhibitors. Breast cancer treatment is a comprehensive and evolving field that combines surgical, radiation, medical, and targeted therapies to effectively manage the disease (Ahn *et al*, 2023). While these advanced treatments are pivotal in the fight against breast cancer, it's also worth exploring alternative and complementary approaches that have historically supported health and healing. One such area is the use of medicinal plants, which have been relied upon for centuries for their therapeutic properties.

2.3.5. Medicinal plants

Before chemical or pharmaceutical medicines, man relied on the healing properties of medicinal plants and people valued these plants due to the ancient belief that plants were created to supply man with food because of their nutritional value and medicinal capacity to treat a lot of many medical conditions and ailments (Al-Snafi, 2015). Plants have formed the basis of sophisticated traditional medicine systems that have been in existence for thousands of years and continue to provide mankind with new remedies (Gurib-Fakim, 2006). Medicinal plant practitioners recognise that respecting plants is often essential to the efficacy of medicines, which should not be seen as ‘miracle’ cures based on chemical compounds, but due to curative energy that draws its medicinal qualities founded on a relationship between the plants and the people (Shinwari, 2010).

Medicinal plants have been used for health care since time immemorial and studies have been carried out globally to verify their efficacy and some of the findings have led to the production of plant-based medicines (Sofowora *et al*., 2013). Medicinal plants have been playing an essential role in the development of human culture (Eshete and Molla, 2021). They have always been at the forefront of virtually all cultures of civilizations and they are regarded as rich sources of traditional medicines (Khan, 2014). These plants many of the

modern medicines are developed and produced (Dar *et al*., 2017). Medicinal plants are a source of herbal drugs as they contain specific active biomolecules modulating biological functions (Tadese *et al*., 2020).

Most people on this planet still rely on their traditional *Materia medica* (medicinal plants and other materials) for everyday health care needs (Gurib-Fukim, 2006). Healing with medicinal plants is as old as mankind itself and the connection between man and his search for drugs in nature dates from the far past, of which there is ample evidence from various sources (Petrovska, 2012).

2.3.6. The immune system and immune response

The immune system is a complex network of cells, tissues, and molecules that work together to defend the body against harmful pathogens, such as bacteria, viruses, and parasites, while also recognizing and eliminating abnormal cells and maintaining tissue homeostasis (Moghaddam *et al*., 2018; Yu *et al*., 2020; Hirayama *et al*, 2018). It plays a critical role in maintaining overall health and protecting against infections and diseases. However, in certain circumstances, the immune system can become imbalanced or dysregulated, leading to immune deficiencies, autoimmune diseases, chronic inflammation, or hypersensitivity reactions (Goto & Kiyono, 2011).

The innate immune system is the body's first line of defence against pathogens because it is a rapid and non-specific response that provides immediate protection. It includes physical barriers like the skin, mucous membranes, and chemical defences such as antimicrobial substances and inflammation. The adaptive immune system is a more specialized and targeted response that develops over time (Starling and Mcclure, 2023 and Spiering, 2015).

The adaptive immune system is composed of two components (humoral and cell-mediated) that recognise specific pathogens and mounts a tailored response. The one component, made up of B cells, produce antibodies that bind to specific antigens on pathogens, marking them for destruction by other immune cells and this process is known as humoral immunity. The other component, made up of T cells, which play a crucial role in cell-mediated immunity by recognising infected cells and destroying them or release of signals that activate other immune cells (Chaplin, 2003, Chaplin, 2010 and Vaziri *et al*, 2012).

The immune system consists of different immune responses which are the antigen recognition that recognises foreign substances called antigens (proteins, carbohydrates) present on pathogens. The immune cell activation which upon antigen recognition, will result in immune cells, such as macrophages, dendritic cells, and B cells, becoming activated. These activated cells engulf pathogens, process and present antigens to T cells, or lead to the production of antibodies (Chaplin, 2010, Chistiakov *et al*, 2015)

T cells receive signals from antigen-presenting cells (APCs) and become activated and it involves the interaction between T cell receptors (TCRs) and antigen-MHC complexes

presented by APCs (Shevach *et al,* 2006). Antibody Production where B cells recognize and bind to antigens, triggering their activation and differentiation into plasma cells. Plasma cells produce and release large quantities of antibodies, also known as immunoglobulins that can neutralize pathogens or target them for destruction (Akkaya *et al*., 2019).

Inflammatory response, where Inflammation is a key component of the immune response that involves the release of chemicals, such as cytokines and chemokines that attract immune cells to the site of infection or tissue damage. Inflammation helps eliminate pathogens and promote healing. The last immune response is the memory and long-term protection, one significant aspect of the adaptive immune response is the development of immunological memory (Munn, 2017 and Chen *et al*., 2018) Memory B and T cells are formed during the initial immune response, enabling the immune system to respond more effectively upon subsequent encounters with the same pathogen (Marshall *et al*., 2018).

2.3.7. Immune response modulation

This concept involves the altering or influencing of the immune response in a controlled manner and the manipulation of the immune system to achieve desired outcomes, such as enhancing immune responses to fight infections or cancer, suppressing excessive immune reactions in autoimmune diseases, or achieving immune tolerance in transplant recipients (Dhabher, 2009 and Dhabhar, 2014).

Immune modulation can be achieved through various approaches, including the use of immunomodulatory drugs, biologics, vaccines, or natural products that possess immunomodulatory properties. These approaches aim to selectively target immune cells, molecules, or signalling pathways to modulate immune responses (Vargason *et al*., 2021). For example; Turmeric (*Curcuma longa*) contains curcumin, which has anti-inflammatory and antioxidant properties. It modulates the immune response by inhibiting pro-inflammatory cytokines (Hewlings *et al*., 2017).

The goals of immune modulation can vary depending on the specific context (immunosuppression or immunostimulation). Some common objectives include, boosting the immune response to enhance the ability to fight infections, such as stimulating the production of antibodies or activating cytotoxic T cells, suppressing the immune response to prevent or treat autoimmune diseases, reduce inflammation, or prevent organ rejection in transplant patients (Kennedy and Celis, 2008 and Kang and Min, 2012). This can be achieved using immunosuppressive drugs or biologics, promoting immune tolerance to prevent or treat hypersensitivity reactions, such as allergies, or autoimmune diseases. This involves modulating the immune system to prevent inappropriate immune responses against harmless antigens or self-tissues.

2.3.8. Different types of immune modulators

There are various types of immune modulators that can be used to regulate the immune response. These modulators can either enhance or suppress immune activities, depending on the specific therapeutic goal. The immune modulators include immunomodulatory drugs which are designed to target specific components of the immune system; thus, these drugs can be used to enhance or suppress immune responses (Julier *et al*., 2017 and Hancock *et al*., 2012).

Second, the utilisation of monoclonal antibodies (mAbs), which are laboratory-produced antibodies that can specifically target and bind to certain molecules on immune cells or pathogens. They can be used to block or activate immune cell functions, alter signalling pathways, or directly target antigens involved in diseases, such as cancer (Faqihi *et al*., 2021).

Thirdly, cytokines, which are small molecular weight proteins functioning as messengers of the immune system and are also involved signalling, are produced by immune cells that regulate immune responses, such as fibroblasts, T helper cells and macrophages. Cytokines can be used therapeutically to enhance or suppress immune activities. For example, interleukins can stimulate immune cell growth and activation, while tumour necrosis factor (TNF) inhibitors can reduce inflammation in autoimmune diseases since TNF may be involved in inflammation-associated carcinogenesis. Some research is focusing on the immunomodulatory peptides, which modulate immune responses and have immunosuppressive or immunostimulatory effects and explore their potential for therapeutic applications, such as treating autoimmune diseases or enhancing immune responses against infections (Franchimont, 2006 and Dorota *et al*., 2014).

2.3.9. Pathways for immune-modulators

Immune modulation involves the modulation or regulation of various pathways within the immune system whose outcome or result may be enhancement or suppression of the immune response. These pathways can be targeted to enhance or suppress immune responses based on the specific therapeutic goals (Catanzaro *et al*., 2020). One of the key pathways involved in immune modulation is the cytokine signalling pathway which involve small molecular weight proteins, cytokines that function as signalling molecule that play a crucial role in immune responses. Immune modulation can target cytokine signalling pathways to modulate immune cell functions and balance pro-inflammatory and anti-inflammatory responses (Tili *et al.,* 2013).

T cell signalling pathways play a critical role in cell-mediated immunity and orchestrating immune responses, especially the T helper cells. Modulating T cell signalling pathways can regulate their activation, proliferation, and differentiation (Nazari *et al*., 2021). Key pathways include the T Cell Receptor (TCR) signalling pathway, co-stimulatory pathways and checkpoint pathways which can be targeted to enhance or suppress T cell responses.

B cell signalling pathways are involved in humoral immunity and antibody production. Modulating B cell signalling pathways can influence B cell activation, differentiation into plasma cells and antibody production and secretion. Key pathways include B Cell Receptor (BCR) signalling, co-stimulatory pathways (e.g., CD40/CD40L), and cytokine signalling pathways.

2.3.10. Phytochemicals as immunomodulators

Phytochemicals are naturally occurring compounds in plants that has been studied for their potential health benefits, including immune-modulating properties (Azmat *et al*, 2023). Phytochemicals are bioactive compounds found in plants that have the potential to modulate the immune system. These natural compounds have been studied for their immunomodulatory effects and their ability to influence immune responses (Jantan *et al*., 2015). Examples of phytochemicals that have been identified as immunomodulatory are Curcumin found in turmeric, which has been shown to possess anti-inflammatory properties and can modulate immune responses by inhibiting pro-inflammatory cytokines and enzymes (Tan and Vanitha, 2004; Mohamed, 2019).

Resveratrol, which is a polyphenol, found in grapes, berries, and other plants, has been shown to have antioxidant and anti-inflammatory properties and thus the ability to regulate immune cell function and modulate inflammatory responses (Vasil *et al*., 2014). Quercetin, which is a flavonoid present in various fruits, vegetables, and herbs, has antioxidant and anti-inflammatory effects and has been reported to influence immune responses, including the modulation of cytokines and immune cell activities (Mlcek *et al*, 2016).

Epigallocatechin gallate (EGCG) is a catechin found in green tea and has been studied for its immunomodulatory properties, including anti-inflammatory effects and regulation of immune cell functions (Kuo *et al*., 2014). Ginsenosides, active compounds found in ginseng have been shown to possess immunomodulatory effects, such as enhancing immune cell activity, regulating cytokine production, and influencing immune responses (Kang and Min, 2012).

Echinacea, a herb known for its immune-enhancing properties, contains various phytochemicals, including alkamides, polysaccharides, and caffeic acid derivatives, has been reported to modulate immune cell functions and enhance immune responses (Kumar and Ramaiah, 2011). Lastly, Astragalus, is a traditional Chinese herb known for its immune-modulating effects due to the polysaccharide found in it and has been studied for their potential immunomodulatory properties, including enhancing immune cell function and promoting immune responses (Liu *et al*, 2017).

2.3.11. Phytochemical immunomodulation in breast cancer

Immunomodulation by phytochemicals has been studied for their potential role in breast cancer treatment. Breast cancer is a complex disease involving dysregulation of the immune system (Yap *et al*., 2021). Phytochemicals derived from plants have shown promising immunomodulatory effects that can potentially be harnessed to influence the immune response against breast cancer cells. Aspects of phytochemical immunomodulation in breast cancer include, the immune activation, phytochemicals like curcumin, resveratrol, and quercetin have been found to activate immune cells, including Natural Killer (NK) cells, T cells, and macrophages, in breast cancer. These compounds can enhance the immune response against breast cancer cells and potentially improve the body's ability to recognise and eliminate tumour cells (Samec *et al*., 2020, Vinay *et al*., 2015 and Alhazmi *et al*., 2021).

One of the influences that phytochemicals had on breast cancer cells was reported to be anti-Inflammatory effects against chronic inflammation associated with breast cancer progression, hence many phytochemicals' anti-inflammatory properties could help modulate inflammatory pathways in breast cancer thus reducing inflammation. These phytochemical compounds could assist in creating a non-toxic and immune-friendly environment that could inhibit tumour growth and metastasis (Ahmed *et al*., 2017*)*. Some phytochemicals, like resveratrol and green tea catechins, have antioxidant properties that can neutralize reactive oxygen species (ROS) and oxidative stress. This antioxidant activity may protect immune cells from damage and promote their optimal function in combating breast cancer disease (Salgado *et al*., 2019).

Certain phytochemicals influence immunomodulatory cytokine production, such as interleukins and interferons, which play a crucial role in immune regulation. These compounds may modulate cytokine signalling pathways and promote an immune response that can target breast cancer cells (Behl *et al*., 2021; Brindha, 2016). Phytochemicals like epigallocatechin gallate (EGCG) and resveratrol have shown to have the ability to induce immunogenic cell death in breast cancer cells. This process stimulates the release of tumour-associated antigens and activates immune responses against the tumour, potentially enhancing the effectiveness of immunotherapy (Casey *et al*., 2015; Deng *et al*., 2020).

Regulation of immune checkpoints, such as programmed death-ligand 1 (PD-L1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), can suppress immune responses against breast cancer (Zhang *et al*., 2021 and Pandey *et al*., 2022). Some phytochemicals, including curcumin and resveratrol, have been studied for their ability to modulate these checkpoints and potentially restore anti-tumour immune responses (Lee *et al*., 2021).

2.3.12. Immunomodulation mechanism and action

Immunomodulation is basically a process which can alter the immune system of an organism and this interference results in either immunostimulation or an enhancement of immune response or immunosuppression, which may result in the reduction or diminished response to infections, environmental and or chemotherapeutic factors (Kumar and Yadav, 2022). Modulation of the immune system denotes any change in the immune response that can involve induction, expression, amplification or inhibition of any part or phase of the immune response. Thus, immunomodulation is a course of mechanism that can be explored and utilised in research for its effect on the immune system (Shantilal, *et al*., 2018).

The immunomodulatory activity of targeted anticancer agents can originate from the interaction of the drug with cancer cells, as well as from the ability of the drug to interact with, and alter the function of, immune cells (Galluzzi *et al*., 2017). Both these general mechanisms of immunomodulation by targeted anticancer therapy can involve direct (i.e checkpoint inhibition and targeted therapy with immunogenic tumour antigens) or indirect (i.e., inhibition of tumour-induced immunosuppression) pathways.

Phytochemicals, natural compounds found in plants, have been shown to influence apoptosis as shown in Figure 2.1 and immune responses in cancer therapy. Like targeted anticancer agents, certain phytochemicals can mediate net immunostimulatory effects by promoting the secretion of anti-inflammatory cytokines or limiting the release or activity of immunosuppressive factors. This immunomodulation can enhance the efficacy of cancer treatments by facilitating the destruction or elimination of malignant or immune cells. Consequently, phytochemicals may contribute to triggering a highly immunogenic variant of cancer cell death, similar to the cancer-immunity cycle initiated by targeted anticancer agents (Galluzzi *et al*., 2017). Additionally, some phytochemicals can selectively promote the depletion of immunosuppressive cells, such as regulatory T (TREG) cells, which are known to favour disease progression (Galluzzi *et al*., 2018).

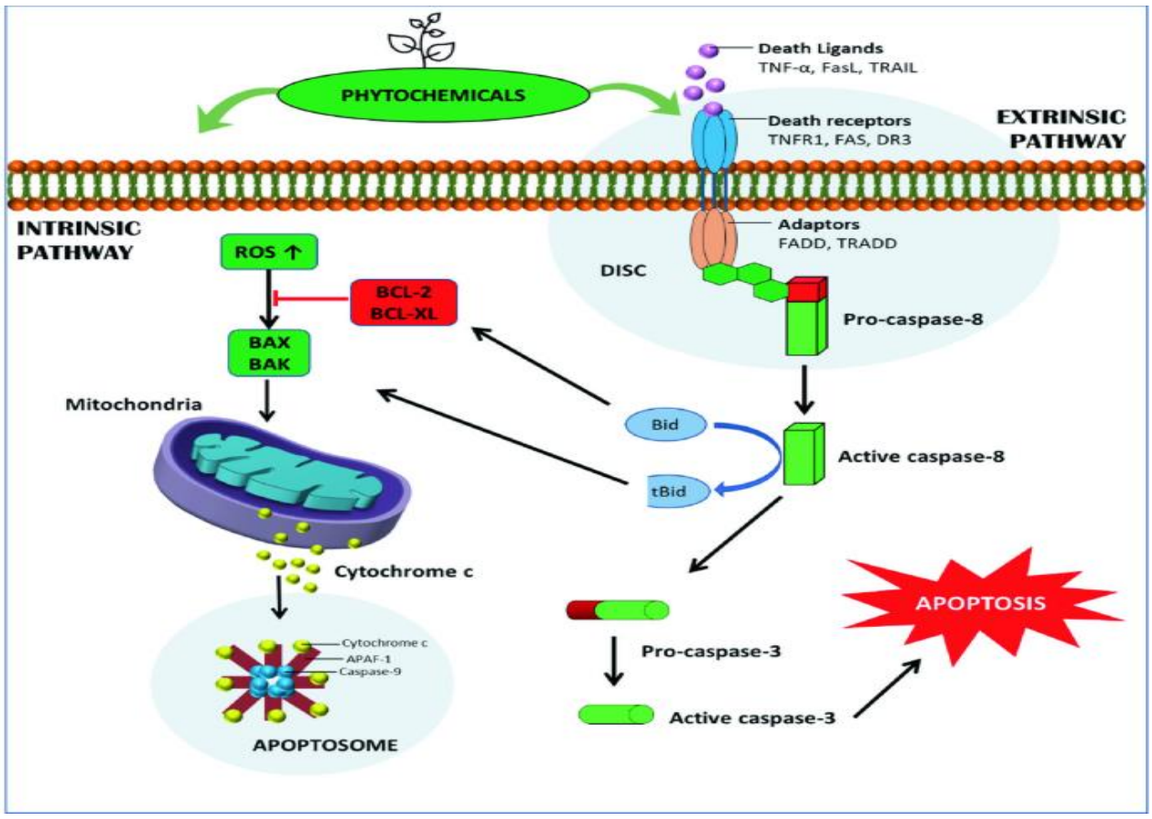

Figure 2.1. - Phytochemicals and Apoptosis: The figure above shows how phytochemical compounds impact Apoptosis in two pathways which is extrinsic which involves the activation of death receptors by death ligands and intrinsic which involve the mitochondrial dysfunction and the activation of pro-apoptotic proteins. This image was obtained from (Cilwyn *et al*. (2021).

2.3.13. A number phytochemicals from plants have been demonstrated to have immunomodulatory activities

Table 2.1 below provides a comprehensive overview of various phytochemicals derived from plants and their documented effects on breast cancer cells. The table presents a diverse array of bio-compounds, such as flavonoids, curcuminoids, and gingerols, derived from *Carpobrotus edulis*, *Curcuma longa*, Ginger, and other plants. These compounds exhibit noteworthy modulation in breast cancer, apoptosis induction, and inhibition of metastasis. The associated cytokines, including IL-6, TNF-alpha, IL-1β, IL-10, IL-8, and IFN-gamma, further elucidate the immunomodulatory and anti-inflammatory properties of these phytochemicals. The citations accompanying each entry provide the sources and studies supporting these findings, offering a scientific foundation for the potential therapeutic roles of these plant compounds in breast cancer treatment. This compilation contributes valuable insights into the multifaceted effects of phytochemicals, paving the way for further research and exploration of natural compounds in the context of breast cancer therapy.

Table 2.1: Phytochemicals and their effect on breast cancer cells

Medicinal plant	**Bio-compound**	**Modulation in breast cancer**	**Cytokines**	**Citation**
Carpobrotus edulis	Flavonoids	apoptosis induction, inhibition of metastasis	IL-6, TNF-alpha	Mohammed *et al*., 2023
Curcuma longa	Curcuminoids	Inhibition of tumour growth, apoptosis induction	IL-6, TNF-α, IL-1β	Vadukoot *et al*., 2022
Camellia sinensis (Green tea)	Epigallocatechin gallate	Anti-proliferative, anti-inflammatory properties	IL-10, IFN-γ, TNF-α	Israel *et al.,* 2018
Vitis vinifera (grape seed)	Resveratrol	Inhibition of metastasis, induction of cell cycle arrest	IL-6, IL-8, TNF-α	Gao and Trygve., 2018
Zingiber officinale (Ginger)	Gingerols	Suppression of cancer cell proliferation, anti-inflammatory effects	Interleukin-1β (IL-1β), Interleukin-6 (IL-6)	Lee *et al.,* 2008
Vitis vinifera (Grape seed extract)	Proanthocyanidins	Induction of apoptosis, inhibition of angiogenesis	IL-6, IL-10, TNF-α	Gao *et al*., 2018
Brassica oleracea (Broccoli)	Sulforaphane	Modulation of oestrogen metabolism, induction of apoptosis	IL-6, IL-8, TNF-alpha	Kuran *et al*., 2020
	Quercetin - Flavonoid	Induction of apoptosis, modulation of oestrogen receptor	TNF-α, IL-6, IL-8	Al-Snafi*, et al.*, 2015
Reseda luteola (Luteolin)	Flavonoid	Anti-inflammatory properties, inhibition of metastasis anti-cancer, neuroprotective, immunomodulatory	IL-6, TNF-alpha, IL-1beta, IL-10, IL-12, IFN-gamma	Cetinkaya, and Baran., 2023

2.3. *2.4. Commelina benghalensis*

Commelina benghalensis also known as Day Flower, or Khotsoana in South Sotho, is a flowering plant species shown in Fig. 2.2 below. The plant grows up to 1 meter tall and has spiderwort-like flowers which have 3 petals, six stamens and 1 pistil. It has fibrous roots and it usually grows in Asia and Africa where it's a most environment, full sun and well-drained soil.

Figure 2.2. - *Commelina benghalensis*: Bright blue coloured flower and the whole plant. The species is native to tropical and subtropical Asia and Africa. The species is a perennial and annual growing and is cultivated or disturbed in places such as roadsides, waste ground as well as in moist grassland, thickets and bushland, woodland, tree plantations and along watercourses; in forest, forest margins and riverine forest. (https://images.app.goo.gl/iBVxTohcnQXvbUqz9)

2.4. *2.1. Use of C. benghalensis as a medicinal plant*

C. benghalensis is a medicinal plant that is used traditionally to treat various diseases. Traditionally it was used by the traditional healers with no knowledge of the specific compounds that could be attributed to the healing ability and powers. The plant was supposedly used for treatment of an array of diseases/conditions, including headache,

constipation, snake bite and jaundice. Its extract was used was to treat warts, acne, eczema, toothaches, night blindness and cataracts due to the biochemical constituents found within the plant extracts. (Pranabesh *et al*., 2019). Interestingly, was thought to influence the reproductive system wherein the plant was used to treat infertility in women (Mohammad *et al*., 2015), whereas in Nepal, the paste of the plant was utilised to treat burns from smoke and juice of the roots utilised to treat acid reflux (Mohammad *et al*., 2015; Pranabesh *et al*., 2019).

Reports on *C. benghalensis* showed the plant to be an excellent antioxidant, with antitumor and anticancer activity and also possessing thrombolytic activity (Mbazima *et al*., 2008; Mohammad *et al*., 2016; Pranabesh *et al*., 2019; Tanvir *et al*., 2015). The plant's therapeutic activities were attributed to phytochemical constituents such as alkaloids, tannins, flavonoids, saponins, polyphenols, glycosides, phytosterols, phlobatannins, resins and balsams which are present in both leaf and stem of the plant (Pranabesh *et al*., 2019; Ibrahim *et al.,* 2010). The plant has also been studied and shown to have pharmacological activities, thus, the plant has been used in the production of Nutraceuticals like vitamin C, proteins, calcium, and iron which are found in *C. benghalensis*. The plant has been show to contain Quercetin which is a flavonoid with anti-inflammatory, anti-cancer, and blood pressure lowering activities and reported to be the most frequent aglycone in *C. benghalensis* (Pranabesh *et al*., 2019).

2.5. *2.2. Phytochemicals from C. benghalensis*

C. benghalensis contains different secondary metabolites, which are organic compounds produced by the organism that are not essential for growth, development, and reproduction. The secondary metabolites included molecules like saponins, tannin, etc. However, these secondary metabolites are primarily involved in the overall maintenance of the organism, while in plants, they serve in defense mechanisms. The presence of tannins could indicate that the plant might have anti-bacterial and antiviral activity and thus credited for the healing of burns and wounds (Ibrahim *et al.,* 2010). Saponins and the glycosides are important classes of secondary metabolites shown to be cardio-

protective as they seemed to lower serum cholesterol and thereby were used for cardiac-related diseases (Ibrahim *et al.,* 2010; Kunle and Egharevba, 2009; Oloyonde, 2005).

2.2.1. Saponins

Saponins were reported to have anticancer and immunomodulatory effects (Oloyonde, 2005; Ibrahim *et al.,* 2010; Kunle and Egharevba, 2009). These immunomodulatory effects were by inducing the production of cytokines, such as interferons and interleukins, which could mediate their immunostimulatory effects (Jie *et al.,* 1984; Kensil, 1996; Rajput *et al.,* 2007). Saponins played a role in inflammation and were reported to have anti-inflammatory effect on the activated macrophages. The saponins inhibit the release of NO and PGE2 by suppressing the transcription of their regulatory genes iNOS and COX2, and the secretion of pro-inflammatory cytokines. This is because saponins affect the translocation of NF-κB from cytoplasm to the nucleus, a key regulator of inflammation as it is a transcription factor of pro-inflammatory genes, modulated by binding to a specific promoter (Jang *et al*., 2013). The saponins inhibit the activity of the NF-κB, which results in the downregulation of pro-inflammatory cytokines and mediators. The saponins inhibit the MAPK signalling pathway in activated macrophages (Figure 2.3) as they inhibit the phosphorylation of p38, JNK and ERK which are responsible for the activation of NF-KB (Jang *et al*., 2013). The inactive NF-KB in the cytoplasm is bound to inhibitory IκB-α protein. The saponins inhibit the activation of NF-κB by inhibiting the phosphorylation of IκB-α protein by IKK, this reduces the availability of free NF-κB p65 which translocate to the nucleus (Wang *et al.,* 2019).

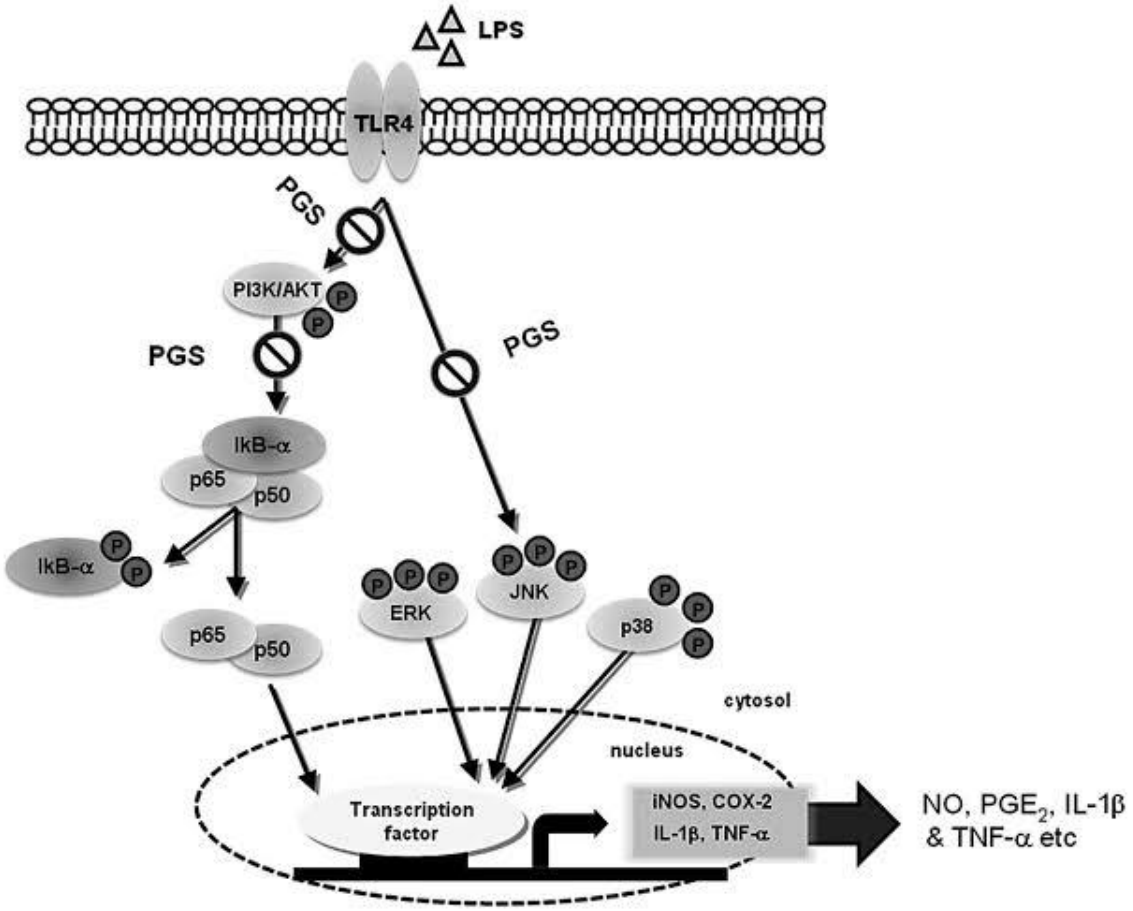

Figure 2.3: Immunomodulatory effect of saponins. A schematic representation of the mechanisms behind the saponins isolated from radix platycodi (PGS)-induced anti-inflammatory response. It shows that PGS inhibited the translocation of NF-KB to the nucleus, followed by blocking phosphoinositide 3-kinase (PI3K)/Akt and MAPK signalling pathway. These effects led to inhibition of the LPS-induced NO and PGE2 production by suppressing the expression of iNOS and COX2 on mRNA and protein expression. PGS also inhibited the production of TNF-α and IL-1β by inhibiting their transcriptional activity (https://images.app.goo.gl/Tb67ZcUWfNzX6SdW9).

2.2.2. Flavonoids

Flavonoids are polyphenolic plant secondary metabolites that contribute to plant pigmentation. The presence of flavonoids implied that the plant might have anti-inflammatory, anticancer, antioxidant, anti-microbial and anti-allergic activity in cells.

Flavonoids have free radical scavenging activity, thus, could be useful in protecting the human body from free radicals (Ibrahim *et al.,* 2010; Kunle and Egharevba. 2009). Flavonoids were potent water-soluble antioxidant and free radical preventing oxidative cell damage and have been shown to possess strong anticancer activity (Yadav *et al*, 2014). Flavonoids inhibit the phosphorylation of IκB-α by IKK thereby inhibiting NF-κB p65 translocation (Figure 2.4). This implies that flavonoids exert their anti-inflammatory effect by inhibiting the NF-κB signalling pathways. Additionally, flavonoids inhibit the expression of the phosphorylated ERK/MAPK, p38 and JNK by signal transduction inhibiting transformation of NF-κB p65 (Li *et al.,* 2017).

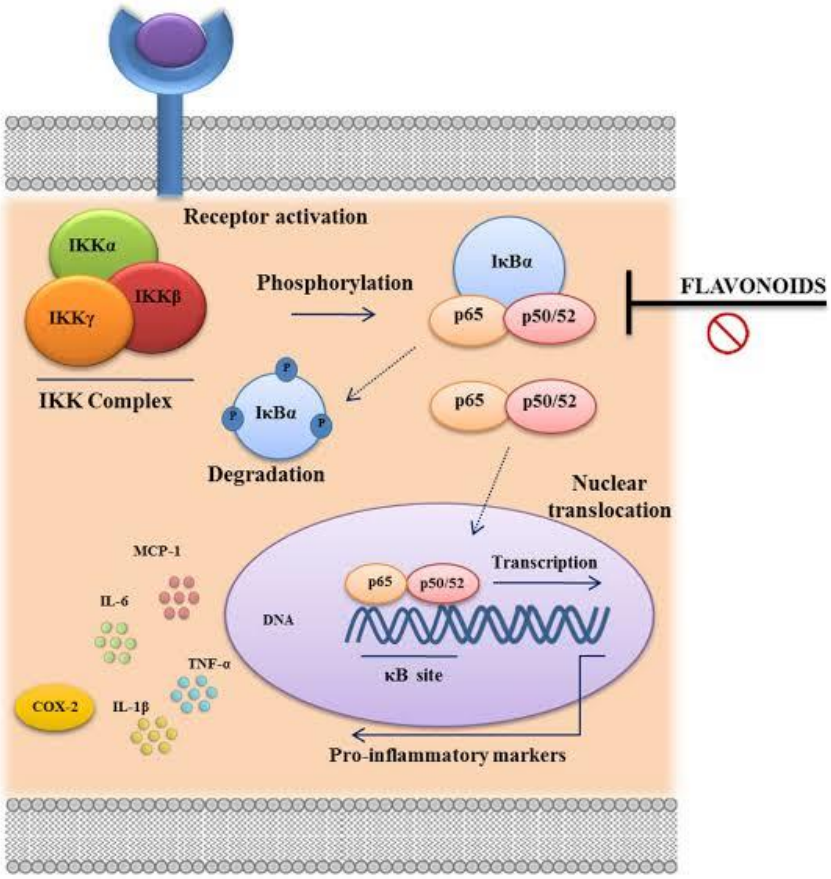

Figure 2.4: Immunomodulatory effects of flavonoids. Representation of the anti-inflammatory effects by flavonoids. The flavonoids inhibit translocation of p65 by inhibiting the phosphorylation of IκB-α by IKK halting the NF-κB signalling pathway (https://images.app.goo.gl/rAynjpbQWP5BrCgY6).

2.6. *2.5. Breast cancer cell lines*

MDA-MB 231 and MCF-7 human derived breast cancer cell lines which are widely studied and used as models for various aspects of breast cancer research, including tumour biology, metastasis, drug testing, and molecular studies (Croker *et al*., 2008). MDA-MB 231 is a triple-negative breast cancer cell line derived from a metastatic site (plural effusion) of a patient with breast adenocarcinoma (Chavez *et al*., 2010). Triple-negative breast cancer lacks expression of oestrogen receptors, progesterone receptors, and human epidermal growth factor receptor 2 (HER2) (Mohieldin *et al*., 2022). MDA-MB 231 cells are highly aggressive and invasive, making them a valuable model for studying metastasis and they are often used to study basal-like breast cancer, which is associated with aggressive behaviour (Mani *et al*., 2007). They have undergone several genetic changes, including mutations in TP53 (p53) and loss of PTEN function (Colombo *et al.,* 2022).

In cancer research, MDA-MD 231 cells are used to study cancer cell invasion, migration, epithelial-mesenchymal transition (EMT) and for testing potential anticancer drugs (Lu *et al*., 2021). Similarly, to MDA-MB 231, MCF-7 cells were derived from the breast tissue of a patient with metastatic breast adenocarcinoma, however MCF-7 cells express oestrogen receptors and are sensitive to oestrogen stimulation and they have been widely used to study oestrogen receptor-positive (ER-positive) breast cancer and its responses to hormonal therapies (Izdebska *et al*., 2021). They are less aggressive and less invasive compared to MDA-MB 231. They are used as a model for ER-positive breast cancer since they carry mutations in TP53 (p53) and have other genetic alterations which might affect their responses to certain treatments (Hasanpourghadi *et al*., 2018). Researchers have used both cell lines to explore various aspects to breast cancer biology, including cell behaviour, signalling pathways, drug sensitivity, and potential therapeutic targets.

3. Introduction

The chapter covers materials including chemicals and consumables, solutions and methodologies including plant harvesting and extraction techniques, determining the bioactive compound molecules that are present within the *Commelina benghalensis (Cb)* using qualitative and quantitative analysis such as Thin Layer Chromatography (TLC), Liquid Chromatography-Mass Spectrometry (LC-MS) and phytochemical screening, further cell culture assays were conducted such as 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) Assay, Muse® cell count and viability, morphological observations, and further Annexin V Assay was conducted to measure the binding of Annexin V to phosphotidylserine on the leaflet of cell membrane during apoptotic process to distinguish between apoptotic and necrotic cells.

3.1. Materials

This section covers all the chemicals and consumables, buffers and solutions and cell lines throughout the project and the companies they were purchased from.

3.1.1.. Chemicals and Consumables

Consumables and reagents were purchased from different companies as tabulated in the table 3.1, below.

Table 3.1: The list of chemicals and consumables used in the study

Reagents/Consumable and kits	Company and country of origin
Cell culture flasks and plates	Cell star, United states of America (USA)
Foetal bovine serum (FBS)	Sigma-Aldrich, Germany
Dimethyl sulfoxide (DMSO)	Saarchem, Republic of South Africa (RSA)
DMEM	Hyclone, USA
Phosphate Buffered Saline (PBS)	Sigma-Aldrich, Germany
Penicillin, Streptomycin, and Neomycin (PSN)	Biowest, USA
Curcumin	Sigma-Aldrich, Germany
Trypsin	Lonza, USA
Muse® Assay Kits (Muse® count and viability Assay, Muse® Cell cycle Assay, Muse® Annexin V and Dead Cell Assay.	Merck-Milipore, Germany

3.1.2. Cell lines

The cell lines that were used in conducting research using *C. benghalensis* aqueous extract were the human breast cancer cell lines; MDA-MB 231 cell line (ATCC HTB-26) representing a mesenchymal stem-like subtype and also a highly aggressive, invasive and poorly differentiated triple-negative breast cancer (TNBC) cell line which lacks oestrogen receptor (ER) and progesterone receptor (PR) expression, as well as HER2 (human epidermal growth factor receptor 2) amplification; and MCF-7 (ATCC HTB-22), representing a luminal subtype, the cell line is oestrogen receptor (ER) positive, progesterone receptor positive .and HER2 negative. As an oestrogen-responsive cell line, MCF-7 is commonly used as a model for studying oestrogen-dependent breast cancers.

2.7. *3.2. Cell lines and Maintenance*

The breast cancer MDA-MB-231 (HTB 26™) and MCF-7 (HTB 22™) cell lines were cultured in Dulbecco Modified Eagle Medium (DMEM), supplemented with 10% of Foetal Bovine Serum (FBS) [Hyclone, USA] and 1% antibiotic mixture containing

penicillin and streptomycin (ThermoFisher Scientific, USA). The cells were maintained in culture flasks at 37°C in a humidified incubator containing 5% CO_2.

2.8. *3.3. Plant collection and extraction of phytochemicals*

Commelina benghalensis (*Cb*) plant was harvested in February 2022 from University of Limpopo (Turfloop) and surrounding areas, Limpopo province, South Africa. The plant was sent to the Larry C Leach herbarium at the University of Limpopo, for identification, confirmation and acquisition of a voucher specimen number (UNIN 1220146). The *Cb* plant was washed with distilled water, the leaves, roots and stems were separated and air-dried under shade on the bench at room temperature. The air-dried leaves, stems and roots of the plant were ground into fine powder. A ratio of 1:20 (plant material: water) was used for the extraction. Fifteen grams (15g) of either leaves, roots and stems were extracted by using 300 ml of boiled distilled water and infused for 45min, then the flask was placed in the mechanical shaker at 200 rpm at 25°C for various intervals (24 hours, 3 hours and 1 hour). After shaking, the dark green or brown extract was filtered to remove insoluble fractions and macromolecules using Whatman filter paper and a funnel into clean storage bottles. The extracts were dried under the cold stream fan. The resultant extract was stored in a refrigerator at 4°C until use, using a protocol previously reported by Laka *et al*. (2021).

2.9. ***3.4.*** *Qualitative analysis of phytochemicals*

3.4.1. Thin layer chromatography (TLC)

The *Commelina benghalensis (Cb)* aqueous (aq) extracts (leaves, roots and stems) were dissolved separately in water to a final concentration of 10 mg/mL for each part of the plant. About 10 µL of the reconstituted *Cb* aq extracts were loaded on aluminium-backed TLC plate and developed in saturated chambers with three solvent systems of different polarity, namely, EMW (ethyl acetate/methanol/water with the ratio of 10:1.35:1; Polar/neutral), CEF (chloroform/ethyl acetate/formic acid with the ratio of 10:8:2; intermediate polarity/acidic) and TEA (toluene/ethanol/ammonia, ratio of 18:2:0.2; nonpolar/basic). The freshly prepared p-anisaldehyde (1 mL p-anisaldehyde, 18 mL ethanol, 1 mL sulphuric acid) and vanillin (0.1g vanillin, 28 ml methanol, 1 ml sulphuric acid) reagent were used to spray then detect various class

of compounds. The plates dipped in p- anisaldehyde, and vanillin reagents were carefully heated at 105°C for optimal colour development. The various classes of compounds were displayed by bands with different colours and the images were captured, using the protocol previously reported by Legerska *et al*. (2020).

3.4.2. Quantitative analysis: Liquid Chromatography–Mass Spectrometry (LC-MS)

Using the protocol by Venter *et al*., (2021), the concentration of 10mg/ml of *Cb* leave-aq extract sample was filtered using a cotton to obtain a clear sample then it was taken to LC-MS analysis at university of Limpopo, chemistry department where the extract was injected into the Liquid Chromatography (LC) system for the different components in the extract to interact with stationary phase of the column and they were eluted at different times based on their polarity to separate the compounds from interfering with the molecules, then the separated molecules were ionised and introduced into the mass spectrometer for the mass-charge ratio of ionized molecule to be determined, then a mass spectrum was created.

3.4.3. Phytochemical screening

The extracts were screened for the presence phytochemicals including; phenols, tannins, flavonoids, alkaloids and lignins using ferric chloride tests, tannic acid test and gallic acid test following the protocol previously reported by Laka *et al*. (2021).

2.10. *3.5. Quantitative analysis*

3.5.1. Total phenolic content determination using Folin-Ciocalteu colorimetric assay

The quantity of phenolics/phenols in each extract of the plant materials was determined using the Folin-Ciocalteu (FC) reagent method described by Singleton *et al*. (1999) with minor modifications. Briefly, 10 mg/mL plant material was reduced to a concentration of 5mg/mL in a test tube, then 100 µL of 5 mg/mL *Cb* aq extracts were diluted with 900 µL of distilled water, followed by the addition of 100 µL of Folin-Ciocalteu reagent. Thereafter 1 mL of 7% Sodium Carbonate (NA_2CO_3) was added and the mixture was incubated in the dark for 30 minutes at room temperature. A blank

was prepared similarly, except that the plant extracts was replaced with acetone. The absorbance for all plant extracts were measured using an Ultraviolet/Visible (UV/VIS) spectrophotometer (Thermo scientific Genesys 105 UV-VIS) at 550 nm. The standard solutions were prepared in the similar manner, except that the plant extracts were replaced by Gallic acid at different concentrations (1.25, 0.63, 0.31, 0.16, 0.08 mg/mL). The total phenolic content of the samples was expressed as milligram of Gallic acid equivalence/gram of extract (mg of TAE/g extract) calculated using linear regression formula from the Gallic acid standard curve (y -0.6814x + 0.0057. R^2 -0.9983). The experiment was conducted in triplicates, using the protocol previously reported by Singleton *et al*., 1999 and Eberhardt *et al*. (2000).

3.5.2. Total tannin content determination using Folin-Ciocalteu colorimetric assay

The tannin content was determined using Folin-Ciocalteu method described by Tambe and Bhambar (2014). Briefly, 50 µL of 10 mg/mL of plant extracts were diluted with 3.8 mL of distilled water in a clean test tube, then 250 µL of Folin-Ciocalteu reagent was added to the mixture and vortexed, thereafter, 0.5 mL of 35% solution of sodium carbonate (Na_2CO_3) was added into the mixture. The mixture in the test tube was transferred to a 10 mL volumetric flask and the volume was made up to 10 mL by the addition of distilled water. The mixture was vigorously vortexed and incubated for 30 minutes at room temperature in the dark, a blank was prepared similarly, except that the plant extracts was replaced with acetone. The absorbance was measured using an ultraviolet/visible (UV/VIS) spectrophotometer (Thermo scientific Genesys 105 UV-VIS) at 550 nm. The standard solutions were prepared in the plant extracts were replaced by tannic acid at different concentrations (1.25, 0.63, 0.31, 0.16, 0.08 mg/mL). The total phenolic content of the samples was expressed as milligram of tannic acid equivalence/gram of extract (mg of TAE/g extract) calculated using linear regression formula from the tannic acid standard curve (y -0.6814x+ 0.0057. R^2 - 0.9983). The experiment was conducted in triplicates, using the protocol previously reported by Makkar (2003).

3.5.3. Total flavonoid content determination using Aluminium Chloride Colorimetric Assay

The aluminium chloride colorimetric method described by Tambe and Bhambar (2014) was used to determine the total flavonoid content in the *Cb* aq extracts Briefly,100 µL of 10 mg/mL of plant extracts were diluted with 4.9 mL of distilled water in a clean test tube, and then 300 µL of 5% Sodium Nitrite ($NaNO_2$) was added to the mixture The reaction mixture was then allowed to stand for 5 minutes at room temperature, after which 300 µL of 10% Aluminium Chloride (AIC) was added to the reaction mixture and vortexed The mixture was allowed to stand at room temperature for 5 minutes, thereafter, 2 mL of 1 M Sodium Hydroxide (NaOH) was added and this was made up to 10 mL with distilled water. A blank was prepared similarly, except that the plant extracts was replaced with 100 µL of distilled water. The absorbance was measured using an ultraviolet/visible (UV/VIS) spectrophotometer (Thermo scientific Genesys 105 UV-VIS) at 510 nm. The standard solutions were prepared in the similar manner, except that the plant extracts were replaced by quercetin (sigma-Aldrich) at different concentrations (0.5, 0.25, 0.125, 0.0625, 0.03125 mg/mL). The total flavonoid content of the samples was expressed as milligram Quercetin Equivalence/ gram of extract (mg QE/g extract) calculated using linear regression formula from the quercetin standard curve ($y = 3.4379x + 0.0017$, $R^2 = 0.9996$). The experiment was conducted in triplicates, using the protocol previously reported by Granato *et al*. (2015) and Ni *et al*. (2012).

2.11. *3.6. Antioxidant Capacity Assay*

3.6.1. Quantitative 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity assay

Free radical scavenging activity of the *Cb* aq extract were quantified and compared using the DPPH assay, following a protocol previously described by Laka *et al*, (2021), with some modification. Briefly, 500µL of *Cb* extract concentrations were prepared and 1000µL of 60µg/mL DPPH (Sigma-Aldrich, USA) solution was added. Ascorbic acid served as positive control. The samples were left at 25°C for 30min in the dark. After the incubation time had elapsed, the absorbance readings of the samples were taken at 517nm using a microtitre plate reader (Promega, USA) and the formula below was used to calculate the percentage inhibition.

$$\%Inhibition = \times \frac{\text{blank absorbance 517 nm} - \text{sample absorbance 517nm}}{\text{blank absorbance 517nm}} \times 100$$

3.6.2. Ferric ion reducing power (FRAP) assay

Various concentrations (0 µg/mL to 1000 µg/mL) of the *Cb* aq extract and ascorbic acid were prepared in 250 µL deionised water. A blank was prepared without extract, with ascorbic acid as the reference standard. These were then mixed with phosphate buffer (250 µL) (0.1 M. pH 7.0) together with 1 % potassium ferric cyanide (250 µL) and incubated at 50°C for 20 minutes. After incubation, 10% of trichloroacetic acid (250µL) was added to the mixture and centrifuged at 3000 rpm for 10 minutes. Five hundred microliltres (500 µL) of the supernatant was mixed with 2.5 ml distilled water and freshly prepared 0.1% ferric chloride solution (500 µL). The absorbance of the samples was measured at 700 nm using a GloMax®-Multi+Detection system microtiter plate reader (Promega. Madison, USA). The below formula was used to calculate the percentage reducing antioxidant power, using the protocol previously reported by Laka *et al*. (2021):

$$\%Reducing\ power = (\text{sample absorbance 700nm} - 1) \times \frac{100}{\text{blank absorbance 700nm}}$$

2.12. *3.7. Cytotoxicity Assay*

3.7.1. Cell Culture and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) Assay

Cell culture and MTT assay were performed following a method as previously reported Laka *et al*., 2021. Dulbecco's Modified Eagle Medium (DMEM) (Hyclone, USA) containing 10 % foetal bovine serum (Hyclone, USA) and 1% antibiotic mixture of Penicillin and Streptomycin (Biowest, USA) was used to culture cells in an incubator containing 5% CO_2 at 37 ◦C. MTT assay was employed to assess the activities of the *Cb* aq extract. Briefly, 1000 cells/well were cultured overnight. Following incubation, treatment was done using different concentrations (31.25− 1000 µg/mL) of the *Cb* aq extract, solvent controls (0.25 % H_2O), and positive control (50 µM curcumin) for 24 h and for 48 hours treated with the leaves part of the plant. Following treatment, the cells in each well plate were subjected to a 5 mg/mL MTT reagent (10 µL) [ThermoFischer Scientific, USA] and incubated in the CO_2 incubator for 3h. After incubation, the formed crystals were dissolved by adding 100 µL/well DMSO and placed in the dark for an

hour at 25 ◦C. Thereafter, measured the absorbance readings using a microtiter plate reader (Promega, USA) at 560 nm. The cell viability was calculated using the formula, using the protocol previously reported by Laka *et al*. (2021):

$$Cell\ viability = \frac{Sample\ absorbance\ 570\ nm}{\text{Untreated Control absorbance 570 nm}} \times 100$$

3.7.2. Cell count and Viability Assay

The MDA-MB 231 cells (1X10^5) were cultured in a 12 well plates with the following the treatment: 750µg/ml *Cb* aq extract, 150µg/ml curcumin, 10% H2O and untreated and incubated for 48 hours. Following treatment, the cells were analysed for their morphological examination then they were trypsinised (1X trypsin) and centrifuged at 300 x g for 5 minutes. The cells were then re-suspended in 50µL of the Muse™ Count and Viability Reagent, following incubation for 5 minutes in the dark at room temperature. The Muse® Cell Analyser (Merck-Millipore, Germany) was used to analyse the samples**.**

2.13. *3.8. The morphological characterisation assay*

The MDA-MB 231 cells (1X10^5) were cultured in 12 well plates with the following the treatments: 750µg/ml Cb aq extract, 150µg/ml curcumin, 10% H_2O and untreated and incubated for 48 hours and following treatment, the morphological changes after treatment were examined and the images were captured on an inverted microscope fitted with a camera. The images were analysed for their morphological changes using LCMicro image analysis software from Olympus.

2.14. *3.9. Apoptosis assay*

To determine if *Cb* aq extract induce apoptosis, Annexin V and Dead Cell Kit (Merck-Millipore, Germany) was employed as per the manufacturer's protocol. Briefly, 2 × 10^3 cells/well were seeded. Following overnight incubation, 48h treatment with IC50 values and the controls was carried. Following treatment, 1X trypsin was used to detach the cells, which was deactivated with a complete medium and centrifuged. Following centrifugation, 100µL DMEM containing 1% FBS was used to re-suspend the pellets. The Annexin V and Dead Cell reagent (Merck- Millipore, Germany) was added, and samples were left in the dark for 20min at 25 ˚C. The Muse® Cell Analyser (Merck-Millipore, Germany) was used to analyse the samples**.**

3. Introduction

Commelina benghalensis (Cb), which is a common weed with medicinal value, was used in this study. The first step was to extract the chemical compounds using aqueous (aq) in its parts separated (roots, stems and leaves). This was followed by the phytochemical analysis to determine whether the *Cb* contain any chemical compounds. This chapter will cover the results from Thin Layer Chromatography (TLC), Liquid Chromatography-Mass Spectrometry (LC-MS) analysis, Phytochemical screening, Antioxidant activity of *Cb,* cell culture and viability, Muse® count and cell viability, Morphological analysis, Annexin-V assay, that were used to address the objectives of the study.

3.1. *4.1. Qualitative analysis*

4.1.1. Profiling Bioactive Compounds using Thin Layer Chromatography (TLC) in Commenlina benghalensis (Cb) extract

The principle of Thin Layer Chromatography (TLC) is based on the separation of a mixture of components between a fixed stationary phase and a liquid mobile phase by the differential affinities between the two phases. The separation of the components is through adsorption. For this project, the separation of the compounds present within *Cb* leaf, root and stems-aqueous extracts, 150mg of the powder extract was dissolved in 15ml tube of distilled water resulting in 10mg/ml concentration, the concentration was reduced to 1000µg/ml and a 10µl was spotted in the plates. The results showed that the Cb aq. extract compounds could be separated in different solvent systems (EMW, TEA and CEF. In figure 4.1A, the plates were sprayed with p-anisaldehyde, the compounds that were separated in a ratio of Ethyl acetate, Methanol and Water (EMW) 10:1.35:1, did not show any compound separation that could be identified, the compounds that were separated in the resolving solvent of Toluene, Ethanol and Ammonia (TEA) 18:2:0.2, showed separation of compounds only in leaves, indicating pink and purple colour, the compounds that were separated in the resolving solvent of Chloroform, Ethyl acetate and Formic acid (CEF) 10:8:2, showed compounds in leaves indicated by purplish colour. The plates were then sprayed with vanillin in Figure 4.1B, the compounds that were separated in the resolving solvent of EMW, showed compound separation in leaves identified by yellow and purple colour, the compounds separated by TEA, showed separation of compounds in leaves indicated by yellow and purple colour, also, roots showed few purple bands indicating compound separated in CEF solvent showed by purple and yellow colours.

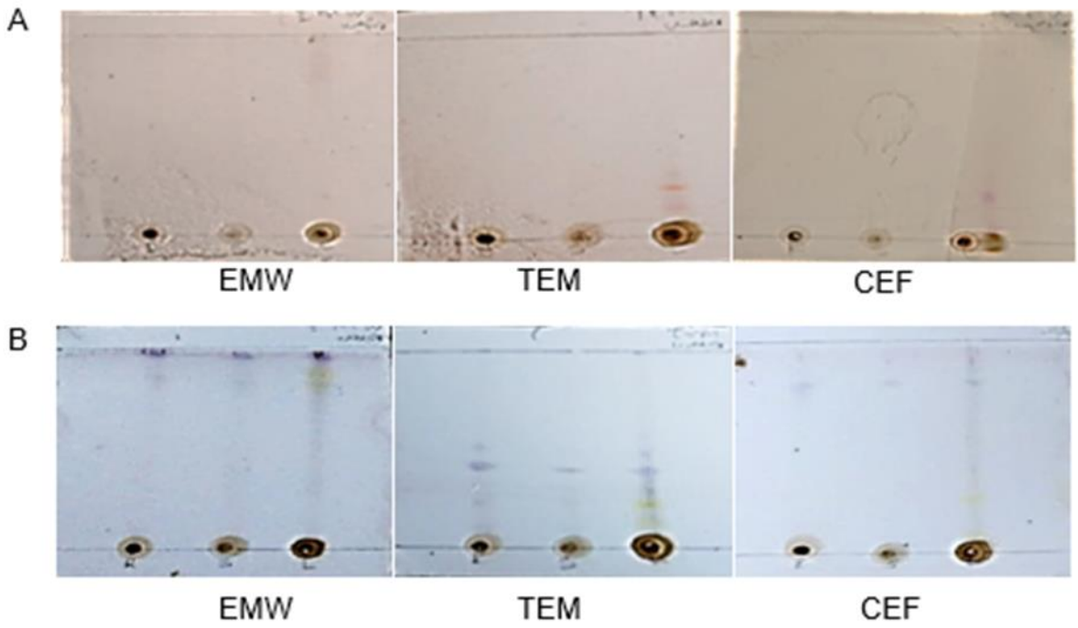

Figure 4.1- Bioactive compounds analysis in *Cb* leaf, root and stem aqueous extract: The results above show the TLC plates of *Cb* leaf, root and stem aqueous extract which were viewed after spraying with p-anisaldehyde (A) and Vanillin (B) and under the following solvent systems; EMW (Ethyl acetate, Methanol and Water), TEA (Toulene, Ethanol, Ammonia) and CEF (Chloroform, Ethyl acetate and formic acid).

4.1.2. Identification of plant metabolites present *Cb* leaf - aqueous extract

Liquid chromatography-Mass spectrometry (LC-MS) is a technique that uses Liquid chromatography (LC) including HPLC as a purification and separation step of the sample components and then introduces them to the mass spectrometer (MS) to identify and quantitate the analyte. The strength of this technique lies in the separation power of LC for a wide range of compounds combined with the capability of the MS to quantify compounds with a high degree of sensitivity and selectivity based on the unique mass/charge (m/z) transitions of each compound of interest.

Thus, for our project we first used LC-MS (Sciex Exion LC coupled to Sciex X500R QTOF system) to identify the compounds present in the *Cb* leaves-aqueous extract sample. Figure 4.2 is a representation of a mass-charge ratio of ions produced by the fragmentation of a molecule in a mass spectrometer. The peaks in the spectrum correspond to the different fragments of molecule that was produced by the mass spectrometer. The names of the molecules in the spectrum were identified by comparing the mass-charge reaction of the peaks to the database of known mass natural product and NIST (National Institute of Standards and Technology) spectral library which was used.

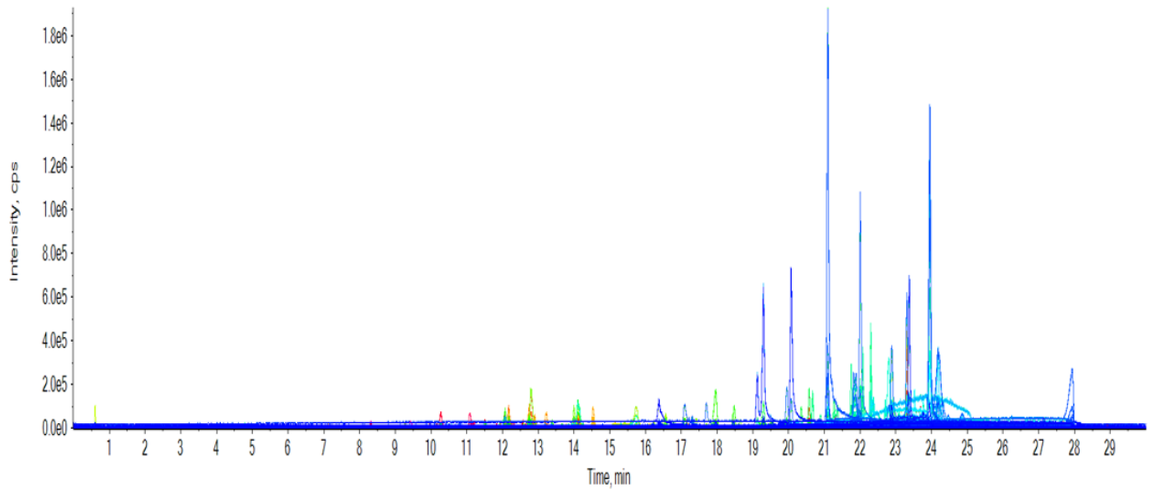

Figure 4.2 - Mass spectrum of *Cb* leaf-aqueous extract substances: The figure shows the numbers of peaks which suggest that the *Cb* leaves-aqueous extract sample is a mixture of different molecules. The spectrum was used to identify the phytochemical compounds in the extract that may be responsible for immunomodulatory activities on breast cancer cells.

4.2. Known chemical compounds from LC-MS spectrum analysis

The LC-MS was run in the Department of Chemistry and the data obtained summarised in Table 4.1 showing compounds identified from the natural product and NIST spectral library. The table includes the biological or chemical properties of each compound identified, providing context for their potential presence in *Cb* leaves-aq extract. There were several entries that highlighted potential anticancer, anti-inflammatory and other medicinal properties based on previous research as cited.

Table 4.1: Known compound molecules from *Cb* leaf-aqueous extract sample from LC-MS spectrum analysis

Compound name	**Group**	**Molecular formula**	**Formula weight**	**Retention time**	**Properties**
1. Demethylzeylasteral	Triterpenoid	C23H36N4O7	480.25696	8.99	Demethylzeylasteral is a natural compound isolated from Tripterygium wilfordii Hook F, which has been reported to have various pharmacological properties including anti-inflammatory and immunosuppressive activities (Li *et al*., 2019)
2. Brucine	Alkaloid	C18H32N2O6	372.22801	9.98	Modern pharmacology studies and clinical practice demonstrate that brucine possesses wide pharmacological activities, such as anti-tumour, anti-inflammatory, analgesic, and the effects on cardiovascular system and nervous system (Lu *et al*., 2020).
3. Sodium glycodeoxycholate	Bile salt	C28H37N7	471.31215	14.12	The compound was shown to have a variety of biological activities, including anti-inflammatory, anti-microbial, and anti-cancer properties (Zou *et al*., 2021)
4. Osthole	Coumarin	C11H12N6O	244.10913	15.46	The compound, a natural compound found in several plants, and has been studied for its potential anti-cancer properties. (Jiang *et al*., 2023)
5. Tuberostemonine	Alkaloid	C14H33N9OS	375.25386	16.03	Combined with adriamycin could time-dependently inhibit the cell proliferation and obviously promoted the cell

					apoptosis. Also the tuberostemonine could inhibit the expression of Survivin (Wang *et al*., 2017)
6. Indigo dye	Organic dye	C11H18OS3	262.05022	16.23	Is a synthetic dye used in various applications, including textile dyeing (Lai and Chang., 2021)
7. 2-Methoxyestradiol	Steroid	C11H22N6S	270.16199	21.39	The compound was shown is a derivative of the hormone oestradiol and has been studied for its potential anti-cancer properties, including breast cancer (Molnar *et al*., 2020)

4.2. Potential bioactive compounds

The LC-MS data also identified compounds that had bioactive or biological potential that were found in *Cb* leaves-aqueous extract. To identify and name the compounds, PubChem and different literature reviews were used to search the names of the compounds, using the chemical formula and molecular mass.

Table 4.2: Potential compound molecules from *Cb* leaf-aqueous extract.

Compound name (IUPAC Name)	Group	Molecular formula	Formula weight/MW	Retention time	Function
1. 1-(hydroxymethyl)-1,4 a-dimethyl-7-propan-2-yl-2,3,4,9,10,10a-hexahydrophenanthren-9-ol is a diterpenoid.	Diterpenoid.	C20H30O2	318.21906	18.04	The compound induces apoptosis, (programmed cell death), in cancer cells. The compound inhibits tumour growth by targeting different signalling pathways, such as TNF-α-induced IL-8 expression (Tundis et al.,2023)
2. (1R,4aR,4bR,10aR)-1,4a-dimethyl-7-propan-2-yl-2,3,4,4b,5,6,10,10a-octahydrophenanthrene-1-carboxylic acid	Abietic acid	C20H30O2	318.21906	18.04	The compound Induces apoptosis by overexpression of apoptotic genes (FasL) (Haffez *et al*., 2022)
3. 3-(4-methoxyphenyl)-8,8-dimethylpyrano[2,3-f]chromen-4-one	Flavonoid	C21H18O4	334.11971	19.23	The compound possess minimal toxicity and produce therapeutic effects such as anti-inflammatory, immune response modulation, and antioxidant effects relieve oxidative stress by regulating reactive oxygen species (ROS) homeostasis,

					triggering apoptotic pathways, and exerting a pro-oxidant effect in cancerous cells. They can scavenge ROS and activate antioxidant enzymes. (Sharma *et al*., 2023)
4. Dodecanamide	Lauramide	C12H25NO	199.19515	19.37	The compound has been studied for its potential antimicrobial properties (Turovskiy *et al*., 2012).
5. 3-(5,6-dihydroxy-1H-indol-3-yl)-1H-indole-5,6-diol	Biindole	C16H12N2O4	296.07751	20.12	The compound exhibited more potent cytotoxic activity against cancer cell lines compared to monoindole compounds (Wang *et al*., 2012). Additionally, biindole-based SMAC mimetics had demonstrated synergistic cytotoxicity with widely used anti-cancer agents in pre-clinical analyses (Rettinger *et al*., 2014)
6. 11.4-[(1E,3S)-3-ethenyl-3,7-dimethylocta-1,6-dienyl]phenol	Phenol	C18H24O	256.18199	20.94	The compound had shown inhibitory effects on lung cancer by modulating the tumour microenvironment and the expression of PD-L1 (Lin *et al*., 2023). Additionally, the compound had been reported to repress Notch 3 expression, exerting an inhibitory effect on the self-

					renewal capacity of breast cancer stem cells and metastasis of breast cancer cells (Li *et al*., 2017).
7. 2,6-ditert-butyl-4-ethylphenol	Alkylbenzene	C16H26O	234.19995	20.98	The compound had been studied for its antioxidant properties as it exhibited strong antioxidant activity (Weng & Huang, 2014)
8. (2E,4E,8Z,11Z)-N-(2-methylpropyl)tetradeca-2,4,8,11-tetraenamide	Alkylamides	C18H29NO	275.22398	21.25	The compound has been reported to inhibit nitric oxide (NO) production in macrophage cells, indicating potential anti-inflammatory properties (Xu *et al*., 2021). The findings suggested that it may have significant implications in modulating immune responses and inflammation, highlighting their potential in therapeutic applications related to immunomodulation and anti-inflammatory effects.
9. Ethane;(NE)-N-[4-methyl-1-[2-[methyl-[2-[(7-methylquinazolin-4-yl)amino]ethyl]amino]ethylamino]thioxanthen-9-ylidene]hydroxylamine	Thioxanthone derivatives	C36H54N6OS	618.40923	10.26	The compound had been shown to possess a variety of biological activities, including anti-inflammatory, anti-microbial, and anti-cancer properties (Al-Bari *et al*., 2021)

10. 2-ethyl-N-(2-ethylhexyl)hexan-1-amine	Amines.	C16H35N	241.27824	8.49	The compound had biological activities including anti-inflammatory, anti-microbial, and anti-cancer properties (Kanehashi *et al*., 2015).
11. 4-(N-(4-tetradecylphenyl) anilino) benzaldehyde	Anilino compounds	C33H43NO	469.33546	10.28	The compound was shown to have a variety of biological activities, including anti-inflammatory, anti-microbial, and anti-cancer properties (Ataz *et al*., 2020)
12. Cyclopentadecane-1,4,7,10,13-pentathiol	Macrocyclic ligands	C15H30S5	370.09634	10.31	Thiols have been shown to have a variety of biological activities, including anti-inflammatory, anti-microbial, and anti-cancer properties by inhibiting the growth of cancer cells in vitro (Zilong *et al* ., 2023)
13. (propan-2-ylideneamino) N-(4-hexoxyphenyl)carbamate	Carbamates	C16H24N2O3	292.17842	10.32	The compound was shown to have a variety of biological activities, including anti-inflammatory, anti-microbial, and anti-cancer properties (Sharanya., 2019)
14. (4-nonylphenyl) 4-[4-(4-nonylphenoxy)carbonylphenyl]benzoate	Liquid crystal	C44H54O4	646.40307	11.12	The compound was shown to have a variety of biological activities, including endocrine-disrupting properties. Additionally, it has shown to have carcinogenic properties, (Andujar *et al*., 2019)

15. 4-tert-butyl-7H-cyclopenta[b]pyridine	Cyclopentabpyridines	C48H60N4	692.48435/	11.12	It inhibit the growth of a variety of cancer cells, including breast cancer cells, leukaemia cells, and colon cancer cells (Donadel *et al*., 2005)
16. (2-octanoyloxy-3-sulfanylpropyl) octanoate	Sulfhydryl	C19H36O4S	360.23456	11.54	Thioesters have been shown to have a variety of biological activities, including anti-inflammatory, anti-microbial, and anti-cancer properties (Nguyen *et al*., 2021)
17. 3-[4-(2-phenylmethoxyethyl)piperazin-1-yl]propanoic acid	Hemihydrate	C16H24N2O3	292.17842	10.32	The compound mediate the anti-proliferative effect in breast cancer cells (Nirgude *et al*., 2022)
18. 1-chloro-2,4-dinitrobenzene	Dinitrochlorobenzene	C14H12O4	244.07285	14.75	The compound was shown as a potential therapeutic agent that can alleviate Atopic dermatitis by modulating Th1/Th2 immune balance and restoring skin barrier function. (Oh *et al*., 2021)
19. N-methyl-N-octyloctan-1-amine	Tertiary amino compound.	C17H37N	255.29397	16.07	The compound was evaluated for anti-oxidant activity and one of these derivatives was tested for cytotoxic activity against breast cancer using MTT assay (Rehan *et al*., 2021)

20. 5-[2-(2-methoxyphenyl)ethyl]-2-methylbenzene-1,3-diol, Stilbostemin D	Alkaloids	C16H18O3	258.12495	17.14	The compound had been extensively studied for its diverse biological activities, including anti-inflammatory, anti-microbial, and anti-cancer properties (Djaldetti., 2021 and Souza *et al*., 2020)
21. (Z)-Docos-13-enamide	Erucamide Fatty amide	C22H43NO	337.33630	27,89	The compound was shown to have huge potential to eliminate binding affinity biases and thus accelerate drug discovery in infectious diseases, autoimmunity, vaccine design, and cancer immunotherapy (Kopicki *et al*., 2022)
22. Methyl octadecanoate	Octadecanoate ester	C19H38O2	298.28848	24.85	The compound was shown to induce apoptosis, however, without having any cytotoxicity on the normal Vero cell-lines. (Hussein *et al*., 2020)
23. N'-hydroxy-N-phenyloctanediamide	Vorinostat	C14H20N2O3	264.14632	10.17	An inhibitor of class I and II of Histone deacetylase inhibitors (HDACs), and is an effective anticancer agent which, individually and/or in combination with other conventional chemotherapeutics, exhibit anti-neoplastic properties through inhibition of proliferation, migration and invasion,

					induction of differentiation and apoptosis as well as cell-cycle arrest, in many types of Breast Cancer cells (Wawruszak *et al*., 2021)

3.2. *4.3. Cytokines targeted by C. benghalensis phytochemicals with immunomodulatory effects*

Table 4.3 shows cytokines that may be targeted by *C. benghalensis* phytochemicals with immunomodulatory effects. The identified phytochemicals with immunomodulatory effects include Abietic acid, Vorinostat and Lauramide. Abietic acid regulates VEGF, TGF-*b*1, IGF1R and ATG1, C-myc, TNF-*α* and NF-*k*B; Vorinostat regulates the expression of IFNγ, IL-2, IL-4, IL-10, IL-13 and *Lauramide* regulates *IFN-γ, TNF-α, IL-2, and IL-10.*

Table 4.3: Cytokines targeted by potential phytochemicals with immunomodulatory effects.

Compound	Regulated Cytokine/s	Immunomodulatory effect	References
1. (1R,4aR,4bR,10aR)-1,4a-dimethyl-7-propan-2-yl-2,3,4,4b,5,6,10,10a-octahydrophenanthrene-1-carboxylic acid (Abietic acid)	VEGF, TGF-*b*1, IGF1R and ATG1, C-myc, TNF-*α* and NF-*k*B.	Downregulation of target genes such as VEGF, TGF-*b*1, IGF1R and ATG12 and levels of oncogenes C-myc, TNF-*α* and NF-*k*B.	Haffez, *et al*, 2022
1. N'-hydroxy-N-phenyloctanediamide (Vorinostat)	Increased gene expression of IFNγ, decreased gene expression of IL-2, IL-4, IL-10, IL-13	IL-10 has both pro- and anti-inflammatory effect on breast cancer, however, IL-2 and IL-10 have	Li, *et al*, 2017

		synergistic effect of enhancing CD8+T cells cytotoxicity to induce apoptosis in breast cancer cells-	
Dodecanamide (Lauramide)	IFN-γ, TNF-α, IL-2, and IL-10	Glycerol monolaurate (GML) inhibits TCR-induced cytokine production of IFN-γ, TNF-α, IL-2, and IL-10	Fosdick M, *et al*, 2021

3.3. *4.4. Phytochemical screening*

Performing phytochemical screening affords researchers to evaluate the plant extract bioactive compounds or phytochemicals, including how prevalent certain of those bioactive compounds may be in that particular plant. The phytochemical or bioactive compounds could then be used for production of therapeutic drugs or understanding biological effects these compounds may have on different cell lines.

Thus, qualitative phytochemical screening was done to find out whether the *Cb* aq extract contained phytochemical compounds including phenols, tannins, flavonoids, lignins or alkaloids. It was noted that the *Cb* aq extracts showed presence (positive) tests (+) for different chemical constituents and in some showed absence (absence) tests (-) of biological constituents as seen in Table 4.3. Our results also showed that the roots extract of *Cb* contained phenols, tannins, flavonoids, lignins and alkaloids because they were positive (showing presence), while the stems extract of *Cb* contained phenols, flavonoids as shown by the positive test; finally, the leaves extract of *Cb* contained phenols, tannins and flavonoids as shown by presence of these molecules.

Table 4.4: The presence/absence of various secondary metabolites in the *Cb* leaf, root and stem-aqueous extract

Phytochemical screening tests	***Cb* aqueous extract**		
	Leaves	**Roots**	**Stems**
Detection of phenols			
Ferric chloride test (2%)	+	+	+
Detection of tannins			
Ferric chloride test (5%)	-	-	-
Detection of flavonoids			
Ferric chloride test (10%)	+	+	+
Detection of Alkaloids			
Tannic acid test	+		-
Detection of lignin			
Gallic acid test	-	+	-

Key: (+) = Presence (-) = Absence

3.4. *4.5. Total phenolic content (TPC), total tannin content (TTC) and total flavonoid content (TFC)*

The total amount (concentration) of phenolic content in *Cb* aq extract was evaluated. As shown in Figure 4.3 A the linear equation of gallic acid was found to be y = 0.0003x +0.0507 and R2 = 0.995. The TPC in extracts was expressed as gallic acid equivalents and the leaves were found to contain a significantly high phenolic content with $p < 0.005$, compared to stems and roots. Quercetin was used as the positive control. The TTC of each extract was determined as tannic acid equivalent using the linear regression from the standard curves as it is shown in Figure 4.3B. The linear equation of tannic acid was found to be y = 0.0003x +0.0605. The roots, stems and leaves contained a significantly low content of tannin ($p < 0.005$) compared to stems. The TFC as shown in Figure 4.3C was expressed in terms of quercetin equivalents and the p value was found to be $p < 0.005$. Phenols and Flavonoids content were found to be higher in roots and leaves, with tannin, the results did not show any significant value for roots, stems and leaves with extremely low content.

Table 4.5: Concentration-dependent total phenolic, content of *Cb*-aqueous extract

Concentration (µg/ml)	**Total Phenolic Content (GAE µg/ml)**			
	Leaves	**Roots**	**stems**	**Quercetin (Positive control)**
0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
62.500	3.269 ± 0.661	2.798 ± 0.566	2.976 ± 0.558	2.593 ± 0.638
125.000	5.922 ± 1.307	4.993 ± 1.054	4.936 ± 1.064	4.696 ± 1.134
250.000	9.816 ± 2.447	8.011 ± 1.966	7.700 ± 2.039	7.776 ± 2.017
500.000	19.420 ± 5.445	15.224 ± 4.412	14. 536 ± 4.600	15.256 ± 4.406
1000.000	34.633 ± 9.265	28.340 ± 7.458	25.909 ± 8.072	27.464 ± 7.596

Table 4.6: Concentration-dependent total flavonoid and content of *Cb*-aqueous extract

Concentration (µg/ml)	**Total Flavonoid Content (QAE µg/ml)**			
	Leaves	**Roots**	**Stems**	**Gallic acid**
0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
62.500	3.319 ± 0.146	4.589 ± 0.577	1.467 ± 0.351	1.600 ± 0.219
125.000	6.744 ± 0.371	7.544 ± 0.273	3.144 ± 0.306	2.867 ± 0.493
250.000	11.100 ± 0.833	9.933 ± 0.384	4.900 ± 0.410	5.744 ± 0.577
500.000	15.144 ± 0.577	15.011 ± 0.467	11.389 ± 0.577	10.922 ± 1.245
1000.000	32.922 ± 1.453	29.808 ± 0.635	19.033 ± 0.786	16.611 ± 0.296

Table 4.7: Concentration-dependent total tannin content of *Cb*-aqueous extract

Concentration (µg/ml)	**Total Tannin Content (TAE µg/ml)**			
	Leaves	**Roots**	**Stems**	**Gallic acid**
0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
62.500	1.855 ± 0.306	2.255 ± 0.000	1.147 ± 0.000	1.223 ± 0.000
125.000	2.720 ± 0.289	2.344 ± 0.240	0.666 ± 0.176	1.919 ± 0.296
250.000	2.623 ± 0.306	2.275 ± 0.240	0.315 ± 0.035	5.151 ± 0.296
500.000	2.279 ± 0.219	1.659 ± 0.318	0.272 ± 0.084	9.610 ± 0.577
1000.000	2.779 ± 0.467	1.573 ± 0.2651	0.332 ± 0.065	10.175 ± 0.521

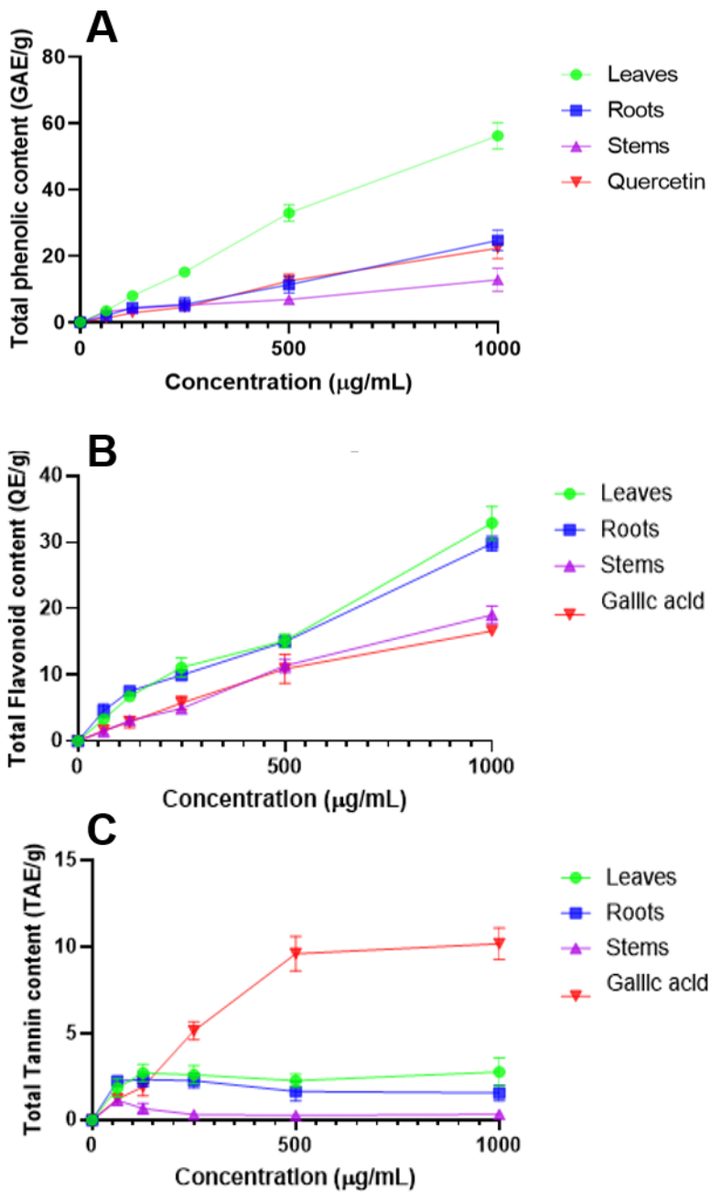

Figure 4.3 – Total Phenolic, flavonoid and tannin content of *Cb* leaf, root and stem aqueous extracts: The total phenolic (A), total flavonoid (B) and total tannin (C) contents of the aqueous extract (AE) represented as Gallic acid, tannic acid and quercetin equivalents in µg/ml respectively and the experiments was done in triplicates and the differences were found to be statistically significant (*** $P < 0.001$) compare to the blank control. Quercetin and gallic acid was used as positive control.

3.5. *4.6. Quantitative 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) and free radical antioxidant power (FRAP) assays*

The antioxidant activity of *Cb* aq extracts was evaluated using both DPPH and FRAP assays. The roots and leaves *Cb* aq extracts showed the antioxidant activity. The roots and leaves exhibited the lowest half-maximum effective concentration (IC_{50}) value of 56% at 125 µg/mL for the DPPH scavenging activity which represents the concentration of the *Cb* aq extract required to scavenge 50% of the free radicals in the DPPH solution as shown in Figure 4.4A. The stems have value of lower than 50%, which means that the stems do not have the concentration required to scavenge 50% of free radicals in the

DPPH solution. The DPPH antioxidant activity started from the lowest concentration gradually increasing as the concentration of *Cb* aq extract increased (0 to 1000µg/mL). FRAP method is another approach to measure the ability of a substance or chemical/compound to scavenge free radicals. Similar to DPPH, the FRAP antioxidant activity also started from the lowest concentration and increased (63 to 1000µg/mL). With the higher concentration of the *Cb* aq extracts indicating that the leaves had the highest absorbance with an increase in concentration.

Table 4.8: DPPH antioxidant activities of different parts of *Cb*-aqueous extracts

Concentration (µg/ml)	**DPPH (%)**			
	Leaves	**Stems**	**Roots**	**Quercetin**
0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
62.500	10.555 ± 1.155	2.888 ± 1.155	35.563 ± 2.082	90.148 ± 2.028
125.000	23. 752 ± 0.882	10.500 ± 1.55	43.454 ± 2.404	92.057 ± 4.655
250.000	55. 577 ± 1.202	17.422 ± 1.453	55.341 ± 2.082	95.487 ± 1.202
500.000	43.512 ± 2.028	23.837 ± 2.333	44. 915 ± 2.333	89.121 ± 2.963
1000.000	44.944 ± 2.309	18.773 ± 3.055	34.085 ± 2.404	136.435 ± 3.055

Table 4.9: FRAP antioxidant activities of different parts of *Cb*-aqueous extracts

Concentration (µg/ml)	**FRAP (700nm)**			
	Leaves	**Stems**	**Roots**	**Ascorbic Acid**
0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
62.500	0.180 ± 0.044	0.059 ± 0.005	0.077 ± 0.007	0.086 ± 0.003
125.000	0.310 ± 0.021	0.061 ± 0.006	0.090 ± 0.000	0.232 ± 0.033
250.000	0.383 ± 0.044	0.093 ± 0.021	0.150± 0.000	0.543 ± 0.032
500.000	0.560 ± 0.031	0.112 ± 0.019	0.210 ± 0.000	0.617 ± 0.037
1000.000	0.793 ± 0.058	0.127 ± 0.042	0.270 ± 0.000	1.370 ± 0.118

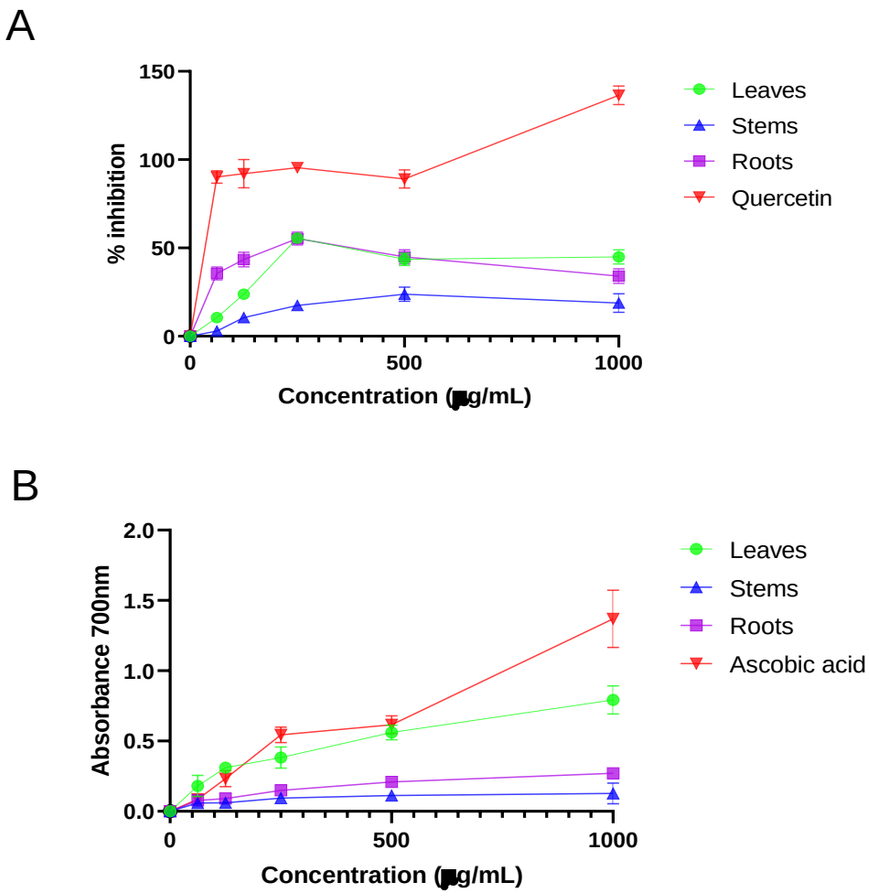

Figure 4.4 – The antioxidant properties of *Cb* leaf, root and stem aqueous extracts: DPPH scavenging activity (A) and reducing antioxidant power activity (B) of *C benghalensis* aqueous extract where ascorbic acid was used as positive control. The experiment was done in triplicates and the differences were found to be statistically significant (*** $P < 0.001$) compare to the blank control. Quercetin and ascorbic acid was used as positive control.

3.6. *4.7. MTT Analysis on MDA-MB 231*

The MTT assay is a method that can be used to measure or distinguish between viable and dead cells whether due to natural effect or after treatment with different compounds. The viable cells have active metabolism and therefore can convert the MTT compound to a purple formazan that has an absorbance maximum at 570nm. The amount of formazan produced can be measured using a spectrophotometer and is proportional to the number of viable cells. In order to determine the safety and toxicity of *Commelina benghalensis* on breast cancer cells, cell viability was determined using MTT assay. As shown in Figure 4.5 and Table 4.6, the cell viability results showed that the *Cb* roots and stems aqueous extract treatment of MDA-MB 231 cells with differing concentrations after 24 hours. There was significant effect on MDA-MB 231 cells with more than 80% cell viability observed in the higher concentrations (500 and 1000µg/mL), (**P>0.01 and ****P>0.00001). The trend was also observed on the same cells when they were exposed to different concentration of curcumin which was the positive control and there was significant effect (****P>0.00001). Curcumin was used as a positive control for benchmarking due to its pharmacological effects and its known anticancer activities (Fioravanti *at al*., 2024).

Table 4.10: The effect of Curcumin, *Cb* root and stem-aqueous extracts on MDA-MB 231 breast cancers cells

Concentration	Curcumin	Roots	Stems
Untreated	100.000 ± 0.000	100.000 ± 0.000	100,000 ± 0,000
10%H2O	104,632 ± 3,046	101,713 ± 0,397	104,190 ± 0,254
31.25	71,040 ± 7,116	103,792 ± 0,833	100,936 ± 0,547
62.5	58,305 ± 7,701	101,934 ± 3,0885	105,418 ± 2,259
125	52,711 ± 1,886	104,803 ± 1,503	97,688 ± 6,476
250	45,927 ± 2,728	95,940 ± 0,988	97,046 ± 6,511
500	30,981 ± 0,858	87,012 ± 1,409	101,472 ± 3,827
1000	22,027 ± 0,650	88,443 ± 0,548	95,777 ± 0,433

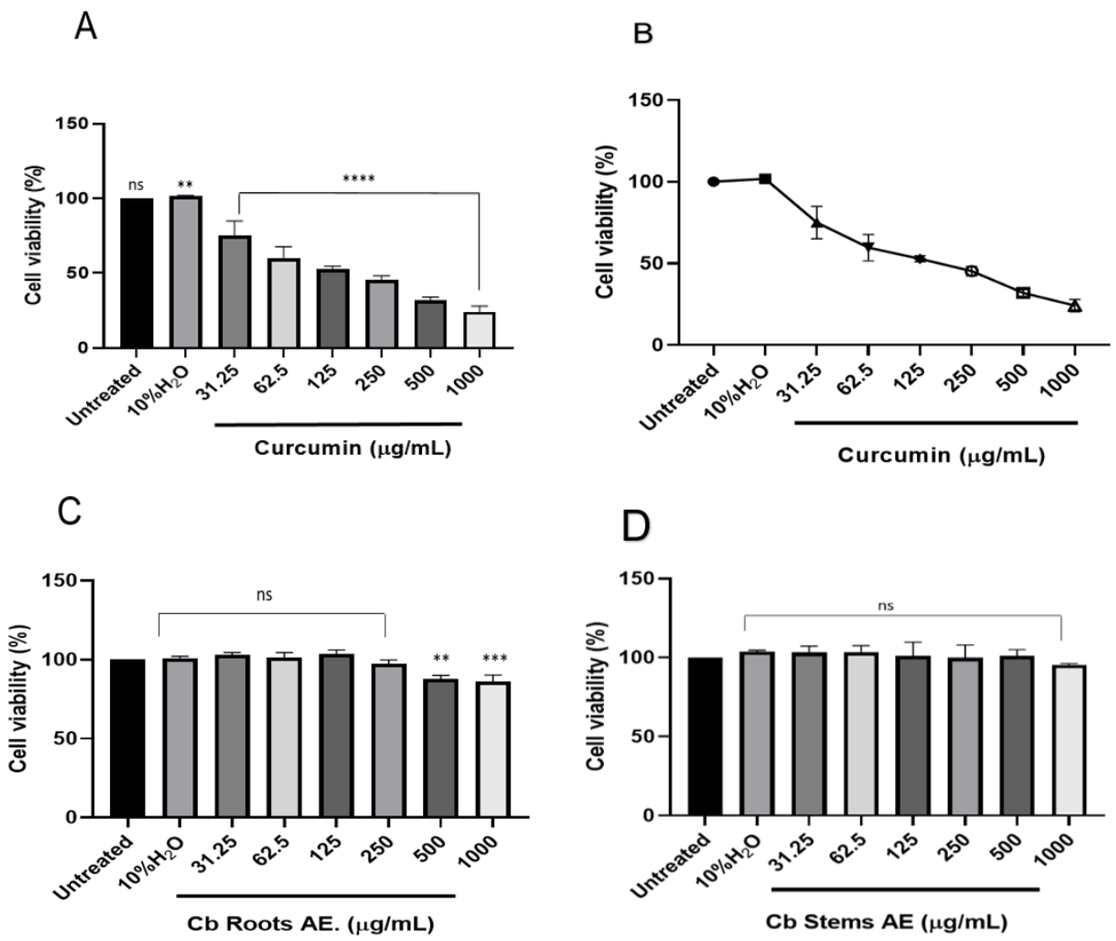

Figure 4.5 - The cytotoxic effect of the *Cb* root and stem-aqueous extract-treated MDA-MB 231 cells after 24 hours: the MDA-MB 231 breast cancer cells were treated with curcumin (A&B), Cb roots (C) and Cb stems (D) with different concentrations, 24h-treatment. The experiment was done in triplicates. The statistical analysis was carried out and the root extract significantly (**P < 0.01 and ***P <0.001) reduced the viability of the MDA-MB 231. Curcumin was used as positive control.

3.7. *4.8. MTT Analysis on MDA-MB 231 and MCF-7*

Table 4.7 presents a detailed analysis of the impact of varying concentrations of *Cb* aq leaves extract on MCF-7 and MDA-MB 231 breast cancer cells, with observations recorded at both 24-hour and 48-hour treatment intervals. The table includes a comparative assessment of cell viability percentages in relation to an untreated control group, 10% H_2O (water), and a positive control represented by 150µg Curcumin. The concentrations of *Cb* aq leaves extract range from 31.25 to 1000, and corresponding cell viability percentages are provided for each treatment condition. The data elucidates the concentration-dependent effects on both MCF-7 and MDA-MB 231 cells, offering valuable insights into the potential cytotoxic or inhibitory properties of the *Cb* aq leaves extract on breast cancer cells over different treatment durations. This comprehensive presentation of experimental results serves as a foundation for further interpretation and discussion regarding the potential therapeutic implications of *Cb* aq leaf extract in the context of breast cancer treatment.

Table 4.11: The effect of *Cb* aqueous leaf extract concentration on MCF-7 and MDA-MB 231 breast cancers cells

Concentration	MCF-7		MDA-MB 231	
	24h treatment	**48h treatment**	**24h treatment**	**48h treatment**
Untreated	100,000 ± 0,000	100,000 ± 0,000	100,000 ± 0,000	100,000 ± 0,000
10%H2O	104,190 ± 0,254	104,190 ± 0,254	104,147 ± 1,621	90,208 ± 5,043
150µg Curcumin	48,880 ± 5,593	48,880 ± 5,593		
31.25	100,936 ± 0,547	100,936 ± 0,546	105,083 ± 1,249	85,176 ± 4,770
62.5	105,418 ± 2,258	102,418 ± 0,741	105,832 ± 2,076	76,449 ± 6,918
125	104,590 ± 0,220	102,0897 ± 0,720	102,734 ± 2,490	60,189 ± 6,023
250	104,183 ± 0,626	99,546 ± 4,011	101,705 ± 0,996	56,335 ± 1,277
500	94,973 ± 0,327	97,473 ± 2,827	85,944 ± 4,522	49,592 ± 0,719
1000	85,777 ± 0,433	90,277 ± 2,067	80,878 ± 0,889	49,831 ± 0,958

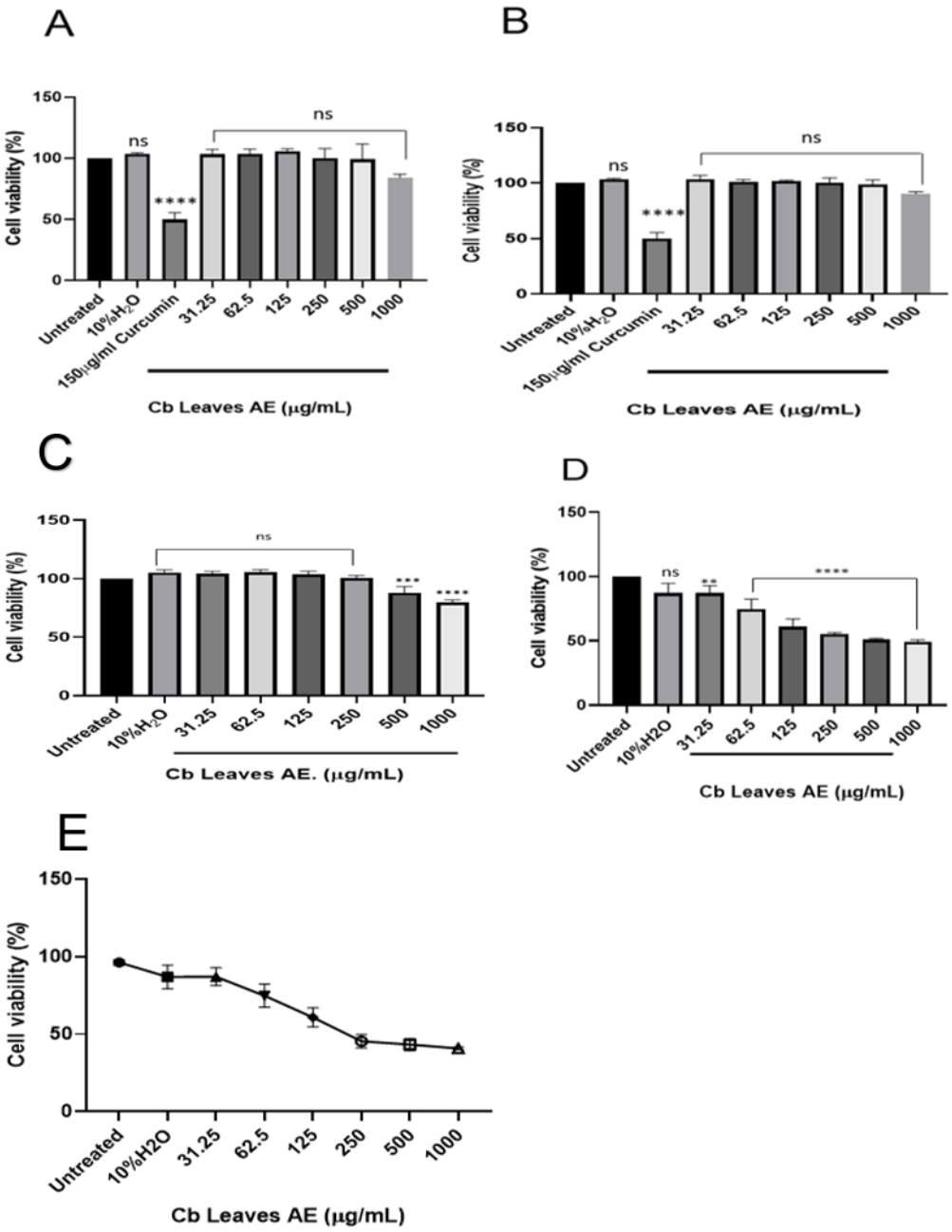

Figure 4.6 - The cytotoxic effect of the *Cb* leaf-aqueous extract-treated MCF-7 and MDA-MB 231 after 24 and 48 hours: The MCF-7 breast cancer cells were treated with *Cb* aq extract in different concentration and incubated for 24 hours (A) and 48 hours (B) and the MDA-MB 231 breast cancer cells were treated with leaves (C&D) in different concentrations and were incubated for 48 hours after treatment. The experiment was done in triplicates. The statistical analysis was carried out and the leave extract

significantly (**P < 0.01, ***P <0.001 and ****P <0.0001) reduced the viability of the MDA-MB 231.

3.8. *4.9. Muse cell viability profile analysis*

The principle for the Muse™ Count and Viability assay utilizes the Muse™ Count and Viability Reagent that will differentially stain viable (live) and non-viable (dead) cells based on the cells' permeability to the two dyes present in the reagent that bind to DNA. The DNA-binding dye in the reagent will stain cells that have lost the integrity of the membrane which will then allow the dye to stain the nucleus of dead and dying cells. This parameter is displayed as viability and is used to discriminate viable (live cells that do not stain) from non-viable (dead or dying cells that stain).

Thus, for further analysis and confirmation of the MTT results (Figure 4.5D), Muse™ Count and Viability Assay (Figure 4.6A-E) showed that the *Cb* leaf-aq extract decreased the viability of MDA-MB 231 cells. The cell viability of MDA-MB 231 cells was found to be significantly reduced (****P<0.0001) after treatment with 750 µg/ml of the *Cb* leaf-aq extract.

Table 4.12: Muse™ cell count and viability Results of *Cb* leaf-extract treated MDA-MB 231 cells after 48 hours

Treatment	**Mean (%) ± SEM**	
	Live	**Dead**
Untreated	93,675 ± 0,925	6,325 ± 0,925
10%H2O	91,175 ± 0,275	8,825 ± 0,275
150µg/mg Curcumin	49,800 ± 0,300	50,200 ± 0,300
750 µg/mg *Cb* leaves	54,000 ± 0,400	46,000 ± 0,400

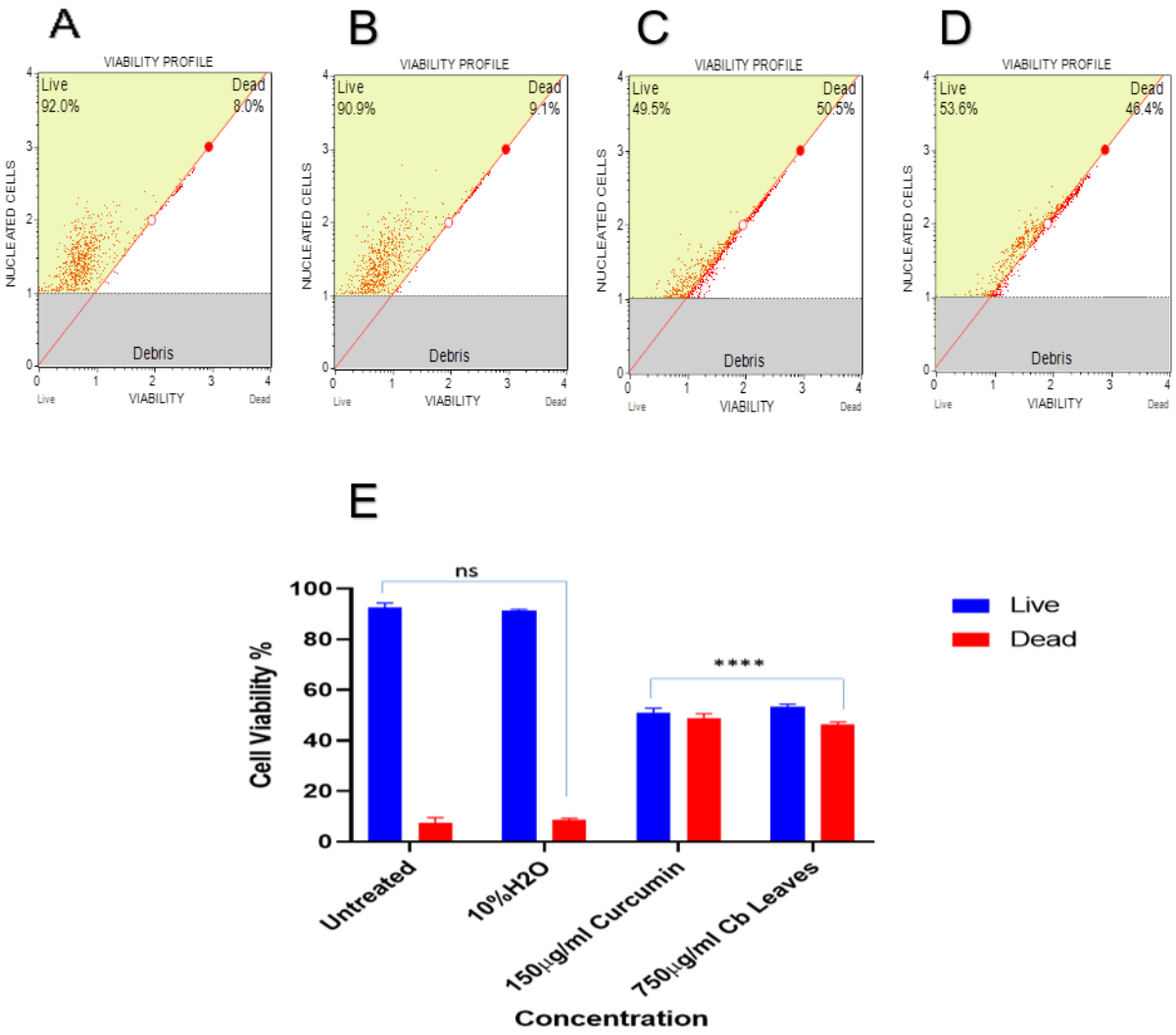

Figure 4.7 - Muse® count and viability profile of the *Cb* leaf-aqueous extract-treated MDA-MB 231 cells after 48 hours: Untreated (A), 10%H_2O (B), 150µg/ml curcumin (C) and 750µg/ml Cb leaves-aq extract (D). The cell viability percentage of the MDA-MB 231 cells treated with Cb and incubated for 48 hours (E), the results shown are the representative data of the profiles (A, B, C &D). The experiment was done in triplicates. The statistical analysis was carried out and the leave extract significantly (****P <0.0001) reduced the viability of the MDA-MB 231.

3.9. *4.10. Morphological characteristics of MDA-MB 231 cells after exposed to Cb leaves-aq extract for 48h*

There was an effect on the growth of the MDA MB231 cells after 48h treatment with Cb leaf-aq extract. This corroborated with the MTT results (Figure 4.5D). The observed morphological changes included cell shrinkage (Figure 4.8). The untreated cells and solvent control (10% H_2O) showed normal epithelial shape, while both the positive control (Curcumin) and Cb treated cells showed cell shrinkage and reduction in cell numbers.

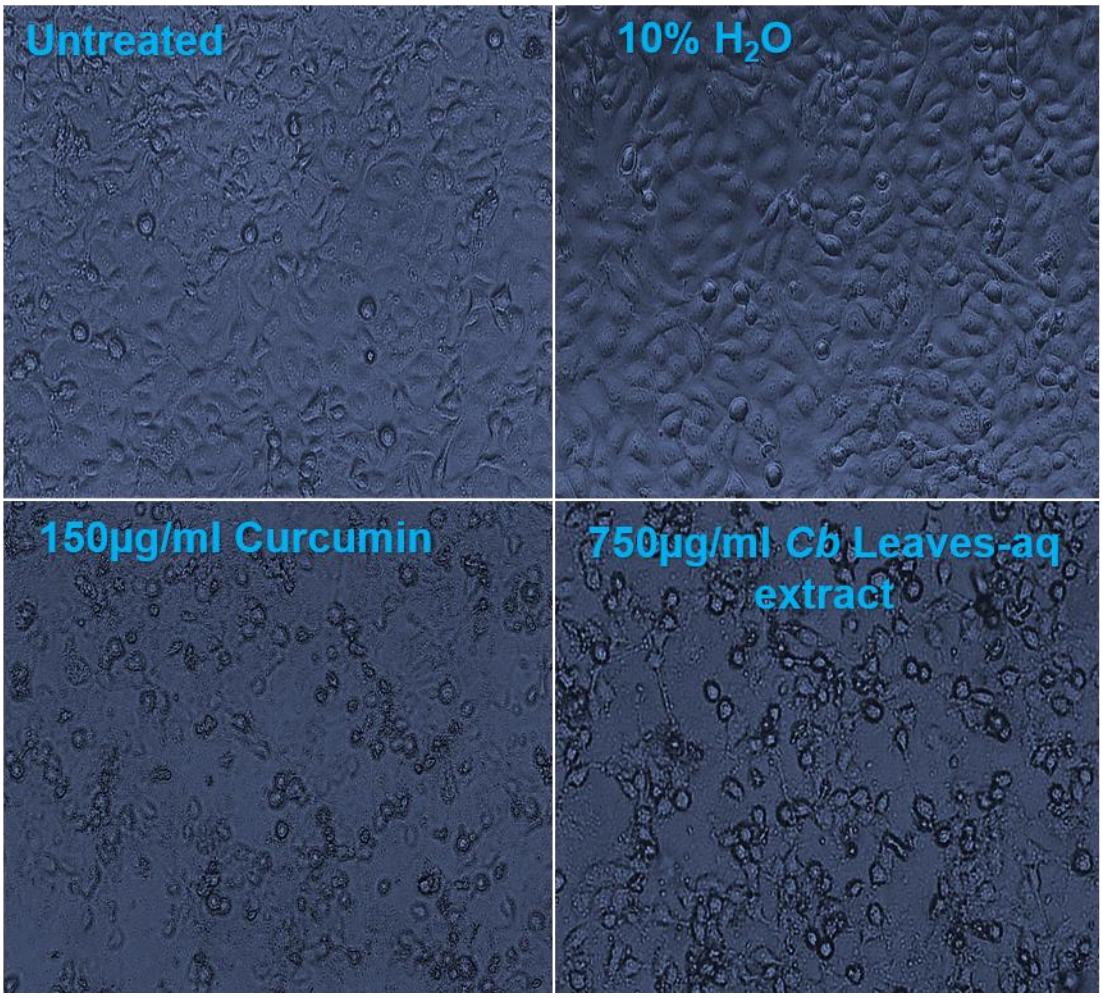

Figure 4.8 - Morphological characteristics of MDA-MB 231 cells. The cells were exposed to only complete media (untreated), 10%H_2O, 150µg/ml Curcumin, and 750µg/ml *Cb* Leaves-aq extract, and the images were captured using an inverted microscope fitted with camera and images analysed with LCMicro software from Olympus.

3.10. *4.11. Apoptosis assessment of Cb leaves aq extract on MDA-MB 231 cells*

The Muse® Annexin V & Dead Cell Kit is a method that is utilised to detect cells in various stages of apoptosis (programmed cell death). The assay relies on the binding of fluorescently labelled Annexin V to phosphatidylserine (PS), which upon the onset of apoptosis, will translocate to the outer surface of the cell membrane. The assay uses a single reagent and two stains to stain and differentiate live, dead and apoptotic cells. Early in the apoptotic pathway, molecules of PS are translocated to the outer surface of the cell membrane. Annexin V-PE is used then to detect PS on the external membrane of apoptotic cells. 7-AAD, because it is excluded from live and healthy cells, permeates the late-stage apoptotic and dead cells, thus, the read-out is a grouping of 4 distinct cell populations: dead cells, live cells, early and late apoptotic cells.

Shown in Table 4.9. is a detailed assessment of cell viability and apoptotic events in MDA-MB 231 breast cancer cells under various treatment conditions. The recorded mean percentages ± standard error of the mean (SEM) depict the distribution of live cells, dead cells, and those undergoing early and late apoptosis. The untreated control group exhibited a baseline cell status, while 10% H2O (water) served as a comparative control. Additionally, the table includes data for a positive control represented by 150µg/mg curcumin and the experimental treatment group with 750 µg/mg *Cb* leaves aq extract. The results highlight the concentration-dependent effect of these treatments on breast cancer cells, shedding light on the potential apoptotic induction properties of curcumin and *Cb* leaves aq extract. The observed variations in live, apoptotic, and dead cell percentages provide essential insights into the impact of these treatments on the cellular dynamics of breast cancer cells, forming the basis for further discussions on the therapeutic implications of the tested agents in cancer treatment.

Table 4.13: The apoptosis mean percentages and SEM of *Cb* leaf-extract treated MDA MB 231 cells after 48 hours

Treatment	Mean (%) ± SEM				
	Live	**Dead**	**Early apoptosis**	**Late Apoptosis**	**Total-apoptotic**
Untreated	94,025 ± 1,475	0,000 ± 0,000	4,950 ± 1,100	1,225 ± 0,175	5,975 ± 1,475
10%H2O	95,500 ± 1,010	0,000 ± 0,000	3,250 ± 0,800	1,250 ± 0,300	4,500 ± 1,100
150µg/mg Curcumin	47,450 ± 1,350	0,000 ± 0,000	38,175 ± 3,175	14,050 ± 1,500	52,500 ± 1,400
750 µg/mg *Cb* aq leaves	47,475 ± 1,375	0,000 ± 0,000	38,475 ± 2,875	14,025 ± 1,475	52,525 ± 1,375

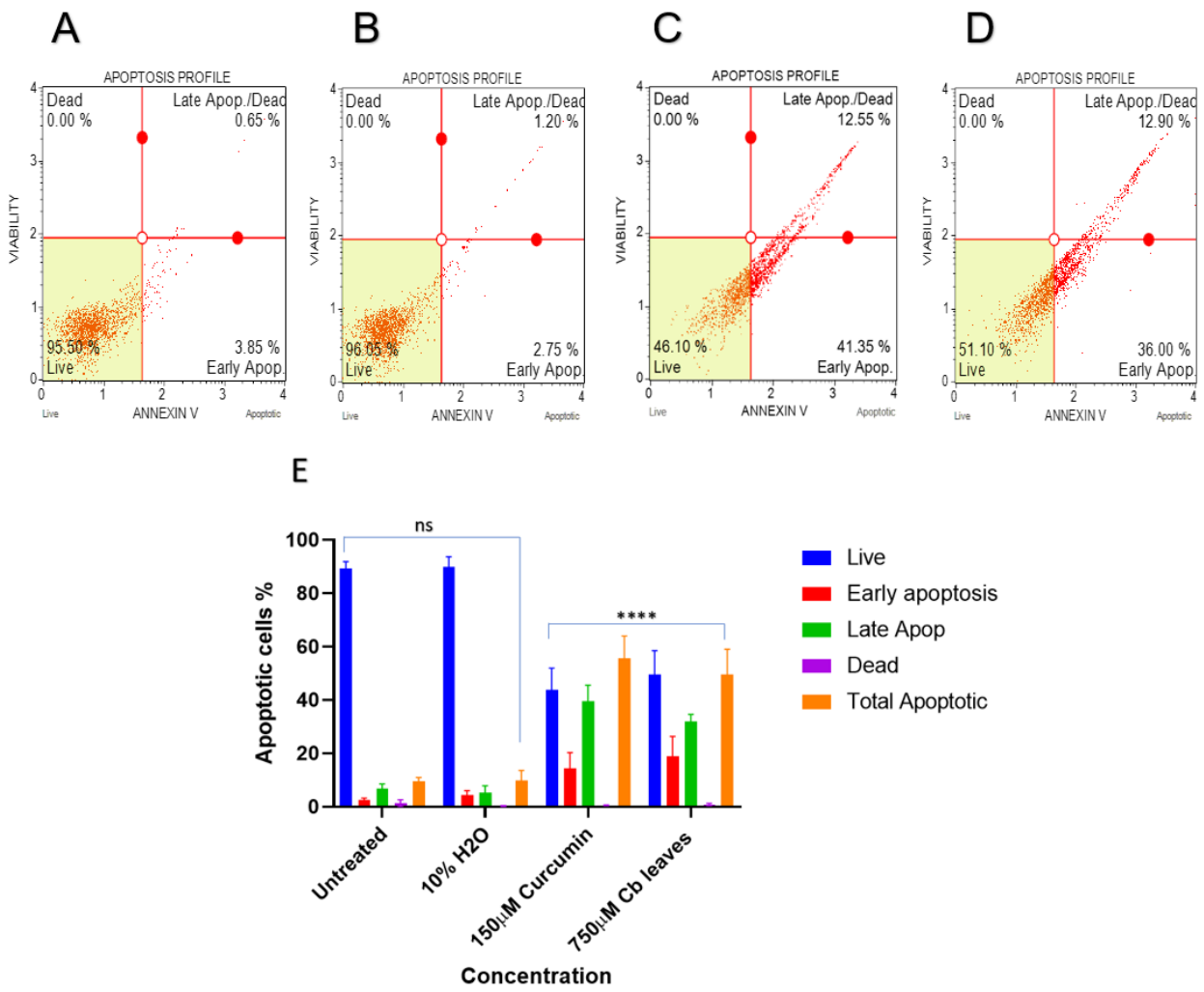

Figure 4.9 - Apoptosis percentages of the *Cb* leaf- aqueous extract-treated MDA-MB 231 cells induced after 48 hours: untreated (A), 2%H_2O (B), 150µg/ml curcumin (C) and 750µg/m Cb leaves (D). The data was analysed using Muse® Cell Analyser. The experiment was done in triplicates and the statistical analysis was carried out. The statistical analysis was carried out and the leave extract significantly (****P <0.0001) induced apoptosis of the MDA-MB 231.

5. Introduction

In this chapter of the study, the anticancer activity of *Commelina benghalensis* (*Cb*) aqueous (aq) extract on breast cancer cells will be discussed in order to elucidate different characteristics of the plant's potential therapeutic effects. The discussion commences with plant extraction, a fundamental process that lays the groundwork for subsequent analyses. Thin Layer Chromatography (TLC) and Liquid Chromatography-Mass Spectrometry (LC-MS) techniques are then employed to identify and characterise e the bioactive compounds present. The investigation extends to phytochemical screening, providing insights into the diverse chemical constituents within *Cb* aq extract. The quantification of total phenolic, tannin and flavonoid Content, coupled with Radical Scavenging Assays such as 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) and Free Radical Antioxidant Power (FRAP), contributes to understanding the antioxidant potential of the *Cb* aq extract. Moving towards a cellular perspective, cytotoxic analysis sheds light on the impact of *Cb* aq extract on breast cancer cell line (MDA-MB 231 and MCF-7). Muse® count cell viability, alongside morphological characteristics, provide crucial information on cellular responses towards *Cb* leaf-aq extract-treated. The apoptotic assay helped to understand whether the observed effects of *Cb* lea-aq extract-treated on MDA-MB 231 cells involve the activation of apoptotic pathways. By measuring apoptotic percentages, the assay provided insights into the potential mechanisms through which *Cb* aq extract may exert its impact on MDA-MB 231 cells. Through a synthesis of these diverse assays, this discussion chapter aims to present a nuanced understanding of the anticancer activities of *Cb* aq extract, offering valuable insights into its potential as a therapeutic agent in breast cancer contexts

3.11. *5.1. Profiling Bioactive Compounds Cb root, leaf and stem-aq extract using Thin Layer Chromatography (TLC)*

Thin Layer Chromatography (TLC) is a separation technique that allows the identification and characterization of different compounds within a mixture based on their affinity for the

stationary phase and mobile phase (Wu *et al*., 2019). The results obtained when *Cb* root, leaf and stem-aq extract were analysed, under the following solvent systems; EMW (Ethyl acetate, Methanol and Water), TEA (Toluene, Ethanol, Ammonia) and CEF (Chloroform, Ethyl acetate and formic acid), revealed a distinctive pattern of pink-purple colour after the plates were sprayed with p-anisaldehyde (Figure 4.1A) and yellow and purple colours after the plates were sprayed with vanillin (Figure 4.1 B), providing information about the composition of the plant extract. In the context of the appearance of yellow and purple colours after spraying, indicated the presence of specific classes of compounds with distinct chemical properties. The yellow coloration which was mostly observed in the leaf-aq extract part of the plant under TEM and EMW, in Fig 4.1B indicate the presence of flavonoids, which are a group of polyphenolic compounds known for their antioxidant properties (Cimpoiu *et al*., 2005). The observation of purple coloration which was mostly observed in the leaf-aq extract part of the plant, under TEM and EMW, indicates the presence of phenolic compounds, highlighting the potential antioxidant capacity of the plant (Pontieri *et al*., 2021). This finding aligns with the broader understanding of the medicinal and therapeutic properties often associated with plants rich in phenolic compounds, reinforcing the potential significance of *Cb* aq extract in the context of its anticancer activity, especially in the realm of breast cancer cells as per the investigation's focus. The coexistence of yellow and pink - purple colours as shown in Fig 4.1A, mostly observed in TEM, indicates a rich and diverse phytochemical profile in *Cb* aq extract. These compounds, particularly flavonoids, are well-documented for their potential health benefits, including antioxidant and anti-inflammatory properties (Panche *et al*., 2016). Therefore, the observed colours signify the potential presence of bioactive compounds in *Cb* aq extract that could contribute to its therapeutic properties. Further characterization and identification of these compounds through additional analyses, such as liquid chromatography-mass spectrometry, provide a more detailed understanding of the specific chemical constituents responsible for the observed colours and potential medicinal properties.

3.12. *5.2. Phytochemical compounds present in Cb leaf-aq extract*

The LC-MS is a technique for identification and quantification of individual compounds within a sample. In this context, the LC-MS enables the precise characterisation of the molecular composition of *Cb* leaf-aq extract by comparing the identified compounds with existing literature on their biological activities. In Figure 4.2, LC-MS spectrum showed the mass-to-charge ratio (m/z) of the ions produced when the *Cb* leaf-aq extract sample was hit with electrons. Each peak in the spectrum corresponded to different molecules in the sample. The peaks in the mass spectrum were used to identify the compound molecules in the *Cb* leaf-aq extract sample by comparing them to the spectral library. The results unveiled a diverse array of compounds identified by the spectral library, which were present in *Cb* leaf-aq extract, including demethylzeylasteral, brucine, sodium glycodeoxycholate, osthole, and tuberostemonine as shown in Table 4.1. Demethylzeylasteral, has been previously associated with anti-inflammatory properties, suggesting a potential contribution to the immunomodulatory effects observed (Sun *et al*., 2022). Brucine, known for its cytotoxic properties, may influence cellular pathways involved in the regulation of immune responses (Lu *et al*., 2020). Osthole, recognised for its anti-cancer properties, contributes to the reported impact on breast cancer cells (Park *et al*., 2019 and Shokoohinia *et al*., 2018). Tuberostemonine, an alkaloid, could play a role in cellular signalling pathways related to immunity, including the inhibition of cell proliferation (Eun *et al*., 2014). The phytochemicals presented in Table 4.2 were unidentified in the phytochemical library used, and they were identified several literature reviews through provided molecular formula and mass.

The results have unveiled a diverse array of compounds including diterpenoids, abietic acid, flavonoids, Biindole, phenol, alkalbenzene, alkylamides, alkaloids, triazole derivatives, thioxanthone, amines, Lauramide, Anilino compounds, macrocyclic ligands, carbamates, liquid crystal, cyclopenta pyridines, sulfhydryl, hemihydrate, Dinitrochlorobenzene, tertiary amino compound, Erucamide fatty amide, octadecanoate ester Vorinostat, collectively exhibit significant immunomodulatory effects in breast cancer, including the inhibition of inflammatory cytokines (TNF-α) (Hosseinzade *et al*., 2019, Zhang &Chun-hai, 2020), induction of apoptosis, and modulation of key signalling pathways (Zhang *et al*., 2018). These findings highlight the potential of these compounds

as promising candidates for the development of targeted immunomodulatory and anti-cancer therapeutic interventions for breast cancer.

As shown in Table 4.3, abietic acid, Vorinostat and Lauramide were found to regulate the expression of a number of cytokine genes. These genes included *VEGF, TGF-b1, IGF1R, ATG1, C-myc, TNF-α, NF-kB,* IFNγ, IL-2, IL-4, IL-10, IL-13, *TNF-α, IL-2,* and *IL-10.* Their deregulation is associated with the carcinogenesis process. IL-10 has both pro- and anti-inflammatory effect on breast cancer; however, IL-2 and IL-10 have synergistic effect of enhancing CD8+T cells cytotoxicity to induce apoptosis in breast cancer cells (Li, et al, 2017).Treatment with Cb leaf extract, may regulate cytokine genes, enhancing immune system-mediated anticancer activities.

3.13. *5.3. Phytochemical screening of compounds present in Cb root, leaf and stem-aq extract*

Phytochemical screening involves the qualitative analysis of plant extracts to identify and characterize the presence of various bioactive compounds where a series of tests are used to detect the presence of specific classes of compounds such as alkaloids, flavonoids, tannins, saponins, terpenoids, and phenolic compounds (Altemimi *et al*., 2017 and Mallikharjuna et al., 2007). The results of the phytochemical screening conducted in *Cb* aq-extract unveil a rich chemical profile inherent in various parts of the plant (root, leaf and stem). The application of the ferric chloride test demonstrated the ubiquitous presence of phenolic compounds across all examined plant parts, including leaves, roots, and stems at shown in Table 4.3. The detection of phenols is indicative of the potential antioxidant properties of *Cb*, as phenolic compounds are renowned for their ability to neutralize free radicals and confer health benefits (Shu *et al*., 2005 and Zymanczyk-Duda *et al*., 2018). Furthermore, the ferric chloride test revealed the widespread occurrence of flavonoids in leaves, roots, and stems. Flavonoids, known for their diverse biological activities, including anti-inflammatory and immunomodulatory effects, signify an additional dimension to the therapeutic potential of *Cb* (Kumar & Pandey., 2013 and Panche *et al*., 2016). The Tannic acid test, however, provided distinct results, indicating the presence of alkaloids in leaves and roots, however, in stems, alkaloids were notably

absent. Alkaloids are a class of secondary metabolites known for their pharmacological activities, and their identification in *Cb* suggested potential bioactive properties that could contribute to the immunomodulatory effects (Gurjar *et al*., 2016). It is noteworthy that the absence of alkaloids in the Tannic acid test contradicted the positive result obtained from the ferric chloride test. Discrepancies in test outcomes may arise from variations in the chemical specificity of each assay or the presence of specific alkaloids that do not react with tannic acid. In the detection of tannins, it was observed that neither of the three parts (root, leaf and stem aq-extract) of the plant contained tannin, in other studies it was observed that the tannins were detected only in *Cb* ethanol-extract not in aq-extract (Harini *et al*., 2022). In summary, the phytochemical screening results underscored the complexity of *Cb* aq extract chemical composition, revealing the presence of phenols, flavonoids, and alkaloids in various plant parts. These findings lay the groundwork for understanding the potential therapeutic benefits of the plant in the context of anticancer activity.

3.14. *5.4. Total phenolic content (TPC), total tannin content (TTC) and total flavonoid content (TFC) of Cb aq-extract*

The investigation into the anticancer activity of *Cb* yielded compelling results regarding the plant's phytochemical composition. The assessment of Total Phenolic Content (TPC), Total Tannin Content (TTC), and Total Flavonoid Content (TFC) has provided valuable insights into the distribution of these bioactive compounds across different parts of the plant. The TPC in *Cb* extract was expressed as Gallic acid equivalent. The results indicate a noteworthy variation in the phenolic composition among various plant parts. Notably, Figure 4.3A, the *Cb* leaf-aq extract exhibit a significant high TPC of 29.087 ± 9.580 ($P < 0.001$) content of phenols compared to the roots and stems. Quercetin was used as positive control. This elevated level of phenolic compounds in the leaf-aq extract showed a potential source of potent antioxidants and bioactive molecules (Shi *et al*., 2022). Similar to phenols, the *Cb* leaf-aq extract showcased a notable significant high TFC of 23.936 ± 4.134 ($P < 0.001$) content of flavonoids in Figure 4.3B in comparison to the roots and stems. Flavonoids are known for their antioxidant and immunomodulatory properties, suggesting that the *Cb* leaf-aq extract harbour key bioactive compounds contributing to

its observed effects on the anticancer activity (David *et al*., 2022). The TFC was expressed in terms of quercetin equivalence and Gallic acid was used as positive control. Furthermore, the observation revealed low content of tannins in Figure 4.3C within *Cb*. In Figure 4.3C the *Cb* root and leaf-aq extract display low levels of tannins, while the stems exhibited much lower tannin content where it was expressed in tannin acid equivalence and Gallic acid was used as positive control. The disparity in tannin distribution highlighted the differential accumulation of these compounds in different plant tissues. In summary, the results underscore the significance of considering different plant parts when evaluating the immunomodulatory potential of *Cb*. The phenolic and flavonoid content in the leaf, adds granularity to the understanding of the plant's phytochemical profile and its implications for harnessing its therapeutic properties, the leaves extract was the one used to conduct further studies for the project.

3.15. *5.5. Antioxidant activities of Commelina benghalensis (Cb) aqueous (aq) extract*

The 2,2-Diphenyl-1-picrylhydrazyl (DPPH) is based on measuring the ability of antioxidants to scavenge the stable free radical DPPH involving the reduction of the purple-coloured DPPH radical to the yellow-coloured DPPH-H by antioxidants, and the degree of discoloration is used to quantify the antioxidant capacity of the compounds or extracts being tested and the free radical antioxidant power (FRAP) assay measures the reducing power of antioxidants. The assays were used to evaluate the antioxidant potential of compounds of *Cb* root, leaf and stem-aq extract to assess its potential health benefits. In the context of *Cb* aq-extract, the root and leaf showed high percentages of inhibition indicating a potent antioxidant activity, was observed in the root and leaf as it is shown in Figure 4.4A, in comparison to the stems. It exhibited the highest half-maximum effective concentration (EC50) values of 250 µg/mL for the DPPH scavenging activity. The higher absorbance observed in the leaves compared to the roots and stems as it was shown in Figure 4.4, signified a greater reducing power in the leaf extracts of *Cb* aqueous aq extract. A higher absorbance (1000 µg/mL) indicated a stronger reducing power, implying a higher capacity to donate electrons and counteract oxidative stress. In the context of immunomodulation, this suggests that the leaf extracts of *Cb* possess robust

antioxidant potential, potentially contributing to the regulation of immune responses by mitigating oxidative damage (Song *et al*. 2010).

3.16. *5.6. Anticancer activities of Cb leaf-aq extract*

The assay is based on viable cells converting MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) into a formazan product, and the intensity of the produced colour being directly proportional to the number of living (viable) cells. The cytotoxicity analysis, conducted using MTT assay, served as a crucial component in evaluating the impact of *Cb* root, leaf and stem-aq extract on MDA-MB 231 and MCF-7 breast cancer cells where curcumin was used as positive control. The results showed that stems-aqueous extract of *Cb* do not have any significant effect on MDA-MB 231 after 24 hours of incubation, as it is shown in Figure 4.5D. The roots-aqueous extract of *Cb* had significant (**P < 0.01 and ***P <0.00) effect on MDA-MB 231 after 24 hours of incubation, as shown in Figure 4.5C (Widyanto et al., 2020). The *Cb* leaf-aq extract possessed significant (**P < 0.01, ***P <0.001 and) potent anti-cancer properties against MDA-MB 231 cells at higher concentration (500 and 1000µg/mL) as shown in Figure 4.6C after 24 hours of incubation, attributed to the rapid interaction of the bioactive compounds present in the extract with the breast cancer cells. Potential mechanisms may involve induction of apoptosis, disruption of cell cycle progression, or interference with vital cellular processes. The increased efficacy observed at 48 hours at higher concentration (62.5 to 1000µg/mL) as shown in Figure 4.6D indicates a sustained and possibly cumulative significant (****P <0.0001) effect. The prolonged exposure may allow for deeper penetration of the bioactive compounds into the cells, resulting in a more pronounced impact on cell viability making it a promising candidate for further exploration in cancer therapeutics. Further investigations into the specific bioactive compounds responsible for the observed effects and their mechanisms of action will be instrumental in unravelling the full therapeutic potential of *Cb* in modulating the immune response against breast cancer cells.

5.6.1. Muse^{TM} cell count and viability

Muse^{TM} cell viability and count assay assessed the survival of cells. The assessment of Muse^{TM} cell viability and count involve techniques such as flow cytometry for viable cell identification. The Muse^{TM} cell count and viability profile for MDA-MB 231 cells *Cb* leaf-aq extract-treated, after a 48-hour incubation has yielded significant (****P <0.0001) results, by reducing the viability of the cells. The untreated cells in figure 4.7A served as a baseline reference, exhibiting more 80% (after 48 hours of incubation) of viability and count under normal (untreated) conditions. Similarly, treatment with 10% H2O Figure 4.7B did not seem to induce significant alterations in the Muse^{TM} count and viability, where cell viability of more than 80% was observed after 48 hours of incubation implying that the results were non-significant. A noteworthy outcome was observed with the treatment using 150µg/ml curcumin (positive control) as shown in Figure 4.6C where there was significant reduction in viable cells. The most significant (****P<0.0001) findings were observed from the treatment with 750µg/ml of *Cb* leaf-aq extract as shown in Figure 4.7D where 53.6% were viable cells. The results demonstrated significant effect at a concentration of 750µg/ml, particularly in comparison to the untreated group with higher viability, underscoring the potential therapeutic relevance of *Cb* leaf-aq extract in modulating the behaviour of breast cancer cells. Furthermore, the findings on cell viability provided a foundation for further investigation into mechanisms that lead to reduction in viability of aq- leaf treated breast cancer cells, such as apoptosis analysis

5.6.2. Morphological characteristics of treated MDA-MB 231 cells

To analyse the shape, size and cell shrinkage, the cells were seeded and treated with 750µg/ml *Cb* aq extract, 150 µg/ml curcumin, 10% H2O and the untreated and the cells were incubated for 48 hours. The morphological analysis was observed without staining, the cells were observed in their natural state without interference from dyes or other chemicals. As shown in Figure 4.8, untreated cells maintained the normal morphology including shape and size. *Cb* aq extract had an effect on the MDA MB 231 cells as demonstrated on Figure 4.8-750µg/ml *Cb* leaf-aq extract, the results showed cell shrinkage, ie, smaller size when compared to the untreated cells.

5.6.3. Apoptosis analysis

Apoptosis is a tightly regulated biological process that eliminates damaged or unwanted cells. The investigation into the apoptotic effect of *Cb* leaf-aq on MDA-MB 231 cells following a 48-hour treatment extract yielded notable and statistically significant (****P<0.0001) results as shown in Figure 4.9. The data, representative of three independent experiments, demonstrated a substantial increase in apoptotic percentages in treated cancer cells. These results have an implications for the broader study on the anticancer activities of *Cb* leaf-aq extract on MDA-MB 231 cells. The observed increase in apoptotic percentages of 50+% at the concentration of 750µg/ml as shown in Figure 4.9E indicated that the *Cb* leaf-aq extract induced programmed cell death in MDA-MB 231 breast cancer cells (Orni *et al*., 2018 and Kansagara and Pandya., 2019). The results were considered a favourable outcome, as the extract hindered uncontrolled cell proliferation and growth. In conclusion, the significant increase in apoptotic percentages in MDA-MB 231 cells treated with *Cb* leaf-aq extract underscore the potential anticancer properties of the *Cb* leaf-aq extract on MDA-MB 231. The outcome aligns with the broader goal of identifying natural compounds with therapeutic potential against breast cancer, specifically by inducing programmed cell death in cancerous cells. The apoptotic assay, through its examination of cell death, serve as a crucial tool in unravelling the mechanistic aspects of *Cb* impact on breast cancer cells in the context of anticancer activity.

3.17. *5.7. Conclusion*

The investigation into effect bioactive compounds found in *Commelina benghalensis* in the treatment of breast cancer yielded noteworthy findings, unveiling the potential therapeutic properties of this plant. The results also yielded through LC-MS a number of compounds that possessed potential immunomodulatory capacity that required further study and analysis. The notable decrease in cell viability observed in MDA-MB 231 breast cancer cells treated with *Commelina benghalensis* leaves after 48 hours implied a possible inhibitory effect on tumour cell growth. Additionally, the increased incidence of apoptosis suggested a potential mechanism through which *Commelina benghalensis* may induce programmed cell death in breast cancer cells, a critical aspect in cancer treatment strategies.

5.7.1. Limitations of the study

Despite the promising findings using the *Cb* medicinal plant, some limitations must be acknowledged. The immunomodulatory effect of the leaves extract influenced by *Commelina benghalensis* beyond the antioxidant and apoptotic effect remain to be fully elucidated. Cytokine profiling after treatment with *Cb* extracts could assist in elucidating pathways imperative to comprehending the underlying molecular mechanisms of the medicinal plant effect on breast cancer cells.

5.7.2. Recommendations and Future studies

Future research endeavours need to focus on the cell cycle so as to determine the specific phase in the cell cycle where the cells were arrested, thus preventing MDA-MD 231 cells from progressing through the cell cycle and dividing and stopping the uncontrolled proliferation of cancer cells. Furthermore, the research on the identified potential bioactive compounds through LC-MS, could be conducted on breast cancer cells to determine their effect on cytokine gene expression and their potential as novel anti-cancer drugs.

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LC-MS DATA

The following results showcase a comprehensive analysis of *Commelina benghalensis* through Liquid Chromatography-Mass Spectrometry (LC-MS), providing a detailed profile of both known and potential chemical compounds present in the plant. The known chemical compounds, as identified by LC-MS, reveal specific peaks corresponding to distinct components such as Demethylzeylasteral, Brucine, Sodium glycodeoxycholate, Osthole, Tuberostemonine, Indigo dye, and 2-Methoxyestradiol. Each entry includes information on the peak's retention time, area, molecular formula, precursor mass, library hit, library score, and formula finder results. Additionally, the table presents potential chemical compounds with corresponding details, although these entries lack matches in the available libraries. The identification and understanding of these chemical components contribute significantly to unravelling the therapeutic potential of *Commelina benghalensis*. Further investigations into these compounds, including their biological activities and interactions, will be instrumental for harnessing the plant's medicinal properties. This LC-MS analysis sets the stage for future research aimed at elucidating the pharmacological relevance of the identified compounds and exploring their potential applications in various fields, including medicine and natural product-based drug discovery.

Table A1: Known chemical compounds

#	Analyse Peak Name	Component Name	Area	Formula	Precursor Mass	Library Hit	Library Score	Formula Finder Results
1. 1904	481.2637 / 8.99	481.2637 / 8.99	5.906e+03	{480.25696}	481.264	Demethylzeylasteral	58.9	C23H36N4O7
2. 2284	395.2167 / 9.98 [M+Na]+	395.2167 / 9.98 [M+Na]+	1.691e+04	{372.22801}	395.217	Brucine	50.3	C18H32N2O6
3. 4631	472.3189 / 14.12	472.3189 / 14.12	5.137e+04	{471.31215}	472.319	Sodium glycodeoxycholate	24.3	C28H37N7
4. 5228	277.1421 / 15.46 [M+CH3OH+H]+	277.1421 / 15.46 [M+CH3OH+H]+	1.871e+04	{244.10913}	277.142	Osthole	22.7	C11H12N6O
5. 5486	376.2606 / 16.03	376.2606 / 16.03	1.205e+04	{375.25386}	376.261	Tuberostemonine	10.4	C14H33N9OS
6. 5568	263.0569 / 16.23	263.0569 / 16.23	6.396e+03	{262.05022}	263.057	Indigo dye	71.6	C11H18OS3
7. 7658	303.1949 / 21.39 [M+CH3OH+H]+	303.1949 / 21.39 [M+CH3OH+H]+	3.423e+04	{270.16199}	303.195	2-Methoxyestradiol	12.7	C11H22N6S

Table A2: Potential Chemical compounds

#	Analyte Peak Name	Component Name	Area	Formula	Precursor Mass	Library Hit	Library Score	Formula Finder Results
1. 8149	303.2305 / 22.53 [M+H]+	303.2305 / 22.53 [M+H]+	1.667e+05	{302.22380}	303.231	No Match	0.0	C20H30O2
2. 6749	335.1264 / 19.23	335.1264 / 19.23	1.310e+05	{334.11971}	335.126	No Match	0.0	C21H18O4
3. 6809	200.2019 / 19.37 [M+H]+	200.2019 / 19.37 [M+H]+	1.513e+05	{199.19515}	200.202	No Match	0.0	C12H25NO
4. 7111	297.0842 / 20.12	297.0842 / 20.12	2.880e+03	{296.07751}	297.084	No Match	0.0	C16H12N2O4
5. 7445	257.1887 / 20.94	257.1887 / 20.94	2.554e+04	{256.18199}	257.189	No Match	0.0	C18H24O
6. 7603	276.2307 / 21.25	276.2307 / 21.25	7.220e+04	{275.22398}	276.231	No Match	0.0	C18H29NO
7. 7458	235.2067 / 20.98	235.2067 / 20.98	2.099e+04	{234.19995}	235.207	No Match	0.0	C16H26O
8. 2429	321.2023 / 10.26 [M+H+Na]2+	321.2023 / 10.26 [M+H+Na]2+	1.280e+05	{618.40923}	321.202	No Match	0.0	C36H54N6OS

9. 4706	242.2853 / 14.25	242.2853 / 14.25	6.879e+03	{241.27853}	242.285	No Match	0.0	C16H35N
10. 2450	470.3422 / 10.28	470.3422 / 10.28	1.125e+04	{469.33546}	470.342	No Match	0.0	C33H43NO
11. 2459	371.1031 / 10.31	371.1031 / 10.31	4.566e+02	{370.09634}	371.103	No Match	0.0	C15H30S5
12. 2466	293.1852 / 10.32 [M+H]+	293.1852 / 10.32 [M+H]+	9.457e+03	{292.17842}	293.185	No Match	0.0	C16H24N2O3
13. 2867	647.4098 / 11.12 [M+H]+	647.4098 / 11.12 [M+H]+	3.590e+05	{646.40307}	647.410	No Match	0.0	C44H54O4
14. 2887	355.7622 / 11.12 [M+H+NH4]2+	355.7622 / 11.12 [M+H+NH4]2+	1.361e+05	{692.48435}	355.762	No Match	0.0	C48H60N4
15. 3152	361.2413 / 11.54 [M+H]+	361.2413 / 11.54 [M+H]+	1.146e+04	{360.23456}	361.241	No Match	0.0	C19H36O4S
16. 2466	293.1852 / 10.32 [M+H]+	293.1852 / 10.32 [M+H]+	9.457e+03	{292.17842}	293.185	No Match	0.0	C16H24N2O3
17. 4925	245.0796 / 14.75	245.0796 / 14.75	5.172e+04	{244.07285}	245.080	No Match	0.0	C14H12O4

18.5504	256.3007 / 16.07	256.3007 / 16.07	8.539e+04	{255.29397}	256.301	No Match	0.0	C17H37N
19.5901	259.1317 / 17.14	259.1317 / 17.14	2.364e+04	{258.12495}	259.132	No Match	0.0	C16H18O3
20.10087	338.3430 / 27.89 [M+H]+	338.3430 / 27.89 [M+H]+	4.734e+04	{337.33630}	338.343	No Match	0.0	C22H43NO
21.8660	299.2955 / 23.74	299.2955 / 23.74	1.265e+05	{298.28875}	299.295	No Match	0.0	C19H38O2
22.2365	265.1530 / 10.17	265.1530 / 10.17	1.167e+04	{264.14632}	265.153	No Match	0.0	C14H20N2O3