ANTIBACTERIAL ACTIVITY AND IMMUNOMODULATORY EFFECTS OF FRACTIONS ISOLATED FROM *COTYLEDON ORBICULATA*

By

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DECLARATION

I, Lucille Deanne van Schalkwyk, declare that the contents of this dissertation/thesis represent my own unaided work, and that the dissertation/thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

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ABSTRACT

The increasing resistance to conventional antibiotics and urgent need for alternative immunomodulatory therapies have driven growing interest in plant derived bioactive compounds. Medicinal plants have historically been a rich source of bioactive compounds with diverse pharmacological properties, yet a large number of species remain unexplored for their therapeutic potential. Among these, *Cotyledon orbiculata* has shown promising traditional uses, suggesting the presence of biologically active constituents. However, scientific validation of their antimicrobial and immunomodulatory properties remains limited. This study aimed to investigate the antimicrobial activity and immunomodulatory effects of solvent-derived fractions from *Cotyledon orbiculata* plant leaves.

Water and methanol extracts were prepared and then fractionated. After fractionation, all fractions were spotted on TLC plates to either pool together as a combined sample or kept individually. Separation was based on observed visual TLC profiles. The water extract yielded 32 fractions whereas the methanol extract yielded 46 fractions which were collected in glass vials. The resulting fractions were evaluated for antimicrobial efficacy against a panel of microorganisms namely, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Methicillin Resistant Staphylococcus aureus*, and *Pseudomonas aeruginosa* using standardized microbiological assays. The selected fractions showed notable antimicrobial activity, and the immunomodulatory effects were assessed through cytokine profiling.

KEYWORDS: antibiotics, anti-inflammatory, antimicrobial activity, antimicrobial resistance, infectious diseases, medicinal plants, skin infections, traditional medicine.

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Dedication

To my mother, my fiancé, my teachers, my mentors and all those who helped me, whose love, support and belief in me has made this possible.

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LIST OF ABBREVIATIONS

ABSSSi	Acute bacterial skin and skin structure infections
AIDS	Acquired immunodeficiency syndrome
AMR	Antimicrobial resistance
APC	Antigen presenting cells
ARG	Antibiotic resistance genes
CDC	Centre of Disease Control and Prevention
CNS	Coagulase-negative staphylococci
CSSTI	Complicated skin and soft tissue infection
DC	Dendritic cells
DMSO	Dimethylsulfoxide
ELISA	Enzyme-linked immunosorbent assay
HGT	horizontal gene transfer
HIV	Human immunodeficiency virus
LB	Luria-Bertani medium
LC	Langerhans cells
LMIC	Low to middle class income countries
LPS	Lipopolysaccharide
MAE	Microwave assisted extraction
MBC	Minimum bactericidal concentrations
MHB	Müller-Hinton agar
MIC	Minimum inhibitory concentrations
MRSA	Methicillin-resistant *Staphylococcus aureus*
NKC	natural killer cells
SSTI	Skin and soft tissue infection
THP-1	Tamm-Horsfall Protein 1

CLARIFICATION OF TERMS

Antibiotic resistance: The ability of microorganisms to persist or grow in the presence of treatment that was previously effective at killing the microorganism.

Anti-inflammatory activity: Cytokines that inhibit or reduce inflammation.

Antimicrobial activity: The ability of a substance to kill or inhibit the growth of microorganisms.

***Cotyledon orbiculata*:** A succulent plant native to South Africa, often used in traditional medicine for treating skin ailments, wounds and infections.

Crude extract: A non-purified extract containing a complex mixture of bioactive and inert plant constituents.

Cytokines: Small proteins released by immune cells that regulate inflammation and immune responses.

Fractionation: A process of separating crude plant extracts into different groups of compounds based on polarity or solubility**.**

Immunomodulation: The process by which a substance modifies the immune response, either enhancing or suppressing it.

Pathogen: Any microorganism such as bacteria or fungi that can cause disease.

Phytochemicals: Naturally occurring chemical compounds in plants, such as flavonoids, tannins, alkaloids, and saponins, often responsible for medicinal effects.

Plant extract: A concentrated preparation obtained from plant material using solvents such as ethanol, methanol, water, used to isolate bioactive constituents.

Pro-inflammatory activity: Cytokines that promote inflammation, these are elevated during infections.

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CHAPTER ONE

1.1. Introduction

A vast number of secondary metabolites such as phenols, tannins, quinones, alkaloids, terpenoids and flavonoids can be found in medicinal plants. These have been used in the treatment of infectious diseases and illnesses worldwide (Ababutain, 2011). The use of plants as medicine is a practice that has been around for centuries, and over several decades has assimilated into allopathic and traditional medicine (Sen & Batra, 2017). Approximately 60% of all plants have medicinal properties. Plant organs or tissues can be used for the generation of health promoting or therapeutic substances (Hao & Xiao, 2018).

Across numerous cultures worldwide, traditional medicine remains a widely utilized healthcare practice. Customs, songs, traditional foods, habits and taboos are all cultural forms of expression for different ethnic groups. As such, the manner in which diseases or illnesses are treated are also a form of heritage passed down from each generation (Teka *et al*., 2020). South Africans still treat primary health care needs with the use of medicinal plants. In developing countries such as South Africa, medicinal plants play an important role in basic health care (van Wyk & Prinsloo, 2018).

In developing countries, 80% of the population make use of traditional medicine, with 50% of the medicines in clinical use. In 2019, global spending on pharmaceuticals was US$1,25 trillion with the USA spending US$455,9 billion on pharmacological drugs (Hardy, 2021). According to more recent data, a report conducted in 2024 concluded that the global pharmaceutical sector's revenue reached US$ 1.48 trillion in 2022 (Stacciarini, J., 2024). Many novel drug components have been isolated from natural plant sources (Obeidat *et al*., 2012). Dried root of *Paeonia lactiflora Pall* has been used for centuries as a medicinal herb in traditional Chinese medicine, an investigation on the water/ ethanol extract of the dried root were conducted and the results of this investigation indicated that compounds isolated from *Paeonia lactiflora* have significant antimicrobial, antiflammatory and antiviral properties (He & Dai, 2011). Other investigations where traditional medicine have proven scientific value, such as *Martynia annua Linn* and its wound healing properties of isolated fractions (Lodhi & Singhai, 2013), the antimicrobial and anti-inflammatory properties of genus *Kalanchoe* ,(Milad *et al*., 2014), and the antimicrobial activity of a family of Fabaceae (Mickymaray, 2019) have shown the value of medicinal plants in various population groups around the world.

According to Gupta & Chaphalkar (2017), immunological disorders or diseases such as rheumatoid arthritis are also an example of when such medicinal products were used as an

effective treatment.A total number of 17 million people die due to bacterial infections, in fact bacterial infections are listed as the fourth leading killer in the US, third in developing countries, and second in the rest of the world. A systematic review that covered antimicrobial resistant pathogens in the context of the COVID-19 pandemic re-affirmed that more than 35 000 deaths and more than 2.8 million antibiotic resistant infections occur in the US annually (Ba *et al*., 2023).

Recent research has begun to validate the traditional use of *C. orbiculata* for skin-wound management and suggests it may have real therapeutic potential against skin infections and impaired wound healing. In a 2022 in-vitro study, *C. orbiculata* aqueous extract and *C. orbiculata*-derived silver nanoparticles (Cotyledon-AgNPs) significantly promoted skin-cell (keratinocyte and fibroblast) proliferation and migration, and up-regulated genes linked to wound-healing processes, confirming for the first time that the plant exerts measurable wound-healing activity under laboratory conditions (Tyavambiza *et al.*, 2022).

Ethnobotanical work conducted in South Africa's Eastern Free State also listed *C. orbiculata* among the most frequently cited plants used for skin diseases and wounds, and detected phytochemicals such as flavonoids, terpenoids, tannins and alkaloids in its extracts - compounds commonly associated with antimicrobial and anti-inflammatory effects (Xaba *et al., 2024)*.

Biological studies performed on this plant have indicated promising antimicrobial and immunomodulation effects (Tyavambiza *et al*., 2023) , (Tyavambiza *et al*., 2022), (J. Azmir, I.S.M. Zaidul, M.M. Rahman, K.M. Sharif, A. Mohamed, F. Sahena, M.H.A. Jahurul, K. Ghafoor, N.A.N. Norulaini, 2013). When esophageal and colon cancer cells were treated with extracts from *Cotyledon orbiculata*, the extract changed how the cells processed certain genes (like hnRNPA2B1 and BCLX). These changes caused the cells to make more of the gene version that triggers cell death, leading to increased apoptosis of the cancer cells. This suggests that the extract could influence tumor growth. However, there is still very limited research on the chemical properties of *Cotyledon orbiculata* (Makhafola *et al*., 2020).

Other investigations on *C. orbiculata* have indicated that use of the plants leaf sap and roots show pharmacological properties such as antibacterial, anticancer, anticonvulsant and anti-inflammatory (Maroyi, 2019). Amabeoku and Kabatende have produced data that this plant possesses antinociceptive and anti-inflammatory properties (Amabeoku & Kabatende, 2012). They showed that this plant (methanol extract) contains saponins, tannins, triterpene steroids, cardiac glycosides and reducing sugars. *In-vivo* and *in-vitro* assays performed indicated that it exhibits pro-angiogenic activity that aids in wound healing by promoting the proliferation

phase and fibroblast proliferation stimulation (Mhlongo, Cordero-maldonado, *et al*., 2022) . The zebrafish pro-angiogenesis assay of *Cotyledon orbiculata* ethyl acetate (500µg/ml) extract indicated pro angiogenic activity. Investigation of traditionally used medicinal plants showed that this plant extract had a MIC value of 1.25 mg/ml against *Staphylococcus aureus*, 1.25 mg/ml against *Enterococcus faecalis*, 2.5 mg/ml against *Escherichia coli* and 5 mg/ml for *Klebsiella pneumonia* (Emamzadeh-Yazdi, 2013).

Nevertheless, despite these promising findings, there remains a research gap: very few studies have tested *C. orbiculata* in in vivo wound-infection models or with clinically relevant, drug-resistant pathogens such as MRSA, *Pseudomonas* aeruginosa in skin-infection settings. The most available data are from cell-based assays or nanoparticle preparations; comprehensive preclinical (animal) studies and controlled topical-formulation trials are lacking. As antimicrobial resistance (AMR) continues to rise globally- undermining conventional antibiotics - further work on *C. orbiculata* (especially standardized extracts or formulations) may help identify novel, effective, plant-based alternatives for skin and wound infections

In this study, the medicinal properties of *Cotyledon orbiculata* fractions will be investigated. The antimicrobial activity of chloroform, methanol and water extracts was tested against *Staphylococcus aureus*, *Staphylococcus epidermidis*, methicillin-resistant *Staphylococcus aureus* (MRSA), and *Pseudomonas aeruginosa* by determination of minimum inhibitory concentrations (MIC). MIC testing is a method used to measure the lowest plant extract concentration capable of inhibiting visible bacterial growth. Results obtained from water, methanol and chloroform extracts produced MIC values ranging from 3.13 to 50 mg/ml and minimum bactericidal concentrations (MBC) of 6.25 to 100 mg/ml. Silver nanoparticles had MIC values ranging from 5-80 µg/ml and MBC's of 20- 160 µg/ml. The determination of immunomodulatory activity showed that all prepared extracts had good anti-inflammatory properties, water extract had better activity against cytokines compared to chloroform and methanol extract (Tyavambiza *et al*., 2021).

1.2. Statement of Research Problem

Skin infections have become a major problem by complicating the wound-healing process. The main layers of skin: the dermis and epidermis, can be a breeding ground for microorganisms when physically or chemically damaged. These microorganisms can lead to severe infections. Skin infections can also occur due to improper care after surgery, burns, microbial infections, metabolic dysfunction or skin diseases, which can all give rise to a wound. Proper wound healing requires the completion of mechanisms such as inflammation, tissue regeneration and remodelling to restore damaged tissue. Opportunistic pathogens such as *Staphylococcus aureus* interrupt this process by colonizing skin wounds. Even though a broad variety of antimicrobial drugs are available for the treatment of such infections, the evolution of microorganisms has led to microbial resistance.

Cotyledon orbiculata, a medicinal plant commonly used to treat skin-related conditions possess properties required to improve or better manage skin diseases. A previous study conducted on the antimicrobial and immunomodulatory effects of the whole plant extracts of *C. orbiculata* showed favourable antibacterial and antifungal activity. The current study aims to investigate the antimicrobial properties of fractions isolated from *C. orbiculata*, which could potentially lead to the development of a more effective antimicrobial agent.

1.3. Aim

To evaluate the antibacterial and immunomodulatory effects of fractions isolated from *Cotyledon orbiculata* extracts.

1.4. Objectives

- Prepare different solvent extracts (water and methanol) of *Cotyledon orbiculata*.
- Prepare fractions of the *Cotyledon orbiculata* solvent extracts.
- Determine which solvents best separate each extract.
- Determine if the fractions have antimicrobial activity against *Staphylococcus aureus*, Methicillin-resistant *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Staphylococcus epidermidis*.
- Determine if the fractions have immunomodulatory effects by investigating their effects on cytokine responses in the human macrophage THP-1 cell line.

1.5. Research Questions

- Can the prepared solvent extracts produce *Cotyledon orbicu*lata fractions?
- Do *Cotyledon orbiculata* fractions have antimicrobial activity against *S. aureus, MRSA, P. aeruginosa,* and *S. epidermis*?

- Do *Cotyledon orbiculata* fractions possess immunomodulatory properties?

1.6. Hypothesis

- Isolated fractions of methanol and water extracts may have antimicrobial activity against selected microorganisms.
- The fractions of either extract may possess the ability to influence cytokine responses in the human macrophage THP-1 cell line.

CHAPTER TWO
LITERATURE REVIEW

2.1 Background of Research

Skin infections or diseases are a worldwide occurrence and said to amount to approximately 34% of all occupational diseases encountered (De Wet *et al*., 2013). Incidences of skin diseases have become an area of major concern due to their co-occurrence with HIV (human immunodeficiency virus) and AIDS (acquired immunodeficiency syndrome). In more than 90% of HIV-infected individuals, the development of skin and mucosal complications occurs (Afolayan *et al*., 2014) . Burn wounds carry high risk of local and systemic infection because thermal injury disrupts the skin barrier, impairs local immunity and creates necrotic tissue that promotes bacterial growth, infection and burn-associated sepsis remain leading drivers of mortality in modern burn patient groups (Greenhalgh & Kiley, 2024). In South African hospitals infection/ sepsis frequently complicates large burns and is a major contributor to in-hospital deaths, an example of this is a 2023 single-centre series reported overall adult mortality >25% with many deaths associated with infectious complications (Mathonsi *et al*., 2023). National-level reporting and recent South African reviews also emphasize a large but incompletely quantified burden of burn injury and related infectious complications across provinces and call for improved surveillance and wound-care resources (Motsepe, 2025). Regionally, the World Health Organization notes that almost two-thirds of global burn deaths occur in the African and South-East Asia regions, underscoring that low- and middle-income settings carry a disproportionate share of burn mortality — much of which is infection-related (Anon, 2023). Peer-reviewed studies and public health reports from 2022-2025 show that burn victims remain highly vulnerable to skin and systemic infections, that infection is a leading proximate cause of death in many burn cohorts in South Africa, Africa and globally, and that strengthening early infection detection and burn wound care is essential to reduce mortality (Greenhalgh & Kiley, 2024).

Skin infections have become a major problem which complicates the wound-healing process. The main layers of skin: the dermis and epidermis, can be a breeding ground for microorganisms when physically or chemically damaged. These microorganisms can lead to severe infections. Skin infections can also occur due to improper care after surgery, burns, microbial infections, metabolic dysfunction or skin diseases, which can all give rise to a wound (Bijnen *et al*., 2014) . Proper wound healing requires the completion of mechanisms such as

inflammation, tissue regeneration, and remodeling to restore damaged tissue. Opportunistic pathogens such as *Staphylococcus aureus* interrupt this process by colonizing skin wounds. Even though a broad variety of antimicrobial drugs are available for the treatment of such infections, the evolution of microorganisms has led to the problem of microbial resistance (Aitcheson *et al*., 2021; Han & Ceilley, 2017). Lack of completion of antibiotic treatment courses as well as the abuse of antibiotics, are some of the factors that aid in the emergence of drug-resistant microorganisms (Mobarki *et al*., 2019). More recent (2023–2025) data confirm that antibiotic resistance in skin, wound, and soft-tissue infections in Africa is widespread: approximately 1 in 3 infected-wound samples grow *S. aureus*, and of these over 40% are MRSA. Other common Gram-negative pathogens (*E. coli*, *K. pneumoniae*) also show very high resistance to first-line antibiotics. This suggests that SSTIs - including those following burns, wounds or surgeries - remain at high risk for antibiotic treatment failure in Africa, including South Africa, making effective infection prevention and antimicrobial stewardship critically important (Id *et al.*, 2024).

The failure of antibiotics to treat infections requires identification or discovery of better treatment options. Probable cause identification of root problem and the pursuit of the introduction of new methods to increase the effectiveness of current infection therapies. Antibiotic resistant bacteria can be the result of improperly administered medication as well as drug selection pressure experienced by professionals (Kowalska-Krochmal & Dudek-Wicher, 2021) . In India, throughout the Covid-19 pandemic, a rise of unnecessarily prescribed antibiotics not being monitored is stated to possibly have a negative impact on antimicrobial resistance. This has also been observed in the United Kingdom and the Lanc*et* Microbe has reported the abuse of antimicrobials within the Covid-19 first wave and stated that low-income and middle-income countries experience this problem at a larger level. This directly shows the hazard antimicrobial resistance poses to public health and the progress already accomplished in the health care industry. Annually, 700 000 deaths are caused by drug-resistant infections, and without the identification of a novel treatment or solution, this could lead to a projected economic cost of approximately US $100 trillion by 2050 (Manesh & Varghese, 2021) .

South African tradition is saturated with indigenous use of medicinal plants as treatment method for various types of infections. A history rich with phytotherapy, the practice of traditional medicinal treatment dates back to 4000 years, being the only healthcare system before the introduction of western medicine (Dyubeni & Buwa, 2012) .
To gain further knowledge of the medicinal properties of this plant, research on identification, isolation and purification of compounds is necessary. The use of new and novel bioactive products derived from plants like *Cotyledon orbiculata* is a field that is relatively unexplored.

2.2 Human Skin

The skin weighs up to 5 kg and a surface area of 2 m^2 (Summerfield *et al*., 2015; Vecino *et al*., 2018),and it is a complex organ that serves as a physical barrier between foreign objects or substances and the human body (Sorg *et al*., 2017) . Various embryonic layers such as the mesoderm, ectoderm and neural crest are stated to be the origin of skin (Kobayashi *et al*., 2019). The skin of a newborn is already colonized by microorganisms from the mother to create a good immune tolerance, this early skin colonization process is necessary (Dréno *et al*., 2016). Commensal skin microorganisms as well as microorganisms from the environment continue to colonize the skin and scalp in the attempt to create a balanced relationship with host skin cells. A balanced relationship or equilibrium is reached with a diverse scalp and commensal skin microbiota once adulthood is reached (Scharschmidt *et al*., 2015).

2.2.1 Structure of Skin

As illustrated in Figure 1, the epidermis, dermis and subcutaneous tissue are the three layers that form skin (Annaidh *et al*., 2012; Gerhardt & Derler, 2012; Dabrowska *et al*., 2016). The epidermis is comprised of cells known as keratinocytes, a major cell type made up of filamentous proteins and keratins, as well as dendritic cells, which primarily functions as a protectant against external entities and water loss control (Zhong *et al*., 2010). Keratinocytes also produce cytokines in defense of the skin once they are injured. The epidermis houses other cells such as melanocytes, Langerhans cells and Merkel cells with keratinocytes cell types comprising the majority (Kolarsick *et al*., 2011) . These cells are present in the first layer of the epidermis.

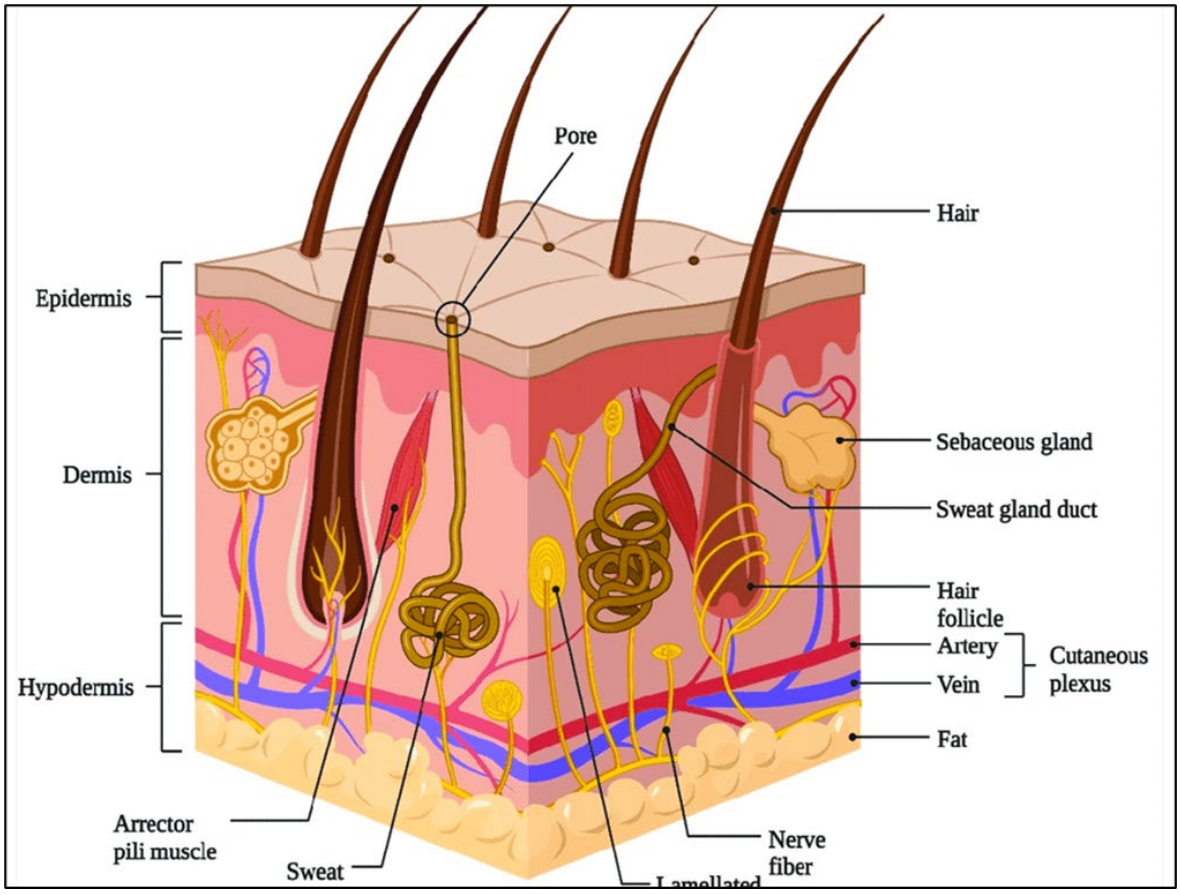

Figure 1: The human skin structure (Kolimi *et al*, 2022).

The skin is divided into four layers named: basal (stratum germinativum), squamous (stratum spinosum), granular [stratum granulosum (SG)] and cornified layer [stratum corneum (SC)] with stratum lucidum present on the hands and the fe*et* (Swaney & Kalan, 2021). Basal cell layer contains mostly keratinocytes with 5-10% being melanocytes. The stratum spinosum is comprised of Langerhans cells (LC). It is stated that the LCs are derived from the prenatal yolk sac and are present in the epidermis preceding birth (Kobayashi *et al*., 2019) . Information is exchanged between LCs and lymph nodes present in the epidermal microenvironment, where the LCs constantly audit, to induce host-protective and immune responses (Doebel *et al*., 2017) . The granular cell layer contains cytoplasm with smaller granules, lipid components are discharged into the intercellular space adding to the functionality of cohesion of the stratum corneum. Migrated cells from the stratum granulosum can be found in stratum corneum. No nuclei and cytoplasmic organelles give these cells a flat surfaced appearance. Body parts such as the hands, palms and fe*et* are areas where these cells have a thicker layer of cells when compared to any other parts of the body (Venus *et al*., 2010).

The dermis makes up the biggest part of the skin. Elasticity, pliability and tensile strength of skin is due to the dermis. Receptors of sensory stimuli are integrated within the dermis, which

also binds water, controls thermal regulation and serves as protection barrier from any mechanical damage. The dermis and epidermis collaborate to maintain properties within both tissues. Working in conjunction and interacting as a single unit once the skin is wounded to repair and reconstruct during the healing process. Collagen, the main component of the dermis is a group of fibrous proteins (Blair *et al*., 2020; Dabrowska *et al*., 2016). Present in the human body inside tendons, lining of bones and ligaments. Constituting 70% of the dry weight of skin, collagen fibres are constantly in a state of flux and then degraded by proteolytic enzymes and replaced with new fibres. A unique helical polypeptide chain is integrated by fibroblasts and then the cell secretes these fibroblasts causing them to assemble into collagen fibrils. Hydroxylysine, glycine and hydroxyproline are some of the amino acids that aid in the enrichment of collagen. Type 1 collagen is the main type of collagen present in the dermis. Papillary and the adventitial dermis contains loosely integrated collagen fibres with sturdy collagen bundles located in the reticular dermis (Kolarsick *et al*., 2011).

The reticular layer of the dermis is right above the subcutaneous tissue. It is structurally described as a loose connective tissue mutating into subcutaneous adipose tissue. Functioning as a shock absorber adipose tissue promotes thermal insulation as well as energy preservation (Arda *et al*., 2014) . Various microorganisms can make entry into this first layer via surgery, biomedical implants, burns and sepsis. Thus, making this organ vulnerable to the creation of a wound that allows interaction between internal defense and the external environment (Kaul *et al*., 2022).

Serving as a barrier, the skin is colonized by a variety of microorganisms such as bacteria, fungi and viruses in symbiosis. This symbiotic relationship is beneficial as it protects against invasion of more harmful organisms. This relationship can be described as an intricate balance and the slightest disturbances between host and microorganism can result in skin disorders or skin infections. Structurally the skin is cool, acidic and desiccated with the habitat being dictated by factors such as skin thickness, density of hair follicles, glands and folds of skin (Grice & Segre, 2012). Pain, skin temperature, disturbance, strain, strain rate and rapid skin oscillations are all detected by receptors present in skin. Object compliance, surface asperities, substance and texture are factors skin encounters and due to these receptors skin can be conscious of it (Dahiya *et al*., 2019).

According to Hay *et al* (2014), skin diseases were the leading cause of nonfatal disease burden in 2010. Listed as one of the most common diseases affecting up to 70% of all people with at risk populations at elevated rates. Skin disease receives a lot less focus within the global health industry when compared to other diseases. In 2010, one of the most common

diseases was skin related, listing within the top 10 worldwide. Fungal skin diseases, three skin conditions, acne, subcutaneous and other diseases are all part of the top 10. Developed and developing countries suffer this burden or strain and thus skin disease management and treatment needs to be included in global health strategies (Hay *et al*., 2014). A spike in the infection rate has been attributed to the development of AIDS, with skin infections becoming common with approximately 95% of patients with HIV/AIDS diagnosis experiencing skin infections (Sounouvou *et al*., 2021).

2.2.2 Skin Infections

In the rural areas of South Africa dermatological diseases frequently occur (Naidoo & Coopoosamy, 2011). Tuberculosis, malaria, and human immunodeficiency virus are found to be the primary focus for research in Africa and only as of recently included neglected tropical diseases. *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Enterobacteriaceae* are common causative infection agents (Schaumburg *et al*., 2014). Wound healing is often compromised or impeded by infection. *Staphylococcus aureus* is stated to be the frequent invading pathogen causing the bacterial infection (Sun *et al*., 2020). Human cells inside and on our bodies are ten times less compared to the number of microbial cells. This indicates the importance of these microorganisms and how they influence disease, diversity within human cells, metabolic processes and immunity (Dahiya *et al*., 2019).

As one of the most frequent bacterial infections in human skin and soft tissue infections (SSTI) are presenting resistance against antibiotics (Suaya *et al*., 2014). Occasionally, when not requiring antibiotic treatment, bacterial SSTIs can be self-limiting mild infections or superficial and complex, leading to more fatal results like sepsis. The occurrence of a SSTI indicates the inflammatory microbial invasion of the epidermis, dermis and subcutaneous layers presented in the form of swelling, pain, heat, redness, and possibly discharge. SSTIs are classified into two categories, namely, complicated SSTI and uncomplicated SSTI , (Renteria *et al*., 2014; Schaumburg *et al*., 2014). Healthcare systems across the world have been placed under great stress due to the increase of complicated skin and soft tissue infection (cSSTIs) with the additional strain since the rise of acute bacterial skin and skin structure infections (ABSSSIs). ABSSSIs have become a global health concern as they place an elevated increase cost both directly and indirectly on medical systems and society (Dryden, 2010).

Several classifications of a cSSTI have been proposed disregarding the extent and severity of the disease and still placed within the category of a cSSTI. Absence of lucid evidence for patients who recovered in a short time frame led the Food and Drug Administration (FDA) to issue an updated guideline on developing drugs for cSSTI treatment in 2013. The new

definition of ABSSSI was introduced and included major skin abscesses, wound infections with 75 cm^2 as a minimum lesion surface area, erysipelas and cellulitis and excluded diabetic foot ulcers, chronic wound infections, and burns (Russo *et al*., 2016).

Patients with other pre-existing conditions such as diabetes, immunosuppression or neurological diseases often suffer from complicated skin and soft tissue infections (cSSTIs) which necessitate surgical intervention or antibiotic therapy. Frequent reasons for hospitalization such as cSSTI have been a source of financial strain on the health care system due to ever evolving causative pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA). Due to recurrence within communities on a regular basis such causative pathogens are complicating the management and treatment of cSSTI (Zervos *et al*., 2012). According to (Talan *et al*., 2011) new MRSA strains are the uppermost source of community associated SSTI in many areas of North America.

2.2.3 Skin Pathogens

The relationship between the microorganisms on skin can change from a commensal to a pathogenic relationship depending on factors such as immune status, genetic variation of host, and microbial dysbiosis (Findley & Grice, 2014). Various infections are prevented due to commensal microbiota that is suspected to contribute to host health. An example of this form of defense is the production of bacteriocins. Synthesized in the ribosomes, these heat stable antimicrobial peptides are broad and narrow inhibitors (Sullivan *et al*., 2019). Described as an immunologic rich organ, skin commensal bacteria and lymphocytes present on it are approximately a million per square centimeter (Lehtimäki *et al*., 2018). The way the skin immune system responds to a pathogen differs based on the pathogen. Information obtained on skin immunology have mostly been based upon the investigations or research received from murine infection models and skin samples from biopsy from infected skin and plastic surgery (Brothwell *et al*., 2021). Different skin regions are populated by different types of microorganisms, factors such as hair follicles, density and moisture of these areas create a different physical and chemical terrain for microorganisms to occupy. An example of this is subcutaneous areas which are mostly occupied by *Staphylococcus* species and *Cutibacterium* and more moist areas occupied by *Staphylococcus,* beta-Proteobacteria and *Corynebacterium* (Chen *et al*., 2018).

Anaerobic and aerobic diptheriod bacilli, aerobic spore forming bacilli (gram positive), Enterococci, α-hemolytic Streptococcus and non-haemolytic aerobic staphylococci are primary microorganisms of skin (Adeleye *et al*., 2003). β-haemolytic streptococci and *Staphylococcus.aureus* of Lancefield groups A, C and G are the most dominant cause of

SSTIs (Kaye *et al*., 2019). Lancefield group B are mostly present amongst diabetics and the elderly (Dryden, 2010). Humans spend most of their time in a closed environment, approximately 80% and this has an impact on the skin and its health, (Li *et al*., 2022; Liu *et al*., 2020).

The frequent occurrence of SSTIs can vary from mild to dangerous, Beta haemolytic streptococci and *Staphylococcus aureus* are suggested to often times be the cause. *Pseudomonas aeruginosa, Enterococcus, Escherichia coli, Staphylococcus aureus* and Beta haemolytic streptococci are culture confirmed causes of SSTI in the USA (Ray *et al*., 2013). In the USA most of ABSSSI cases are due to gram positive pathogens with a notable percentage of them being MRSA. SSTI are a common infection type amongst visiting emergency room patients. Approximately 80% of ABSSSI are caused by gram positive bacteria causing wound infections, abscesses and cellulitis.

Human skin possesses coagulase-negative staphylococci (CNS) as a normal part of the microbiome. Some are present on all parts of the body whereas others are only present on certain parts because of their preferences. Some gram-positive bacteria are stated to be transient occupants, with the *Staphylococcus aureus* being the most frequent cause of infections and *S. epidermidis* a resident being part of the skin microflora (Vecino *et al*., 2018). Sustainability of a healthy skin flora depends on *S. epidermidis* population and interaction with other potentially pathogenic bacteria such as *S. aureus*. Receiving the title of an opportunistic pathogen *S. epidermidis* when invading the epithelial barrier can lead to remarkable damage (Talan *et al*., 2011). Impetigo and infected abrasions are external infections caused by *S. aureus*. It can also cause internal infections such as folliculitis, cellulitis, subcutaneous abscesses and ulcers. Approximately 500 000 hospital admissions per year and 11.6 million emergency room visits and outpatient care is due to *S. aureus* infections in the U.S.A. *S. aureus* colonize the skin by existing on the surface of the skin as a commensal organism. Upon infection of the epidermis the invading pathogen causes impetigo characterized by its honey-crusted sores and erosion present on the exterior of the skin (Krishna & Miller, 2012). Depending on the site of hair follicle infection folliculitis can either be deep or superficial. Numerous small papules and pustules with a central hair indicates superficial folliculitis. Deep folliculitis presents as plaques and nodules accompanied by an elevated pain level and scars after the healing process is completed (Laureano *et al*., 2014).

In Africa and other developing countries information regarding the characteristics and frequency of MRSA is limited (Maina *et al*., 2013). Considered a significant pathogen globally 2% of hospitals in developing countries reported isolation of MRSA in 1970’s compared to the

30% in the 1990's. African countries such as Nigeria, Cameroon and Kenya showed MRSA prevalence and antibiotic susceptibility of 21-30% with Tanzania at ≥10% (Maina *et al*., 2013). Reports of the occurrence of SSTIs within South Africa is increasing. It is now the most common reason for the use of antibiotics in South African hospitals with approximately 12.97% of patients receiving prescriptions for treatment, (Makwela *et al*., 2023; Black & Schrock, 2018). Records indicate that countries such as Tanzania, Ethiopia, Uganda and Malawi have infectious dermatoses numbers of 85%, 71%, 40% and 78% respectively. The most frequently reported dermatoses in black South Africans are infections, eczema and acne based on epidemiological studies (Dlova *et al*., 2015). For this study the microorganisms listed below were selected due to their common affiliation with SSTIs and other complicated skin infections. *Staphylococcus aureus* and gram-positive cocci and *Streptococci* with *Pseudomonas aeruginosa, Esherichia coli* and *Enterococcus* are stated causative agents of SSTIs in hospitalised patients and burn victims (Makwela *et al*., 2023). Complicated SSTIs affect the population with pre-disposition conditions such as diabetes, immunodeficiencies and vascular deficiency affecting deep within the subcutaneous tissue and muscles (Ioannou *et al*., 2018).

2.2.3.1 *Staphylococcus aureus*

Staphylococcus aureus is a microorganism commonly present on the human skin. The methodology of how this microorganism's presence changes from commensal to pathogenic is not completely understood (Yang *et al*., 2018). As seen in **Figure 2**, the cells are spherical and typically appear in clusters, a gram-positive microorganism it retains the crystal violet stain and appears purple under light microscopy with individual cocci measuring approximately 1 micrometer. When cultured on blood agar plates, it forms round, golden yellow convex colonies that are approximately 1-4 mm in diameter.

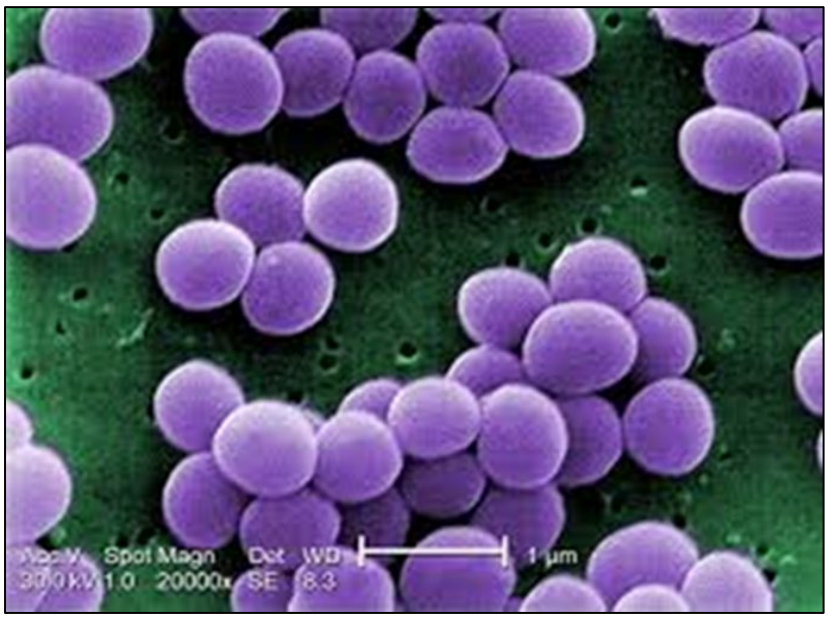

Figure 2: Scanned electron micrograph of *Staphylococcus aureus* (Croes *et al*.,2009).

This microorganism is described as a very versatile pathogen, *S. aureus* has a large array of virulence factors these include chemotaxis inhibitors, neutrophil killing toxins, anti-killing and anti-phagocytic surface molecules, immune evasion proteins and superantigen (Chen *et al*., 2018) . Once introduced into the bloodstream this organism will spread and enter organs possibly resulting in significant illness and disease (Choi *et al*., 2014). The human immune defense system adequately responds via antimicrobial host factors during colonization and infection. Host defense efficiency is affected by the resistance mechanisms of *Staphylococcus aureu*s (Ryu *et al*., 2014).

2.2.3.2 *Staphylococcus epidermidis*

Staphylococcus epidermidis is stated to be the most frequent species in CoNS infection via the formation of highly resistant biofilms and sustaining a low inflammatory profile thus preventing an immune response (Swaney & Kalan, 2021). As seen in Figure 3**,** when viewed under the microscope this microorganism has a spherical shape, appearing as grape like clusters and is gram positive similar to the microorganism *S. aureus*. When cultured on blood agar colonies appear as smooth and round, small white to gray colonies that are between 0.5-1.5 micrometres in size. With dual function, they can interact as a favorable colonizer, and capable of leading to significant infections when the host epithelial barrier is damaged (Nguyen *et al*., 2017).

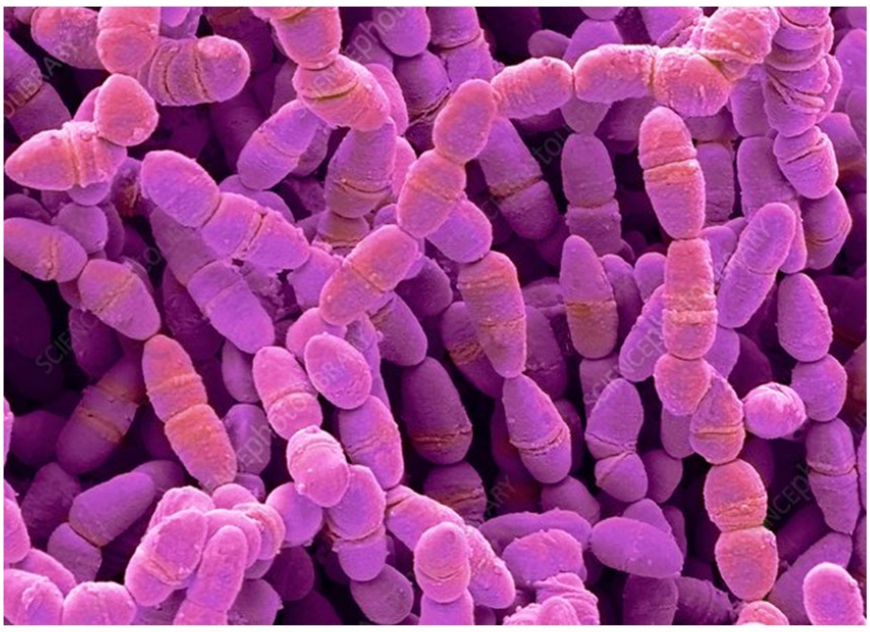

Figure 3: Coloured scanned electron microscope image of dividing *Staphylococcus epidermidis* (Gschmeissner, 2017).

Staphylococcus epidermidis is part of the coagulase-negative Staphylococci (CoNS) and stated to be one of the most prevalent CoNS. Present in areas such as the groin, toe webs, conjunctive anterior nares and armpit this microorganism is present as a commensal on all human skin surfaces (Büttner *et al*., 2015), but is capable of causing significant infections when breaking the skin barrier (Skovdal *et al*., 2022). This group of *Staphylococcus* species does not possess coagulase the blood clotting enzyme. Various research conducted on *S. epidermidis* has shown that esters such as geranyl, medium chained esters and unsaturated esters can be produced by *S. epidermidis* lipase enzyme without organic solvents (Hosseini *et al*., 2014). In cases where medical devices such as prosthetic joints or fracture fixation devices are implanted this microorganism behaves as an opportunistic pathogen. *S. epidermidis* is capable of accumulating and adhering to the surface of the medical device and once this device is implanted and introduced to the surgical site can cause an infection (Brescó *et al*., 2017). This microorganism does not possess the most of the hostile virulence factors that *S. aureus* produce, but *S. epidermidis'* mechanism of action is the formation of deep seated biofilms on mentioned medical devices and native host tissues (Le *et al*., 2018).

2.2.3.3 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a member of the family Pseudomonadaceae, and described as a gram-negative, rod-shaped gram-negative bacterium as seen in Figure 4, it is classified both as a facultative aerobe capable of anaerobic respiration (Wood *et al*., 2023). Present in environments such as water, soil, oral mucosa, skin and vegetation, this microorganism is biochemically versatile with the ability to catabolize a variety of organic molecules for nutrients (Wood *et al*., 2023). *Pseudomonas aeruginosa* is stated to be the principal factor causing sepsis and bacteremia in recipients of cancer treatment, a fatal cause of chronic infection of cystic fibrosis in patients,a regular cause of infections in diabetic ulcers, corneal ulcers, surgical wounds, and burn wounds (Wood *et al*., 2023). Infections caused by *Pseudomonas aeruginosa* have a fatality rate of 40%, despite the variety of antibiotic therapies available, statistics have shown no improvement over the years due to the elevated degree of acquired and intrinsic resistance *Pseudomonas aeruginosa* exhibit towards a broad variety of antibiotics (Nathwani *et al*., 2014; Miragaia, 2018).

Figure 4: Colour SEM image of *Pseudomonas aeruginosa,* capable of causing serious infections (Science photo library, nd).

2.2.3.4 Methicillin resistant *Staphylococcus aureus*

MRSA is highly prevalent in hospitals and clinics worldwide and is said to be globally recognized as one of the causative pathogens that are responsible for hospital and community acquired infections. **Figure 5** shows *MRSA* cells, coccus shaped bacterium resistant to many antibiotics.

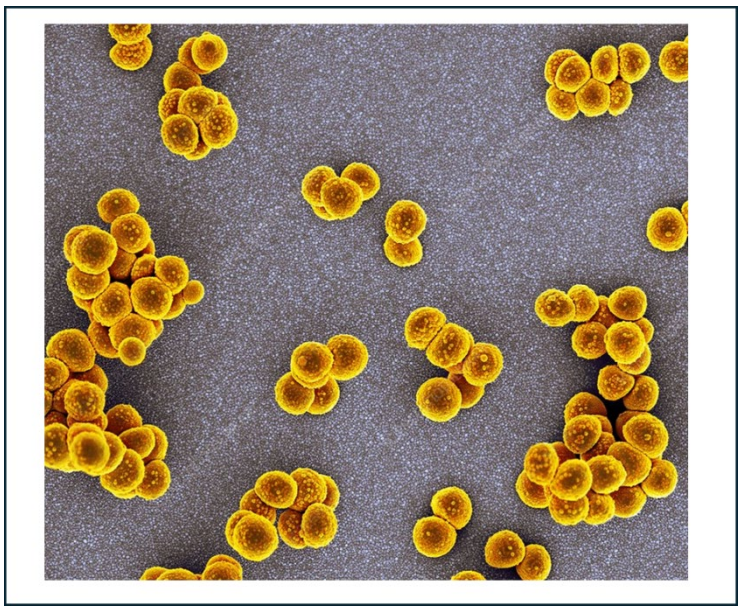

Figure 5: Coloured scanning electron micrograph (SEM) of methicillin-resistant *Staphylococcus aureus* (MRSA) bacteria (Science Photo Library, nd).

The emergence of *MRSA* is attributed to the acquisition of the Staphylococcal cassette chromosome mec (SCCmec) by methicillin-susceptible *S.aureus.* This genetic element carries the *mecA* gene, which encodes penicillin-binding protein 2a-(PBP2a), rendering the bacterium resistant to all bet-lactum antibiotics (Sit *et al*., 2017). Staphylococcal cassette chromosome mec (SCCmec) is one of 10 other SCCmec types that exist, with only types I to V distributed globally and remaining types uncommon and existing native to the country of origin (Miragaia, 2018; Sit *et al*., 2017).

Penicillin was discovered by Sir Alexander Fleming in 1928, by 1941 a purified form of penicillin was available and used during World War II and within 2years of launching penicillin *S. aureus* resistance was discovered and the first resistant strain identified in 1942 (Nandhini *et al*., 2022). In the 1950's semisynthetic antibiotic methicillin was designed and in the 1960's methicillin resistant *S. aureus* detected. PBP is a penicillin binding protein that is produced by MRSA strains which can be encoded by an obtained gene named mec-A. The inclusion and acquirement of the mobile genetic elements into chromosomes and vulnerable strains has led to the emergence of methicillin resistant strains of staphylococci (Serra *et al*., 2015; Wood *et al*., 2023b). Resistant to many antibiotics such as methicillin, oxcillin, cefoxitin, cloxacillin and other frequently used antibiotics, *MRSA* can spread via close contact with the already infected, transmitting from an object to human or human to human. Nosocomial infections are caused by hospital acquired *MRSA* strains which are associated with SCC*mec* type I, II and III (Sit *et*

al., 2017). Community acquired *MRSA* strain can lead to skin and soft tissue infections (SSTI's), necrotizing pneumonia, sepsis, and osteomyelitis.

2.3. Antimicrobial Resistance

Globally instances of antimicrobial resistance (AMR) are increasing and becoming a great concern (White & Hughes, 2019; Chokshi *et al*., 2019). Defined as the ability of bacteria to evolve or change in a manner which reduces the effectiveness of antibiotics (Kraker *et al*., 2016). Both developed and developing countries are affected by AMR, it is essential that antibiotic resistance development is investigated worldwide. If the increase of AMR continues it is estimated that by 2050 approximately 10 million deaths will be attributed to AMR infections (White & Hughes, 2019). Approximately 4.95 million deaths globally were attributed to AMR in 2019. In the US only treatment cost associated with AMR is stated to be US$4.6 billion. An investigation into the burden of AMR in 2019, in sub-Saharan Africa was the highest and higher in lower and middle class countries when compared to Western Europe and East Asia (Ikhimiukor *et al*., 2022). Microorganisms such as *Klebsiella* and *Escherichia coli* were the most frequent bacteria in new-borns with sepsis in 2011 in India. An outbreak in India caused the deaths of 4 babies whom developed New Delhi metallo-beta-lactamase 1 (NDM-1) which produced *E.coli* bacteremia and sepsis. Antibiotics are of great importance for procedures such as joint replacement surgeries, stem cell treatment and transplantation practices (Subramaniam & Girish, 2020).

ESKAPE is an acronym used to classify a group of highly antibiotic-resistant bacteria responsible for nosocomial infections including *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species (Murray *et al*., 2015). India is a country that is said to be a high rated consumer of antibiotics which directly contributes to antimicrobial resistance reporting up to 10.7 units per person. Reduced treatment options and ineffective patient outcomes are a result of prescriptions of broad-spectrum antibiotics which in turn is due to antibiotic resistance. This problem is due to a combined list of factors such as inadequate diagnostics, cross infections, fraudulent activities amongst medical staff to promote certain medicines and lack of awareness. India has since established the Antimicrobial Resistance Surveillance Research Network. Clinical research regarding drug resistance infections involving academia, the private sector and government are required to address the problem effectively (Manesh & Varghese, 2021).

Approximately an estimated 2 million of the population suffer infections with antibiotic pathogens each year, with 23 000 people dying per year due to these infections stated in a publication by Centres of Disease Control and Prevention (CDC) (Maddox, 2022). Investigations into antimicrobial use indicated that 30-50% of instances these drugs are incorrectly prescribed when it comes to treatment, duration, and agent of choice. Extended use of antibiotics or use and management of contaminating microorganisms. Likely impediments of antimicrobial treatment with no benefit occurs when this medication is incorrectly prescribed and unnecessarily used. Another risk factor is introduced when health care employees transfer these antibiotic-resistant pathogens between patients. Optimal hygiene is a requirement for all health care providers to prevent any form of cross contamination. Prevention of the occurrence of infections and patient treatment improvement can minimize the impact of antibiotic resistant infections. New antibiotic and diagnostic test promotion, better use of current antibiotics, monitor and tracking of resistant bacteria as well as cross contamination prevention are the actions outlined by CDC to reduce antibiotic resistant infections (Lushniak, 2014). Discovery of novel antimicrobial treatment, development of new forms of therapies and initiatives by the medical research society is necessary to alleviate the increase of antibiotic resistance. Resistant gram positive and gram-negative bacteria have led to untreatable infections. In various health care institutions, early identification of causative pathogens and the susceptibility method is lacking leading to the unnecessary use of broad-spectrum antibiotics (Frieri *et al*., 2017).

2.3.1 Causes of Antimicrobial Resistance

Natural products are said to be the origin of antimicrobials, usually these natural products are from microbes themselves. After the necessary protocols to commercially produce a viable drug, a compound is isolated and modified for elevated antimicrobial activity. It can thus be concluded that as a defense mechanism against said natural products microbes evolved to produce antibiotic resistance genes against other microbes (Amarasiri *et al*., 2020).

It is stated that although AMR instances have increased the discovery or progress of novel agents, agents that can combat resistance have declined (Lee *et al*., 2013), (Richardson, 2017). AMR can arise from genetic mutation spontaneously resulting in the inheritance of such genes by the following generation. Mobile genetic elements such as plasmids can lead to transference of resistant genes amongst bacteria and this horizontal gene transfer (HGT) can happen between different bacterial species. Antibiotic resistance genes (ARG) can occur naturally and is categorized as acquired or intrinsic, with some antibiotic bacteria possessing the potential to evolve into new emerging pathogens (Hu *et al*., 2017),(Zhuang *et al*., 2021). AMR within environmental bacteria can also be a result of mutagenesis via antibiotic targ*et*

alteration, gene amplification etc. antibiotics, antidepressants and mutagenic disinfectant by-products contribute to this type of mutagenesis (Baharoglu *et al*., 2013).

For the increase of agricultural yield, be it, crop or livestock, antimicrobial treatment is often used to eliminate bacterial parasites as well as for the augmentation of antibiotic-resistant bacteria which then is introduced and consumed by humans as food supplements (Subramaniam & Girish, 2020). Meat and meat products are one of India's export products to the world and is stated to be the fourth on the list of countries that consumes antimicrobials for animal usage, with China, USA and Brazil as the top 3 consumers. Food animal products such as chicken and milk have been reported to possess antibiotic residue. Biocides are contributing factors of AMR, disinfectants, insecticides, and fertilizers are proclaimed to be capable of increasing the amount of resistant organisms in an environment when administered at non-fatal concentrations (Aggarwal *et al*., 2012).

Massive growth in population, with 7.7 billion in April 2019, compared to the 200000 years the population took to reach 1 billion combined with the fact that travel to and from countries used to be a lot less frequent or easy as it is currently thereby allowing globalisation of unwanted passengers such as microbes or pathogens. Factors such as overpopulation, selection pressure, increased global migration and poor sanitation are stated to be a causative agent of the resistance (Aslam *et al*., 2018). In low to middle class income countries (LMICs), a drastic increase in population allows for higher probability of transmission of bacteria and antimicrobial access. Improper health regulations in such areas or environments intensify the spread of resistance. Medline- index investigations performed and published before 2021, sampling approximately 100 latrines, confirmed the presence of antimicrobial resistance in pit latrines and sanitation systems in LMICs (Ikhimiukor *et al*., 2022). With *Echerichia coli* isolates, approximately 75% were resistant to at minimum one antimicrobials and 45% resistant to 3 or more antimicrobials and two isolates confirmed to carry beta-lactamase genes (Mahmud *et al*., 2019) (Ikhimiukor *et al*., 2022).

The use of expired antimicrobials is also stated to increase the resistance rate between 2 to 6 times when compared to the use of antimicrobials within the acceptable shelf-life range (Chokshi *et al*., 2019). The storage conditions can affect the drug and can lead to degradation if the storage instructions are not adhered to (Ogunshe & Adinmonyema, 2014).

2.4 Traditional Medicine

Natural products have always been used to treat diseases, plants, micro-organisms, marine organisms, or animals have all been utilized to treat diseases. Dating back to an estimated 60

000 years based on unearthed fossil records, global usage of traditional medicines for many years in Chinese and Korean populations with the addition of time flourished to regulate medicine systems. Introduction of Western medicine in China occurred in the sixteenth century but only developing in the nineteenth century. Prior to development, the leading medicinal treatment method was the use of traditional Chinese medicine. The abundance of data from experimental distribution or usage of traditional medicines, established on the 5000 years of medical experience and practise is stated to assure its potency and validity (Yuan *et al*., 2016). It is stated that approximately 70% of the South African population with 60% regularly consulting of the 200 000 traditional healers (Mhlongo, Cordero-Maldonado, *et al*., 2022). Plants as medicine is a practise that has been around for centuries, and over several decades has been assimilated into allopathic and traditional medicine (Sen & Batra, 2017). The primary health care needs of the Southern African population are still being treated by the use of medicinal plants. The use of indigenous medicinal plants is fairly common amongst numerous cultures (Majeed, 2017).

Traditional medicine within the South African population is a combination of different beliefs, practices, approaches and knowledge. Well-being (Impilo) is maintained and diseases prevented by making use of spiritual therapies, animal, plant and mineral-based medicines (Mutola *et al*., 2023). There has been a global interest in traditionally used medicinal plants with various initiatives to explore Southern Africa's botanical resources with the intent to seek out potential pharmacologically-active compounds by identifying and determining their chemical profiles. Conducting research and looking at the development of South African medicinal plants avails opportunities to discover novel and useful biological activities (Street & Prinsloo, 2013). The South African population is stated to have close to 27 million of the population that use traditional medicine because of its accessibility through local traditional healers and its affordability (Cock *et al*., 2023). Approximately 500 000 is the gauged number of species of the plant kingdom with a minimal amount of these plants having been investigated for antimicrobial and or immunomodulatory effects. Due to the overall safety and efficacy of plants it is possible to grow traditional medicinal plants without structured standards or analysis. Toxicity screening or testing of bioactive compounds is stated to be more successful when derived from traditional medicinal plants as compared to the chemically synthesized compounds (Mickymaray, 2019).

In developing countries, it is estimated that between 70-95% of the population depend on plants for basic health care (Maria *et al*., 2016). Despite the popularity of the use of medicinal plants for health care within recent years, in rural areas it is often the only available treatment

for ailments. In these areas skin infections are frequently treated with such plants due to the reoccurrence and contagiousness as well as immunocompromised patients. Dermatological uses of plants are due to their wound healing properties, reduction of other skin ailments and its ability to stop bleeding (Mabona & Van Vuuren, 2013).

According to (Mundy *et al*., 2016) the distinctiveness of herbal medicine can be attributed to the way the constituents synergistically or antagonistically interact. As of recently, copious efforts are being made to conduct research on identification and isolation of secondary metabolites from plants with reported antimicrobial activity.

2.5 *Cotyledon orbiculata*

2.5.1. Physical Characteristics

The leaves of *Cotyledon orbiculata* are bright to light green as seen in Figure 6, grey or grey-green in colour outlined with a red margin with a smooth waxy layer (Nthatisi Innocentia Molefe, 2013). Dark purple red with a bell-like structure grow on the slender stalks and the common names in different languages for *Cotyledon orbiculata* are as follows: plakkie, varkiesblaar (Afrikaans), pig's ear (English), imphewula (Xhosa) and seredile (Sotho, Tswana) (Duarte *et al*., 2014).

Figure 6: Leaves of *Cotyledon orbiculata (Bonet, 2021)*

Tyledoside C, cotyledosides, and orbicusides are some of the beneficial bioactive compounds produced from the leaves of this species. The presence of flavonoids, reducing sugars, saponins, condensed tannin, phenolics, cardiacglycosides, gallotannin, and triterpene steroids in the leaf extract has been proven by phytochemical analysis. Due to popularity of use amongst traditional healers and medicinal merit of this plant, wild populations are collected vigorously approaching an almost threatened level in some parts of South Africa (Zengin *et al*., 2023).

Equipped with the anatomy and structure to withstand extreme temperatures succulents survive in arid habitats related to approximately 12 500 species. Crassulaceae family with the synonym Sedaceae is a big group comprised of dicotyledons. Receiving praise for their unique xeromorphic structure with both the leaf and stem containing water storage tissue. Used primarily in horticulture requiring minimal care most members of this family have an appealing appearance. Structurally described as erect plants that are fleshy, sessile or stalked with yellow, purple or scarl*et* flowers these plants are mostly large, a calyx that is four parted with the corolla tube being taller than the narrow tubes (Milad *et al*., 2014). Crassulaceae referred to as the houseleek or stonecrop family, is the third largest family of succulents comprised of genus groups with varied temperature tolerance and habitat adaptabilities. Sub families include *Sedeveria*, *Sedum, Crassula, Echeveria* and *Graptopetalum* with assorted morphological appearances (Nam *et al*., 2016)

Distributed mostly in mountainous areas, this flowering plant contains 35 genera. *Cotyledon orbiculata* L is one of Southern Africa's first succulents to be described with a renowned history. The genus Cotyledon enters south western Arabia but is primarily a African genus when it comes to the distribution of the plant (Adebayo *et al*., 2015). Countries such as South Africa, Macronesia, Mexico and Himalaya, all have an elevated diversity with South Africa being the primary home of the Cotyledon genus (Duarte *et al*., 2014).

USDA (United States Department of Agriculture) classifies *Cotyledon orbiculata* as follows:

Table 1: Taxonomical Classification of *Cotyledon orbiculata*

Kingdom	**Plantae**
Subkingdom	Traccheobionta (Vascular plants)
Superdivision	Spermatophyta (Seed plants)
Division	Magnoliophyta (Flowering plants)
Class	Magnoliosida (Dicotyledons)
Subclass	Rosidae
Order	Rosales
Family	Crassulaceae
Genus	Cotyledon

2.5.2. History

Cotyledon orbiculata, is a commonly used medicinal plant in South Africa and this is a succulent plant which is known as pig's ear. It is a small shrub with fleshy leaves and belongs to the family Crassulaceae (Aucamp, 2014). *Cotyledon orbiculata* is a diverse species divided into 5 varieties named *C. orbiculata* var. dactylopsis, *C. orbiculata* var. flanaganii, *C. orbiculata* var. oblonga, *C. orbiculata* var. *orbiculata*, *and C. orbiculata* var. spuria. Derived from the Greek word "kotyledon" the genus name Cotyledon means cup-shaped hollow referring to the leaves of some species. The Latin word "orbiculata" of the species means round or circular referencing the shape of the segments (Maroyi, 2019).

Traditional medicine practitioners commonly use the leaf of this plant to treat a long list of ailments such as the softening of hard corns, boils or warts (Kumari *et al*., 2016). Skin abscesses as well as skin eruptions have been treated by using the warmed peeled leaves as a poultice (Xaba, 2016). The fresh leaf juice from *Cotyledon orbiculata* has also been used to treat epilepsy and syphilis by using the leaf decoction as an enema, it has also been shown that by using the extract as a lotion it can aid in the management of toothache, earache, and acne. Previous studies conducted on stem and leaf extraction of *Cotyledon orbiculata* indicated antifungal properties against *Candida albicans,* root extraction by Fouche *et al* (2006) showed anticancer effects while methanol leaf extraction presented anticonvulsant effects (Amabeoku *et al*., 2007). Antioxidant and anti-inflammatory properties were reported by (Ondua *et al*., 2019; Bhoite & Thakur, 2022).

Cotyledon orbiculata extracts and biogenic silver-nanoparticles (*Cotyledon*-AgNPs) have demonstrated antimicrobial, anti-inflammatory and in-vitro wound-healing activity against skin-pathogen models and skin fibroblasts in recent studies, supporting its traditional use for cuts, boils and other skin conditions (Tyavambiza *et al*., 2022). Species of the closely related genus *Kalanchoe* (e.g., *K. pinnata, K. blossfeldiana*) show a broader and more extensively characterized wound-healing and antibacterial profile in contemporary literature, including mechanistic data on anti-inflammatory compounds and several in-vivo wound models, which makes *Kalanchoe* a better-documented candidate for topical therapeutics than *Cotyledon* at present (Beatriz *et al*., 2023). Crassula species have also been reported to possess antioxidant and antibacterial activities relevant to skin health, but the data are mainly phytochemical screens or preliminary antimicrobial assays rather than focused wound-healing or infection-challenge studies (Kumar *et al.*, 2024). Comparatively, *C. orbiculata* research in the last five years has moved beyond simple antimicrobial screening to include nanoparticle synthesis, cytotoxicity testing on skin cells, and in-vitro scratch assays however these remain largely lab-based and short-term. A consistent finding across the family is promising antioxidant, anti-inflammatory and antimicrobial potential, but *Kalanchoe* benefits from more animal-model and mechanistic studies while *Cotyledon* evidence is emerging and more limited in scope (Beatriz *et al*., 2023). There is a clear need for well-designed in-vivo wound-infection models, standardized extract characterization, dose-response studies, as well as controlled clinical (topical) trials for *Cotyledon orbiculata* to directly compare efficacy and safety against established Crassulaceae candidates (such as *Kalanchoe pinnata*), particularly focusing on clinically relevant skin pathogens (MRSA, *P. aeruginosa*) and on formulation/stability for topical use.

2.6 Immunomodulation

The term immunomodulation is defined as the alteration of the immune response either to increase or decrease responsiveness (Mukherjee *et al*., 2014; Akraml *et al*., 2014). Immuno-stimulation and immunosuppression refer to the enhancement in immune responsiveness and reduction in immune responsiveness, respectively. A substance, be it synthetic or biological, capable of stimulating, modulating or suppressing innate and adaptive components of the immune system are defined as immunomodulators (Majeedi *et al*., 2015). Investigations into *Cotyledon orbiculata* extracts have indicated that this extract possess both antimicrobial and immunomodulatory properties, with water, methanol, and chloroform extracts demonstrating inhibitory activity against skin-infection–associated pathogens such as *Staphylococcus aureus*, MRSA, and *Pseudomonas aeruginosa* (Tyavambiza *et al*., 2022). These extracts also

reduced pro-inflammatory cytokine expression, indicating that *C. orbiculata* may modulate immune responses involved in skin-infection progression and wound inflammation (Tyavambiza *et al*., 2022). Subsequent studies further confirmed that the plant's fractions display immunomodulation alongside antimicrobial activity, reinforcing its potential for infections in which dysregulated inflammation delays healing (Tyavambiza *et al*., 2022). Together, these findings position *C. orbiculata* as a promising dual-action therapeutic candidate for managing skin infections where both pathogen control and immune regulation are required.

Serving as a defense mechanism against foreign invading pathogens, upon recognition the host immune response is activated and acts to stop invasion (Reza *et al*., 2023). The immune system consists of two types of immunity namely: Innate (Nonspecific) and Adaptive (Specific) Immunity. According to (Kumar *et al*., 2011) the innate immune response is the first response mechanism of defense against physical, biochemical and cellular components, it is nonspecific and occurs the moment invasion takes place (Iwasaki & Medzhitov, 2015). It includes natural killer cells (NKC's), mast cells, macrophages, granulocytes and dendritic cells (DCs). There are four forms of protection for innate immunity, namely, the skin or mucous membranes, physiological conditions such as temperature or pH, and inflammation and phagocytosis (Ali Reza *et al*., 2023). In this type of immune response natural killer cells (NKCs) play a vital role. Cytokines and complement secretion, barrier function, activation of NKCs and phagocytes are defense mechanisms that occur upon invasion. Once infection occurs phagocyte cells accumulate, polymorphonuclear cells respond to the site to destroy the pathogen. This step of migration of phagocytic cells occur in phases, namely, rolling, adhesion to endothelial cells, diapedesis, migration to infection site and phagocytosis and destruction of pathogen (Harun *et al*., 2015). The process of attachment to endothelial cells occurs via a specific membrane receptor named beta 2 integrin. Endothelial cell receptors, intercellular adhesion molecules 1 and 2 attach to phagocyte cells due to mediation of the beta 2 integrin receptor (Schmidt *et al*., 2013).

The adaptive immune response is a more complex process, that is, antigen specific and dependent which means that when exposed to an antigen it requires time to respond. Adaptive immunity differs from innate immunity in the manner of how it allows memory of a previously intruding antigen, this enables the host to respond in a timely and efficient manner. The cells involved with adaptive immunity are B cells which differentiate into plasma cells and antigen specific T cells that are s*et* to proliferate through antigen presenting cells (APC) once activated (Marshall *et al*., 2018). Failure of innate immunity to respond triggers adaptive immunity to activate once infection occurs. The primary responsibility of this type of immunity is recognition

of non-self specific antigens, that is while self specific antigens are present, elimination of pathogenic cells and specific pathogens as well as immunologic memory development in the instance of future infections (Warrington *et al*., 2011).

A vast amount of medicinal plants have been used for immunomodulation, for example, *Astronium urudeuva*, *Coclospermum* v*itifolium*, *Terminalia amazonic*a and *Citrullus colocynthis* (Akraml *et al.,* 2014). The identification of immunomodulators with plants as the source is termed immunopharmacology and results obtained from a study on fractions isolated from *Tinospora crispa* showed that it significantly induces the expression of cytokines, thus, showing its immunomodulatory effect (Abood *et al*., 2014). Several studies conducted on plants and their immunomodulatory activity have showed improvement properties on the immune system. Due to all the secondary metabolites such as proteins, alkaloids, steroids, flavonoids, and phenolic substances present, plants are able to restore and improve health (Sarraf *et al*., 2016). Advantages of the use of plant sources as immunomodulators are relatively reduced toxicity, activation of immune function via biologically active compounds and a lenient immunomodulating effect (Nfambi *et al*., 2015; Shinkovenko *et al*., 2018).

2.7 Phytochemistry

2.7.1. Classification of Phytochemicals

The different health effects experienced by humans or animals when plant material is consumed is due to phytochemicals that are present. Plants used as remedies for ailments and diseases are used because of their phytochemical constituents. Phytochemical constituents such as terpenoids, flavonoids, tannins, alkaloids and saponins are stated to be the most valuable bioactive constituents. Medicinal plants, flowers, leaves, and vegetables are said to contain phytochemicals which are defined as natural bioactive compounds (Sharma *et al*., 2014). According to the function of the constituent in plant metabolism these phytochemicals are further separated into two groups, namely, primary and secondary constituents. Phytochemicals of great medicinal value include alkaloids, phenolics, tannins, saponins, steroids, flavonoids, terpenoids, and glycosides found in different parts of the plant. The medicinal use of phytochemicals can serve as antioxidant, antimicrobial, anti-inflammatory, antihypertensive, and even antidiarrheal purpose. For the plant itself, phytochemicals such as phenolics might give the impression that they serve a trivial purpose where they in fact aid in the survival of plant by interacting with the surrounding environment like competitors, pollution, the protection against illness, and environmental stress (Shaikh & Patil, 2020).

Plants possess the ability to rapidly produce distinct bioactive compounds. The valuable phytochemicals present in plants can serve as supplements for humans as antioxidants. Previous studies conducted have indicated that plant constituents such as lignans, flavonoids, tannins, and vitamins A, C as well as vitamin E operate as antioxidants. Other phenolics as well as acids such as ascorbic acid and beta carotene are highly involved in processes that influence aging, prevention of cancerous cells as well as the reduction of inflammation (Altemimi *et al*., 2017).

Approximately more than 4000 phytochemicals have been catalogued and are classified based on physical and chemical characteristics, with up to 150 phytochemicals being documented and researched in detail. The role of the plants metabolism indicates whether it will be classified as primary or secondary constituents. Amino acids, proteins, simple sugars, pyrimidines, and purines of nucleic acids are examples of primary constituents whereas the secondary constituents are glycosides, phenolics, flavonoids, alkaloids, terpenes, and saponins (Saxena *et al*., 2013).

2.7.2 Role of Phytochemicals in Plant Defense

Serving a two-dimensional purpose secondary metabolites are active when protection against herbivores or pathogens are necessary. They can serve as a protectant when there is inter-plant competition but when the environment is surrounded by favourable microorganisms they act as attractants. When the plant experiences stresses such as extreme temperatures such as drought or cold, UV exposure, salinity, increase or decrease of light, lack of nutrients, these secondary metabolites behave in a protective manner. Previous studies indicated that secondary products serve as modulators of gene expression and act as cellular levels of plant growth regulators and research has shown that it is essential in the ecophysiology of plants (Borokini & Omotayo, 2012).

The biosynthetic pathway is used to separate the three major groups of secondary metabolites, terpenes, nitrogen containing compounds and phenolic compounds (Rabizadeh *et al*., 2022). Secondary metabolites consist of groups such as terpenoids and steroids, alkaloids, fatty acid derivatives, phenylpropanoids, non-ribosomal polypeptides, and enzyme cofactors. Frequently extracted with petroleum ether, terpenoids are lipid soluble compounds. Terpenoids are the most ubiquitous secondary metabolites with approximately 30 000 compounds used as a treatment method for various illnesses. Terpene-based drugs used in pharmaceutical industry are Taxol the anticancer drug, and Artimesinin, the antimalarial drug (Dhanarasu, 2012). It is stated that approximately 23000 are known active compounds and are synthesized via the mevalonic acid pathway.

Secondary metabolites containing simple nitrogen atoms stated to be alkaloids with a small variety of compounds containing weak acid characteristics. Produced also by micro-organisms such as bacteria, fungi and animals. There is no clear apparent classification of alkaloids that separates it from other nitrogen containing compounds with the recent classification being based on the similarity of the carbon skeleton. Anti-carcinogenic, anti-inflammatory and antioxidant properties are the known biological properties of phenolic compounds present in practically all plants. Classified according to hydroxylic groups, chemical composition and substitutes in carbon skeleton (Kabera *et al*., 2014). To detect various phytochemicals or constituents in plant materials different chromatography methods must be employed to firstly identify which solvent system would be best for a specific type of plant as well as what compound the researcher is testing for.

2.7.3 Chromatography

Chromatography is defined as a group term that combines various techniques that are all based on separation of mixtures of compounds into smaller or single components. Chromatography was discovered as a separation technique used to separate was discovered in 1901 by a Russian botanist Mikhail S. Tsvet, discovered this separation method for separating plant pigments.

Separation of mixtures, the purification and analysis of different components such as pharmaceuticals, plant extracts, water samples as well as pesticides can be achieved by chromatography. Due to factors such suitability and precision, chromatography is used in important industries like drug manufacturing. High-performance chromatography is a prime method employed for separation of compounds as prerequisite step to characterization. Identifying the composition of different compounds present in the drug or sample. Chromatography techniques are also employed by forensic analysts for drug tests for police investigations. Vaccine production makes use of chromatography to purify vaccines, an example of this is the recent isolation of the SARS coronavirus spike protein by use of liquid chromatography (Talreja & Tiwari, 2022).

Column chromatography is an isolation and purification technique for natural products via solid-liquid extraction. High separation efficiencies and high biomolecule recovery and yield is commonly associated with this type of chromatography; however, it can be time consuming and challenging to scale up (Roque *et al*., 2020). Crude plant extracts are often purified by using column chromatography to isolate or identify the present compound. Adsorption strength of the solute molecules in a mobile phase and stationary phases directly affects the separation

of the solutes. Adsorption sites on the stationary phase enables mobile phase to compete with solute molecules for attachment. Solute molecules with low adsorption strength will elute out of column whereas a higher adsorption strength will cause some solute molecules to reside in the column (Ngo *et al*., 2018). The discovery of historical texts from China showed evidence of the use of medicinal herbs , isolated from plants as a medicine and in recent years plants have frequently been isolated for the discovery and development of novel drugs (Petrovska, 2012). Techniques and analytical methodologies such as electrophoresis, chromatography, enzymology as well as isotope techniques have led to the successful clarification of the biosynthetic pathways and the precise structural formulas (Kabera *et al*., 2014) and (Saxena *et al*., 2013) . Biomolecule extraction and determination or investigation into the potential medicinal properties plants possess is vital for use by pharmaceutical and agricultural industries. It is, therefore, important to continue phytochemical studies on plants as their nutrients are beneficial for overall health, a phytochemical constituent of such importance or benefit is terpenoids (Batool *et al*., 2019).

The following **Table 2.1** summarizes major differences between thin layer chromatography and paper chromatography (Talreja and Tiwari, 2022).

Table 2.1: Types of chromatography and their differences.

Paper chromatography						
Stationary phase	**Cost**	**Mobile Phase**	**Prep time**	**Heat**	**Time**	**Amount Sample**
Cellulose paper	Cost effective	Hydrophilic or hydrophobic	Minimal	No	Quick	Minimal
Thin layer chromatography						
Stationary phase	**Cost**	**Mobile Phase**	**Prep time**	**Heat**	**Time**	**Amount Sample**
Silica coated glass plate	Less cost effective	Glycerol, pyridine, acetone or Carbon tetrachloride	More Time required	Yes	Longer	More

Column chromatography is frequently used to purify molecules. The preferred sample is placed first onto a column (which is marked the stationary phase) to follow is the mobile phase. The flow of these phases within the column material in the samples eluting at the results bottom of column to be collected in a flask. When chromatography was initially discovered it is stated that the primary purpose was for the separation of substances based on colour.

Over time this purpose has evolved. In recent years chromatography has been identified as an efficient and precise method to separate mixtures with column chromatography as one of the methods employed to do so. The value of chromatography in various applications and professions has expanded since its initial discovery and it can be used in clinical laboratories for research studies by biochemists and an example of this is the determination of metabolic disorders by studying bodily fluids of individuals. To determine the amount of hormones like steroids or barbiturates or lipids gas chromatography is often used (Coskun, 2016).

2.8. Methods for Plant Extraction, Isolation and Purification

To determine the medicinal properties of plants, it is imperative to efficiently prepare the plant or parts of the plant such as the leaf, stem, flower, or roots for experimental testing processes. This is done by performing extractions and screening for possible compounds present in the extract (Abubakar & Haque, 2020). Development and discovery of new therapeutics from plants for the advancement of medicine is stated to be due to the chemical constituents within plants. These compounds have shown significant potential in pharmacological research and can lead to the development of novel therapeutic agents for the treatment of various ailments. Their diverse biological activities, including anti-oxidant, anti-inflammatory, and antimicrobial properties, make them essential candidates for drug discovery and development. It is stated that flavonoids, alkaloids, tannins, and phenols are the most valuable bioactive components (Yadav *et al*., 2014). Flavonoids and tannins have strong anti-oxidant, anti-inflammatory and antimicrobial properties, flavonoids by neutralizing free radicals and protecting cells from damage and tannins are good at binding proteins (Gupta *et al*., 2021).

2.8.1. Solvents

For the successful extraction of phenolic compounds from plants the use of solvents with different polarities is necessary. A study conducted by (Anokwuru *et al*., 2011) showed that *N.N*-dimethylformamide successfully extracted antioxidants whereas a comparative study by Koffi *et al* showed that extraction of compounds from walnut fruit was more effective when using methanol as solvent instead of ethanol. To extract phytochemicals multiple solvents have been employed by professionals. It is preferred that the extract is in a dried powder form to cancel the inference of water when the solvent is used. Choice of solvent depends on the polarity of the solute (Altemimi *et al*., 2017).

2.8.2. Microwave Assisted Extraction (MAE)

This involves the combination of solvent extraction and the use of a microwave by heating up the solvent of choice and the plant extract. This method is said to result in an increased number

of phytochemicals released. The use of heat causes immense pressure on the cell wall and causes evaporation. Due to pressure on the cell wall, it ruptures, and a higher yield of active phytochemicals is observed (Ingle *et al*., 2017).

Stated to be the most efficient method for flavonoid extraction, electromagnetic radiation is applied at frequencies ranging from 300 MHz and 300 GHz with wavelengths at 1 cm and 1 m. Applied heat causes the solvent to penetrate the sample. The disadvantage of this method is that the heat can cause degradation of compounds like tannins and anthocyanins (Abubakar & Haque, 2020).

2.8.3. Ultrasonic Assisted Extraction (UAE)

Used in different applications ultrasound assisted extraction similarly to MAE disrupts the plant cell wall enabling the improved penetration of solvent to cells using ultrasonic levels greater than 20 kHz. Stated to be a simple extraction method maintaining high extract quality of compounds, a mixture containing the solvent and extract, or sample is placed in ultrasonic bath and parameters such as time and temperature is s*et* and controlled. An ultrasonic probe can also be used but this has indicated problems when it comes to reproducibility. In previous studies conducted to compare ultrasonic bath extractions, shaking water baths, use of different solvents, and ultrasonic probes results showed that a lower operation time was only observed with ultrasonic bath and ultrasonic probe systems was employed (Altemimi *et al*., 2017).

2.9 Plant Extract Preservation

The freeze dry process is a complex process which stabilizes a material by dehydration and is quite expensive with the added advantage of not requiring heat (Tarafdar *et al*., 2017; Saikia *et al*., 2015). Freezing of a liquid into a solid state and drying, which involves the removal of both the frozen and unfrozen solvent from the product are the two major steps involved in freeze drying. Freeze drying is further sub-divided into primary and secondary drying. Sublimation is used to remove the frozen solvent, and secondary drying is a step to ensure the removal of the unfrozen solvent through a process referred to as desorption (Assegehegn *et al*., 2021).

CHAPTER THREE

RESEARCH DESIGN AND METHODOLOGY

The primary objective of this study was to prepare fractions of *Cotyledon orbiculata,* water and methanol extracts and to determine the antimicrobial and immunomodulatory properties of the prepared fractions.

3.1 Type of study

This research project was a quantitative, analytical, experimental laboratory study.

3.2 Ethics Approval

Minimal risk ethics approval was granted by the Health and Wellness Sciences Research Ethics Committee at the Cape Peninsula University of Technology (CPUT).

3.3. Study Sites

The Department of Biomedical Sciences laboratory at CPUT as well as the Departments of Biotechnology and Chemistry laboratories at the University of Western Cape (UWC) were the sites at which experimental work was conducted.

3.4 Plant Material

Fresh plant material of *Cotyledon orbiculata* was purchased from Van Der Berg Garden Nursery in Stellenbosch, Cape Town. Before the extraction process, plants were kept inside their vases to maintain freshness.

3.5 Chemicals

The following solvents used in this study were purchased from Lasec.

Dichloromethane

Chloroform

Ethanol

Hexane

Ethylacetate

Butanol

Phorbol 12-myristate 13-acetate and silica gel used in this study were purchased from Merck Life Science South Africa.

3.6 Preparation of Plant Extracts

The *Cotyledon orbiculata* leaves were cleaned by washing them with distilled water. The washed leaves were dried using paper towels. Plant leaves were then subsequently cut into smaller pieces to ease the blending process. The water extract was prepared by weighing out 300 g of cut leaves and these were blended with 600 ml of distilled water using the kitchen blender, as can be seen in Figure 7. This was carried out in batches a total of 6 times, thus, resulting in the use of 1.8 kg of plant leaves.

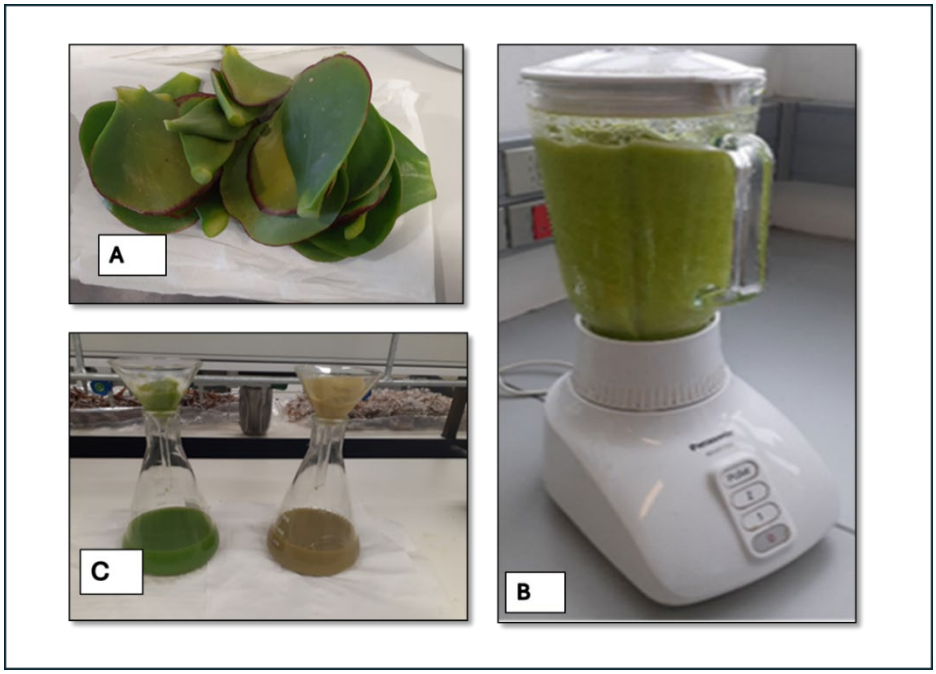

Figure 7: Plant extraction process. (A) Cleaning and washing of leaves, (B) Blending of leaves with solvent, (C) Use of filter paper and cotton wool to filter bigger plant particles, green liquid is the water extraction filtrate and the brown liquid is the methanol extract filtrate.

For the methanol extract, 300 g of cut leaves were blended with 600 ml of methanol. Blended until methanol and all solid plant material was evenly mixed with the solvent. Similarly with the water extract, the process was done a total of 6 times resulting in the use of 1.8 kg plant leaves. Both extracts were left at room temperature for 24 hours on magnetic stirrer to ensure efficient mixing of plant with the solvent.

After extraction, filtration followed, cotton wool was used first to remove bigger plant particles, and after that Whatman filtration paper No. 1 was used to ensure all unwanted particles were removed. Extracts were then centrifuged at 3000 rpm for 5 minutes followed by the use of a 0.45 µm syringe filter. The water extract was kept frozen at -80 °C until further use. After filtration the methanol extract was evaporated by rotary evaporation using the Buchi Rotavapor

11 with the temperature of the water bath maintained at 45°C and 14 mbar (Figure 8 B). The methanol and water extracts were then freeze dried and stored in the refrigerator at -20 °C awaiting analysis (Figure 8 C).

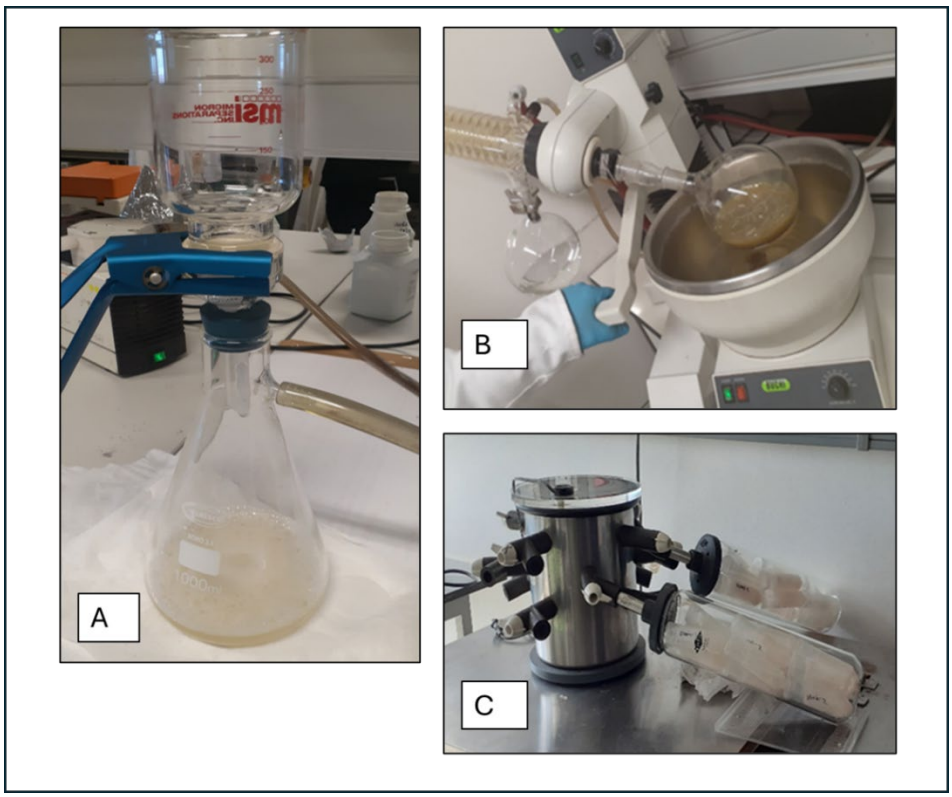

Figure 8: (A) Filtration process via use of pump and filter paper, (B) Rotary evaporation of methanol extract, (C) Freeze drying process of the water and methanol extracts of *C. orbiculata*.

3.7 Phenol Sulphuric Acid Test

The phenol sulphuric acid test was done to confirm the presence of carbohydrates such as mono-, di-, oligo- and polysaccharides within the extract. The sample tested was the water extract. Precisely 0.036 g of glucose, polysaccharide and water extract (sample) was individually weighed and allocated into separate vials. Subsequently 1 ml of distilled water was added to each vial and mixed to ensure it is dissolved in distilled water. The solutions were transferred to labelled test tubes (S, G and P). A control test tube was added and labelled as W. 1 ml of phenol was added to each test tube, followed by 5 ml sulphuric acid.

3.8 TLC Spotting

To determine the purity of a substance or identify unknown compounds present in a mixture, TLC chromatography is commonly used. The solute and mobile phase compete for a binding place, separating compound within (Bele *et al*., 2011). Thin layer chromatography (TLC) studies were carried out to determine which solvent system would best separate compounds or components within the dried extract. Precoated TLC Silica gel aluminium 60 F_{254} with 0.2 mm thickness sheets were used. TLC spotting was done by vertically ascending sheets inside a glass chamber saturated with the chosen solvent system. Glass capillaries were used to spot on TLC silica aluminium plates. Plates were cut to sizes of 5×20 cm by use of a pencil. A line was drawn 1.5 cm from bottom end of plate and top end of plate. Spots were lightly made approximately 1.0 cm apart to allow for proper uninterrupted movement of extract. Extracts were prepared by mixing 0.1 mg with approximately 1 ml of distilled water. For visualization of TLC spots UV light was used at 254 nm or 366 nm. For further visualization vanillin spray was used. If required, the sheet was sprayed with vanillin reagent (vanillin preparation is the combination of 15 g vanillin with 250 ml ethanol and 2.5 ml concentrated sulphuric acid). After spraying, a heat gun was used to dry the sheet until visible spots appeared.

3.9 Fractionation

After the use of silica gel TLC sheets/ plates were used to spot dried extract to determine a suitable solvent system to use for flash column chromatography, a column was prepared with cotton wool, loaded chromatography column with prepared extract and silica mixture. The stationary phase, silica gel 60 (0.040-0.063 mm) with about 230-400 mesh particle size (Merck Life Science South Africa) was packed in glass columns (20-25 mm diameter) and used for column chromatography. Precisely 5 g of each extract was pre-absorbed onto silica gel by gravity elution using a combination of the chosen solvent system.

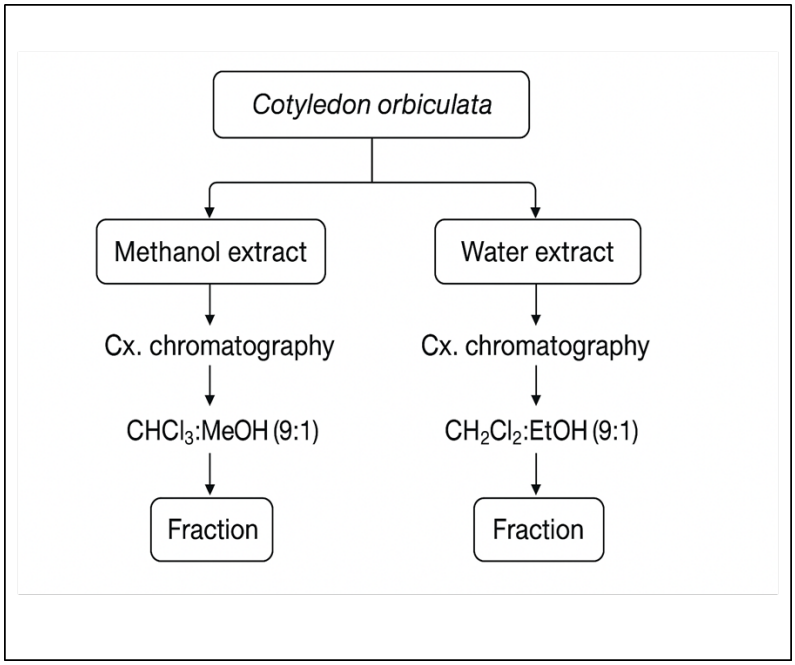

Figure 9: Image illustration of fractionation scheme.

Dichloromethane and ethanol at a ratio of 9:1 was chosen as solvents for fractionation of the water extract. Fraction scheme of extracts is illustrated in **Figure 9**. Fractionation was done using the following solvent mixtures of dichloromethane and ethanol (9:1), (8:2), (7:3), (6:4), (5:5), (4:6), (3:7), (2:8), (1:9) and finally washed with 1 L of ethanol. The methanol extract was fractionated using solvent mixtures of chloroform and methanol at a ratio of 9:1. 1 L volumes of 9:1 were added to the column and elution began. This was repeated at the following ratios (8:2), (7:3), (6:4), (5:5), (4:6), (3:7), (2:8), (1:9). Thereafter, complete 1 L of methanol for methanol extract and,1L of ethanol for water extract were added to the column. The collected fractions were analyzed by use of TLC.

Retardation factor or Rf value, stated to be a crucial concept in TLC. It is defined as the ratio of the distance travelled by the solute/ sample analyzed to the distance travelled by the solvent front on the TLC plate (Lundanes *et al*., 2014). The Rf value is a dimensionless number that can be used to identify compounds based on their movement on the TLC plate. Different compounds have different affinities for the stationary phase and the mobile phase, this results in different Rf values, making these values an important factor for comparing and identifying compounds in a mixture (Vasta *et al*., 2009). In thin layer chromatography the Rf values help in determining the polarities, relative masses and solubilities of the compounds, with particular use in organic chemistry for purification of mixtures and monitoring chemical reactions (Xu *et al*., 2022).The eluted solutions were collected and separated based on color and profile on TLC and then combined and evaporated. Fractions with similar Rf values were collected,

pooled together and evaporated. The fractions were evaporated with rotary evaporator and stored in the fume hood. After fractions were evaporated the fractions were spotted on TLC, to visually determine if similar TLC profile could be seen on the plate and then combined if it had the same profile. Fractionation was done over a 7-day period for each extract and added to the column in increasing polarity. All the fractions were stored in the fume hood for evaporation, once completely dried all fractions were weighed, and all masses were recorded.

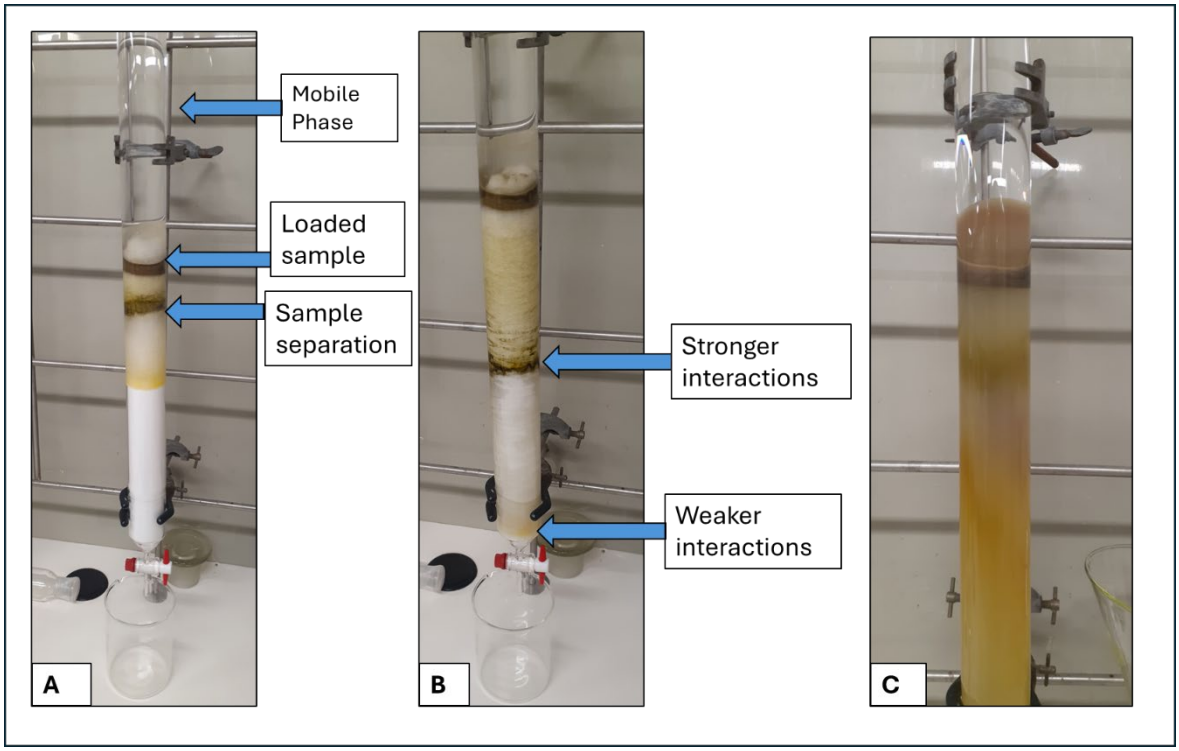

Figure 10: Column chromatography (A) Loaded chromatograph with added solvent (mobile phase), (B) Start of fractionation, collection of elute, weaker interactions first to elute, (C) Clear separation as solvent concentration is adjusted.

3.10 Evaluation of Antimicrobial Activity of Fractions Derived from *Cotyledon orbiculata* Extracts

The fractions isolated from the water and methanol extracts of *Cotyledon orbiculata* were evaluated for antimicrobial activity against Gram positive bacteria *Staphylococcus aureus* (ATCC 25923), Methicillin resistant *Staphylococcus aureus* (ATCC 33591), *Staphylococcus epidermidis* (ATCC 12228), and Gram-negative bacterium *Pseudomonas aeruginosa* (ATCC 27853). These bacterial strains were obtained from the American Type Culture Collection (ATCC).

These microorganisms were selected due to their common affiliation with SSTI and other complicated skin infections. *Staphylococcus aureus* and gram-positive cocci and *Streptococci* with *Pseudomonas aeruginosa, Esherichia coli* and *Enterococcus* are stated causative agents of SSTIs in hospitalised patients and burn victims. Complicated SSTI affect the population with pre-disposition conditions such as diabetes, immunodeficiencies and vascular deficiency affecting deep within the subcutaneous tissue and muscles .

3.10.1 Culturing of Microorganisms

All the culture media was prepared according to the manufacturer's instruction manual and sterilized by autoclaving the media at 121 °C for 15 minutes. The culture media was then poured into petri dishes and left to cool down and set in a laminar flow. Set petri dishes were stored at a temperature of 4 °C. The microorganisms were obtained from Thermo Fisher Scientific. All microorganisms were first cultured in nutrient broth at 37 °C for 24 hours and subsequently sub-cultured on Müller Hinton agar (MHA) and incubated at the same conditions. Microorganisms were continuously subcultured on fresh media to maintain them.

3.11. Antimicrobial Activity

3.11.1 Minimum Inhibitory Concentration (MIC)

Minimum Inhibitory Concentration (MIC) is defined as the lowest concentration necessary for an antimicrobial agent to prevent or inhibit visible growth of an organism within in-vitro conditions and is expressed in mg/ml or µg/ml and it is employed to determine an antimicrobial agent's level of strength. Quantitative methods employed to determine MIC values such as microdilution and agar dilution differ as agar dilution can be time consuming and challenging when dealing with limited amounts of test agent. Wells that had bacterial growth appeared more turbid and lighter yellow prior to the addition of dye indicator. Based on the individual mass of each fraction, a stock solution was prepared by dissolving 2 mg of each of the dried fractions in 1 ml 10 % DMSO. The antimicrobial activity of all fractions was determined by testing it against known skin infections causing organisms such as *Pseudomonas aeruginosa*, *MRSA*, *Staphylococcus aureus* and *Staphylococcus epidermidis*. The positive control and negative controls used were 2.5 mg/ml amplicillin and 10 % DMSO respectively.

The broth micro-dilution method was used to determine antimicrobial activity of *Cotyledon orbiculata* fractions. Assays for MIC were run in triplicates. Microbial suspensions of all microorganisms were prepared to match 0.5 McFarland standard. Exactly 100 µl of the

prepared standard solution was diluted in 14900 µl Mueller Hinton Broth (MHB) and mixed. This was repeated for each microorganism.

MHB was dispensed (50 µl) into all wells of a 96 well plate. 100 µl of each fraction was dispensed into the first row of a 96 well plate in triplicates. A multichannel pipette set at 50 µl was used to serially dilute all fractions and the last 50 µl obtained from the last well was discarded, to ensure all wells contain only 50 µl. Then 50 µl of prepared microbial suspension was added to all wells. The 96 well plate was then sealed and incubated at 37 °C for 24 hours. Controls were also included in the well plate.

Alamar Blue was used as an indicator, and after the incubation period lapsed,10 µl of alamar blue was added to all the wells and the plate was sealed and incubated for 3 hours at 37 °C.The growth of bacteria in the wells will reduce the blue colour from the dye to a bright pink colour (Rampersad, 2012; Tyavambiza *et al*., 2021). The wells that retained the blue colour are indicators of inhibited bacterial growth. The wells that had the pink colour indicated that the bacteria present in the well had reduced the blue colour to a pink colour. The MIC values were recorded as the concentrations in the last wells which retained the blue colour. The MIC was also recorded with a spectrophotometer at fluorescence intensity 600 nm and was considered as the last concentration before a sharp increase in fluorescence intensity.

3.11.2 Minimum Bactericidal Concentrations (MBC)

The wells that indicated no bacterial growth (blue wells) during MIC analysis were used. A loopful of media from such wells were sub-cultured onto prepared agar plates and incubated at 37 °C for 24 hours. The MBC value was then recorded. A MBC value, that is higher, when compared to MIC, indicates that the tested extract or fraction is only bacteriostatic and not bactericidal and a MBC, that is similar to the MIC, indicates that it is bactericidal at the tested concentration and can cause death.

3.12. Assessment of Immunomodulatory Properties of Fractions isolated from Water and Methanol Extracts of *Cotyledon orbiculata*

3.12.1 Cell Culture

The human monocytic leukaemia cell line, THP-1 was used and obtained from Department of Pharmacy, University of the Western Cape. A combination of Roswell Park Memorial Institute (RPMI), 10 % Fetal Bovine Serum (FBS) and 1 % Pen Strep (Penicillin and Streptomycin) was

prepared. The prepared media was used to culture and grow THP-1 cells in cell culture flasks at 37 °C in a humidified temperature of 5 % CO_2 in a SHEL LAB incubator.

3.12.2 THP-1 Differentiation

Phorbol 12 myristate 13 (PMA) was used to differentiate THP-1 monocytes to macrophages. PMA was obtained from Sigma Aldrich. THP-1 cells were seeded at a density of 2×10^5 cells/ml per well in a 24 well sterile cell culture plate with a concentration of 50nM PMA. After an incubation period of 3 days at 37 °C at 5 % CO_2 in a humidified atmosphere in a SHEL LAB Incubator. Subsequently after incubation period of 3 days the media was replaced with PMA free media for 24 hours.

3.12.3 Cell Viability

The WST-1 assay was used to determine cell viability. The purpose of this assay was to evaluate the toxicity of the fractions isolated from *Cotyledon orbiculata* extracts on the THP-1 cell line. This assay was performed by use of 96 well culture plates. Cells were seeded into a 96 well plate at a cell density of 1×10^5 cells/ml and was differentiated with 50 nM PMA as mentioned above (section 3.11.2). The differentiated cells were then treated with different concentrations of fractions isolated from the water and the methanol extracts. The cell culture plates were then incubated for a period of 24 hours at 37 °C in a humidified atmosphere of 5 % CO_2. After the incubation period, the treatment was removed from all wells and replaced with media containing the 10% WST-1 reagent and incubated for 3 hours at 37 °C in a humidified atmosphere of 5% CO_2. After 3 hours the absorbances were measured by spectroscopy at 440 nm (reference 630) by use of the POLARstar Omega plate reader. The cell viability was expressed as a percentage of the absorbance of treated cells to control (untreated) cells.

3.12.4 Immunological Assay (cytokine determination)

Lipopolysaccharide (LPS) from *Escherichia coli* 0111: B4 purchased from Sigma Aldrich, was used for cell stimulation for the determination of cytokine production. Described as an endotoxin by clinicians, LPS is more accurately defined as a microbe-associated molecular pattern (MAMP) capable of activating the host innate immune response by inducing signal transduction which in turn results in the release of pro-inflammatory cytokines (Huszczynski *et al*., 2020). Once the THP-1 cells are exposed to LPS, antibacterial defense begins and the production of inflammatory mediators begin.THP-1 cells that were differentiated with PMA in 24 well plates were stimulated with 1 µg/ml LPS for a period of 6 hours. The stimulated cells were then incubated with fractions from methanol and water extracts at 37 °C for 24 hours.

Wells used as a control were stimulated with LPS but not treated with the fractions. After the incubation period, the cell supernatants were collected from the wells and transferred to Eppendorf tubes and centrifuged for 10 minutes at 1500 rpm (Centrifuge 5417R (Eppendorf AG, Hamburg, Germany)). The supernatants were collected and transferred to a new Eppendorf and stored at -80 °C for further cytokine measurement. The ELISA Kits from Bio Scientific were used to determine the production levels of cytokines IL-6, IL-10 and TNF-α. The manual provided by the manufacturer was followed for cytokine determination.

3.13. Statistical Analysis

GraphPad Prism version 9 software was used for statistical analysis. The results were expressed as mean ± standard error of the mean (SEM). Immunomodulatory effects of fractions were compared by conducting a two-way Analysis of Variance (ANOVA) using Tukey's Multiple comparisons test. All analysis performed were done in triplicate.

CHAPTER FOUR

Results and Discussion

4.1 Plant Extracts

After extraction, the resulting mass after freeze drying each extract was weighed and recorded as is seen in Table 4.1. The same weight of plant material was used for extraction, but methanol resulted in a higher mass (25g) of freeze-dried extract compared to the water (19g) as shown in table below.

Table 4.1. Weight of leaves prior to freeze drying and the resulting freeze-dried extract

Plant Extract	Weight of fresh leaves (kg)	Freeze dried extract (g)
Water	1.8	19
Methanol	1.8	25

4.2 Phenol Sulphuric Acid Test

Figure 11 is an image showing the phenol sulphuric acid test result.

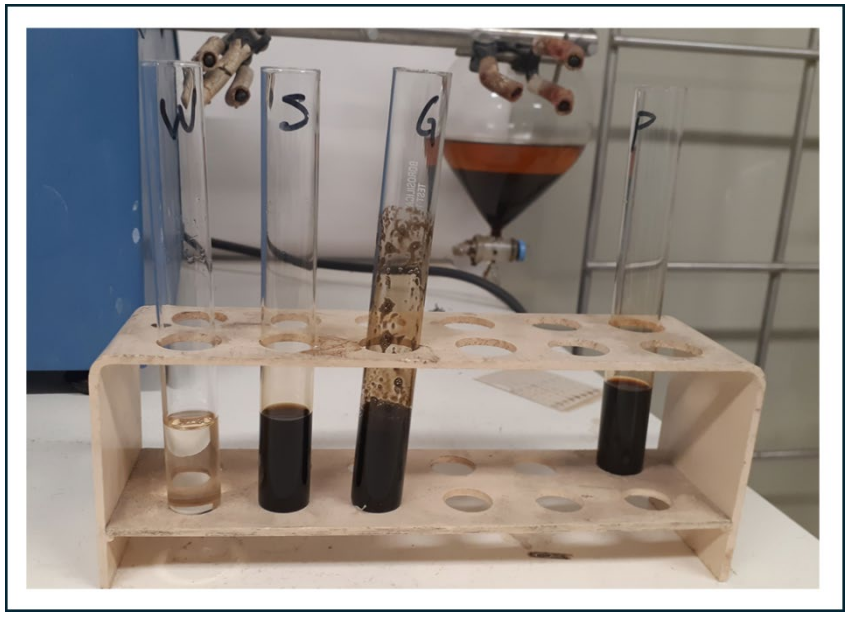

Figure 11: Visual result of sulphuric acid test. Test tubes labelled as W=Control, S=Water extract, G=Glucose and P= Polysaccharide

The Phenol sulphuric acid method of determining carbohydrate content is a commonly used quantitative approach to detect the presence of carbohydrates present in a solution or extract

(Jain *et al*., 2017). This test was performed to confirm the presence as well as indicate the degree of carbohydrates in water extract and is often used to analyse complex mixtures. Carbohydrate content of extracts or mixture have a significant effect on the antimicrobial ability or effect of stated plant extract. Constituents such as polysaccharides and oligosaccharides both play significant roles in antimicrobial activity, polysaccharides can inhibit bacterial growth via disruption of the cell wall whereas some oligosaccharides promote the growth of beneficial bacteria (Dobrin *et al*., 2023).

Visual result for sulphuric acid test showed a positive result for test tubes S, G and P. Color change was a deep dark red-brown colour. This was done to detect carbohydrates that might contribute to bioactivity. Many polysaccharides and oligo saccharides possess immunomodulatory properties, capable of suppressing or stimulating the immune system. This was also done to further investigate the plant extracts solubility for chromatography and to determine which solvent would best suit or allow proper fractionation. The result obtained indicated that the water plant extract was rich in carbohydrate-based phytochemicals.
A positive test result for the Dubois Phenol-sulphuric acid test appears as a deep orange or deep brown colour in the tested sample solution as stated. A high concentration of carbohydrates would present visually as a higher intense brown/orange colour. The formation of biofilms by microorganisms is interrupted by carbohydrate polymers as well and this function is of great importance as this is a defence mechanism employed by microorganisms upon treatment and thus contributes to resistance of microorganisms to treatment. The presence of carbohydrates in an extract also causes stability and solubility of other potentially bioactive components such as phenolic compounds (Inamuddin *et al*., 2021). As seen in **Figure 11**, a deep dark brown colour was observed and serves as confirmation of the presence of carbohydrates. High polysaccharide content (intense red-brown) may contribute to antioxidant activity, wound healing, or immune-modulating properties. A low to moderate intensity (pale yellow to yellow-orange) may indicate presence of simple sugars that could support microbial growth if extracts are applied to wounds or could be metabolically active compounds in herbal remedies. This method is not strictly stoichiometric when applied to mixtures of different sugars or complex extracts. For accurate carbohydrate quantification, one must build a standard calibration curve (typically using a known sugar like glucose, or a sugar representative of the expected composition) under the same conditions (reagent volumes, acid concentration, incubation time, wavelength). The colour intensity is directly proportional to carbohydrate concentration only if all reaction conditions are consistent. Complex biological extracts such as plant extracts, algae, pigmented samples, the interfering substances (pigments, polyphenols, other organic compounds) or suboptimal hydrolysis / reagent addition can skew the results (overestimate or underestimate total carbohydrates).

Therefore, while this method is useful for rapid total carbohydrate screening or estimation, the results should be interpreted as approximate glucose-equivalent or total sugar-equivalent rather than absolute- especially when the sugar composition is unknown or diverse.

4.3 Fractionation

The fractionation process resulted in 46 methanol fractions and 32 water fractions. Each fraction was collected at different intervals, and the separation efficiency is evident from the visible differences in color and composition among the fractions. The rotary evaporator was used to evaporate and combine fractions with similar profile on TLC plate. indicates the resulting fractions. It can be seen that color profile of fractions varies, different compounds moved through the mobile phase at different times, a dark green fraction was collected and afterwards a bright yellow color fraction as can be seen in below in Figure 12 image A. The water fractions showed no obvious difference for most of the fractions, with only different shades of yellow.

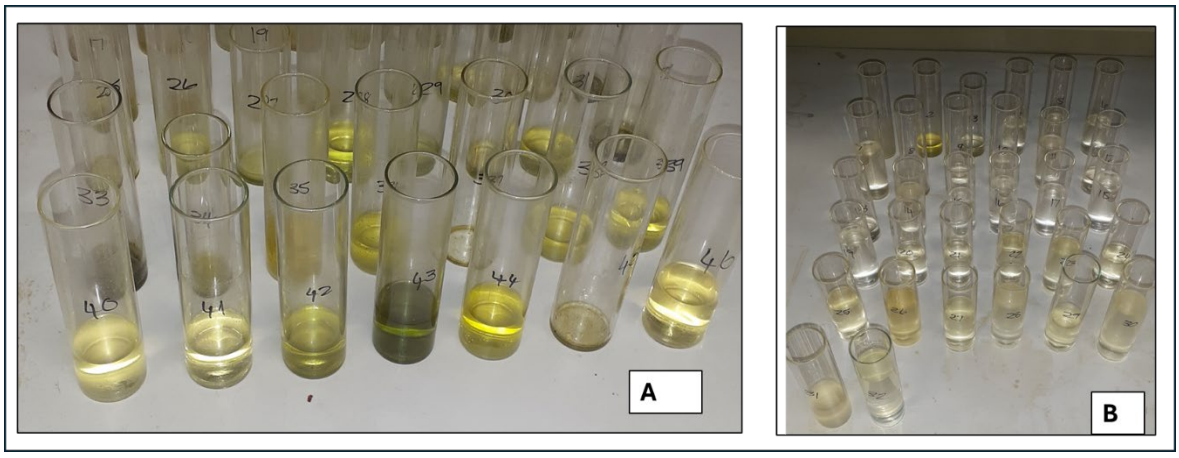

Figure 12: The different fractions after column chromatography and evaporation processes (A) 46 methanol fractions, (B) 32 water fractions.

To successfully study and investigate plant materials and their bioactive compounds, it is imperative to use an efficient extraction method. Extraction was done by dissolving leaves in water and methanol.

The separation of a plant extract into different fractions is defined as fractionation, it is then isolated further into more groups or compounds, this process is repeated until a pure compound is isolated (Abubakar & Haque, 2020). Methods are grouped into physical and chemical methods. Fractional distillation, crystallization, liberation, chromatographic techniques, and the separation funnel method are physical methods. Chemical reactions are used to split and purify mixtures of compounds and are based on the functional groups that are present or suspected to be present in the sample (Dhanarasu, 2012). Due to the

complexity of samples, fractionation is used to simplify or reduce the complexity of samples before employing mass spectroscopy for identification.

Fractionation of plant extracts is a method used to obtain a pure compound by continuously adding the selected solvent to plant extract. It is stated that when different solvents are used, solvents must be added in order of increasing polarity. Once the functional group of the compound is known a chemical method can be used to selectively react with or modify the group, aiding in identification, quantification or further characterization of the compound. However, with extracts where the compounds present are unknown or uncertain, physical methods are employed such as separation funnel, fractional liberation and chromatographic techniques. It is necessary for pre-screening tests or analysis due to the variety of bioactive compounds present in plants.

The use of chloroform and methanol as solvents for plant extraction is frequently employed due to their complementary polarities that allows the dissolution of broad spectrum of phytochemicals. These solvents were chosen for fractionation of the methanol extract. The polar solvent methanol effectively extracts polar compounds such as phenolics, flavonoids and alkaloids whereas the non-polar solvent chloroform is adept at dissolving lipophilic substances like terpenoids and certain lipids. It is stated that when used in combination this solvent system can extract both polar and non-polar compounds from plant materials, an example of this is use of a method developed by Jordi Folch Pi to effectively extract lipids from brain tissue known as the Folch method.

A study that evaluated the use of different solvents for compound extraction from *Schinus buxifolia* produced results that found that methanol had the highest extraction yield for extracting phenolics, flavonoids, terpenoids and alkaloids (Truong *et al*., 2019). Most recently some studies that made use of the Folch method are the lipid extraction from shale samples, as well as a study that made use of green microalgae for the extraction of total lipids and lastly a study that investigated various defatting procedures for the enhancement of protein extraction from green microalgae (Akondi *et al*., 2017; Axelsson & Gentili, 2014; Le *et al*., 2025). These studies demonstrate the continued use and relevance as well as efficacy of the Folch method utilizing chloroform and methanol in extracting lipids and other compounds from biological compounds like plants and microalgae.

For methanol fractions M3–M10 showed clearly visible bands of green and yellow color on plate. These bands were present at different Rf levels. To fractionate the water extract, the

solvents dichloromethane and ethanol were used. For the water extract, the chosen solvent system was dichloromethane and ethanol as it had the best separation upon spotting.

Ethanol, a polar solvent, possesses the ability to successfully fractionate hydrophilic compounds such as saponins, flavonoids and tannins. Dichloromethane on the other hand, is less polar than ethanol and is efficient as the extraction of lipophilic substances such as some terpenoids and essential oils, the combination of a polar and less polar, an extraction that is polarity based, can cause the isolation of broad spectrum of phytochemicals (Kumar *et al*., 2023).

Various studies have made use of dichloromethane and ethanol as solvents and resulted in fractions that exhibited significant antibacterial properties. An example of such a study is a study that focused on the stem bark of *Piliostigma reticulatum,* the ethanol extract of this plant was fractionated and the dichloromethane fraction showed antibacterial activity against susceptible and resistant bacterial strains (N'Guessan *et al.*, 2015), another study using a dichloromethane fraction of a ethanol extract from *Piliostigma reticulatum,* a medicinal plant used to treat inflammation, contained bioactive compounds that were anti-inflammatory and healing properties *(Dosso et al*., 2020). Employing ethanol and dichloromethane as solvents for the fractionation of a water extract allows the separation of a diverse array of bioactive compounds, enhancing ant-inflammatory, antimicrobial and healing activities. The use of these two solvents was to maximize the extraction of both hydrophilic and lipophilic compounds present.

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4.3.1 TLC Spotting

TLC spotting during the fractionation process of the methanol extract revealed distinct separation patterns in all fractions. The TLC plates show well defined spots of different colours, indicating the different mixtures of compounds present. M2 had a clear spot, whereas M3, M4 and M5 showed varying Rf values and different colours as well. Plate 3, in Figure 12 shows, clear visible spots at different Rf values suggesting the presence of different compounds, plate 5, M11-M13 shows 2 spots at similar strengths and Rf values and fractions thereafter (plate 6 -plate 9) showed minimal spots. Based on the profiles visualized on the TLC plates, fractions displaying similar chromatographic patterns were combined. Table 4.2 indicates the lists the new fraction label as well as which fractions were combined, and which were kept as a individual fraction. This approach ensured that compounds with closely related characteristics were pooled together, thereby facilitating more efficient subsequent analysis and possible characterization.

The separation via funnel method was initially used to fractionate extracts but yielded minimal fractions with ≥0.001 mg per vial. Thin layer paper chromatography was done for both extracts for determination of a suitable solvent, and it was then decided to perform column chromatography for increased mass as well as diversity of fractions. After fractionation all fractions were left to evaporate. This resulted in a partly solid viscous substance in glass vial. It was not possible to individually remove and weigh separately. All fractions were spotted on TLC and combined based on visual results.

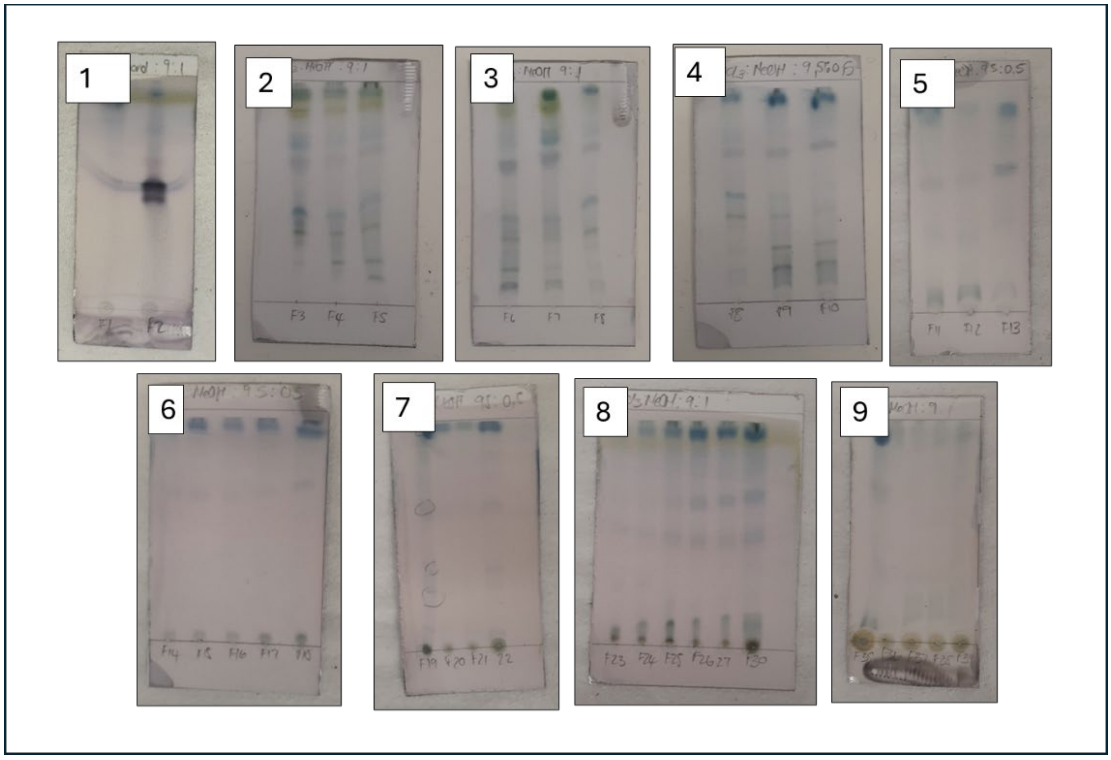

Figure 13: TLC plates of spotted Methanol fractions

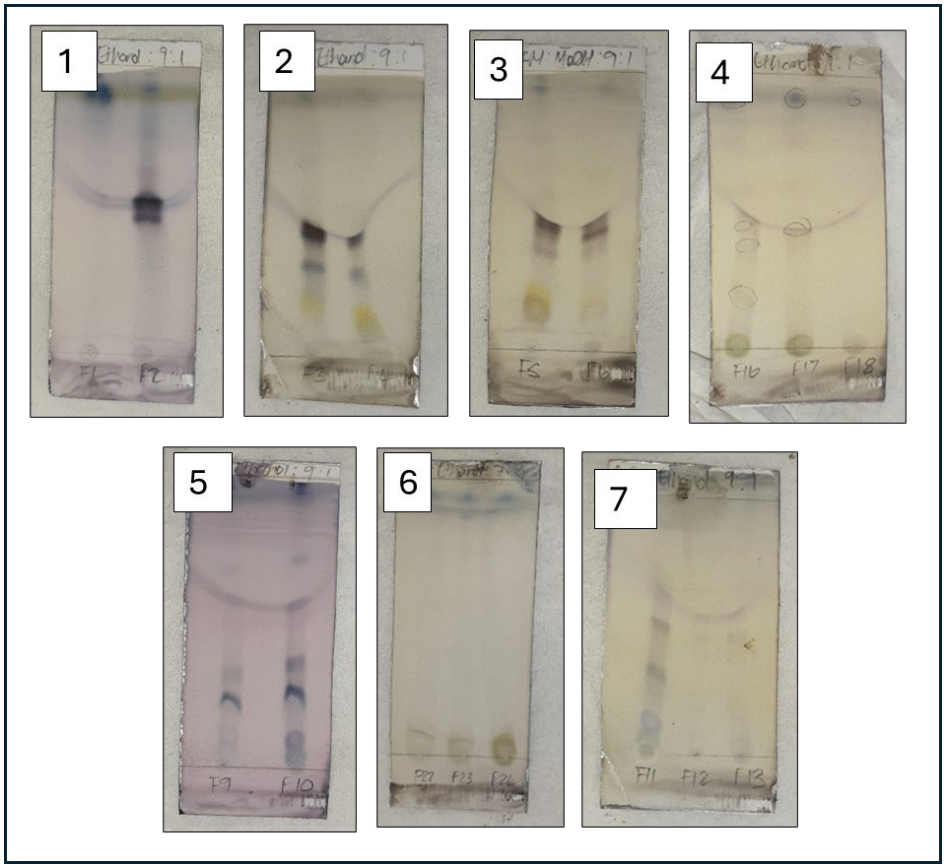

Figure 14: TLC plates of spotted Water fractions

Fractionation of water extract and methanol extracts resulted in a total of 74 individual vials of fractions; 32 water fractions and 42 methanol fractions.

Due to the high number of fractions, it was a challenge to combine solely on visual results due to faint results in some instances. After the use of a rotary evaporator, samples were left in fume hood to further evaporate the remaining liquid, this took approximately two and a half weeks.

Figures 13 and 14 are the TLC plate results of TLC spotting that was done during the fractionation process. For the methanol extract chosen solvent system was chloroform and methanol as it yielded the most favorable results when methanol extract was spotted on a TLC plate with this solvent system. Chosen ratio to start fractionation process with was a ratio of 90:10 $CHCl_3$: MeOH. On TLC plate, M3–M8 it can be deduced that the solutions/elute contains a variety of compounds. For methanol fractions M3–M10 showed clearly visible bands of green and yellow color on plate. These bands were present at different Rf levels. After M13 the bands/spots are reduced, only showing one band. M9 and M10 show a clear compound present with similar Rf value indicating that it is the same compound. Continuous collection and continuous spotting occurred and TLC plates with profiles for M9 to M10 had the same visual profile, showing one clear compound, with streaks of different shades visible which could indicate the presence of some impurities or pigmentation. M14-M22 showed one

clear spot at different intensities, no spots of other colors was visible, indicating one sole compound or mixture of compounds. M23 to M28 had a clear visible compound, three bands reappeared after M25 most likely the same compound present with faded impurities or pigments present. M36 to M39 shows no presence of compounds within the solution. Table 4.2. shows all collected fractions, and which pairs or sets were combined as they had similar visible color profile or spotted TLC profile. It also indicates the new resulting fraction number.

The water fractions collected from column chromatography resulted in 32 different fractions, with the TLC profile displayed in **Figure 13**, labelled plated 1-7. Water fraction W2, had a clear spot, condensed with a dark blue colour whereas plate 2, water fractions W3 and W4 each showed 3 compounds of different colour and similar Rf values. Plate 4 showed no indication of a compound and Plate 5 indicated 4 different spots (W10) whereas W9 showed only 1 spot. The water fractions with similar chromatographic profiles were combined as shown in Table 4.3 and it was observed that the water fractions showed a less complex and condensed TLC profile than the methanol fractions.

Table 4.2 Methanol fractions after fractionation

Methanol extract	Chromatography using chloroform & methanol as solvents	Fraction Label
M1-M2	Combined	M1
M3-M5	Combined	M2
M6-M7	Combined	M3
M8-M10	Combined	M4
M11-M13	Combined	M5
M14-M18	Combined	M6
M19-M22	Combined	M7
M23-M24	Combined	M8
M25-M27	Combined	M9
M28-M30	Combined	M10
M31	Kept separate	M11
M32-M34	Combined	M12
M35-M36	Kept separate	M13-M14
M37-M39	Combined	M15
M40-M44	Kept separate	M16-M19
M43-M45	Combined	M20
M46-M49	Kept separate	M21-M24

M50-M51	Combined	M25
M52-M64	Kept separate	M26-M38
M65-M66	Combined	M39
M67-M69	Kept separate	M40-M42

Table 4.3 Collected water fractions after fractionation

Water extract	Chromatography using dichloromethane & ethanol as solvents	Fraction label
W1-W6	Kept separate	W1-W6
W7&W8	Combined	W7
W9-W11	Kept separate	W8-W10
W12-W13	Combined	W11
W14-W15	Combined	W12
W16-W24	Kept separate	W13-W21
W25-W26	Combined	W22
W27-W28	Combined	W23
W29-W30	Combined	W24
W31-W32	Combined	W25
W33-W34	Combined	W26
W35-W37	Combined	W27
W38-W40	Combined	W28
W41-W43	Combined	W29
W44-W46	Combined	W30
W47-W50	Combined	W31
W51-W54	Combined	W32

4.4. Antimicrobial activity

4.4.1 Minimum Inhibitory Concentration (MIC)

Minimum Inhibitory Concentration (MIC) is defined as the lowest concentration necessary for an antimicrobial agent to prevent or inhibit visible growth of an organism within in-vitro conditions. It is employed to determine an antimicrobial agent's level of strength. Quantitative methods employed to determine MIC values such as microdilution and agar dilution differ as agar dilution can be time consuming and challenging when dealing with limited amounts of test agent (Elshikh *et al*., 2016). The antimicrobial activity of all fractions isolated from both

the water and the methanol extracts was evaluated by determining the MIC values using the broth microdilution assay. To enhance the microdilution, resazurin dyes can be used to improve the visual determination of an MIC, for this study, Alamar blue dye was used. On a spectrophotometer the MIC was interpreted as the last concentration before a significant increase in fluorescence values and visually as the last wells with the blue colour. Table 4.4 and Table 4.5 list the MIC & MBC values of the water and methanol fractions, respectively.

Table 4.4. MIC (µg/ml) values of Water Fractions.

Treatment	***Pseudomonas Aeruginosa***		***Staphylococcus Aureus***		**Methicillin resistant *Staphylococcus aureus***		***Staphylococcus Epidermidis***	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
Positive Control	0.625	0.625	0.625	×	1.25	×	0.625	×
W1 -W2	2 000	×	-	•	-	•	-	•
W3	1 000	×	-	•	-	•	-	•
W4-W7	2 000	×	-	•	-	•	-	•
W8	500	×	-	•	-	•	-	•
W9-W10	1000	×	-	•	-	•	-	•
W11	2 000	×	-	•	-	•	-	•
W12-W13	1 000	×	-	•	-	•	-	•
W14	2 000	×	-	•	-	•	-	•
W15	450	×	-	•	-	•	-	•
W16	1 000	×	-	•	-	•	-	•
W17	950	×	-	•	-	•	-	•
W18	750	×	-	•	-	•	-	•
W19-W20	1 000	×	-	•	-	•	-	•
W21-W32	2 000	×	-	•	-	•	-	•

× = No MBC **• = N/A** **- = No MIC**

The results listed show that water fractions exhibited a MIC value exclusively against *Pseudomonas aeruginosa*. In contrast, the tested methanol fractions demonstrated MIC values against all tested microorganisms, indicating a broader spectrum of antimicrobial activity. The results for the water fractions were rather promising against the gram-negative bacterium. All fractions 1 through to 32 of the water extract exhibited some antimicrobial activity against *Pseudomonas aeruginosa*. *Pseudomonas aeruginosa* is a gram-negative bacterium and is stated to be a highly drug-resistant microorganism. The water fractions showed antimicrobial activity against only *Pseudomonas aeruginosa* and not against any of the gram-positive microorganisms. This result was interesting as gram positive bacteria is stated to generally be more susceptible to antimicrobial agents (Subramaniam *et al*., 2023). However, the activity against only the gram-negative bacteria might be due to their thin peptidoglycan wall, gram-negative bacteria possess a thin peptidoglycan layer of approximately 10 nm or less, in between the cytoplasmic membrane and the outer membrane (Ortega Morente *et al*., 2013). *Pseudomonas aeruginosa* is stated to have one of the thinnest peptidoglycan layers of about approximately 3 nm ± 0.54 nm (Matias *et al*., 2003). The outer membrane of gram-negative bacteria act as a barrier to antibiotics especially to hydrophilic compounds and thus have reduced sensitivity when compared to gram positive bacteria. However the outer membrane contains porin proteins that permit selective passage of hydrophilic molecules (Zeinab *et al*., 2023). It is due to the thinness and porous nature of the gram-negative peptidoglycan that, once an antibiotic agent gains access via porins, they reach and disrupt their molecular targets. An example of this is penicillin, a beta-lactum antibiotic that diffuses faster through porins and then bind to penicillin-binding proteins (PBP's), weakening cell wall integrity and causing lysis (Maher & Hassan, 2023). If an antibiotic agent can bypass the outer membrane of gram-negative bacteria through mechanisms such as porins the thin peptidoglycan offers less structural resistance and thus making gram-negative bacteria susceptible to cell wall inhibitors and oxidative treatments. In a previous study where cold atmospheric plasma (CAP) was used, it was found that gram-negative bacteria were more sensitive to physical disruption due to the leaky outer membrane and reduced support against electrostatic or oxidative stress (Mai-Prochnow *et al*., 2016; Oliveira Paiva *et al*., 2025).

The MIC value for each fraction was different, as shown in **Figure 14**, Fractions W1-W2, W4-W7, W11, W14 and W21-W32 had a MIC of 2 mg/ml whereas fractions W3, W9, W10, W12, W13, W16 and W19-W20 had a MIC of 1 mg/ml and the remaining W17, W18, W8 and W15 and a MIC of 0.95, 0.75 and 0.45 mg/ml respectively.

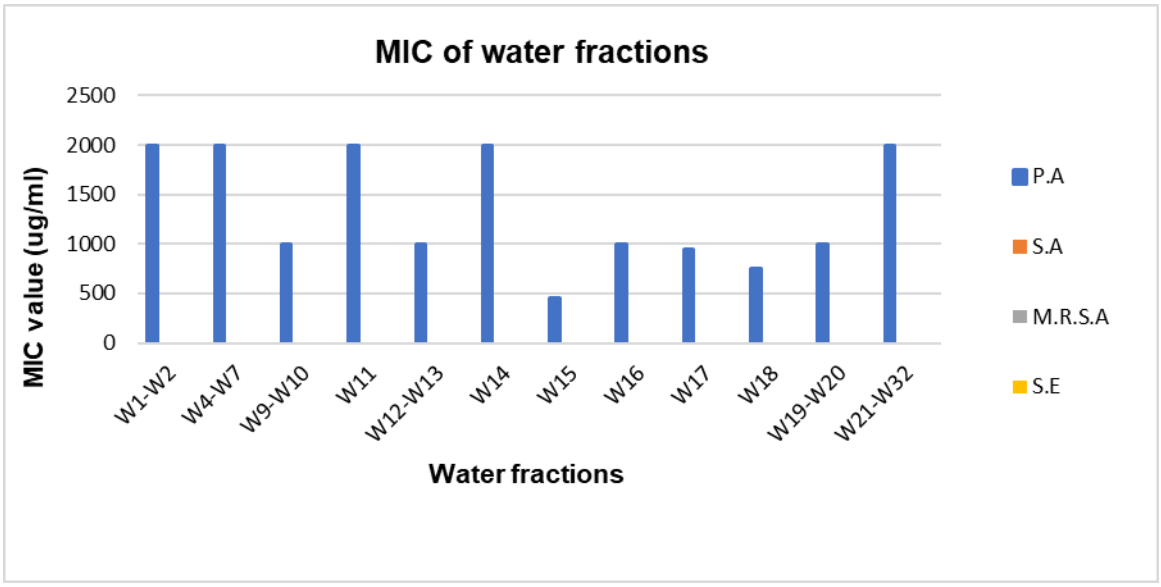

Figure 15: Column graph comparison of the MIC of different water fractions.

Possible reasons for activity against *Pseudomonas aeruginosa* can be the cell wall structure and composition, efflux pumps, biofilm formation and phytochemical specificity. The phytochemicals present in the water extract fractions can potentially cause the cell wall to be more susceptible to the water extract. These phytochemicals are bufadienolides, cardiac glycosides, flavonoids and phenolics, saponins, tannins, triterpene steroids, and alkaloids. Cardiac glycosides-like compounds, flavonoids and phenolics are known for biological activity, this includes cotyledosides, orbicusides and tyledoside C found in parts of *Cotyledon orbiculata* (Xaba *et al*., 2024; Zengin *et al*., 2023)
DCM and ethanol was used as a solvent for fractionation of the water extract. A previous study has listed that the use of these solvents for extraction and consequent antimicrobial investigations resulted in activity against *Pseudomonas aeruginosa.* The polarity of these solvents allow a wide range of extraction of potentially novel compounds (Mombeshora & Mukanganyama, 2019). This microorganism possess the ability to form biofilms however the water extract fractions of *C orbiculata* was able to disrupt and interfere with biofilm formation, thus resulting in antimicrobial activity, a previous study conducted on *T. welwitschii* showed results that indicate that the plant extract was able to cause significant nucleic acid leakage by increasing cell permeability (Subramaniam *et al*., 2023).

Minimum Inhibitory concentration (MIC) values were determined for the methanol fractions of *Cotyledon orbiculata* against various gram positive and gram-negative bacterial species. These

values indicate the lowest concentration required to inhibit visible microbial growth as presented in **Table 4.5.**

Table 4.5. MIC (µg/ml) values of Methanol Fractions

Treatment	*Pseudomonas Aeruginosa*		*Staphylococcus Aureus*		Methicillin resistant *Staphylococcus aureus*		*Staphylococcus Epidermidis*	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
Positive Control	0,625	2.5	0,625	0,3125	0,625	0,625	0,156	0,156
M1	1 000	×	1 000	×	1 000	1 000	500	×
M2	1 000	×	1 000	1 000	1 000	×	500	×
M3-M4	1 000	×	1 000	×	1 000	1 000	500	×
M5	1 000	×	1 000	1 000	1 000	1 000	500	×
M6	1 000	×	1 000	1 000	1 000	1 000	500	1 000
M7	1 000	×	1 000	×	1 000	1 000	500	1 000
M8	1 000	×	1 000	1 000	1 000	1 000	500	1 000
M9	1 000	×	1 000	×	1 000	1 000	500	1 000
M10	1 000	×	1 000	×	1 000	×	500	1 000
M11	1 000	×	1 000	×	1 000	1 000	500	1 000
M12-M32	2 000	×	-	•	-	•	-	•
M33	2 000	×	2 000	×	2 000	×	2 000	×
M34-M42	2 000	×	-	•	-	•	-	•

× = No MBC **• = N/A** **- = No MIC**

The methanol fractions all indicated antimicrobial activity against all tested microorganisms. All the fractions from M1-M11 had an MIC of 1 mg/ml and fractions from M12-M42 with a MIC of 2 mg/ml against *Pseudomonas aeruginosa.* Fractions M1-M11 had a MIC of 1 mg/ml and M33 a MIC of 2 mg/ml against *S. aureus* and *MRSA*. Fractions M1-M11 all had a MIC value of 0.5 mg/ml when tested against *S.epidermidis* and M33 once again a MIC of 2 mg/ml. Fractions M12 to M32 and M34-M42 had no MIC value for microorganisms *S. aureus* and *MRSA*. It is interesting that all the first 11 fractions of the methanol extract showed activity

against all four tested microorganisms but fractions M12-M32 only showed activity against the Gram-negative *Pseudomonas aeruginosa*. These microorganisms have shown to be the most susceptible to methanol fractions M1-M11. This can be attributed to the phytochemical composition of fractions M1-M11, as well as the solvents used for the fractionation of methanol plant extract. The solubility of the phytochemicals present can also affect antimicrobial activity and the solvents that were used for fractionation of methanol plant extract were chloroform and methanol, with methanol stated to be effective at extracting flavonoids, phenolic acids and tannins, chloroform on the other hand can better extract non-polar phytochemicals such as alkaloids and terpenoids. A study conducted on the antioxidant and antimicrobial activities in chloroformic and methanolic extracts 6 medicinal plants, showed antimicrobial activity against *Pseudomonas aeruginosa* (Hadadi *et al*., 2020) (Ashraf *et al*., 2011). The data in **Figure 16** demonstrates that some methanol fractions exhibit MIC activity against specific microorganisms, while other methanol fractions show MIC activity against different microorganisms. This indicates the selective antimicrobial efficacy of the methanol fractions against the different microorganisms.

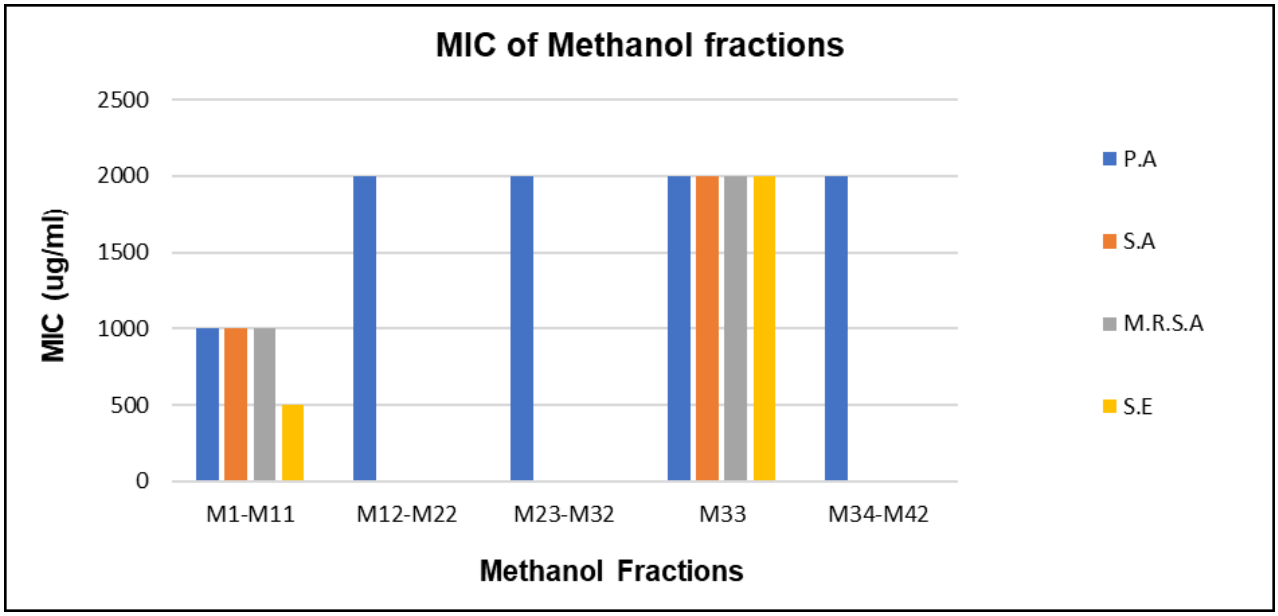

Figure 16: Column graph comparison of the different MIC of methanol fractions against the microorganisms.

4.5. Immunomodulatory Effects of Fractions Isolated from *Cotyledon orbiculata* water and methanol extracts

4.5.1 Differentiation of THP-1 monocytes into Macrophages

There are various cell lines that can be employed in the study of macrophages *in vitro*, most frequently used include monocytic cell lines of a variety of differentiation levels and also primary peripheral blood mononuclear cells (PBMC). The benefit of using monocytic cell lines for immunomodulatory studies is that they can be maintained as a continuous homogenous population whereas the use of PBMC's present with issues concerning availability of samples, inconsistency and purity (Biswas *et al*., 1998; Solati *et al*., 2015). THP-1 monocytes were cultured and differentiated over a 2-day period. **Figure 17** shows the change in cells after cell differentiation has occurred.

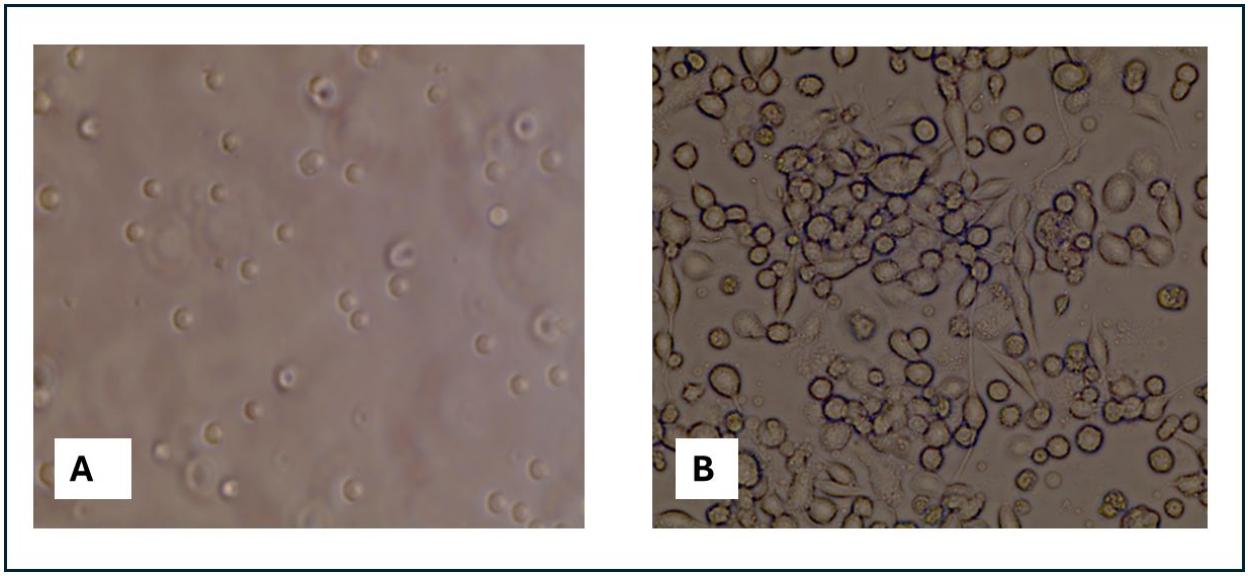

Figure 17: (A) Undifferentiated THP-1 cells, (B)THP-1 cells after differentiation.

To conduct immunomodulatory studies the THP-1 cells were cultured and incubated and grown in cell culture medium. The cells were then treated with PMA for differentiation. Noticeable morphological changes such as increased cell size and increased cytoplasmic volume can be seen in **Figure 17**. The increase in the cell size is due to the expansion of cytoplasm and that results in a reduced nucleus to cytoplasm ratio. PMA is a common agent used for differentiation of THP-1 cells to macrophage cells (Aldo *et al*., 2014). Morphological features vary and are dependent on incubation period and concentration of PMA used. Various methods have been used to induce differentiation of THP-1 cells into macrophages, commonly PMA would be used but at different concentrations. Frequently used concentration of PMA is stated to be 100 nM. Lower concentrations ranging between 10-50 ng/ml result in incomplete

or partial differentiation of THP-1 cells. In this study different concentrations of PMA were evaluated namely 20 nM and 50 nM, the results are not shown. A concentration of 50 nM resulted in the most favorable morphological change without damage to cells and this concentration was selected to conduct further studies.

4.5.2. Cell Viability

Water-Soluble Tetrazolium -1 is a colorimetric method used to assess cell viability, proliferation and cytotoxicity based on mitochondrial metabolic activity. It is a Tetrazolium salt that is cleaved by cellular mitochondrial dehydrogenases in viable, metabolically active cells that produce a water-soluble formazan dye, which shows an orange colour measurable at 440-450nm. The amount of formazan formed is directly proportional to the number of living cells enabling quantification cytotoxic effects of test compounds or fractions (Id *et al*., 2020).

To determine if the water and methanol fractions were toxic to cells, the WST-1 cell viability assay was performed. *Cotyledon orbiculata*, treated cells were treated with various concentrations of both methanol and water fractions. This was done to determine what concentration, or the highest concentration that would be safe for the cells. There are different cell viability assays such as WST-1, MTT and ATP that can be used to determine cytotoxicity effects of mixtures/ compounds. In this study the WST-1 assay was used. The toxicity assay performed indicated that when exposed to higher concentrations of extract the viable cell count was reduced due to cell death. Both extracts indicated that at a concentration of 500 µg/ml , the fraction has a toxic effect on cells resulting in significant cell death. **Figure 18** illustrates shows cell viability of methanol fractions treated THP-1 cells.

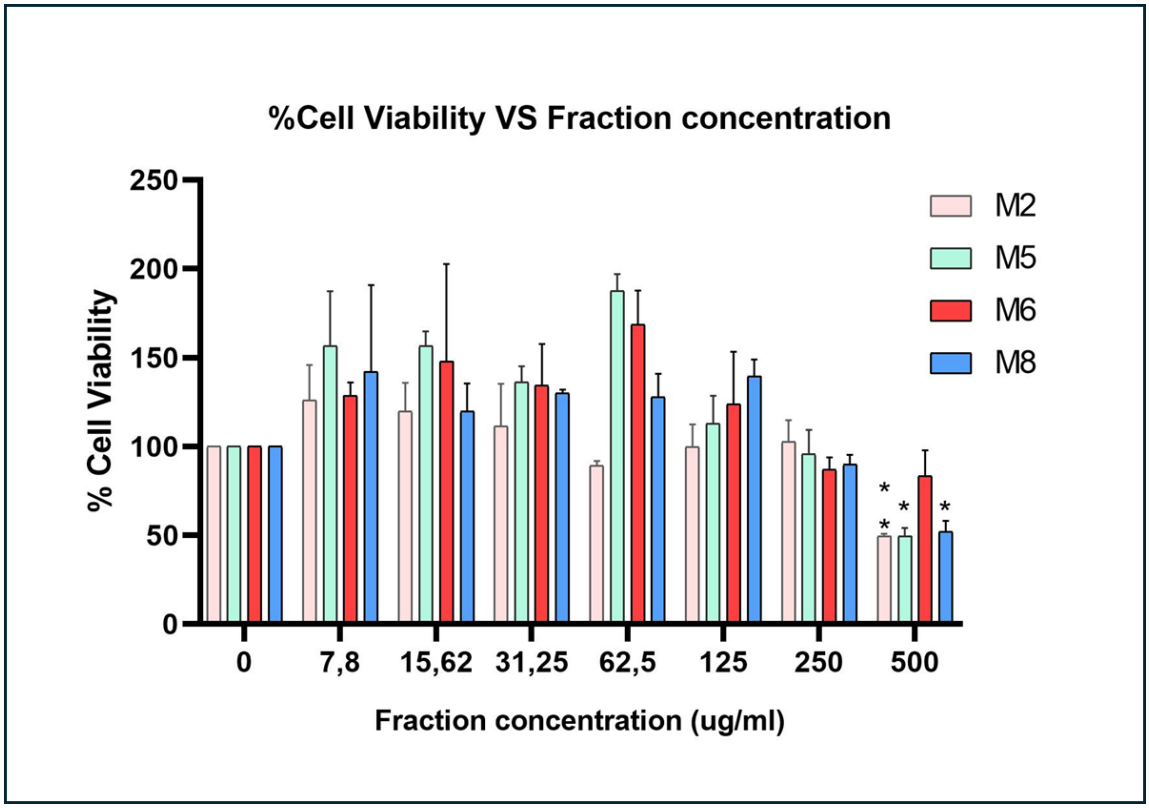

Figure 18: % Cell Viability against the different methanol fraction concentrations (µg/ml).

For the methanol fractions, at a concentration of 7.8 µg/ml, cell viability is high indicating minimal toxic effect and at a concentration of 15.62 µg/ml fractions M5 and M8 demonstrated an increased viability, and this can potentially indicate proliferative effect at low doses and at a concentration of 31.25 µg/ml M6 showed the highest viability. At a fraction concentration of 62.5-125 µg/ml , M2 showed a significant drop in cell viability, M6 and M8, declined below 110 % whilst M5 maintained a high cell viability. At the highest concentration of 250-500 µg/ml all fractions showed a drastic decline, confirming that higher concentrations of these fractions affect the cell viability as it reduces cell survival, most likely due to it's cytotoxicity effects. Lower doses of the methanol fractions may possibly possess the ability to stimulate some cell lines, while a concentration of 500 µg/ml is highly toxic.

Figure 19 illustrates the percentage cell viability of water fractions treated THP-1 cells.

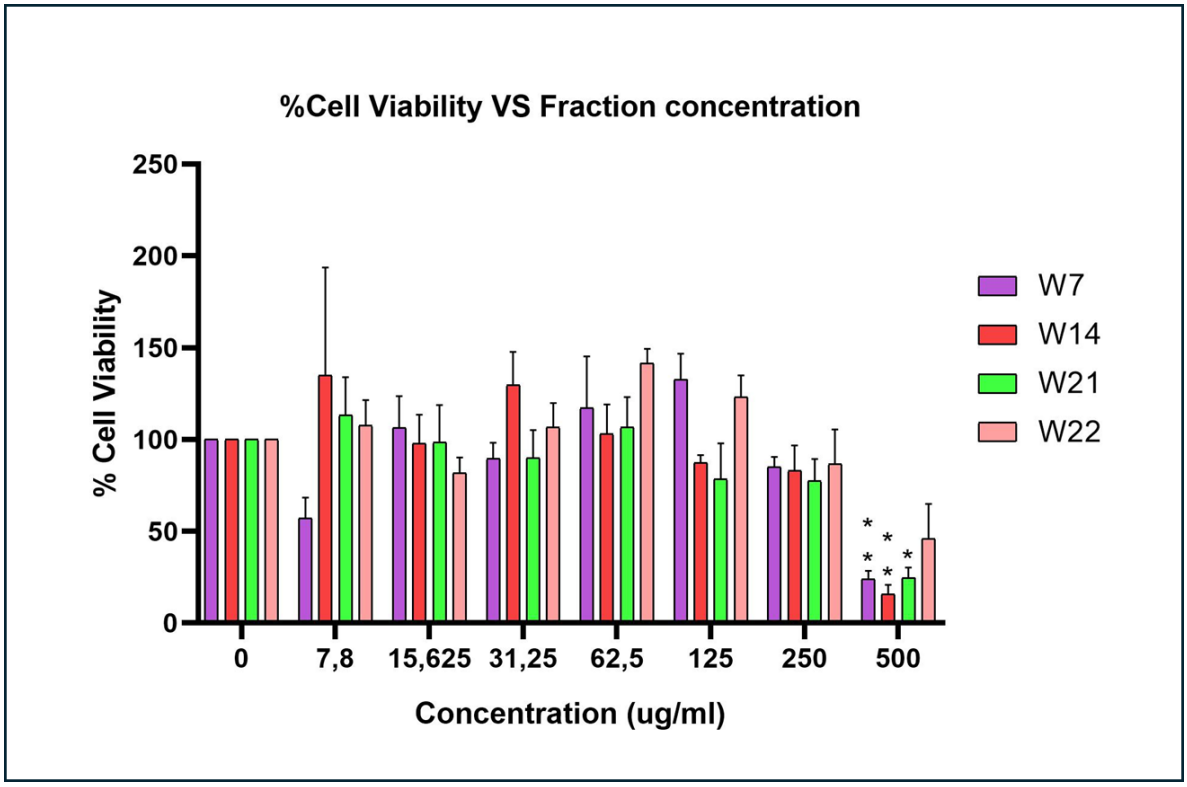

Figure 19: %Cell Viability VS water fraction concentrations µg/ml .

The water extract fractions general observed trend showed that at low to moderate concentrations of 7.8-31.25 ug/ml cell viability increased. At the concentration range of 125-500 ug/ml cell viability was reduced. W7, at a concentration of 7.8 µg/ml, had reduced cell viability when compared to viability of W14, W21 and W22. The fractions remained near untreated levels at a concentration of 15.625 µg/ml. W14 and W22 indicated the highest viability at concentration of 31.25 µg/ml, this indicated a strong proliferation response. For W14 the cell viability percentage indicated a drop at a concentration of 62.5 µg/ml, while W21 and W22 indicated an increase from 31.25 µg/ml to 62.5 µg/ml high viability. These two fractions showed delayed toxicity (W21 and W22). Statistics indicated that the most significant decrease in cell viability when compared to untreated cells, was between water fractions W7, W14 and W21 at a fraction concentration of 500 µg/ml and P values of 0.0094, 0.0098 and 0.0154 respectively.

4.5.3. Cytokine Determination

To determine the effects of the fractions on cytokine IL-6, TNF-alpha and IL-10 production in THP-1 cells for this study, the ELISA assay was used. After the THP-1 cells were differentiated with PMA, the cells were stimulated using LPS and then the cells were treated with the methanol and water fractions. Adding LPS to the cells increased the levels of cytokine

production as shown in **Figures 20**, **21** and **22** in the UN(LPS) bar. However, addition of the fractions to the cells decreased the levels of the IL 6 and TNF-alpha cytokines. Contrary to this finding, addition of the fractions to macrophages resulted in an increase in the production of the cytokine IL10.

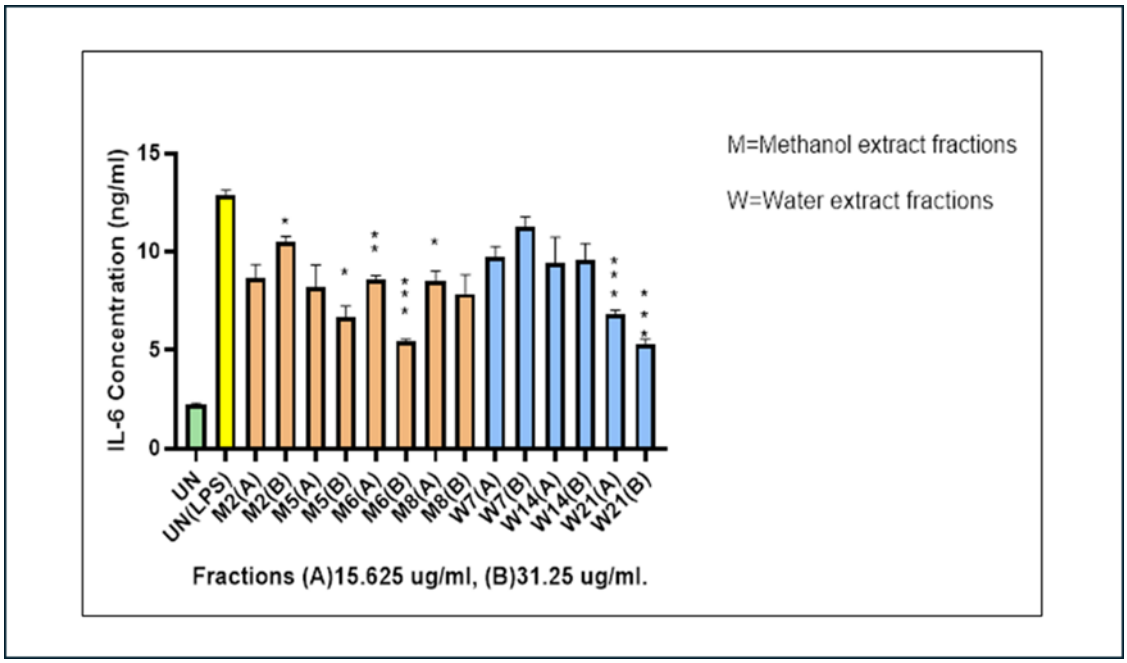

Figure 20: IL-6 secretion in THP-1 cells treated with Methanol and Water fractions.

The THP-1 cell line was used to determine the immunomodulatory effects of methanol and water fractions isolated from *Cotyledon orbiculata.* Directly after the differentiation of THP-1 cells the cells were treated with LPS and incubated prior to treatment with water and methanol fractions. LPS treatment is used to induce the production of cytokines such as TNF-alpha, IL-6 and IL-10. The results for cytokine IL-6 secretion showed varying results for methanol fractions. The general conclusion was that all the fractions possess anti-inflammatory properties as they all inhibited the production of IL-6. The results show that for the methanol fractions at a higher fraction concentration the greater the IL-6 suppression, increased inhibitory effect at higher fraction concentration except for fraction M2. M6B showed the most significant inhibitory effect on IL-6 secretion of the methanol fractions. The water extract fractions showed a better inhibitory effect at lower fraction concentrations, except for fraction W21, which showed a reduction in IL-6 secretion at a concentration of 31.25 µg/ml .

The observed inhibitory effects of both methanol and water fractions on IL-6 secretion suggests that the extracts contain bioactive phytochemicals capable of modulating key intracellular inflammatory pathways. A primary mechanism possibly involves suppression of NF-κB activation, as many plant-derived flavonoids and phenolic acids inhibit IκB kinase

activity and prevent NF-κB nuclear translocation, thereby reducing IL-6 transcription (Makwela *et al.*, 2023). Another plausible pathway is inhibition of MAPK signaling, since compounds such as flavonoids and terpenoids commonly block phosphorylation of ERK, JNK, and p38, which subsequently decreases AP-1-mediated IL-6 gene expression. Modulation of the NLRP3 inflammasome may also contribute, as polyphenols are known to suppress ROS-mediated inflammasome priming and downstream cytokine amplification.

Some fractions may interfere with JAK/STAT3 signaling, a pathway frequently inhibited by flavones like luteolin, resulting in reduced IL-6 mRNA stability and diminished autocrine inflammatory feedback. Antioxidant activity is another likely contributor, as polyphenolic constituents can reduce intracellular ROS levels and thereby limit activation of redox-sensitive transcription factors responsible for IL-6 synthesis.

If IL-6 secretion was LPS-induced, inhibition of TLR4-MyD88 signaling by flavonoids or saponins may also explain the cytokine suppression observed in both extract types. These combined mechanisms may account for the strong inhibitory activity of methanol fraction M6B at higher concentrations and the pronounced low-dose activity seen in water fractions such as W21, both suggesting the presence of potent NF-κB or TLR4 inhibitors. Collectively, the literature supports that phytochemicals commonly extracted in methanol and water are capable of reducing IL-6 production through simultaneous modulation of different pathways.

IL-6 is a multifunctional cytokine with a central role in immune regulation, inflammation, and hematopoiesis. The IL-6 patterns revealed in this study showed a variation in immune response. Fraction 2 (M2) showed an increase in IL-6 secretion at a higher concentration then at lower fraction concentration. This suggests a dose-dependent immunostimulatory effect, leading to the possibility that components within M2 may enhance pro-inflammatory responses or activate immune cells at higher doses. Other fractions such as M5, M6 and M8, exhibited a decrease in IL-6 secretion at a higher concentration relative to a lower dose, this trend indicates a dose dependent immunosuppressive or anti-inflammatory effect. M6 and M8 demonstrated a similar response with lower IL-6 secretion levels at high fraction concentrations, this reinforces the potential of these fractions to suppress or inhibit the pro-inflammatory cytokine production. IL-6 secretion of the water fractions also yielded fraction specific results. Both fractions W7 and W14 showed increased IL-6 secretion with increased fraction concentration, better inhibitory effect at a lower fraction concentration, mirroring an immunostimulatory pattern similar to methanol fraction M2. Fraction W21 on the other hand exhibited lower IL-6 levels at higher fraction concentration indicative of an anti-inflammatory or immunosuppressive response similar to M5, M6 and M8.

The variation in secretion of IL-6 levels, as seen in **Figure 20**, can be due to many factors such as differences in phytochemical composition of individual fractions, the solubility and extraction method used, dose dependent and synergistic effects. Different extraction solvents yield fractions with distinct phytochemical profiles, an example of this is a study of black rice leaf extracts, where the ethyl acetate fraction showed greater inhibition of IL-6 secretion than other tested fractions, suggesting that the specific phenolic compounds present in the ethyl-acetate fraction is responsible for its anti-inflammatory effects (Thepthanee *et al*., 2021).

Hexane and ethyl acetate fractions of *Hibiscus noldeae,* inhibited IL-6 production in LPS induced RAW 264.7 cells, compared to other fractions. Compounds and fractions indicated varied levels of inhibition which shows that phytochemicals within the fractions modulate IL-6 secretion differently (Tomani *et al*., 2020). The higher IL-6 levels can be an indication of pro-inflammatory activity as the treatment may stimulate the inflammatory pathway.
The untreated control group showed near-zero levels of IL-6 secretion, confirming the absence of spontaneous inflammatory activity under basal conditions. This serves as a appropriate negative control, reinforcing the validity of the observed cytokine responses in the LPS-treated and plant extract fraction treated groups. The substantial IL-6 upregulation in the LPS group validated the assay's sensitivity to immune activation. In contrast the varied responses in plant extract fractions- ranging from marked IL-6 induction to strong suppression- emphasizes their fraction-specific immunomodulatory potential. These outcomes were clearly distinguishable from the resting state of the untreated cell, demonstrating that the observed effects were not due to baseline variability or culture artifacts.

Interleukin-10 is an anti-inflammatory cytokine that plays a central role in the regulation of the immune system, particularly in controlling inflammation. Various stimuli, including microbial infections and inflammatory signals causes a reaction that instructs T helper cells, regulatory T cells, B cells, monocytes and macrophages to produce IL-10. IL-10 is of interest in studies examining immunomodulation, especially natural plant extracts, as plant derived compounds that promote IL-10 secretion may have therapeutic potential for treating inflammatory disorders or modulating immune responses to various stimuli. The secretion of IL-10 can act as an indicator of immune regulation, suggesting that a particular fraction of a plant extract may suppress excessive inflammation or promote immune tolerance.

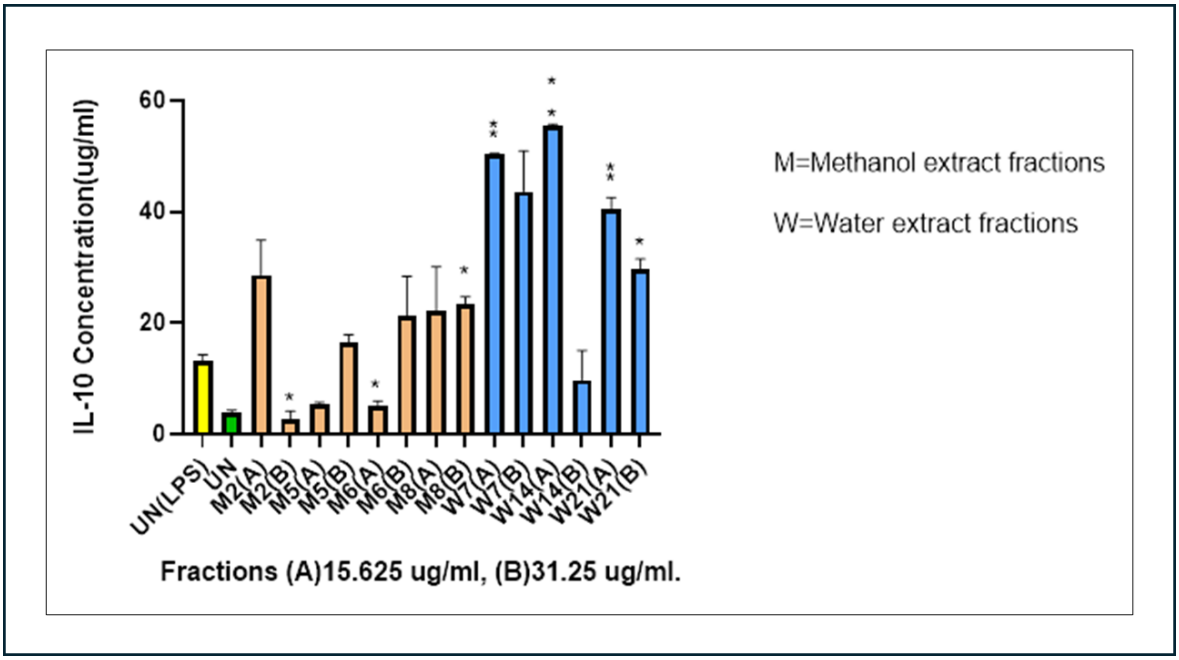

Figure 21: IL-10 secretion of THP-1 cells after being treated with water and methanol fractions.

Figure 21 illustrates how the water fractions possess the ability to induce high IL-10 levels when compared to the methanol fractions. Previous studies have shown that polyphenols, flavonoids, and alkaloids in plant extracts can induce IL-10 secretion. At higher fraction concentrations it was observed that W14 and W21 had significant IL-10 levels. This indicates that these fractions may contain bioactive compounds that are able to strongly induce IL-10 production. Results obtained for IL-10 showed that several plant extract fractions demonstrated a capacity to upregulate IL-10 secretion suggesting immune-regulatory effects that could help balance pro-inflammatory responses. M2 showed a high secretion at low fraction concentration. Fractions M5 and M6 showed higher secretion at a higher fraction concentration, suggesting that these fractions may have the potential to act as immune modulators and aid in promoting a balanced inflammatory response. The fractions that are possibly indicative of pro inflammatory properties because they are decreasing the levels of IL-10 are M2B, M5A and M6A, however this does not correspond to the previous results and further investigation might be necessary.

The water extract fractions W7, W14 and W21 also induced high secretion at a higher fraction concentration further reinforcing the possibility that these fractions could be effective for the promotion of immune tolerance. Fraction W14B showed reduction on IL-10 secretion, these findings suggest that different fractions contain varying phytochemicals that can modulate IL-10 secretion, a key anti-inflammatory cytokine involved in immune responses. The observed differences between fractions may be attributed to the differential solubility and polarity of bioactive compounds during fractionation and further investigation are necessary.

Figure 22 is the graph indicating the effect of fractions on TNF-alpha secretion. Methanol fractions had the most significant effect on secretion of cytokines as fractions M2, M5 and M6 had inhibitory effects on TNF-alpha secretion. The strong inhibitory effect as seen in **Figure 22**, by M6, W7 and W14 is consistent with research indicating that certain plant extracts can suppress pro-inflammatory cytokines. As with *Pseuderanthemum palatiferum* leaves where a dose dependent supression of TNF-alpha was observed. W14 and W21 indicated varying and inconsistent results which suggests variability in the anti-inflammatory potential and this can be due to factors such as extraction methods and plant part used.

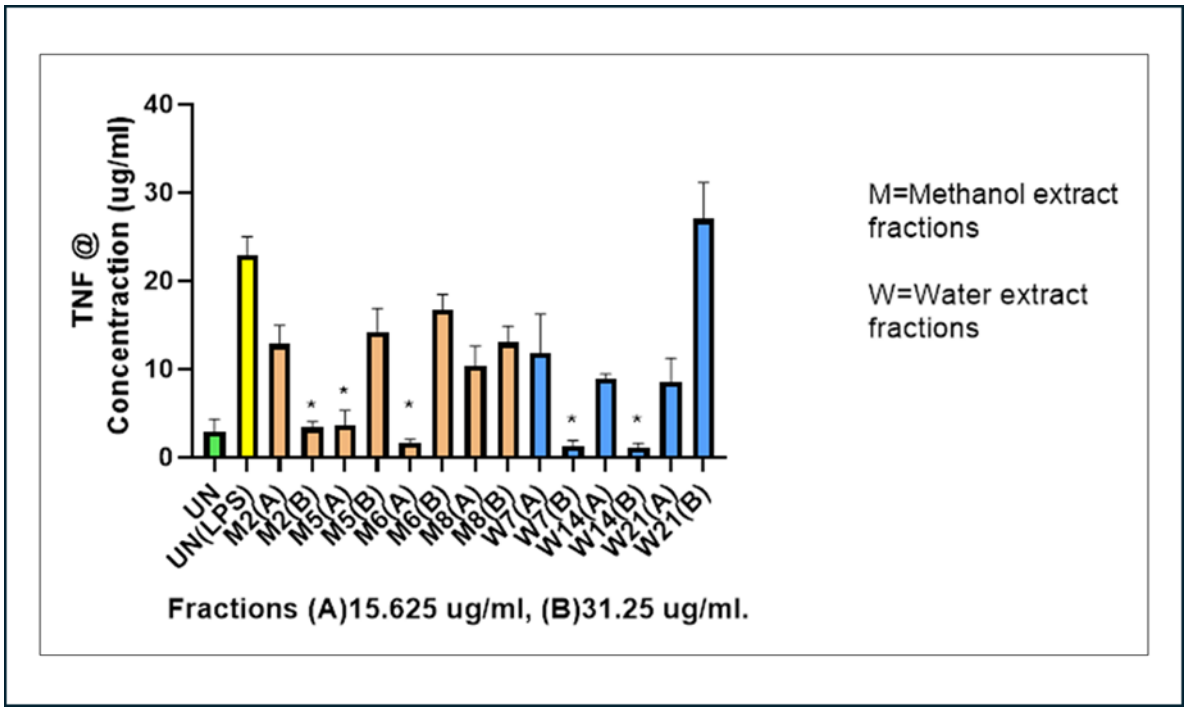

Figure 22: TNF@ secretion of THP-1 cells when treated with water and methanol fractions

TNF-alpha is a pro-inflammatory cytokine involved in the immune response to infection, trauma and other inflammatory conditions. As seen in **Figure 22** the results revealed that several fractions from both the methanol and water extracts significantly increased TNF-alpha secretion, this indicates immune activation.

M2 showed a higher TNF-alpha secretion at low fraction concentration. M5, M6 and M8 demonstrated higher TNF-alpha secretion at higher plant extract concentrations, this indicated higher inhibitory effect, resulting in higher anti-inflammatory properties. When compared to the LPS treated cells all fractions for water and methanol showed anti-inflammatory properties. The water fraction W21 showed results indicating possible pro-inflammatory properties.

CHAPTER FIVE
CONCLUSION

5.1. General Conclusions

The main purpose of this study was to determine if the fractions isolated from the methanol and water extract of *Cotyledon orbiculata* plant have antimicrobial and immunomodulatory properties. This plant has shown that it has antimicrobial and immunomodulatory properties (Xaba *et al*., 2024; Mabona & Van Vuuren, 2013). The methanol and water extract of this plant were prepared and fractionated by column chromatography, yielding 32 water fractions and 42 methanol fractions. These fractions were then individually tested against the most frequent skin infection causing microorganisms. Results obtained from antimicrobial tests showed that both extract fractions had antimicrobial activity, with W8 and W18 from the water extract having MIC values of 0.5 and 0.75 mg/ml respectively against *Pseudomonas aeruginosa*.
Most of the methanol fractions indicated antimicrobial activity against all four tested micro-organisms, with the lowest MIC of 0.5mg/ml against *S. epidermidis*. Based on the results obtained from the water extract fractions it can be concluded that this extract can possibly contain compounds that are especially effective against *Pseudomonas aeruginosa*, leading to the assumption that this extract contains selective bioactive compounds.

The cell viability studies of both water and methanol fractions showed a clear reduction in cell viability at increased fraction concentrations. The methanol fractions had a significant drop in cell viability, at 250 ug/ml cell viability was over 80% for all fractions but at 500 µg/ml the cell viability dropped to below 50%. Water fractions only showed a minimal reduction as fractions still maintained 100% viability. Concentrations of 15.625 µg/ml and 31.25 µg/ml were selected for both water and methanol fractions to continue immunomodulatory studies. An objective of this study was to also determine if the fractions isolated from the water and methanol extracts influence cytokine response on the human macrophage THP-1 cell line. The cytokine production (TNF-alpha, Il-6, and IL-10) in THP-1 cells was determined using the ELISA assay. The results from the ELISA assay showed that IL-6 production was inhibited in cells treated with all fractions. The level of inhibition, however, varies among tested fractions, at different concentrations. The methanol fractions had increased inhibitory effects at higher fraction concentrations compared to lower concentration, except for one methanol fraction (M2) whereas the water fractions showed results where the lower fraction concentrations had better inhibitory activity than higher fraction concentrations, except for W21. This leads to the conclusion that the listed fractions do have anti-inflammatory and immunosuppressive activity.

The water fraction W21 also had significant inhibitory effect whereas W7, W14 showed some inhibitory effect, but not as significant. It can be concluded that some fractions, may in fact, contain phytochemicals that induce pro-inflammatory response.

Several of the fractions indicated significant inhibitory effects on TNF-alpha secretion, this suggests strong anti-inflammatory activity. These findings are consistent with existing research on anti-inflammatory potential of this particular medicinal plant. M5, M6 and W7 are strong TNF-alpha inhibitors, which suggests anti-inflammatory effects, M2 and M8, show moderate inhibition and W14 and W21 warrant further investigation. The tested plant extract fractions (water and methanol) in this study show anti-inflammatory and immunomodulatory potential. This suggests that the fractions possibly possess a stimulatory effect on the cells leading to increased cytokine production. It can also be deduced from the results that these particular fractions are capable of eliciting a pro-inflammatory response in cells, this is useful in situations where an immune response is desired (Khumalo *et al*., 2024; Burns *et al*., 2010).

5.2 Significance of Study

This study aimed to evaluate the immunomodulatory and antimicrobial effects of *Cotyledon orbiculata* plant, methanol and water extract fractions, specifically examining their potential to modulate the immune response and combat common skin pathogens. This has significant implications for skin infection treatments, particularly in light of the increasing resistance to conventional antibiotics and the need for novel therapeutic agents

The results obtained in this study have shown that it is possible to fractionate the water and methanol extracts of *Cotyledon orbiculata.* The antimicrobial activity of all obtained fractions tested was also confirmed at various fraction concentration levels. The investigation into the antimicrobial properties of all fractions yielded results that indicated that all methanol fractions had antimicrobial activity against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *MRSA* and *Staphylococcus epidermidis.* This suggests that the methanol fractions may contain bioactive compounds that can serve as alternative or adjuvants to current antibiotics to combat the limitations of drug-resistant pathogens. Water fractions tested demonstrated antimicrobial activity against *Pseudomonas aeruginosa* only, this selective antimicrobial activity implies the presence of a specific compound in the water extract fractions that are selectively active against certain pathogens. This is significant due to the potency of a microorganism such as *Pseudomonas aeruginosa* that has been proved to be a challenging pathogen in skin infections, burn wounds and chronic conditions such as eczema and ulcerations.

The immunomodulatory studies conducted on both water and methanol fractions showed results that demonstrated that the *Cotyledon orbiculata* plant extract fractions are capable of modulating cytokine production, thus highlighting their potential in regulating the immune response. This is very significant in the context of skin infections, which often involves an imbalance between immune activation and regulation. The ability of the plant extract fractions to stimulate pro-inflammatory cytokines such as TNF-alpha and IL-6 whilst promoting IL-10 leads to the implication that these fractions could be useful in managing inflammatory skin conditions. Tested fractions were able to induce IL-10 at low concentrations and this can aid in chronic inflammatory skin conditions where immune suppression is needed to avoid tissue damage and promote wound healing.

In skin infections, the immune response plays a critical role in both pathogen clearance and tissue damage. The immunomodulatory activity observed in this study could complement the antimicrobial effects, helping to enhance the body's defense mechanism while preventing excessive inflammation or immune dysregulation that can cause further tissue damage or chronic inflammation. This is particularly important for the successful treatment of skin wounds, infected ulcers and chronic skin conditions.

5.3. Study Limitations

This study provides valuable information regarding the immunomodulatory and antimicrobial properties of the *Cotyledon orbiculata* plant methanol and water extract fractions, however the following can be described as limitations.

- Lack of detailed phytochemical characterization - the full phytochemical testing of each of the extracts would aid in the determination of a suitable solvent system for the fractionation process. This influences the efficacy of obtaining pure compounds during the chromatography process.
- Absence of chemical profiling: No LC–MS, NMR was performed before or after fractionation. As a result, the specific phytochemical constituents responsible for the observed antimicrobial and immunomodulatory effects remain unidentified.
- Limited phytochemical information: Only the phenol–sulphuric acid assay was conducted, providing basic carbohydrate detection without characterizing key secondary metabolites such as flavonoids, terpenoids, tannins, or saponins.

- Low yield - after the fractionation process, both methanol and water extracts yielded eluent of varying colour and TLC profiles, a few of these fractions resulted in below 0.05mg final weight and could therefore not be used for antimicrobial testing.
- Limited mechanistic understanding: While cytokine inhibition and antimicrobial effects were demonstrated, the underlying cellular pathways, molecular targets, and mechanisms of action were not explored. This limits interpretation of how the fractions exert their biological effects.

5.4. Recommendations for Future Studies

- More detailed phytochemical analysis should be conducted on the water extract.
- Comprehensive chemical profiling: Perform LC–MS, LC–MS/MS, HPLC, or NMR to identify and quantify bioactive constituents in each fraction. .Nuclear Magnetic Resonance (NMR) studies should be done on all fractions isolated from both water and methanol extract. This will aid in identification of all unknown compounds present.
- Biofilm formation – Many of the pathogens involved in chronic skin infections, like *Staphylococcus aureus* and *Candida albicans*, form biofilms that make them resistant to both immune response and antibiotics. Testing the ability of *Cotyledon orbiculata* plant extract fractions for inhibition of biofilm formation could be quite relevant.
- Expand antimicrobial testing: Include a broader range of pathogens, particularly antibiotic-resistant clinical isolates relevant to skin and wound infections for example *Streptococcus pyogenes*).
- Mechanistic studies: Evaluate pathways such as NF-κB, MAPK, oxidative stress markers, or membrane integrity assays for clarification on how the fractions exert their antimicrobial and immunomodulatory actions.

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