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Could traditional African herbal remedies be a source of novel antimicrobial compounds?
An analysis of the antimicrobial and antibiofilm properties of Uvaria chamae and Prosopis africana

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COULD TRADITIONAL AFRICAN HERBAL REMEDIES BE A SOURCE OF NOVEL ANTIMICROBIAL COMPOUNDS?

An analysis of the antimicrobial and antibiofilm properties of *Uvaria chamae* and *Prosopis africana*

By

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ABSTRACT

With the steady increase in global antimicrobial resistance rates, concomitant with a recent paucity of novel antibiotic discovery, it is essential that new antimicrobial compounds be discovered to prevent a return to the pre-antibiotic era over coming decades. Knowledge of the medicinal properties of traditional plants has given rise to many of the modern day medicines. Consequently, the aim of this work was to assess the antimicrobial and antibiofilm activities of a number of traditional African herbal remedies against a range of clinically important bacterial species.

After a pilot study was performed, *Prosopis africana* and *Uvaria chamae* were selected from a range of medicinal plants due to their greater antimicrobial activity against *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Escherichia coli* than the other remedies tested. Crude extracts from *P. africana* (PAM, PAS and PAU) and *U. chamae* (UCM, UCS and UCU) were prepared using maceration, Soxhlet and ultrasound extraction method respectively. Cultures of both aerobic and anaerobic bacterial species were exposed to the crude extracts in agar-well diffusion and broth micro-dilution assays. In addition, semi-quantitative and quantitative antibiofilm assays were performed using PAS, PAU, UCS and UCU extracts against preformed biofilms of *P. aeruginosa*, *S. aureus*, *S. epidermidis*, *Propionibacterium acnes* and *Clostridium difficile* strains. Fractionation of the crude extracts was then performed using column chromatography; with antimicrobial, simple phytochemical and spectrophotometric analyses being performed on the resultant fractions.

It was found that all extracts had both bacteriostatic and bactericidal activity against all of the bacterial species tested, although the Soxhlet and ultrasound extracts demonstrated a significantly greater activity than the maceration extracts. The extracts

demonstrated antibiofilm activity against preformed biofilm of *P. aeruginosa*, *S. aureus*, *S. epidermidis*, *P. acnes* and *C. difficile* strains by reducing the density of biofilm biomass significantly by at least 11.5 % compared to the control. In the antimicrobial activity test, the fractions that were the most effective against *P. aeruginosa* and *S. aureus* with MIC and MBC value ranges of 0.195 % - 2.60 % and 0.391 % - 6.25 % respectively, contained the following phytochemical groups; saponins, quinones, flavonoids, and alkaloids which have been previously shown to be antimicrobial by other workers. Absorption spectrum analysis further suggests that the fractions of *U. chamae* and *P. africana* with the greatest antimicrobial activity might contain flavonoid-related compounds as their major active components.

This study demonstrated that *P. africana* and *U. chamae* extracts are effective antimicrobial and antibiofilm agents and therefore the isolation and evaluation of their active ingredients could yield potentially new leads to fight antimicrobial resisance.

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CHAPTER ONE

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1.0 INTRODUCTION

Antimicrobial resistance (AMR) has become a major global issue, which threatens the health and well-being of all the people in the world, and therefore requires a global plan of action towards curtailing the severity it poses. Global leading health institutions such as the World Health Organisation (WHO) and Centers for Disease Control (CDC), and also the European Union have made it a priority to study the issue of antimicrobial resistance and to produce a plan of action to control this problem (WHO 2014). Globally, countries have developed action plans to address this problem: the UK government has set up a five year action plan to tackle antimicrobial resistance (Hopkins 2016); the US government is intensifying efforts to combat it (Centers for Disease Control and Prevention 2014); and scientists around the world are focusing their research towards finding new therapies for it (Olamide and Agu 2013). Interestingly, an independent review on antimicrobial resistance was commissioned in the UK in 2014, chaired by Lord Jim O'Neill. O'Neill gave his final report and recommendations in 2016, detailing ten interventions for tackling drug-resistant infections globally.

Antimicrobial resistance poses a hazard to the populace, making it hard for infections to be treated and also increasing morbidity and mortality, the risk of adverse effects (when a less safe drug alternative is used), and the duration of any hospital admission. Antimicrobial resistance also increases the cost expenditure of treatment and management of ailments (Zhang et al. 2013; Moorthy et al. 2015). The emergence of drug resistant organisms, which can both spread through the population and transfer their resistance to other non-resistant species, can occur when drug sensitive microorganisms

are suppressed or destroyed by antibiotic therapy (Cosgrove 2006). The development of resistance towards antibacterial drugs by bacteria has caused the treatment of infections to become increasingly complicated (Tenover 2006). Therefore, it is important that action be taken to reduce the emergence of drug resistance in microorganisms against existing antibiotics and find novel antibiotics by exploring other options from natural materials (Cox and Wright 2013).

1.2.0 ANTIMICROBIAL RESISTANCE

Antimicrobial resistance (AMR) is the ability of a microorganism to resist antimicrobial therapy or drug that was originally effective for the treatment of infections caused by it (Tenover 2006). Resistance of an organism to an antibiotic may occur due to several factors or characteristics, so, resistance can be intrinsic or acquired. Over the years, the emergence and severity of antimicrobial resistance has increased drastically, increasing morbidity and mortality in the populace. Two years after the mass production of penicillin in 1945, it was discovered that about 20% of *Staphylococcus aureus* isolates from patients showed resistance against penicillin. Subsequently, other antibiotics such as methicillin and tetracycline were ineffective against *S. aureus* one to four years after they were introduced to the public (Carranza et al. 2015). Past experience indicates that for every newly developed antibiotic, bacterial strains rapidly develop resistance mechanisms against it. Therefore, there is an urgent need for alternative ways to treat bacterial infections and to combat the problem of antimicrobial resistance (Carranza et al. 2015). One of the alternatives that can be utilised for combating antimicrobial resistance is traditional herbal remedies from plants (Matu and Van Staden 2003; Carranza et al. 2015).

1.2.1 Intrinsic resistance

Intrinsic resistance occurs when the organism is not normally susceptible to an antibiotic. This can be attributed to the antibiotic being unable to penetrate the organism's cell and reach the appropriate target site; absence of affinity between the antimicrobial agent and the site of action; or complete lack of action site on the microorganism's cell (Cox and Wright 2013). These processes are said to be inherent. Tenover (2006) highlighted some possible effects caused by the inability of the antimicrobial agent to penetrate and reach the target sites, some of which are: inability of the antimicrobial agent to interfere with both nucleic acid synthesis (fluoroquinolones and rifampin) and cell-wall synthesis (as in the case of β-lactams and glycopeptide agents); inability of the anticrobial agent to inhibit both protein synthesis (macrolides and tetracyclines) and metabolic pathways (trimethoprim-sulfamethoxazole); and the inability of the antimicrobial agent to disrupt the organisms' membrane structure (polymyxins and daptomycin).

Pseudomonas aeruginosa and *Escherichia coli* have been reported to demonstrate intrinsic resistance towards β-lactam antibiotics (Cox and Wright 2013). Being Gram-negative bacteria, their outer membrane is composed of lipid molecules, which is covalently bonded with polysaccharide units. The lipopolysaccharide (LPS) of the Gram-negatives' outer membrane is tightly packed with hydrocarbon chains due to the saturation of the fatty acid chains. This provides a low resistance to many of the bacterial agents by slowing down the permeability of molecules (Cox and Wright 2013). The outer membrane of the Gram-negatives alone cannot provide a substantial level of drug resistance. Other mechanisms of resistance are involved. Furthermore, the presence of β-lactamase in *P. aeruginosa* plays a major role in the resistance to many antibacterial

agents. The presence of this enzyme inactivates β-lactam at an optimal rate as the molecule pass into the periplasm (Nakae et al. 1999).

Efflux pumps are present in the chromosomes of all Gram-positives and Gram-negatives (Cox and Wright 2013). Efflux pumps export toxic molecules out of the bacterial cell. They can either be substrate-specific, exporting one molecule or they can have a broad-spectrum, exporting structurally distinct classes of molecules (Piddock 2006). Certain classes of antibiotics cannot be used to treat ailments caused by Gram-negative bacteria because of the intrinsic resistance caused by the efflux pump. Li et al. (1994) discovered that the ability of *P. aeruginosa* to demonstrate intrinsic resistance towards different classes of antibiotics like tetracycline, chloramphenicol and norfloxacin is because of the presence of efflux pumps encoded in its chromosomes. Furthermore, deleting the components of the presumed efflux system (MexAB-OprM) in *P. aeruginosa*, made the bacteria susceptible to a wide range of bacterial agents (Poole 2004).

1.2.2 Acquired resistance

Antimicrobial-sensitive bacteria (non-resistant species), can acquire resistance genes from other microorganisms (Vanhoek et al. 2011). The process of acquiring new genes by antimicrobial-susceptible microorganisms from resistant ones can occur through mutation and horizontal gene transfer. Mutation is an alteration in the DNA sequence of a gene that can lead to changes in the trait it codes for. In bacteria, alteration in the genomic sequence can lead to the emergence of a resistance trait, which is vertically passed-on to daughter cells, subsequently creating a resistant bacterial population (Gillespie 2001). Horizontal gene transfer can occur through conjugation, transformation, or transduction (Vanhoek et al. 2011). Acquisition of resistance genes through

conjugation occurs when resistance carrying DNA is transferred through sex pilus, requiring cell-to-cell contact. Resistance genes acquired through transformation occurs when naturally transformable bacteria take up short fragments of naked resistance carrying DNA. Through transduction, resistance genes are acquired when the resistance carrying DNA is transferred from one bacterium to another through bacteriophages (Vanhoek et al. 2011).

The acquisition of resistance genes enables the bacterial cell to produce enzymes that aid in: the destruction of the antibacterial agent in the microorganism's cell; reduction in the uptake of the antimicrobial agent or an increase in the rate of excretion of the antimicrobial (via the expression of efflux systems) which in turn lowers the intracellular concentration of the antimicrobial agent; the modification of the target site for the antimicrobial agent that renders it unable to bind to the target site; and the formation of a substitute metabolic pathway which causes the target site to be bypassed (McDermott et al. 2003; Wright 2005).

Methicillin resistant *Staphylococcus aureus* (MRSA) is one of the common Gram-positive bacterial strains that has been found to cause outbreaks of infections in hospitals due to antimicrobial resistance (Rice 2006). The occurrence of MRSA was attributed to horizontal gene transfer through the acquisition of *mecA* gene, which encodes penicillin-binding protein (PBP)-2 that is resistant to β-lactam antibiotics (Enright 2003). The resistance gene *mecA* can be found on a genetic element called Staphylococcal chromosomal cassette (SCC) located at ORF-X on the *S. aureus* chromosome (Hiramatsu et al. 2001). Over the years, different strains of MRSA have been isolated (Enright 2003). Rice (2006) reported on other strains of MRSA, which emerged due to clonal transfer and mutation. These strains are HA-MRSA (hospital acquired MRSA) and CA-MRSA (Community acquired MRSA). HA-MRSA are generally multidrug resistant and known

to emerge due to clonal spread of MRSA from one patient to the other usually through medical personnel as potential intermediaries. CA-MRSA are mostly acquired in the community and they emerged as a result of mutation of the normal MRSA. Soft tissue infections are normally associated with CA-MRSA. Although the processes of methicillin resistance are similar in both HA-MRSA and CA-MRSA, CA-MRSA seem to possess a wider antibiotic susceptibility than HA-MRSA mostly due to difference in susceptibilities to non β-lactam antibiotics (Rice 2006).

The resistance of Gram-negative bacteria towards antimicrobial agents is increasing rapidly, leading to serious life-threatening infections (Paterson 2006). Paterson (2006) reported on the antimicrobial resistance of Gram-negative species such as *Klebsiella pneumoniae*, *Escherichia coli*, and *Salmonella.* Strains of *K. pneumoniae* resistant to cephalosporins are known to have emerged due to the acquisition of plasmids containing genes, which usually encode for extended-spectrum β-lactamases (ESBLs); these plasmids are often known to carry other resistance genes too (Paterson 2006; Frieri et al. 2017). This has also typically been found to occur in other Gram-negative species such as *E. coli* and *Salmonella*, resistant strains of which generally exhibit multidrug resistance and are often found to cause invasive infections in children (Frieri et al. 2017). Other factors that are responsible for the emergence of resistant strains in Enterobacteriaceae are: over-production of AmpC β-lactamases, and chromosomal mutations, which lead to alteration of target enzymes. ESBL and AmpC-producing Enterobacteriaceae are known to exhibit resistance against both third- and fourth-generation cephalosporins, whilst Enterobacteriaceae strains that have undergone chromosomal mutations are known to be resistant towards quinolones. On the other hand, a wide majority of Enterobacteriaceae are known to be resistant to carbapenem antibiotics

(Paterson 2006). Resistance in other Gram-negative bacteria like *Pseudomonas aeruginosa, Neisseria meningitidis* has also been reported (Tenover 2006).

1.2.3 Biofilm: a major contributor to antimicrobial resistance

Apart from the molecular and genetic bases of antimicrobial resistance (intrinsic and acquired) in bacteria, biofilms are major contributors to antimicrobial resistance. The majority of microorganisms in nature exist in a stable and persistent state called a biofilm. The initiation of biofilm development by microorganisms is mainly due to distinct environmental cues like optimum temperature for growth, oxygen tension, low nutrient availability and optimum pH. The continual development of the biofilm is dependent on these factors, but where they are depleted or unavailable, the biofilm detaches, and the bacteria return to free living cells allowing the cells to search for favourable growth conditions (Ramli et al. 2012).

Microorganisms found in nature and medical infections are commonly located on a surface, but function in biofilm-communities bonded by an extracellular-slime, known as extracellular polymeric substances (EPS). This extracellular-slime which is composed of polysaccharides, proteins, extracellular DNA and water, is responsible for the structure of the biofilm and for holding cells together, which is important for cell – cell communication through quorum sensing (QS). Quorum sensing which is significantly important in biofilm formation, is an intercellular signalling system through which bacteria communicate and regulate gene expression by releasing chemicals known as auto-inducers (Sharma et al. 2014)

Biofilm enhances antimicrobial resistance and bacterial survival through various mechanisms. Biofilm matrix acts as a barrier, preventing the entrance of antimicrobial agents, thereby providing a protective effect to the bacteria. Furthermore, high bacterial

cell density in biofilms, encourages horizontal genetic exchanges, which leads to an increase in the number of resistant bacteria within the biofilm when a resistance gene is transferred (Jolivet-Gougeon and Bonnaure-Mallet 2014). In a biofilm, a sub-population of bacteria have the tendency of entering into a reversible slow-growing or starved state. This set of bacterial cells in the biofilm, known as dormant cells or persisters, are less susceptible to antimicrobial agents due to their lower metabolic rate compared to the active, exponential-phase bacteria. Toxin/antitoxin (TA) system in biofilms, induced by environmental starvation and mutation, enhances the formation of persisters. The TA system in biofilm acts by: inhibiting protein synthesis; inhibiting translation leading to mRNA degradation; and reducing metabolic rate by decreasing ATP synthesis. Biofilms with an increased number of persisters tend to be highly resistant to antimicrobial agents (Lewis 2012). In addition, bacteria in biofilms respond to hyperosmolarity (increased osmolar concentration of fluids) and outer membrane changes induced by antimicrobial agents or environmental deprivation by activating stress response genes that promote antimicrobial resistance and upregulate the efflux pumps (Guyard-Nicodeme et al. 2008).

The presence of a microbial biofilm on a wound surface is a barrier to its healing (Rhoads, Wolcott and Percival 2008). When a wound is formed due to damage to the skin (e.g. abrasion or laceration), host defences are compromised thus permitting microbial adhesion and colonisation of the underlying tissues. The primary host's immune system attempts to prevent microorganisms seeded into the wound from developing a chronic infection (Niyonsaba et al. 2006). The establishment of a biofilm in a wound makes the repair of the wound and suppression of the infection difficult especially in individuals with a compromised immune system. The bacteria and their extracellular components may thus be able to prolong inflammation indefinitely, delaying the normal healing process. With this, a possible assertion could be given that the biofilm communities

present on the tissue get their nutrients from plasma and exudate percolating from the wound bed (Rhoads, Wolcott and Percival 2008). It is the presence of a biofilm on the wound surface that, most likely, contributes to its chronic state (Wolcott, Rhoads and Dowd 2008). Therefore, an antimicrobial-containing wound dressing may have an effect on biofilms found in recalcitrant chronic wounds, which will help to tackle antimicrobial resistance (Percival et al. 2008).

1.3.0 IMPORTANCE OF MEDICINAL PLANTS IN COMBATING ANTIMICROBIAL RESISTANCE

In developing countries, the dependence on herbal medicines for treatment of disease and infections in rural areas can be linked to both cultural and economic factors (Matu and van Staden 2003). From an economic standpoint, the high cost of drugs and the poor accessibility of good healthcare services are two of the major reasons why people in rural areas utilise traditional herbal medicine as the only form of healthcare. Furthermore, with the prevalence of microbial infections in developing countries due to poor sanitary, hygiene and living conditions, antimicrobial agents are needed to curb the increasing rate of antibiotic-resistant infections. Fortunately, herbal remedies could be used to serve this purpose. This is attributed to the fact that native plants and herbs are accessible and available to the people at little or no cost. From a cultural point of view, traditional medicine is seen as an efficient, reliable, cheap and acceptable form of healthcare which has existed in a particular area for many generations (Munguti 1997) and is therefore accepted by the people even when a modern healthcare system co-exists (Shaheen et al. 2015).

Over the last few decades, a number of studies have been carried out on herbal products to determine their pharmacological and medicinal values. Traditional herbal

products are believed to possess medicinal values; therefore, these could serve as a potentially valuable source for drug development (Shaheen et al. 2015). Consequently, screening of these medicinal plants for bioactive compounds is likely to prove highly reliable (Shaheen et al. 2015). Phytochemicals from herbal extracts such as quinone, alkaloid and flavonoids are used by pharmaceutical companies for drug production and development due to their antimalarial, antibacterial, antifungal and antiviral properties (Atanasov et al. 2015).

In developing countries like The Gambia, ailments like fever, gastro-intestinal disorders, burns, sores, acne and a host of others are being treated traditionally in rural areas using plants like *Prosopis africana, Cassia occidentalis*, *Uvaria chamae, Ficus carica, Pterocarpus erinaceus* and *Dracaena mannii*. These plants are usually consumed directly through eating of the leaves; steeping of the plant parts in hot water and drinking the water; cooking of food with the leaves; direct application to wound dressing; and inhalation of steam from cooked plant parts (Costa et al. 2015). According to Nascimento et al. (2000) and Costa et al. (2015), plants used as traditional medicine possess phytochemicals such as oxygenated terpenes, tannins, alkaloids, aldehydes and cardiac glycosides, which are responsible for the plant's biological properties, even though limited studies on the identification and classification of these phytochemicals in plants have been done. Nonetheless, some herbal materials used as traditional medicines are known to possess antimicrobial capacities and can serve as potential candidates for combating antimicrobial resistance (Walter et al. 2011; Ismail et al. 2012). A selection of these plants: *P. africana*; *C. occidentalis*; *U. chamae*; *F. carica*; *P. erinaceus*; and *D. mannii*, used as traditional medicine in The Gambia, vary in physical attributes (Table 1.1) and usage, even though some of them are poorly characterised. For a better understanding of these plants, profiles of these plants are discussed below.

1.3.1 *Uvaria chamae* (*Sambe*)

Uvaria chamae, also known as *Sambe* in The Gambia, belongs to the Annonaceae plant family. *Uvaria chamae* is a climbing plant, which is found in the tropical rain forests of West Africa, although it is also widely distributed in parts of Asia, South America and Australia. The *Uvaria chamae* plant grows to about 3.6 m - 4.5 m in height, and is extensively branched with broad, sweet, aromatic and alternate leaves (Figure 1.1a) (Okwu and Iroabuchi 2009). The flowers consist of 3 - 6 green petals, which are 3 cm long for each petal and 4 – 6 green sepals (Figure 1.1a). Fruits from *U. chamae* are oblong and velvety with acidulous fleshy pulp, which is used as a preservative and edible food source (Figure 1.1b) (Irvine 1961).

Figure 1.1: (a) Leaves and flowers of *Uvaria chamae* (Sambe) (b) Fruits of the *Uvaria chamae* (Sambe) plant containing seeds (Brunken et al. 2008).

Uvaria chamae is an important plant of interest due to its considerable importance in traditional medicine. Extracts from the roots of *U. chamae* have been reportedly used in the following ways: during child delivery in the rural parts of West Africa; and for the treatment of fevers, stomach disorders, abdominal pains, and piles (Oluremi et al. 2010). The leaves and stems of *U. chamae* have also reportedly been used for the treatment of

malaria, wounds and sores; haematuria; and eczema (Okwu and Iroabuchi 2009). *Uvaria chamae* plant parts have reportedly been used for the treatment of bacterial infections with some degree of success in remote parts of West Africa where medical amenities are limited (Oluremi et al. 2010). Interestingly, *U. chamae* has been reported to possess antifungal properties. Okorie (1977) in her study reported that chamuvaritin, a benzyl dihydrochalcone, which was extracted from *U. chamae*, is used in the drug, benzyl benzoate, for the preparation of antifungal agents. Additionally, Hufford et al. (1979) in their experiments showed that the ethanolic extract from the stem bark of *U. chamae* demonstrated cytotoxic activity *in vivo* against P-388 lymphocytic leukaemia and *invitro* against human carcinoma cells from the nasopharynx.

1.3.2 *Prosopis africana* (*Kembo*)

Prosopis africana, also known as *Kembo* in The Gambia, belongs to the Mimosaceae plant family. *Prosopis africana* is a medium sized savannah tree mostly found in the tropical regions of Africa, ranging from West Africa to East Africa (Orwa et al. 2009). Weber et al. (2008) reported that *P. africana* trees could vary in height and size depending on the geographical area where the tree is located. The tree has a height range of 4 m – 20 m, with a deep tap root, which grows fast and spreads extensively into the ground (Table 1.1). The tree also has dark and scaly bark, and leaflets which are bi-pinnate with 9 – 16 oblong leaf pairs with a size range of 12 mm – 30 mm (Figure 1.2a). The brown hard seeds of *P. africana* are enclosed in brown pods (5 seeds – 10 seeds in a pod) with a yellow interior when the pods are dried and opened (Figure 1.2b).

Prosopis africana is one of the most important trees in countries like Benin, Senegal, The Gambia and parts of West Africa due to its economic value in wood and paper production (Houetchegnon et al. 2015). Nevertheless, the significant nutritional and

medicinal value of *P. africana* in these countries cannot be over-emphasised. *Prosopis africana* plant parts are important for the traditional treatment of a variety of infections, whilst the leaves are used as analgesics for the treatment of headache, toothache and general body pain. Both the bark and the leaves are combined for the treatment of rheumatism. Skin infections, dental caries, fevers and eye infections are all treated using the bark of the plant. The roots are used for the treatment of gonorrhoea, toothache, stomachache, dysentery and bronchitis. In Mali for example, parts of the plant like the leaves, stem and roots are used for the treatment and management of a range of infections, and of disease conditions like dermatitis, bronchitis, tooth decay, malaria, stomach cramps and dysentery (Kolapo et al. 2009).

One important medicinal use that *P. africana* has been associated with is in the area of wound management and treatment. In rural and remote areas of Ghana, Senegal, The Gambia and some of the West African countries, where the use of traditional medicine is prominent, the bark and the root are used in the treatment and management of wounds and cuts to avoid infections, and also in the delivery of babies to speed up the healing of the umbilical cord (Orwa et al. 2009). Wound healing is a complex process of tissue restoration, with dynamic processes involving inflammation, proliferation and differentiation of cells to enable tissue repair. The presence of bacteria in wounds could interfere with any of the processes involved in wound healing, thereby slowing down the rate of tissue repair (Ezike et al. 2010). Ezike et al. (2010) showed that a methanolic extract of *P. africana* significantly reduced bleeding and coagulation time and also promoted the epithelialisation of excised wounds in rats. Furthermore, it was shown that a methanolic extract of *P. africana* inhibited the growth of *Staphylococcus aureus, Pseudomonas aeruginosa, Salmonella Typhi, Bacillus subtilis* and *Klebsiella pneumoniae* (Ezike et al. 2010).

Figure 1.2 (a) Leaves of the *Prosopis africana* (Kembo) plant (b) *Prosopis africana* (Kembo) seed pod (Brunken et al. 2008)

1.3.3 *Cassia occidentalis* (*Kassala*)

Cassia occidentalis, which is also known as *Kassala* in The Gambia, belongs to the Fabaceae plant family. *Cassia occidentalis* is a plant that grows in most tropical parts of the world including parts of Africa, Asia, South, Central, and North America, although it originates from Brazil (Chukwujekwu et al. 2006). *Cassia occidentalis* is a branched smooth woody shrub with a height range of 0.8 m – 1.5 m. The stem is reddish-purple in colour, and the leaves are alternately bi-pinnate with 4 – 6 pairs of leaflets on each stem (Table 1.1). The leaves are ovate, 4 cm – 6 cm long, and 1.5 cm – 2.5 cm in width, with a pointed tip and white hairs on the margin (Figure 1.3a). The flowers consist of 5 – 8 yellowish petals, which are 2 cm long for each petal and 4 – 6 green sepals (Figure 1.3a). The seeds of *C. occidentalis* are small and enclosed in a green pod (20 seeds – 40 seeds in a pod) which eventually turns brown when dried (Figure 1.3b) (Orwa et al. 2009).

Dating back to the 10th century, *Cassia* plants have been believed to possess medicinal properties when they were used as laxatives and purgatives. *Cassia occidentalis* has been used in the treatment of dermatitis, bacterial infections, liver disorders such as hepatitis, jaundice, cirrhosis, the eradication of intestinal parasites and

worms and as an immune stimulant. In most parts of Africa, it is still used as an antimicrobial agent to treat a wide range of bacterial infections (Yadav et al. 2010). In a study performed by Abo et al. (2000), the ethanolic extract of the different parts of *C. occidentalis* demonstrated antimicrobial activity against *E. coli* and *S. aureus*. They also showed that seed extracts demonstrated more antimicrobial activity than the leaf and the stem bark.

Figure 1.3 (a) Leaves and flowers of *Cassia occidentalis* (Kassala) (b) Seed pods of the *Cassia occidentalis* (Kassala) plant containing seeds (Brunken et al. 2008).

1.3.4 *Ficus carica* (fig)

Normally referred to as the common fig, *F. carica* belongs to the Moraceae plant family. *Ficus carica* is known to have originated from the Middle East and Asia, although it can also be found in most parts of the world including North, Central and South America, Africa and Australia. Due to its unique features, *F. carica* can grow in harsh conditions like a dry climate and nutritionally poor soil (Aref et al. 2010). The *Ficus carica* plant grows to a height of 7 m – 10 m and has a smooth light brown bark, with a leaf of 12 cm – 25 cm long and 10 cm – 18 cm wide (Figure 1.4a). The fruit is usually 3

cm – 5 cm long with a green outer skin (Figure 1.4a), which changes to purple when it is fully ripe (Figure 1.4b) (Wang et al. 2003).

Figure 1.4: (a) Leaves and unripe seed pods of *Ficus carica* (b) Ripe seed pods of the *Ficus carica* containing seeds (Brunken et al. 2008).

Ficus carica plants have been used for centuries for medicinal, nutritional and economic purposes. Due to its high nutrient content and its phytochemical content, *F. carica* has been used for the treatment and management of disease conditions and infections for centuries. It has been believed that consuming fig prevents thrombosis (Wang et al. 2003) and it can be also used to treat constipation as a laxative due to its high fibre content (Aref et al. 2010). *Ficus carica* has been used traditionally for the treatment of infections such as gastro-intestinal infection, dysentery and wound infections (Oliveira et al. 2009).

1.3.5 *Pterocarpus erinaceus*

Pterocarpus erinaceus, which is also known as Senegal rosewood or African teak, belongs to the Fabaceae plant family. *Pterocarpus erinaceus* is a medium sized tree that grows to between 12 m – 15 m tall (Table 1.1). It originates from West Africa and is

predominantly found in countries like Senegal, The Gambia, Nigeria, Benin, Sudan and Ghana. The tree usually grows in hot climatic conditions and can withstand drought for a considerable period of time. The brownish-black bark is scaly and thick and also produces a red sap when cut. The leaves of *P. erinaceus* are alternate and grow up to 3 cm in length (Figure 1.5a), the flowers are irregular, yellow in colour and the petals are 10 mm – 12 mm long and whilst usually hairy (Figure 1.5b). The seeds of *P. erinaceus* are enclosed in a green pod when fresh but this turns brown when ripe and dry. The pods are usually 5 cm – 8 cm long and contain 3 – 5 kidney-shaped seeds (Orwa et al. 2009).

Figure 1.5: (a) Leaves of *Pterocarpus erinaceus* (b) flowers of *Pterocarpus erinaceus* (Brunken et al. 2008).

Pterocarpus erinaceus is used as a food due to its nutritional value, and it is also used for fuel, timber, gum, and medicinally. For medicinal use in rural areas where healthcare services are poor, *P. erinaceus* has reportedly been used for the treatment of severe diarrhoea and dysentery, urethral discharge, gastro-intestinal infection and in dressings for chronic ulcers and wounds (Noufou et al. 2012). Several studies have demonstrated the antimalarial activity (Karou et al. 2003), antiprogestative activity (Benie

and Thieulant 2004), and antimicrobial activity (Nuhu et al. 2000) of *P. erinaceus.* In their study, Gabriel and Onibanjo (2010) were able to demonstrate the antimicrobial activity of crude methanolic extracts of *P. erinaceus* against *Pseudomonas aeruginosa*, *Staphylococcus aureus, Escherichia coli, Bacillus subtilis* and *Candida albicans*.

1.3.6 *Dracaena mannii*

Dracaena mannii, which is also known as Bafe in The Gambia, belongs to the Dracaenaceae plant family. *Dracaena mannii* is a shrub that grows up to 5 m tall (Table 1.1) and is predominantly found in tropical Africa in countries like Tanzania, The Gambia, Angola, Senegal, Kenya, Cameroun, Gabon and Liberia. The tree usually grows in hot climatic conditions and can withstand drought for a considerable period of time. The leaves of *D. mannii* are oblong-elliptic with a flaring base clinging to the stem for half its circumference, up to 400 x 20 mm (Figure 1.6), the flowers are greenish to creamy white in colour which can be up to 37 mm long. The pedicel bases of *D. mannii* are up to 4 mm and the receptacles are up to 2 mm long. The fruits which are 30 mm in diameter, are brown in colour at the early stage and subsequently turns to scarlet when ripe (Figure 1.6) and the seeds enclosed in in the fruit are 7 – 20 mm in diameter (Jstor 2019).

Figure 1.6: Leaves and fruits of *Dracaena mannii* (Jstor 2019).

Dracaena mannii has been used as traditional medicine in the treatment of infections in different parts of Africa (Malan and Neuba 2011). Inspite of the traditional relevance of this plant, a few number studies showed the antimicrobial activity of *D. mannii.* Sofowora and Olaniyi (1975) demonstrated the antimicrobial activity of *D. mannii* extract against *Staphylococcus aureus*. Okunji et al. (1990) demonstrated the antifungal properties of *D. mannii* against *Candida albicans*.

Table 1.1 Summary of the physical profile and dimensions of the plants used in the current study.

Plant	Height	Leaves	Flowers	Fruits/Pods	Reference
PA	4 m - 20 m	Leaves alternate, bi-pinnate; rachis 10-15 cm long with 3-6 pairs of opposite pinnae (5-8 cm long); 9-16 pairs of leaflets, oblong-lanceolate (Figure 1a)	Greenish-white to yellow in colour, fragrant, in dense 6-10 cm long axillary spikes; calyx pubescent but petals glabrous	Pods are dark brown, cylindrical, thick and hard with a measurement of 15 cm x 3 cm, with woody walls, about 10 loose rattling seeds per pod (Figure 1b)	Orwa et al., (2009)
CO	5 m - 8 m	Pinnate, pale green in colour and measuring about 1.5 cm - 5 cm (Figure 2a)	Flowers are erect, circular-shaped with pointed ends and yellowish in colour	Seeds of this plant are found in long pods (Figure 2b)	Yadav et al., (2010)
UC	5 m - 15m	Pinnate and wide leaves measuring about 2 cm - 6 cm	3 - 6 green petals which are 3 cm long for each petal and 4 – 6 green sepals	Oblong and velvety with sour tasting pulp	Okwu and Iroabuchi, (2009)
FC	7 m - 10 m	12 cm – 25 cm long and width of 10 cm – 18 cm	Flowers not visible	3 cm – 5 cm long with green outer skin. Changes to purple when ripe	Wang et al. (2003)
PE	12 m – 15 m	Leaves are alternate and grow up to 30 cm.	10 mm – 12 mm long. Yellow in colour and hairy	Pods are usually 5 cm – 8 cm long and contain 3 – 5 seeds which are kidney-shaped	Orwa et al. (2009)
DM	5m	Leaves are narrowly oblong-elliptic which measure up to 400 mm in length and 20 mm in width.	Greenish to creamy white in colour and measures up to 37 mm long.	Fruits are brown in colour and later turns scarlet when they are ripe, measures 30 mm in diameter. The seeds found in the fruits measures 7 – 20 mm in diameter.	Jstor (2019)

UC - *Uvaria chamae* PA - *Prosopis africana,* CO - *Cassia occidentalis,* FC-*Ficus carica,* PE - *Pterocarpus erinaceus,* DM - *Dracaena mannii*

1.4.0 STUDIES ON ANTIMICROBIAL ACTIVITIES OF PLANTS

Research into the antimicrobial activities of plant extracts has increased over the years due to the importance of herbal remedies in traditional healthcare. Rios and Recio (2005) undertook a systematic review of studies that had assessed the antimicrobial activity of medicinal plants that have been performed in the past four decades between 1966 – 2005 based on 3 main study criteria: mode of isolation of the active compounds from the medicinal plant; natural plant of a specified area or region; and the activity of a

medicinal plant against a specific microorganism. In their study, Rios and Recio (2005) found, using PubMed that 115 - studies of the antimicrobial activities of medicinal plants against microorganisms were published during the period of 1966 to 1994. The number of studies published during the decade between 1995 and 2004 doubled to 307 as (determined using PubMed). Rios and Recio (2005) then proposed that the number of studies could well triple during the following decade. However, there was no follow-up study to validate the proposal made by Rios and Recio (2005). This shows potential of medicinal plants as sources for new agents and to combat antimicrobial resistance when problems arise with bacterial resistance against existing antibiotics.

One of the major problems observed when comparing studies that analysed the antimicrobial activity of plants has been the lack of uniformity in selecting important criteria such as experimental protocols, bacterial species, and plant parts used. This has given rise to contradictory results from different studies of the same samples (Rios and Recio 2005). One of the ways in which this problem has been solved has been by reviewing the different experimental protocols used over the decades, and then recommending the protocol that provided the most reproducible results (Rios et al 1988). The protocols that were recommended have been widely accepted by research groups due to the number of times this protocol was cited (Rios and Recio 2005). These protocols were the agar-well diffusion assay, the solid dilution assay (overlay assay) and the liquid dilution assay that is now known as the broth micro-dilution assay. However, the most highly recommended amongst these three methods was the liquid dilution assay. This is because the liquid dilution assay was the most sensitive method to identify the potency of pure compounds against specific microorganism (Rios and Recio 2005). Furthermore, Jansen et al. in their 1987 review, recommended that to improve uniformity in future studies: the strain number and growth conditions for the test microorganism be stated;

that details of the plant parts to be used, including where they were obtained should be noted; that details of the extraction methods and conditions used be given; and that details of the antimicrobial assay used, should all form a fundamental part of the experimental report.

1.4.1 Selected plant antimicrobial studies from last decade

Interestingly, the number of plant antimicrobial studies has increased from the last decade to date, due to the quest in search of new compounds for the treatment of bacterial infections and the fight against antimicrobial resistance. Table 1.2 summarises a selection of plant antimicrobial studies performed from the last decade. Also, a few of the selected studies have been briefly explained below.

Matu and van Staden (2003) investigated some plants that have been used in Kenya as traditional antimicrobials. Their results indicated that *Maytenus senegalensis*, one of the plants that they analysed possessed the greatest antimicrobial activity. Antimicrobial activity was only observed against Gram-positive bacteria, and *Staphylococcus aureus* was the most susceptible bacterial species. These authors attributed the lack of activity against Gram-negative bacteria to their thick murein cell wall, which could prevent entry of plant-based inhibitors. Their findings proved that the high antibacterial activity justified the use of *Maytenus senegalensis* in the treatment of bacterial infections in rural Kenya, due to its strong antibacterial activity.

A similar study, carried out in Mexico by Yasunaka et al. (2005), showed that out of the 22 Mexican plants analysed, 17 plants demonstrated antimicrobial activity against *Staphylococcus aureus* (methicillin-sensitive and methicillin-resistant) and *Escherichia coli*, whilst only 5 plants showed no activity. The plants analysed by Yasunaka et al. (2005) were used widely in Mexico for the treatment of a variety of ailments ranging from

stomachache and diarrhoea to fever, measles and gonorrhoea. In their study, they isolated and purified two active compounds: jacareubin and 1, 3, 5, 6-tetrahydroxy-2-(3, 3-dimethylallyl) xanthone, from plant extracts that demonstrated antimicrobial activity. Each of the purified compounds showed a high bactericidal effect against *S. aureus* and *E. coli*. Consequently, Yasunaka et al. (2005) concluded that the two isolated compounds were responsible for the plants' antimicrobial activity and therefore recommended these compounds for further studies to help in the development of new antibiotics.

Akinpelu et al. (2014) investigated the antimicrobial potency of *Alchornea laxiflora*. Their results demonstrated potent antimicrobial activity of *Alchornea laxiflora* against both Gram-positive and Gram-negative bacteria. *Pseudomonas aeruginosa, S. aureus, E. coli* and *K. pneumoniae* were all found to be susceptible to leaf extracts of *Alchornea laxiflora*. Although specific compounds in the plants were not analysed, Akinpelu et al. (2014) suggested that the antimicrobial activity in the plant was due to the presence of phytochemicals like alkaloids, tannins, flavonoids, saponins and reducing sugars observed in the plant extract. Further recommendations were made in order to analyse those specific antimicrobial compounds responsible for the antimicrobial activity.

In a similar study, Moorthy et al. (2015) analysed the antimicrobial activities of two medicinal plants: *Aristolochia krisagathra* and *Thottea ponmudiana,* which were used in the treatment of a range of ailments in India. Their study showed that the methanolic extracts of *A. krisagathra* and *T. ponmudiana* demonstrated antimicrobial activity against *Staphylococcus epidermidis, S. aureus, B. subtilis* and *E. coli*. They further showed that methanolic extracts of the plants were more effective against the susceptible bacteria than the petroleum ether extract, which showed no effect.

Table 1.2 A summary of selected plant antimicrobial studies, highlighting the plants used extraction method, microorganism used and the antimicrobial activity observed.

Plants used	Extraction method	Micro-organism used	Antimicrobial Activity	Reference
Maytenus senegalensis, Plectranthus barbatus, Zanthoxylum chalybeum, Zanthoxylum usambarense and *Spiranthes mauritianum*.	Sonication using hexane, methanol and water separately as extraction solvents	*Bacillus subtilis, Micrococcus luteus, S. aureus, E. coli* and *K. pneumoniae*	Activity observed against Gram positive bacteria only but not against Gram-negative bacteria	Matu and Van Staden (2003)
Bursera simaruba (L.) Sarg. (Burseraceae), *Haematoxylum brasilettoH.* Karst. (Fabaceae), *Calophyllum brasiliense* Cambess. (Clusiaceae), and *Mammea americana* L. (Clusiaceae)	Conventional extraction using methanol as the solvent.	*E. coli* MSSA strains MRSA strains	Activity was observed against *E. coli*, MSSA and MRSA.	Yasunaka et al. (2005)
Cassia occidentalis (kassala)	Extraction of dry roots using ethanol with sonication for 1h	*S. aureus, E. coli, K. pneumoniae* and *B. subtilis*	Emodin, an antibacterial anthraquinone was found to possess antibacterial activity, especially against *S. aureus*	Chukwujekwu et al. (2006)
Cussonia zuluensis, Vepris reflexa, Curtisia dentata, Trichilia emetica, Terminalia phanerophlebia, Terminalia sambesiaca and *Kigelia africana*	Conventional extraction using acetone, n-hexane as solvents	*S. aureus, Enterococcus faecalis, P. aeruginosa* and *E. coli*	Plant species had varying degrees of antimicrobial activity. *C. dentata* shows greater antimicrobial activity than any of the other six species screened.	Shai et al. (2008)
Garcinia kola and *Vernonia amygdalina*	Hot water extraction mixed with 30ml of honey	*E. coli, Proteus mirabilis, S. aureus, Pseudomonas aeruginosa, K. pneumoniae* and *C. albicans*	The extract from both plants showed antimicrobial activity against all of the bacterial species tested, although no specific component was positively shown to possess antimicrobial properties.	Mboto et al. (2009)

Table 1.2 Cont'd A summary of selected plant antimicrobial studies, highlighting the plants used extraction method, microorganism used and the antimicrobial activity observed.

Plants used	Extraction method	Micro-organism used	Antimicrobial Activity	Reference
Annona senegalensis	Extraction with a mixture of methanol: methylene chloride (1 : 1) using Soxhlet extractor	*E. coli*, *B. subtilis*, *P. aeruginosa*, *Salmonella paratyphi* and *S. aureus*, *Aspergillus niger* and *C. albicans*	The diterpenoid, kaur-16-en-19-oic acid or kaurenoic acid, has been identified as the phytochemical constituent responsible for the antibacterial effects of root-bark of *Annona senegalensi*	Okoye et al. (2012)
Khaya senegalensis	Conventional extraction using ethanol, methanol and water as solvent.	*Salmonella* Typhi.	Possess antimicrobial ability against *Salmonella* Typhi	Ugoh et al. (2014)

1.5.0 MECHANISMS OF ACTION OF PLANT COMPOUNDS AGAINST MICROORGANISMS

Phytochemicals protect plants against attack by predators like microorganisms, insects and birds. Plants produce a variety of compounds such as: terpenoids, which are responsible for the plant's odour; quinones and tannins that give the plant its pigmentation; flavonoids and alkaloids, which are responsible for the flavour of the plants. Other phytochemicals like proteins, cardiac glycosides, reducing sugars and steroids play important roles in the biological properties of plants. For the purpose of this study, phytochemicals like tannins, phenols, saponins, quinones, alkaloids, flavonoids and terpenoids will be elucidated due to their antimicrobial activities. These phytochemicals have specific activity (Table 1.3) against microorganisms, which can act either individually, or synergistically (Cowan 1999).

1.5.1 Phenols

Phenols are phytochemicals, which can be found in a variety of plants. Cinnamic acid and caffeic acids are phenolic phytochemicals. These are predominantly found in

tarragon and thyme, respectively, and are reported to have antimicrobial properties against viruses (Wild 1994) and bacteria like *S. aureus* and *E. coli* (Brantner et al. 1996). Catechol and pyrogallol are examples of hydroxylated phenols, with catechol having two –OH groups and pyrogallol having three –OH groups. The number of –OH groups, and the positions of the –OH groups, correlate with the toxicity of these types of phenolics against microorganisms, which implies that the greater the number of –OH groups on the phenolic, the greater the toxicity. The mechanism of action of phenols is substrate deprivation, which leads to bacterial cytoplasmic disruption (Cowan 1999) (Table 1.3). Mason (1987) in his research, discovered that the toxicity of phenols against microorganisms was due to the inhibition of enzymes by oxidised substrate.

1.5.2 Quinones

Quinones are phytochemicals found in most fruits and vegetables and are responsible for the brown colouration in cut or injured fruit and vegetables due to the reaction they undergo on exposure to oxygen. Quinones are also responsible for melanin synthesis in the human skin (Cowan 1999). Quinones are known to provide stable free radicals, which form irreversible complexes with amino acids in proteins (Figure 1.6). The formation of these complexes can inhibit or inactivate enzymes for specific reactions. The presence of quinones is lethal and toxic to microorganisms due to disruption of important chemical reactions. Possible targets for quinones within the microbial cell are structures such as exposed adhesins, polypeptides and membrane bound enzymes (Cowan 1999) (Table 1.3).

Figure 1.6 Structures of some important phytochemicals with antimicrobial properties (Cushnie and lamb 2005)

1.5.3 Flavones, Flavonoids and Flavonols

Flavones, flavonoids and flavonols are phytochemicals, which are quite similar structurally and functionally to quinones. The only difference they have from quinones is that they possess one carbonyl group (a carbon atom double bonded to an oxygen atom) while the quinones possess two carbonyl groups (a carbon atom with two double bonded oxygen atoms) (Figure 1.6). Flavones are not hydroxylated, but flavonols have a hydroxyl group at position 3 on the carbonyl group and flavonoids have hydroxyl groups at positions 3 and 7 on the carbonyl group (Figure 1.6) (Cowan 1999). According to Dixon et al. (1983), these phytochemicals are synthesised in plants for their protection against environmental changes and in response to microbial infection. Therefore, their antimicrobial activity does not come as a surprise. The mechanisms of action of flavones, flavonoids and flavonols are quite similar to those of the quinones. Due to the possession of free radicals, flavones, flavonoids and flavonols have the ability to bind to proteins in the bacterial cell, thereby causing enzyme inhibition and inactivation (Cushnie and Lamb 2005) (Table 1.3). Sakagami et al. (1998) demonstrated the mechanism of action of sophoraflavonone G against MRSA. Using spectrophotometry, these researchers showed that on exposing MRSA strains to sophoraflavonone at 6.25 µg/ml, the MIC of this compound, an increase in fluorescence occurred. Their findings suggest that the presence

of sophoraflavonone increases the permeability of the bacterial membrane due to the destruction of the membrane thereby inhibiting membrane function (Sakagami et al. 1998).

1.5.4 Tannins

Non-hydrolysable tannins can be formed by either the condensation of flavan sub-units, or the polymerisation of quinone derivatives. Hydrolysable tannins on the other hand are formed from mixtures of gallic acid esters and D-glucose. Tannins are present in all the parts of a plant: the stem, bark, roots, leaves, fruits and flowers (Lim et al. 2006). One of the main attributes of tannins is their ability to form complexes with proteins and polysaccharides through hydrogen bonding and covalent bonding (Table 1.3). The formation of complexes and the ability to adhere to surface proteins by tannins, causes enzyme inhibition and inactivation. It also leads to substrate deprivation and membrane disruption due to the formation of complexes (Lim et al. 2006).

1.5.5 Alkaloids

Morphine is an example of an alkaloid and was first isolated from the plant *Papaver somniferum* in 1805, with codeine and heroin as its derivatives (Cowan 1999). Since then, other types of alkaloids have been isolated and used medically. Alkaloids are also known to possess antimicrobial activity. In a study, Karou et al. (2006) found that the alkaloid extracted from *Sida acuta* showed antimicrobial activity against a diverse range of both Gram-positive and Gram-negative bacteria. Alkaloids have been reported to inhibit RNA and protein synthesis (Amin et al. 1969). Berberine is an alkaloid derivative that was found to inhibit RNA and protein synthesis in *Vibrio cholerae* and *Staphylococcus aureus* at a concentration of 50 μg/ml, thereby causing lysis of the

bacterial cells (Amin et al. 1969). Cowan (1999) also noted that alkaloids have the ability to intercalate with cell membrane and microbial DNA leading to lysis of the bacterial cell (Table 1.3).

1.5.6 Terpenoids and Essential oils

Terpenoids and essential oils are compounds synthesised from acetate units, which are found in most plant parts and are responsible for the plant's odour. The only difference between terpenes and fatty acids is that terpenes are cyclised and extensively branched while fatty acids are linear and are not branched (Cowan 1999). The antimicrobial mechanism of action of these phytochemicals involves the disruption of the bacterial membrane. The activity and mechanism of action of essential oils isolated from *Melaleuca alternifolia* (tea tree) against *S. aureus, E. coli* and *Candida albicans* were elucidated by Cox et al. (2000). This group reported that on exposing these microorganisms to their corresponding Minimum Bactericidal/Fungicidal Concentrations, an uptake of propidium iodide was observed indicating disruption of the cell membrane of the microorganisms (Table 1.3). Furthermore, the workers observed potassium ion leakage, which signifies the loss of chemiosmotic control in the microorganisms (Cox et al. 2000). In a different study, electron microscopy was used to elucidate the mechanism of action of essential oils isolated from cinnamon against *S. aureus,* and *E. coli* (Zhang et al. 2016). It was observed that when *S. aureus* and *E. coli* were exposed to Cinnamon essential oil at the Minimum Bactericidal Concentration (MBC) of 2.0 mg/ml and 4.0 mg/ml, respectively, there was a leakage of electrolyte, which led to an increase in the electrical conductivity of the samples. In addition, there was a change in the structure of the bacterial cells when viewed under the electron

microscope which led them to conclude that the essential oils caused the permeability and the destruction of the bacterial cell membrane (Zhang et al. 2016).

1.5.7 Saponins

Saponins belong to a group of phytochemicals that serves as one of the major defence systems for plants against microbial, fungal, and insect attack. They can be found in most plant species although the amount and the concentration of saponins in plants varies from species to species (González-Lamothe et al. 2009). Depending on the structure of their aglycone, saponins are grouped into triterpenoids, and steroidal glycoalkaloids (Osbourn 1996). Saponins act by forming complexes with sterols or polysaccharides within the microbial cell membrane so destroying the cytoplasmic membrane integrity (Morrissey and Osbourn 1999) (Table 1.3). Morrissey and Osbourn (1999) reported that avenacosides A and B which are saponins extracted from oat leaf are normally inactive. However, when exposed to fungal attack, avenacosides form complexes with glucose molecules on the fungal cell membrane producing antifungal 26-desglucoavenacosides, which are responsible for loss of membrane integrity to the fundal cells. Electron microscopy and electrical conductivity analyses gave evidence for the development of transmembrane pores when fungal cells were exposed to this saponin (Morrissey and Osbourn 1999).

Table 1.3 Mechanisms of action of some of the phytochemicals found in some antimicrobial plants

Phytochemical	Mechanism of action	References
Phenols	- Membrane disruption - Substrate deprivation	Mason and Wasserman (1987) Cowan (1999)
Quinones	- Formation of complexes with protein - Inhibition and inactivation of enzymes	Cowan (1999)
Flavonones, Flavonoids and Flavonols	- Protein binding to form complexes - Enzyme inactivation - Destruction of bacterial cytoplasmic membrane	Cushnie and Lamb (2005)
Tannins	- Metal-ion complex formation leading to membrane disruption - Substrate deprivation - Enzyme inhibition - Inhibition of RNA and DNA synthesis	Lim et al. (2006) Amin et al. (1969)
Alkaloids	- Intercalation with cell membrane and DNA	Cowan (1999)
Terpenoids and essential oils	- Disruption of bacterial membrane	Cox et al. (2000)
Saponin	- Formation of complexes with sterols, leading to bacterial cytoplasmic disruption	Morrissey and Osbourn (1999)

1.6.0 THE ANTIBIOFILM ACTIVITY OF MEDICINAL PLANTS

One of the main factors that influences the antimicrobial resistance of bacterial cells is the presence of biofilm. Microbial biofilm formation goes through a complex cycle of stages, which consist of initiation, maturation, maintenance and dissolution (Ramli et al. 2012). Recent studies suggest that phytochemicals from plants can interfere with the formation of microbial biofilms and influence the degradation of preformed biofilms (Niu and Gilbert 2004). There are several modes of action through which these plant compounds interfere with, and influence, microbial biofilm formation.

Interestingly, plant extracts have been shown to interfere with quorum sensing thereby disrupting biofilm formation and degrading preformed biofilms. A study showed that halogenated furanones isolated from seaweeds are capable of interfering with quorum sensing in marine algae leading to degradation of the algal community, therefore reducing

biofouling (Steinberg et al. 1997). In another study, Gao et al. (2003) extracted active substances from young seedlings of *Medicago truncatula* and then further fractionated this extract using reverse-phase C18 high-performance liquid chromatography (HPLC). Although they did not identify the fractions chemically, this group showed that all the fractions inhibited the secretion of N-acyl homoserine lactone (AHL) signal in the quorum sensing reporter bacterium *Pseudomonas putida* CepR. A similar study performed by Packiavathy et al. (2012) demonstrated antibiofilm property of *Cuminum cyminum* against *P. aeruginosa, Pr. mirabilis* and *Serratia marcescens* through inhibition of quorum sensing. With a minimum inhibitory concentration of 2 mg/ml, *C. cyminum* extract interfered with AHL activity through inhibiting the production of the AHL molecules responsible for quorum sensing, leading to the inhibition of exopolysaccharide (EPS) production, swarming motility and inhibition of *invitro* biofilm formation (Packiavathy et al. 2012).

Degradation of the extracellular polymeric substances (EPS) bacterial biofilm matrix and prevention of bacterial adhesion by active compounds is another way in which plants act against biofilm. Recent studies have shown that phytochemicals like essential oils and phenols have the ability to degrade EPS in bacterial biofilm. By using a scanning electron microscope, Kim et al. (2015) were able to demonstrate the antibiofilm activity of cinnamon bark oil against *P. aeruginosa* preformed biofilm, through the degradation of the biofilm matrix. They also reported that cinnamon bark oil and cinnamaldehyde at a concentration of 0.01% (v/v) were both able to significantly inhibit bacterial adhesion and so prevent biofilm formation. Similarly, essential oils from thyme have been shown to cause biofilm degradation of *Sphingomonas* species, *Acinetobacter* species and *Strenotrophomonas* species at a lethal concentration of 0.001% (w/v) (Szczepanski and Lipski 2014). Structural changes to the biofilms, and also a reduction and dispersal of the

biofilm of these species, when exposed to this concentration of thyme essential oil, suggest that it has antibiofilm activity against these bacteria (Szczepanski and Lipski 2014).

Studies suggest that most of the compounds present in plant extracts are able to inhibit bacterial adhesion and so prevent biofilm formation, whereas degradation of preformed biofilm is more difficult to achieve. For example, *Mentha piperita* (peppermint) extract was shown to be able to reduce bacterial attachment and colonisation of *P. aeruginosa* and *C. albicans* by 50% at a concentration of 0.38 mg/ml (v/v), but it was not effective against preformed biofilms of these species (Sandasi et al. 2009). Similarly, extracts from *Rosmarinus officinalis* (rosemary leaf), *Melaleuca alternifolia* (tea tree) and *Mentha piperita* (peppermint) were reported to reduce initial bacterial cell attachment of *Listeria monocytogenes* by at least 50 % over a concentration range of 2.5 mg/ml – 4.0 mg/ml but were less effective at removing preformed biofilm of *L. monocytogenes* (Sandasi et al. 2009). Sandasi et al. (2009) attributed this observation to the fact that bacterial cells in biofilms are more resistant to antimicrobial agents than planktonic bacteria, in agreement with the observations of Krysinski et al. (1992). In contrast, Selim et al. (2014) reported that plant extracts did have the ability to significantly reduce preformed biofilm density depending on the phytochemicals present in the extract. From their study, Selim et al. (2014) demonstrated that the combination of essential oils and flavonoids present in *Cupressus sempervirens* leaf extract, at a concentration of 0.5 mg/ml (v/v), totally eradicated preformed biofilms of *K. pneumoniae* from the surface of intravenous infusion tubes. Similarly, Teanpaisan et al. (2017) reported that *Piper betle* extracts both prevented the formation of biofilm by oral pathogens and eradicated preformed biofilm of oral pathogens at a concentration of 8.33 mg/ml (v/v). They attributed the antibiofilm activity of the *Piper betle* extract to the high concentration of

4-chromanol which is the main constituent of the extract. Therefore, these workers suggested that the amount of an active compound in a plant extract might influence its antibiofilm properties against specific bacterial strains (Teanpaisan et al. 2017).

1.7.0 EXTRACTION AND IDENTIFICATION OF BIOACTIVE COMPOUNDS FROM PLANT MATERIALS

Extraction and identification of bioactive compounds is an important and integral part of the analysis of medicinal plants.

1.7.1 Preparation of sample for extraction

For the preparation of the extracts, plants that have been selected based on their medicinal usage, ought also to be prepared and extracted as described by the traditional medicine healer so as to emulate the original herbal drug (Fabricant and Farnsworth 2001). Consequently, preparation of the sample involves washing of the plant parts, drying (air-drying or freeze-drying) of the plant, grinding of the dried plant sample into smaller particles to have a homogenous plant sample. Grinding the plant material improves the kinetics of the extraction process and also increases the surface area of the sample for optimal contact with the solvent system (Sasidharan et al. 2011). Adequate measures must be taken to ensure that the active components in the medicinal plants are not destroyed or lost during extraction (Sasidharan et al. 2011).

1.7.2 Solvent selection

Selecting the appropriate solvent for the extraction process is solely dependent on the characteristics of the active compounds to be extracted. A range of solvents can be used for the extraction of active compounds from herbal materials. Polar solvents like

ethyl acetate, ethanol and methanol are used to extract hydrophilic compounds. In contrast, when extracting hydrophobic or lipophilic compounds, non-polar solvents like hexane, dichloromethane or a mixture of dichloromethane and methanol can be used (Cos et al. 2006). An important point to note is that some of these bioactive constituents may be thermally unstable or easily altered; consequently, it is important to optimise the extraction method so that the active compounds are extracted efficiently without altering them more than can be avoided.

1.7.3 Selecting the appropriate extraction method

Different extraction methods (Table 1.4) have been employed for the optimal extraction of phytochemicals (Sasidharan et al. 2011). Traditional and conventional methods of extraction involve steeping or maceration of the fresh or dried plant material in water or the chosen solvent system. This traditional method is mostly employed due to its ease and simplicity since it requires little or no special skill, although there are limitations associated with this method of extraction as discussed below (Sasidharan et al. 2011). Firstly, many of the solvents used for extraction can be toxic or hazardous, and so their presence in drugs and food is heavily regulated. For example, a reduction in all solvent concentration to 2530 parts per million (ppm) in the final product is expected. Secondly, bioactive compounds that are readily volatile can be lost when evaporating the solvent, which in turn reduces the efficacy of the extract.

Table 1.4 Extraction conditions used in some plant antimicrobial studies

	Conventional (Steeping)	**Sonication (ultrasound)**	**Soxhlet extraction**
Solvents	Ethanol, Methanol,	Ethanol, methanol, Hexane	Ethanol, Methanol, Industrial methylated spirit (IMS)
Temperature	Room temperature	15-60ºC	Boiling point of solvent
Time	24 hours	1 hour	3-12 hours
Volume of solvent (ml)	150-200	50-100	Varies with sample size
Reference	Nielsen et al. (2012)	Briars et al. (2013)	Redfern et al. (2014)

In recent studies, the use of techniques such as Soxhlet extraction or ultrasound extraction for the extraction of phytochemicals has been advocated due to advantages such as low power consumption (De Castro and Capote 2010; Briars and Paniwnyk 2013), the simplicity of the protocol, and the quantity and quality of the extracts obtained, amongst others (Dai and Mumper 2010; Sasidharan et al. 2011; Petigny et al. 2013). In addition to Soxhlet and ultrasound extractions, other methods have been developed and improved to optimise the extraction of bioactive compounds from plant materials. Some of the extraction techniques that have been developed include: pressurised liquid extraction; solid phase extraction; supercritical fluid extraction; microwave-assisted extraction; surfactant-mediated extraction and solid phase micro-extraction. The advantages associated with these extraction techniques include, but are not limited to, a reduction in the amount of solvent used during the extraction process; a reduction in the destruction of vital bioactive compounds; the removal of extra sample clean-up and concentration stages before purification and identification of components; and enhancement of the extraction proficiency, selectiveness and kinetics (Huie 2002).

One method that has recently found particular favour in the extraction of phytochemicals is the use of ultrasound. This method has been used in many studies for

the extraction of active compounds from plants (Briars and Paniwnyk 2013; Petigny et al. 2013). Sonication has also been found to be effective for the extraction of food components like aroma, antioxidants, pigments and mineral compounds from the various matrices of plant tissues (Caldeira et al. 2004; Chen et al. 2007; Ma et al. 2009). This technique benefits from reduced energy consumption, which reduces the cost of extraction, and it also uses less solvent, leading to a shorter extraction time and the recovery of a purer extract (Petigny et al. 2013). The working principle of the ultrasound extraction technique involves the rupturing of the plant cell walls by the action of ultrasonic vibrations thereby aiding the penetration of the solvent and the release of the extractable cellular components (Dai and Mumper 2010; Sasidharan et al. 2011). A study conducted by Briars and Paniwnyk (2013) demonstrated the effect of ultrasound on the extraction of artemisinin from *Artemisia annua*. This study showed a 58% increase in the yield of artemisinin when sonication was used, as compared to the conventional steeping method, and also led to a purer extract (Briars and Paniwnyk 2013). A similar study by Petigny et al. (2013) showed a greater yield of phytochemicals compared with the conventional maceration method.

Since the bioactive compounds present in plant materials consist of multi-component mixtures, their separation and characterisation still create problems. Practically, most of them have to be purified by the combination of several chromatographic techniques, and possibly, by other purification methods, to isolate the bioactive compounds (Sasidharan et al. 2011).

1.7.4 Isolation, purification and identification of bioactive compounds from plant extracts

Natural products are frequently isolated, purified and identified following the evaluation and characterisation of the crude extract in a biological assay. A wide range of techniques can be used to purify and identify active components from plant samples. Some of the techniques that can be used are thin layer chromatography (TLC), gas chromatography–mass spectrometry (GC-MS), column chromatography, high performance liquid chromatography (HPLC), nuclear magnetic resonance (NMR), Fourier-transform infrared spectroscopy (FTIR) and other spectrometric techniques (Nostro et al. 2000; Dai and Mumper 2010; Petigny et al. 2013).

Isolation and purification of active plant constituents involves the use of one or more chromatographic techniques. Thin layer chromatography is one of the methods used in the isolation and purification of plant compounds. It involves the use of an adsorbent on a glass plate or other support to serve as the stationary phase, while the sample to be analysed is dissolved in a suitable solvent to serve as the mobile phase (Harbone 1998). Although only a small amount of sample is analysed using TLC, one of the major advantages of TLC is its speed and versatility. Column chromatography has the same principle with the TLC technique, with an added advantage of analysing samples on a large scale. The combination of TLC and column chromatography can be utilised for the separation of bioactive plant compounds (Harbone 1998).

In identifying bioactive compounds from plants after isolation and purification, it is important to first determine the class of compound, the isolated compounds belong to. An array of methods such as phytochemical test, use of a simple spectrophotometer, NMR, HPLC, GC-MS, and x-ray crystallography, can be used for identifying bioactive compounds and the class to which they belong. It is important to note that, HPLC and

GC-MS can be used to isolate, purify and identify bioactive compounds instantly (Harbone 1998). Most bioactive compounds are present in small proportions in an extract; therefore, a technique with a high resolving power is needed to analyse them (Sasidharan et al. 2011). HPLC is one of those techniques with such potential. Separation of bioactive compounds using HPLC makes use of differences in the rate of migration of compounds on a given column and in a given mobile phase (Petigny et al. 2013). Gas chromatography–mass spectrometry (GC-MS) is another vital technique that is used in separating, identifying and quantifying different compounds in an extract. This technique consists of a gas chromatograph coupled to a mass spectrometer to adequately separate and then identify volatile and thermally stable compounds. Unlike HPLC, which uses liquid as its mobile phase, GC-MS uses gas as the mobile phase for conveying samples through the column for separation. Separation is mostly based on the compounds' interactions with both the column packing material and the mobile phase. Compounds eluted from the column after heating are converted to ions where they are detected and identified with the aid of a mass spectrometer (Sasidharan et al. 2011).

A considerable body of research studies were able to demonstrate the antimicrobial properties of crude extracts made from plants, but only a few of these studies proceeded to identify the active compounds that showed antimicrobial activity. Identifying these active compounds is vital for the development of antibacterial agents, which can be used to combat bacterial species that have acquired or developed antimicrobial resistance. One of the major challenges in identifying and characterising bioactive compounds is their physical and chemical separation. Plant extracts comprise a mixture of different bioactive compounds with different properties and polarities, which makes separating them a challenge (Sasidharan et al. 2011). Chukwujekwu et al. (2005) successfully isolated and identified the antibacterial anthraquinone Emodin from the roots

of *Cassia occidentalis*. These workers used vacuum liquid chromatography (VLC) to separate the extract into sixteen different fractions. Bioautographic assay was performed to obtain the most active antimicrobial fraction. The most active fraction was further purified using gravity column chromatography and subsequently by thin layer chromatography (TLC). Identification of the purified isolate was finally performed by mass spectrometry. The profile of the isolated compound was compared with literature values and was determined to be 4, 5, 7-trihydroxy-2-methylanthroquinone (Chukwujekwu et al. 2005). A second study that successfully identified the active compounds in plant extract was performed by Okoye et al. (2012). These workers adopted the Soxhlet extraction method to obtain their crude extract from *Annona senegalensis* using a mixture of methanol and methylene chloride (1:1) as the solvent system. On fractionation, two fractions (F1 and F2) with antimicrobial properties were produced. Extract F1 was a lipophilic oily liquid whilst extract F2 yielded a crystalline product after the purification using column chromatography and thin layer chromatography. Identification and characterisation of the purified compounds was carried out using gas chromatography–mass spectrometry (GC-MS) for the F1 fraction and proton Nuclear Magnetic Resonance (NMR) and x-ray crystallography for the F2 compound. Using NMR, F2 was found to be a diterpene known as Kaur-16-en-19-oic acid whilst F1 comprised 6 other compounds that included Kaur-16-en-19-oicacid. Consequently, Kaur-16-en-19-oicacid was proposed to be one of the major active components of *Annona senegalensis* (Petigny et al. 2013).

1.8.0 AIMS AND OBJECTIVES

Emergence of antimicrobial resistance is a global issue caused by several factors, including pharmacological and host factors, e.g. the overuse and misuse of antimicrobials.

The search for new compounds from natural materials to fight antimicrobial resistance is therefore important. Consequently, the aim of this research was to determine the antimicrobial and antibiofilm properties of traditional herbal remedies. There is ample evidence that a wide range of traditional medicinal plants are used as alternatives for the treatment of bacterial infections in several parts of the world, which can therefore serve as potential sources for new compounds and may be potentially effective against resistant microbes. The main objectives of this study include:

- Preliminary antimicrobial study on a range of medicinal plants
- Assessing the extract yield from different extraction methods on selected plants and phytochemical screening of extracts
- Antimicrobial study on selected plant extracts using agar-well diffusion and broth micro-dilution assay
- Antibiofilm study on selected plant extracts using semi-quantitative and quantitative antibiofilm assays.
- Preliminary identification and characterisation of the biochemical nature of the active extracted compounds through fractionation, antimicrobial assay, phytochemical screening and spectrometric study of active fractions.

CHAPTER TWO

2.0 PRELIMINARY ANTIMICROBIAL STUDY ON A SELECTION OF AFRICAN MEDICINAL PLANTS

2.1.0 INTRODUCTION

Traditional herbal remedies have been used in different parts of the world as alternative treatments to bacterial infections (Matu and van Staden 2003). Medicinal plants like *Uvaria chamae*, *Prosopis africana*, *Cassia occidentalis*, *Ficus carica*, *Dracaena mannii* and *Pterocarpus erinaceus* have been reportedly used as traditional alternatives for the treatment of infections in parts of Africa as discussed in Section 1.4.0. Consequently, this study seeks to analyse the antimicrobial activities of selected medicinal plants against *Pseudomonas aeruginosa, Staphylococcus aureus* and *Escherichia coli*. These bacterial species are pathogens causing common infections like gastrointestinal infections, skin and wound infections (Thenmozhi et al. 2009). Studies show that various medicinal plants possess antimicrobial activities against bacteria such as *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Escherichia coli* amongst others (Okwu and Iroabuchi 2009; Orwa et al. 2009).

Furthermore, the antimicrobial susceptibility profile of bacteria to be used in the entire study was also determined. Some of the bacterial strains like *Propionibacterium acnes* strains and *Clostridium difficile* strains, which were used for the entire study, are isolates from clinical samples. Therefore, it was important to determine their degree of susceptibility against a range of common antibiotics. One of the methods which was used to determine the antimicrobial susceptibility profile of these bacterial strains in this study was the Kirby-Bauer assay, which is also known as an agar diffusion assay (Drew

et al. 1972). Another antimicrobial assay used in this current study to determine the antimicrobial activity of the plant extracts is the broth microdilution assay, which determines the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the plant extract to be tested against the selected bacterial strain.

The aim of this study was to determine the antimicrobial activity of selected medicinal plants against *Pseudomonas aeruginosa, Staphylococcus aureus* and *Escherichia coli*; and to perform an antibiotic susceptibility test on a range of bacterial species.

2.2.0 MATERIALS AND METHODS

Plant materials: *Uvaria chamae* (dried woody stem); *Uvaria chamae* (dried leaves); *Prosopis africana (*dried stem); *Cassia occidentalis* (dried stem); *Ficus carica* (dried stems); *Dracaena mannii* (dried stems) and *Pterocarpus erinaceus* (dried stem), were collected, air-dried, identified and supplied by Dr Bakary Touray (Agrovet Co. Ltd, P O Box 1271, Banjul, The Gambia).

Plant preparation: The air-dried plant samples were grated into fine powder using a metal grater. This was done to provide a larger surface area to enable optimal extraction of the plants' components.

Media preparation: Mueller-Hinton Agar (MHA) (Sigma-Aldrich UK, 70192-500G), Mueller-Hinton Broth (MHB) (Sigma-Aldrich UK, 70191-500G), Brain Heart Infusion Broth (BHIB) (Sigma-Aldrich UK, 53286-500G), Brain Heart Infusion Agar (BHIA) (Sigma-Aldrich UK, 70138-500G), Fastidious Anaerobic Agar (FAA) (Lab M, UK LAB090) with 7% Horse Blood Defibrinated (TCSbiosciences, HB035) as a supplement, and Fastidious Anaerobe Broth (FAB) (Lab M, UK LAB071). All media were prepared

following the manufacturers' instructions. All media were autoclaved and sterilised at 121^{o}C, 15 psi pressure for 15 min.

2.2.1 Extraction (maceration)

Maceration extraction was performed as previously described by Pratiwi et al. (2015). A 100 ml absolute ethanol solution (Sigma-Aldrich) was added to 10 g of the plant material in a conical flask (10 % w/v) and mixed by swirling. A foam bung was used to seal the conical flask, and the mixture was left at room temperature for 24 h. The plant extract was poured into Universal bottles (20 ml aliquots) and centrifuged using a benchtop centrifuge (Sanyo Gallenkamp centaur 2) at 1500 rpm for 5 min to pellet the debris from the extract. Afterwards, the extract was filtered using 0.22 µm millipore filter (Sartorius 16534K) to remove debris and possible bacterial contamination (0.45 µm millipore filter is small enough to remove bacterial contaminants). To avoid any interference with the antimicrobial effect of the extract, the solvent (absolute ethanol) was removed from the filtered extract using a rotary evaporator (BUCHI waterbath B-480). The extract left after the removal of the solvent was air-dried and removed from the round bottom flask and weighed. Storage of the extract was performed at 4^{o} C for subsequent use.

For subsequent analysis, extracts were re-suspended in 50 % aqueous ethanol and left for 3 h in a 75^{o} C water bath to evaporate the ethanol content. Extract was re-sterilised using a 0.22 µm millipore filter. A negative control was prepared to ensure the removal of ethanol and also ensure that no remnant of ethanol interfered with subsequent results. For the preparation of the negative control, 50 % aqueous ethanol (same volume to the one used in re-suspending the extract) was left for 3 h in a 75^{o} C water bath to evaporate

the ethanol content (usually, the volume of the remaining solution is approximately half its original volume).

A concentration of 10 mg/ml was made for extracts of the following plants: *Uvaria chamae* leaf, *Uvaria chamae* stem, *Prosopis africana*, *Cassia occidentalis*, *Ficus carica*, *Dracaena mannii* and *Pterocarpus erinaceus* for subsequent analysis.

2.2.2 Bacterial Strains and Growth Conditions

Staphylococcus aureus NCTC 6591, *Staphylococcus epidermidis* RP62A, *Klebsiella pneumoniae* NCTC 13865, Methicillin-resistant *Staphylococcus aureus* (MRSA) NCTC 43300, *Escherichia coli* NCTC 8164 and *Pseudomonas aeruginosa* NCTC 8295 were provided by the Health and Life Sciences (HLS) laboratory (Coventry University, UK). They were cultured in Mueller-Hinton Broth (MHB) (Sigma-Aldrich UK, 70192-500G), incubated at 37^{o} C for 24 h and maintained on Mueller-Hinton Agar (MHA) (Sigma-Aldrich UK, 70191-500G) at a temperature of 4^{o} C.

Propionibacterium acnes NCTC 737; Lumbar disc isolates 77 (LI77) and 88 (LI88); Skin isolates 5 (SI5) and 8 (SI8) of these species were provided by Prof Peter Lambert (Aston University, UK). They were cultured in Brain Heart Infusion broth (BHIB) (Sigma-Aldrich UK, 53286-500G), incubated at 37^{o} C for 72 h anaerobically in an anaerobic chamber (Whitley A35 Workstation) which contains anaerobic growth mixture and nitrogen gas. They were maintained on Brain Heart Infusion agar (BHIA) (Sigma-Aldrich UK, 70138-500G) at 4^{o} C.

Clostridium difficile NCTC 11204, ribotypes 001, 002 and 005 were provided by Public Health England (PHE) laboratory (Newcastle, UK). They were cultured in Fastidious Anaerobe Broth (FAB) (Lab M, UK LAB071), incubated anaerobically at 37^{o} C for 24 h and maintained on a fastidious anaerobe blood agar (Fastidious Anaerobic

Agar (FAA) (Lab M, UK LAB090) and 7% Horse Blood Defibrinated (TCSbiosciences, HB035) as a supplement) at 4^{o} C.

2.2.3.0 Antimicrobial assays

2.2.3.1 Antibiotic susceptibility assay (Kirby-Bauer assay)

Kirby-Bauer assay was performed as described by Drew et al. (1972). All overnight bacterial cultures were adjusted to 0.5 McFarland scale (absorbance of 0.085 at 625nm) as previously described by Balouiri et al. (2016). One hundred microlitre aliquots of bacterial cultures were inoculated onto fresh MHA plates (20 ml of molten MHA) by spreading the culture across the plate aseptically. Sterile filter disks containing known concentrations of antibiotics (10 µg Gentamicin (thermofisher CT0024B), 10 µg Penicillin G (thermos-fisher CT0043B), 10 µg Ampicillin (thermofisher CT0003B), 30 µg Erythromycin (thermofisher CT0021B), 10 µg Colistin (thermofisher CT0017B) and 5 µg Vancomycin (thermofisher CT0188B)) were then placed onto the inoculated petri dishes using a disk dispenser, which places the disks a pre-determined and reproducible distance apart from each other. The plates were incubated under the appropriate conditions, as described in section 2.2.2. The disk diffusion assays were observed by measuring the zones of inhibition caused by the antibiotic disks. The zones of inhibition were measured in mm using digital callipers.

2.2.3.2 Agar-well Diffusion Assay

Agar-well diffusion assay was perfomed as described by Ncube et al. (2008). All overnight bacterial cultures were adjusted to 0.5 McFarland scale (absorbance of 0.085 at 625 nm) as previously described by Balouiri et al. (2016). One hundred microlitre aliquots of bacterial cultures of *E. coli*, *P. aeruginosa* and *S. aureus* strains were spread-plated

onto Muller-Hinton Agar (MHA). Wells with a diameter of 6 mm were then cut asceptically into the agar using a sterile cork-borer and 50 µl aliquots (10 mg/ml) of *Uvaria chamae* leaf, *Uvaria chamae* woody stem, *Prosopis africana*, *Cassia occidentalis*, *Ficus carica*, *Dracaena mannii* and *Pterocarpus erinaceus* extracts added to separate wells. An ethanolic solvent control that consisted of 50 % aqueous ethanol alone that had been heated in a 75^{o} C water bath for 3 h to evaporate the ethanol was also used; this was added to the control well. Treated bacterial plates were incubated under the appropriate conditions noted above, after which the zone of clearing around each well was measured using digital callipers.

2.2.3.3 Broth microdilution assay

Minimum Inhibitory Concentration (MIC)

An MIC assay was performed using the micro-dilution method as described by Valgas et al. (2007). All bacterial cultures were adjusted to 0.5 McFarland scale (absorbance of 0.085 at 625nm) as previously described by Balouiri et al. (2016). One hundred microlitres of double-strength Mueller-Hinton broth was added to all the wells in a 96-well plate. A doubling dilution of the broth was performed in the first and twelfth well in a row by adding 100 µl of sterile distilled water to the wells. The first well in this experiment acted as a control with no antimicrobial whilst the twelfth well acted as a control with no bacteria. These doubling dilutions were prepared by mixing the broth and the water using a pipette and then discarding 100 µl of the mixture after the dilution to leave 100 µl of diluted broth in the well. To the second well in the row, 100 µl of the plant extract was added to the 100 µl of broth, and mixed by pipetting up and down. This produces a doubling dilution (1/2) of the stock extract. One hundred microlitres was subsequently transferred from the second well into the next well, producing a further 2-

fold dilution (1/4). This process of transferring 100 µl was repeated, to produce a 2-fold dilution series down to 1/1024 dilution. From the eleventh well, 100 µl was removed and discarded so as to maintain a consistent volume (100 µl) in all of the wells. Four microlitres of bacterial culture was added to each well, except for the negative control well which was reserved as a second control (no bacteria). The bacteria used for this study were *P. aeruginosa*, *S. aureus* and *E. coli* strains. For each bacterial species tested, duplicate rows in the microtitre plate were prepared as above. Inoculated microtitre plates were incubated overnight at 37^{o} C. The microtitre plates were observed for the presence or absence of bacterial growth after incubation. The duplicate rows were observed for any variability, which may arise due to contamination or experimental error, and the mean inhibitory concentration calculated from the two rows of wells. The lowest concentration that inhibited bacterial growth was considered to be the minimum inhibitory concentration of the crude extract. This process was also repeated for all the extracts with the solvent control inclusive. This experiment was repeated in triplicate for each bacterial species tested.

Minimum Bactericidal Concentration (MBC)

An MBC assay was performed as previously described by Ncube et al. (2008). After identifying the dilutions of each extract that inhibited bacterial growth, the MBC was determined. For each dilution which inhibited microbial growth, a sterile swab was used to subculture the test sample onto fresh Mueller-Hinton agar. These plates were then incubated under the appropriate conditions, as noted above. Plates were observed after incubation for the presence or absence of bacterial growth. The MBC was defined as the lowest concentration of extract that permitted no bacterial growth. This experiment was also repeated in triplicate for each bacterial species.

2.2.4 Statistical analyses

Means and standard deviations of the data were obtained using SPSS version 25. Analysis of variance (ANOVA) was initially used to compare the data, with the *post-hoc* Tukey test used to identify specific differences within each data group. A *P*-value of less than 0.05 was considered as statistically significant for the purposes of this work.

2.3.0 RESULTS

2.3.1 Antibiotic Susceptibility assay

Results from the antibiotic susceptibility assay shows that erythromycin demonstrated antimicrobial activity against *S. aureus*, MRSA, *S. epidermidis*, *K. pneumoniae*, *C. difficile* NCTC 11204, *C. difficile* 001, *C. difficile* 002, *C. difficile* 003, *P. acnes* NCTC 737, *P. acnes* LI77, *P. acnes* LI88, *P. acnes* SI5 and *P. acnes* SI8 with zones of inhibition as shown in Table 2.1. There was no significant difference in the antimicrobial activity of erythromycin between these bacteria that showed zones of inhibition ($P > 0.05$). There was no zone of inhibition observed after erythromycin was tested on *Pseudomonas aeruginosa* (Table 2.1)

Table 2.1 Antibiotic susceptibility assay showing the zone of inhibition of erythromycin, gentamicin colistin, penicillin, vancomycin and ampilicillin against *Pseudomonas aeruginosa, Staphylococcus aureus,* Methicillin-resistant *Staphylococcus aureus, Staphylococcus epidermidis, Klebsiella pneumoniae, Clostridium difficile* strains and *Propionibacterium acnes* strains.

Microbial species used	ZONE OF INHIBITION (mm)					
	Erythromycin	Gentamicin	Colistin	Penicillin	Vancomycin	Ampicillin
P. aeruginosa	0.0 ± 0.0	17.4 ± 1.1	13.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
S. aureus	34.0 ± 1.6*	26.1 ± 0.8	21.3 ± 1.2	34.8 ±3.0**	14.6 ± 0.3	33.7 ± 1.1***
MRSA	11.3 ± 1.5*	31.3 ± 0.7	17.4 ± 1.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
S. epidermidis	10.5 ± 1.1*	34.0 ± 0.9	19.6 ± 2.3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
K. pneumoniae	11.2 ± 1.7*	24.1 ± 1.1	13.5 ± 1.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
***C. difficile* NCTC 11204**	10.4 ± 0.2*	16.3 ± 0.8	18.3 ± 0.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
C. difficile 001	10.1 ± 0.4	17.6 ± 0.7	17.3 ± 0.6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
C. difficile 002	10.2 ± 0.7	16.5 ± 0.5	16.6 ± 0.7	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
C. difficile 003	10.1 ± 0.3	15.3 ± 1.1	18.0 ± 0.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
P. acnes NCTC737	10.4 ± 0.5*	36.1 ± 1.7	28.4 ± 1.4	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
P. acnes LI77	10.5 ± 0.6	28.2 ± 1.3	26.4 ± 1.6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
P. acnes LI88	10.1 ± 0.3	26.4 ± 0.4	25.6 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
P. acnes SI5	10.7 ± 0.5	22.3 ± 1.8	27.1 ± 0.8	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
P. acnes SI8	10.3 ± 0.2	25.8 ± 1.2	26.8 ± 1.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

Data represented as mean ± Standard deviation (SD); (-) represents no observable zone of inhibition; n = 3; * =P < 0.05 vs *P. aeruginosa* (erythromycin); ** = P < 0.05 vs *S. epidermidis* (Penicillin); *** = P < 0.05 vs *P. aeruginosa* (Ampicillin).

Pseudomonas aeruginosa, *S. aureus*, MRSA, *S. epidermidis*, *K. pneumoniae*, *C. difficile* NCTC 11204, *C. difficile* 001, *C. difficile* 002, *C. difficile* 003, *P. acnes* NCTC 737, *P. acnes* LI77, *P. acnes* LI88, *P. acnes* SI5 and *P. acnes* SI8 all demonstrated zones of inhibition to the antimicrobial activity of gentamicin. There was no significant difference in the antimicrobial activity of gentamicin against the bacteria that showed zones of inhibition (P > 0.05), although *P. acnes* demonstrated the biggest zones of inhibition while *C. difficile* demonstrated the smallest zones of inhibition (Table 2.1).

Pseudomonas aeruginosa, *S. aureus*, MRSA, *S. epidermidis*, *K. pneumoniae*, *C. difficile* NCTC 11204, *C. difficile* 001, *C. difficile* 002, *C. difficile* 003, *P. acnes* NCTC 737, *P. acnes* LI77, *P. acnes* LI88, *P. acnes* SI5 and *P. acnes* SI8 all demonstrated zones of inhibition to the antimicrobial activity of colistin. There was no significant difference in the antimicrobial activity of colistin against all the bacteria ($P > 0.05$) (Table 2.1).

All of the bacteria, except for *S. aureus*, did not demonstrate zones of inhibition against the activity of penicillin, vancomycin and ampicillin. *Staphylococcus aureus* demonstrated zones of inhibition to penicillin, vancomycin and ampicillin respectively (Table 2.1).

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2.3.2 Agar well diffusion assay

The antimicrobial activities of crude extracts from all of the plant samples (*Dracaena mannii, Ficus carica, Cassia occidentalis, Prosopis africana, Pterocarpus erinaceus, Uvaria chamae* leaf and *Uvaria chamae* woody stem) were analysed using the agar well diffusion assay against *Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus*, as described in Section 2.2.3. The results of these agar well diffusion assays are presented in Table 2.2. Crude extracts from three plant samples - *Prosopis africana*, *Uvaria chamae* leaf and *Uvaria chamae* woody stem - demonstrated antimicrobial activities against *S. aureus*. *Prosopis africana* crude extract was the only extract that demonstrated antimicrobial activity against both *P. aeruginosa* and *S. aureus* with zones of inhibition (Table 2.2). Although there was no significant difference in the antimicrobial activity of *P. africana* crude extract against *S. aureus* and *P. aeruginosa*, *P. africana* crude extracts showed larger zones of inbition against *S. aureus* than *P. aeruginosa* (Table 2.2).

Table 2.2 Antimicrobial activity of selected herbal extracts against *Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus* analysed using the agar well diffusion assay to determine the zone of inhibition.

	DIAMETER OF INHIBITION ZONE (MM)		
Crude extract	***E. coli***	***P. aeruginosa***	***S. aureus***
Dracaena mannii	-	-	-
Ficus carica	-	-	-
Cassia occidentalis	-	-	-
Prosopis africana	-	12.1 ± 0.5	15.3 ± 0.7
Pterocarpus erinaceus	-	-	-
***Uvaria chamae* leaf**	-	-	17.9 ± 0.9
***Uvaria chamae* woody stem**	-	-	21.8 ± 0.8
SCntrl	-	-	-

Data represented as mean ± Standard deviation (SD); (-) represents no observable zone of inhibition; n = 3; Treatment concentration = 10 mg/ml (concentrated by evaporation); SCntrl – Solvent Control.

Uvaria chamae crude extract demonstrated antimicrobial activity against *S. aureus* with a zone of inhibition (Table 2.2). *Uvaria chamae* woody stem and leaf extracts demonstrated antimicrobial activity against *S. aureus* with a zone of inhibition, but no activity was demonstrated against *P. aeruginosa* (Table 2.2). There was no significant difference in the antimicrobial activities of *P. africana*, *U. chamae* leaf or *U. chamae* woody stem extracts against *S. aureus* ($P > 0.05$). Crude extracts from *Dracaena mannii* (Bafe), *Ficus carica* (fig), *Cassia occidentalis* (Kassala), and *Pterocarpus erinaceus* (African teak) did not show any inhibitory activity against the bacteria used in the agar diffusion assay (Table 2.2).

2.3.3 Broth microdilution assay

2.3.3.1 Minimum Inhibitory Concentration (MIC)

The replicate MIC data were used to calculate the mean and standard deviation for MIC as previously demonstrated by Awouafack et al. (2013) and Carranza et al. (2015). All of the crude extracts demonstrated antimicrobial activities against *E. coli*, *P.*

aeruginosa, and *S. aureus* in the broth microdilution assay (Table 2.3)**.** The crude extracts that demonstrated the least antimicrobial activity against *E. coli* were those from *D. mannii*, *C. occidentalis* and *P. erinaceus*; each yielded a minimum inhibitory concentration (MIC) of 5 mg/ml (50 % of the starting concentration of 10 mg/ml). *Prosopis africana*, *U. chamae* leaf and *U. chamae* woody stem crude extracts demonstrated the most antimicrobial activity against *E. coli* (Table 2.3). Furthermore, *F. carica* crude extract also demonstrated antimicrobial activity against *E. coli* (Table 2.3). There was no significant difference between the crude extracts in their antimicrobial activities against *E. coli* ($P > 0.05$). Nevertheless, all of the crude extracts showed a significantly greater antimicrobial activity against *E. coli* when compared to the solvent control ($P < 0.05$) (Table 2.3).

Table 2.3 Antimicrobial activity of selected herbal extracts against *Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus* analysed using the broth microdilution assay to determine the minimum inhibitory concentration (MIC).

	Minimum Inhibitory Concentration (mg/ml)		
Crude extract	***E. coli***	***P. aeruginosa***	***S. aureus***
Dracaena mannii	5.0 ± 0.0	3.33 ± 1.4	1.25 ± 0.0*
Ficus carica	2.5 ± 0.0	1.25 ± 0.0	0.26 ± 0.90*
Cassia occidentalis	5.0 ± 0.0	1.25 ± 0.0	0.21 ± 0.90*
Prosopis africana	1.25± 0.0	1.04 ± 3.6	0.065± 0.23
Pterocarpus erinaceus	5.0 ± 0.0	5.0 ± 0.0	1.25 ± 0.0*
***Uvaria chamae* leaf**	1.25 ± 0.0	1.67 ± 7.2	0.0391 ± 0.0
***Uvaria chamae* woody stem**	1.25 ± 0.0	1.25 ± 0.0	0.0098 ± 0.0
SCntrl	-	-	-

Data represented as mean ± Standard deviation (SD); (-) represents no observable antimicrobial activity; n = 3; Starting concentration = 10 mg/ml; SCNTRL – Solvent Control. **Note**: There was a significant difference between the extract treatments and the control for all the bacteria ($P < 0.05$); * =$P < 0.05$ vs *Uvaria chamae* bark (*S. aureus)*

The crude extracts that demonstrated the least antimicrobial activity against *P. aeruginosa* were those from *D. mannii* and *P. erinaceus* (Table 2.3). *Ficus carica*, *C. occidentalis*, *U. chamae* leaf and *U. chamae* woody stem crude extracts each

demonstrated antimicrobial activity against *P. aeruginosa* (Table 2.3). Although *P. africana* crude extract showed the most antimicrobial activity against *P. aeruginosa*, there was no significant difference in the MIC values when compared to the other crude extracts ($P > 0.05$). However, when compared to the solvent control, all of the crude extracts showed significant antimicrobial activity against *P. aeruginosa* ($P < 0.05$).

The crude extracts that demonstrated the least antimicrobial activity against *S. aureus* were those from *D. mannii* and *P. erinaceus* (Table 2.3). *Ficus carica* and *C. occidentalis* crude extracts demonstrated a significantly lower MIC against *S. aureus* than *D. mannii* and *P. erinaceus* ($P < 0.05$) (Table 2.3). *Prosopis africana* and *U. chamae* leaf crude extracts demonstrated a significantly lower MIC against *S. aureus* than *D. mannii*, *P. erinaceus*, *F. carica* and *C. occidentalis* crude extracts ($P < 0.05$) (Table 2.3). *Uvaria chamae* woody stem crude extract demonstrated the most antimicrobial activity against *S. aureus* with an MIC value which was significantly higher than the other crude extracts ($P < 0.05$). All of the crude extracts demonstrated a significantly lower MIC against *S. aureus* when compared to the solvent control ($P < 0.05$).Furthermore, all crude extracts demonstrated significantly lower MIC values against *S. aureus* compared to *P. aeruginosa* ($P < 0.05$) (Table 2.3).

2.3.3.2 Minimum Bactericidal Concentration (MBC)

The minimum bactericidal concentration of the crude extracts against *E. coli*, *P. aeruginosa* and *S. aureus* was determined (Table 2.4).

The solvent control and the *P. erinaceus* crude extract showed no bactericidal effect against *E. coli*. The crude extracts that demonstrated the least bactericidal effect against *E. coli* were those from *D. mannii* and *C. occidentalis* (Table 2.4). *Prosopis africana* and *U. chamae* woody stem crude extracts demonstrated the greatest bactericidal

effect against *E. coli* (Table 2.4). Furthermore, *F. carica* and *U. chamae* leaf crude extract demonstrated a minimum bactericidal concentration against *E. coli* of 2.5 mg/ml. There was no significant difference between the crude extracts in their minimum bactericidal concentration against *E. coli* ($P > 0.05$) (Table 2.4). However, in comparison with the solvent control and the *P. erinaceus* crude extract, all of the other crude extracts yielded significantly reduced minimum bactericidal concentrations against *E. coli* ($P < 0.05$).

Table 2.4 The minimum bactericidal concentration of the selected herbal extracts against *Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus*.

	Minimum Bactericidal Concentration mg/ml		
Crude extract	***E. coli***	***P. aeruginosa***	***S. aureus***
Dracaena mannii	5.00 ± 0.0	-	2.50 ± 0.0*
Ficus carica	2.50 ± 0.0	5.00 ± 0.0	1.25 ± 0.34*
Cassia occidentalis	5.00 ± 0.0	5.00 ± 0.0	1.25 ± 0.34*
Prosopis africana	1.25 ± 0.0	1.25 ± 3.61	0.625 ± 0.26*
Pterocarpus erinaceus	-	-	2.50 ± 0.0*
***Uvaria chamae* leaf**	2.50 ± 0.0	5.00 ± 0.0	0.156 ± 0.0
***Uvaria chamae* woody stem**	1.25 ± 0.0	2.50 ± 0.0	0.0789 ± 0.0
SCntrl	-	-	-

Data represented as mean ± Standard deviation (SD); (-) represents no bactericidal activity; n = 3; Starting concentration = 10 mg/ml; SCNTRL – Solvent Control. Note: Excluding *D. mannii* and *P. erinaceus*, there was a significant difference between the extract treatments and the control for all the bacteria ($P < 0.05$); * =$P < 0.05$ vs *Uvaria chamae* bark (*S. aureus*).

The crude extracts that did not demonstrate bactericidal activity against *P. aeruginosa* were those from *D. mannii* and *P. erinaceus* (Table 2.4). *Ficus carica*, *C. occidentalis*, and *U. chamae* leaf crude extracts demonstrated the highest minimum bactericidal concentration against *P. aeruginosa* (Table 2.4). *Prosopis africana* crude extract demonstrated the lowest minimum bactericidal concentration against *P. aeruginosa*. On the other hand, the *U. chamae* woody stem crude extract yielded a minimum bactericidal concentration against *P. aeruginosa* of 2.50 mg/ml. Although the

P. africana crude extract showed the lowest minimum bactericidal concentration against *P. aeruginosa*, there was no significant difference between this and the other crude extracts, except for those from *D. mannii* and *P. erinaceus* ($P > 0.05$). Nevertheless, all of the crude extracts - except those from *D. mannii* and *P. erinaceus* - demonstrated significantly lower minimum bactericidal concentrations against *P. aeruginosa* than the solvent control ($P < 0.05$) (Table 2.4).

The crude extracts that were least bactericidal against *S. aureus* were those from *D. mannii* and *P. erinaceus* (Table 2.4). *Ficus carica* bark and *C. occidentalis* crude extracts both demonstrated bactericidal activity against *S. aureus* (Table 2.4). *Prosopis africana* and *U. chamae* leaf crude extracts both yielded lower minimum bactericidal concentrations against *S. aureus* than crude extracts from *D. mannii*, *P. erinaceus*, *F. carica* and *C. occidentalis*, (Table 2.4). *Uvaria chamae* woody stem crude extract yielded a significantly lower minimum bactericidal concentration against *S. aureus* than the other crude extracts ($P < 0.05$). Furthermore, all crude extracts demonstrated significantly lower MBC values against *S. aureus* compared to *P. aeruginosa* ($P < 0.05$) (Table 2.4).

2.4.0 DISCUSSION

2.4.1.0 Antimicrobial assay

2.4.1.1 Antibiotic susceptibility assay

Before using the bacterial strains for experimental analysis, the susceptibility and resistance of these bacterial strains were tested against a selection of known antibiotics (Table 2.1). A manual (M100 performance standards for antimicrobial susceptibility testing) from the Clinical Laboratory Standards Institute (CLSI) (2017) was used as a guide to make appropriate reference after the assay (Appendix 1).

Pseudomonas aeruginosa tested was susceptible to Gentamicin because it had a zone of inhibition, which is greater than 15 mm (Appendix 1). Gentamicin is an aminoglycoside that interferes with the synthesis of protein by binding to the 30S ribosomal sub-unit. The general effect and activities of gentamicin on bacteria is bactericidal (Coyle 2005). Kadurugamuwa et al. (1993) explained that the effectiveness of gentamicin against *P. aeruginosa* and other Gram-negative bacteria was due to its ability to inhibit protein synthesis and perturb bacterial cell surface.

Pseudomonas aeruginosa strain showed an intermediate response against colistin because their zones of inhibition (Table 2.1) fell within the intermediate range of 13mm - 15 mm as specified by CLSI (Appendix 1). Furthermore, CLSI (2017) further advised that colistin should be combined with another antibiotic to have an effective response because of the mode of action of colistin. Being a cationic polypeptide, the mode of action for colistin involves interactions with anionic lipopolysaccharide molecules on the cell membrane, leading to the displacement of calcium and magnesium ions, which leads to the permeability of the cell membrane, disrupting cellular processes within the cell (Falagas et al. 2005).

The *Pseudomonas aeruginosa* strain tested was considered to be resistant against erythromycin, penicillin, vancomycin and ampicillin because no zone of inhibition was observed with these (Table 2.1). Furthermore, CLSI (2017) advised that bacteria with zones of inhibition less than 12 mm for these antibiotics can be termed as resistant (Appendix 1).

The *Staphylococcus aureus* strain (NCTC 6591) was susceptible to erythromicin, gentamicin, penicillin and ampicillin because the zones of inhibition for these antibiotics (Table 2.1) were greater than the specified 25 mm cut off (Appendix 1). The mode of action for erythromycin is similar to gentamicin except that erythromycin interferes with

protein synthesis by binding to the 50S ribosomal sub-unit, whilst gentamicin binds to the 30S ribosomal sub-unit (Coyle 2005). Penicillin and ampicillin also have a similar mode of action to each other in that they interfere with the trans-peptidation reaction, which further inhibits the cross-linking of peptide chains in the cell wall, resulting in the destruction of the cell wall (Erlanger and Goode 1967; Coyle 2005). One of the factors contributing to the susceptibility of *S. aureus* and most Gram-positive bacteria to penicillin and ampicillin is the structure of the cell wall. The cell wall structure of Gram-positive bacteria contains two major components; the teichoic acids and the peptidoglycan layer and is less complex than the Gram-negative bacterial cell wall that contains a complex array of components (Coyle 2005).

Staphylococcus aureus strain (NCTC 6591) was considered to have an intermediate response against colistin and vancomycin because the zone of inhibition for these (Table 2.1) fell within the intermediate range as indicated by the CLSI reference tables (2017). The Methicillin resistant strains of *Staphylococcus aureus* (MRSA) were considered to be susceptible to gentamicin because the zone of inhibition generated by this antibiotic was greater than the 25 mm specified by CLSI (2017). On the other hand, the MRSA was considered to have an intermediate response to erythromycin because the zone of inhibition generated by this was within the intermediate range of 8 mm - 16 mm (CLSI 2017). The MRSA was observed to be resistant to colistin, penicillin, vancomycin and ampicillin (CLSI 2017). For colistin, the zone of inhibition was found to be below 19 mm, whilst for penicillin, vancomycin and ampicillin, no zone of inhibition at all was observed when tested against MRSA, making MRSA resistant to these antibiotics (Appendix 1). Differences in the sensitivity of *S. aureus* strain and MRSA against erythromycin, colistin, penicillin, vancomycin and ampicillin (Table 2.1) can be attributed to the expression of resistance genes in MRSA which are not found in

methicillin susceptible *Staphylococcus aureus* (MSSA) strains (Enright et al. 2002). The resistance genes stop antibiotics from deactivating the relevant enzymes and proteins responsible for cell wall synthesis (Enright et al. 2002).

The *Staphylococcus epidermidis* strain (RP62A) was found to be susceptible to gentamicin because the zone of inhibition generated by gentamicin was greater than 25 mm (Appendix 1). *Staphylococcus epidermidis* showed an intermediate response to colistin because the zone of inhibition (Table 2.1) fell within the intermediate range as specified by CLSI (2017). The *Staphylococcus epidermidis* strain tested was found to be resistant to erythromycin, penicillin, vancomycin and ampicillin as specified by CLSI (2017)

The *Klebsiella pneumoniae* strain tested was found to be susceptible to gentamicin because the zone of inhibition was greater than 15 mm (Appendix 1). On the other hand, *Klebsiella pneumoniae* strain tested showed an intermediate response against colistin (Table 2.1) (CLSI 2017). *Klebsiella pneumoniae* was resistant to erythromycin, penicillin, vancomycin and ampicillin. As specified by CLSI (2017), a zone of inhibition less than or equal to 12 mm generated by erythromycin on *K. pneumoniae* is termed as resistant. Also, as specified by CLSI (2017), zones of inhibition generated on *K. pneumoniae* by penicillin, vancomycin and ampicillin that are less than 15 mm are termed resistant (Appendix 1).

The *Clostridium difficile* and *Propionibacterium acnes* strains used were found to be resistant to erythromycin, penicillin, vancomycin and ampicillin, with zones of inhibition formed at less than 11 mm (CLSI 2017). In contrast, the *Clostridium difficile* and *P. acnes* strains used were found to be susceptible to colistin (CLSI 2017). The *P. acnes* strains was found to be susceptible to gentamicin whilst the *C. difficile* strains gave an intermediate response (CLSI 2017). Some of these clinical isolates might have been

exposed to a range of antibiotics overtime, necessitating the development of resistance against common antibiotics (Costelloe et al. 2010). Jernberg et al. (2010) explained that long-term exposure of susceptible bacterial strains to antibiotics, influences the emergence and colonisation of resistant strains due to adaptability and genetic transfer.

With reference to the M100 performance standards for antimicrobial susceptibility testing (CLSI 2017), most bacteria used showed some degree of resistance against common antibiotics used for the treatment of infections, thereby making them suitable candidates for antimicrobial and anti-biofilm analysis in this present study.

2.4.1.2 Determination of the antimicrobial activities of a selection of medicinal plants from The Gambia

Plant materials and products are known to possess medicinal and health benefits against microbial infections (Muthu et al. 2006). Some of these plants used in the treatment of infections in parts of Africa like The Gambia, were analysed in this present study for their antimicrobial activities against some clinically important bacteria. After performing an agar-well diffusion assay, it was clear (Table 2.2) that *Prosopis africana* crude extract demonstrated antimicrobial activity against the *Pseudomonas aeruginosa* and *Staphylococcus aureus* strains tested whilst *Uvaria chamae* (woody stem and leaf) crude extracts demonstrated antimicrobial activity against only the *S. aureus* strains. In contrast, no antimicrobial activity was observed against the *E. coli*, *P. aeruginosa* and *S. aureus* strains using crude extracts from *Dracaena mannii* (Bafe), *Ficus carica* (fig plant), *Cassia occidentalis* (Kassala), and *Pterocarpus erinaceus* (African teak) using the agar-well diffusion assay. The broth microdilution assay, on the other hand, demonstrated varying degrees of antimicrobial activity for all of the plant extracts against the *E. coli*, *P. aeruginosa* and *S. aureus* strains used. The reason for the difference in the

antimicrobial activity of the extracts after performing the agar-well diffusion and broth dilution might be attributed to the sensitivity of the assay. Valgas et al. (2007) in their study showed that the broth microdilution assay is a more sensitive and reliable method of screening antimicrobial activities of natural products than the agar-well diffusion method, which correlates with the results obtained in this present study. The reason why agar – well diffusion is a less sensitive and less reliable method of screening the antimicrobial activity of plant materials than the broth dilution method, is because of the lower rate of solubility and diffusibility of the extract in the medium in agar – well diffusion method than in the broth dilution method (Vlietinck 1991). Moreover, agar – well diffusion method is qualitative and cannot be utilised to assess the bacteriostatic and bactericidal effect of a plant material whilst the broth dilution method is both qualitative and quantitative, and can be utilised for assessing the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of a plant extract (Eloff 1998).

Dracaena mannii is amongst the plants used in remote parts of Central and West Africa to prevent bacterial infections during childbirth, prevention and treatment of infections in wounds and burns (Malan and Neuba 2011). Despite the use of this plant, only a limited number of studies into the antimicrobial activities of *D. mannii* have been performed. For example, Sofowora and Olaniyi (1975) examined the antimicrobial activity of petroleum ether and ethanol extracts of *D. mannii* against strains of *S. aureus*. The workers found that, the ethanolic extract of *D. manni* demonstrated a greater antimicrobial activity than the petroleum ether extract of *D. manni* against strains of *S. aureus* at an inhibitory concentration range of 5 mg/ml – 12 mg/ml, which has the same inhibitory concentration range to the one obtained in this current study (Sofowora and Olaniyi 1975). In addition, Okunji et al. (1990) reported on the antifungal properties of a saponin (DM-1) isolated from the fruit of *D. mannii* against *Candida albicans*. In this

present study, *Dracaena mannii* crude extract demonstrated antimicrobial activities against all of the strains of *E. coli*, *P. aeruginosa* and *S. aureus* tested in the broth microdilution assay, with *S. aureus* proving to be the most susceptible of the three bacteria. Apart from the two studies mentioned earlier, there was no published study on the antimicrobial activity of *D. mannii* against any bacterial strain to the knowledge of the author. The antimicrobial activity of *D. mannii* crude extract demonstrated in this current study, justifies the use of this plant for the treatment of infections caused by the tested pathogens and gives new opportunities for further antimicrobial study against a wide range of bacterial species.

Ficus carica crude extract has been reported to possess a range of biological activities, including antimicrobial activity (Aref et al. 2010). Aref et al. (2010) demonstrated the antimicrobial activity of an ethanolic extract of *F. carica* against *E. coli*, *P. aeruginosa* and *S. aureus*. In contrast, no antimicrobial activity of *F. carica* was observed against the bacterial species tested in an agar-well diffusion assay. The group attributed this negative result to the sensitivity of this assay compared with the broth dilution assay (Aref et al. 2010). The workers speculated that the lower sensitivity of agar-well diffusion assay when compared to the broth dilution assay might be attributed to the solubility of *F. carica* extract in the media of both assays (Aref et al. 2010), thus agreeing with the suggestion made by Vlietinck (1991), as mentioned earlier. Interestingly, the inhibitory concentration range (0.33 mg/ml – 2.5 mg/ml) of *F. carica* extracts against the tested bacterial strains was the same as the inhibitory concentration range obtained in this current study. Similarly, Oliveira et al. (2009) found no antimicrobial activity against *E. coli*, *P. aeruginosa* and *S. aureus* when *F. carica* crude extract was tested in an agar-well diffusion assay. In this present study, antimicrobial activity of *F. carica* crude extract and the bactericidal activity of *F. carica* crude extracts against *E. coli*, *P. aeruginosa* and *S.*

aureus were observed (Tables 2.3 and 2.4), although no antimicrobial activity was observed when the agar-well diffusion assay was used (Table 2.2). The reason for this, might be purely down to the sensitivity of the assay used. For example, broth micro-dilution method is more sensitive than agar-well diffusion assay due to the ease of diffusibility and solubility of the antimicrobial in the broth micro-dilution than in the agar-well diffusion assay (Eloff 1998). Furthermore, the antimicrobial activity of *F. carica* crude extract demonstrated in this current study, justifies the use of this plant for the treatment of infections caused by the tested pathogens.

Cassia occidentalis has been used in parts of Africa, Asia and South America for the treatment and management of diseases such as fevers, tuberculosis, anaemia, diarrhoea, wound infections, bronchitis and many other ailments (Chukwujekwu et al. 2006). Due to its therapeutic importance in remote parts of Africa, Asia and South America, *C. occidentalis*, known also as Kassala in The Gambia, was selected for the antimicrobial studies described in this thesis. In this study, *C. occidentalis* crude extract demonstrated both inhibitiory activity and bactericidal activity against the strains of *E. coli*, *P. aeruginosa* and *S. aureus* when tested in the broth micro dilution assay. Again, in contrast to this, the *C. occidentalis* extract failed to demonstrate antimicrobial activity in the agar-well diffusion assay. Studies performed by Chukwujekwu et al. (2006) showed that emodin, which is an anthraquinone extracted from the roots of *C. occidentalis*, was more active against Gram positive bacteria like *S. aureus* than Gram-negative bacteria like *E. coli*. Their findings correlated with the findings in this current study, which demonstrated *S. aureus* as the most susceptible bacterial species to *C. occidentalis* crude extract when compared to other bacteria tested. A previous study by Samy and Ignacimuthu (2000) showed that methanolic extracts from the leaves of *C. occidentalis* were active against both Gram-positive bacteria like *S. aureus* and Gram-negative

bacteria like *E. coli*. A review by Yadav et al. (2010) goes further to explain that the use of different solvents for the extraction of *C. occidentalis*, affects the type of active compound being extracted due to the solubility index of such compounds in the solvent. This can cause a variation in the antimicrobial activity of *C. occidentalis* extracts obtained using different solvents, as was the case in the two studies highlighted previously. In addition, the use of different plant parts can lead to variations in the antimicrobial activity of the extracts prepared due to the spatial compartmentalisation of the active compounds into different parts of the plant (Yadav et al. 2010). For example, a study by Castello et al. (2002), showed that the ethanolic leaf extract of *B. orellana* demonstrated a greater antimicrobial activity against *Bacillus pumilus* than the ethanolic root extract of *B. orellana*, therefore suggesting that the compounds with antimicrobial activity against the tested bacteria, might be more concentrated in the leaf than other parts of the plant. In this present study, *C. occidentalis* was active against both Gram-positive bacteria and Gram-negative bacteria, although *S. aureus* was more sensitive than *E. coli* or *P. aeruginosa*.

Pterocarpus erinaceus is reportedly used in parts of Africa for the treatment and management of infections like diarrhoea, toothache, fever and dysentery (Gabriel and Onigbanjo 2010). In the present study, *P. erinaceus* crude extract showed inhibitory activity against the *E. coli*, *P. aeruginosa* and *S. aureus* strains used, but it was only bactericidal against *S. aureus*. In contrast to this, but in line with the other plant extracts tested and described above, the *P. erinaceus* extract failed to exhibit antimicrobial activity against any of the bacterial strains tested in an agar-well diffusion assay. Similarly, Lagnika et al. in 2011 demonstrated the antimicrobial activity of *P. erinaceus* extracts against *E. coli*, *P. aeruginosa* and *S. aureus* at a concentration of 0.31 – 10 mg/ml, which was similar to the MIC range obtained in this current study. Gabriel and Onigbanjo

(2010) also found *P. erinaceus* crude extracts to have antimicrobial activity against *E. coli*, *P. aeruginosa* and *S. aureus.* Interestingly, this group used an agar-well diffusion assay although they suggested that extraction methods and solvents play an important role in the extraction of bioactive compounds, which influences the antimicrobial activity of an extract. In their study, Gabriel and Onigbanjo (2010) showed that methanol extract of *P. erinaceus* had antimicrobial properties against *E. coli* and *P. aeruginosa* while hexane extract and ethyl acetate extract of *P. erinaceus* showed no antimicrobial activity against *E. coli* and *P. aeruginosa*. In this present study, the ethanolic extract of *P. erinaceus* was active against all the bacteria (*E. coli*, *P. aeruginosa* and *S. aureus*) tested. The findings in this current study support the use of *P. erinaceus* for the treatment of infections associated with the tested bacteria.

In the current study, *Prosopis africana* crude extract demonstrated antimicrobial activity against the strains of *E. coli*, *P. aeruginosa* and *S. aureus* tested in the broth microdilution assay used. Moreover, *P. africana* crude extract was the only extract that demonstrated antimicrobial activity against *P. aeruginosa* after performing an agar-well diffusion assay (Table 2.2), and, out of all those tested here, it was one of the extracts that demonstrated inhibitory and bactericidal effects against *E. coli* and *P. aeruginosa* (Table 2.3 and 2.4). Consequently, *P. africana* crude extract was selected as the most promising candidate for further studies into its antimicrobial and antibiofilm activity, most especially against Gram-negative bacteria. One commonly known use of *P. africana* in parts of Africa is in wound care and in the treatment of bacterial infections (Ezike *et al*. 2010). In laboratory studies, *P. africana* crude extract demonstrated antimicrobial activities against *E. coli* and *P. aeruginosa* with an MIC value range of 1.562 mg/ml – 6.25 mg/ml (Ajiboye et al. 2013). This MIC value was similar to the MIC value obtained in this present study. Furthermore, the MIC and MBC values of *P. africana* extract falls

within the cut-off concentration of 1 mg/ml that has been suggested as the level at which plant materials can be considered to be antimicrobial (Carranza et al. 2015). This makes *P. africana* extract a suitable choice for further study. Despite its medicinal uses, there had been limited study into the antimicrobial activity of *P. africana* done to the knowledge of the author.

In this study, both *Uvaria chamae* leaf crude extracts and woody stem crude extracts demonstrated antimicrobial activity against the *E. coli*, *P. aeruginosa* and *S. aureus* strains used in both the agar-well diffusion assay and the broth microdilution assay. With the agar-well diffusion assay though, only the *S. aureus* strain used was sensitive to the *U. chamae* leaf and woody stem crude extracts (Table 2.2). The *U. chamae* leaf and woody stem crude extract yielded the lowest MIC and MBC values of all the plant extracts tested against *S. aureus*, and also one of the lowest MIC and MBC values of all against the *S. aureus*, *E. coli* and *P. aeruginosa* strains (Table 2.3 and 2.4). On comparing the antimicrobial activity of *U. chamae* leaf crude extract with that of *U. chamae* woody stem crude extract, the bark extract demonstrated greater antimicrobial activity than did the leaf extract. As previously highlighted, a possible reason why *U. chamae* woody stem crude extract demonstrated a greater antimicrobial activity than *U. chamae* leaf extract might be due to the spatial compartmentalization of active compounds into different parts of the plant as suggested by Yadav et al. (2010). This can lead to variations in the antimicrobial activity of the extracts prepared from the different plant parts. For example, a study performed by Badakhshan et al. in 2009, showed that the methanolic leaf extract of *Lantana camara* demonstrated the most antimicrobial activity against *B. cereus* and *S.* Typhi than the methanolic flower, stem and root extracts of the plant. The workers suggested that the active compounds with antimicrobial activity against the tested bacterial strains might be more concentrated in the leaf than other parts

of the plant (Badakhshan et al. 2009). Consequently, *U. chamae* crude extract was selected as the most promising candidate for further studies into its antimicrobial and antibiofilm activity. In a comparative assessment of the antimicrobial activity of different *U. chamae* plant parts, Oluremi et al. (2010) reported that an extract prepared from the stem bark of *U. chamae* demonstrated greater antimicrobial activity against *E. coli, P. aeruginosa, S. aureus,* MRSA, *Proteus* spp. and *Klebsiella* spp. than did an extract prepared from the leaf of *U. chamae*. The group reported on the other hand that an extract prepared from the stem bark and an extract prepared from the root of *U. chamae* had a similar degree of antimicrobial activity (Oluremi et al. 2010). Their suggestion was that the phytochemicals responsible for the antimicrobial properties in *U. chamae* might be localised in the stem and bark region of the plant, but further studies would be needed to elucidate this. The findings of the current study, which also showed that extracts from *U. chamae* woody stem possessed greater antimicrobial activity than extracts from its leaf, corroborate those of Oluremi et al. (2010). Furthermore, the MIC and MBC values of *U. chamae* woody stem extracts falls within the cut-off concentration of 1mg/ml that has been suggested as the level at which plant materials can be considered to be antimicrobial (Carranza et al. 2015).

2.4.2 Conclusion

In this study, the medicinal plants used, demonstrated antimicrobial activity against some of the bacterial strains which were resistant to some of the commonly used antibiotics. However, *P. africana* crude extract demonstrated the most antimicrobial activity out of all the plant extracts tested against the Gram-negative bacteria used. In contrast, the *U. chamae* woody stem crude extract, demonstrated the most antimicrobial activity against *S. aureus*, in comparison with the other extracts. Furthermore, the MIC

and MBC values of *U. chamae* woody stem crude extract and *P. africana* crude extract fell within the suggested MIC level for plant antimicrobials. This necessitated the choice of *P. africana* crude extract and *U. chamae* woody stem crude extract as better candidates for more in-depth antimicrobial and antibiofilm studies.

CHAPTER THREE

3.0 EXTRACTION EFFECIENCY OF *PROSOPIS AFRICANA* AND *UVARIA CHAMAE* USING DIFFERENT EXTRACTION METHODS AND THE PHYTOCHEMICAL SCREENING OF EXTRACTS

3.1.0 INTRODUCTION

Extraction is one of the most important steps for the antimicrobial study of plant samples. This process ensures that bioactive components from the plant tissues are released into the extracting solvent for use. In most plant studies, extraction of plant material falls under the "sample preparation" techniques (Azmir et al. 2013). Furthermore, there are common factors that affect the extraction of plant components. Some of these factors are: matrix of the plant part; the solvent used for extraction; temperature applied during the extraction; pressure applied during the extraction; and time taken to run the extraction. Interestingly, the last four factors are responsible for the difference in the extraction conditions amongst different extraction methods (Sasidharan et al. 2011).

Solvent plays an important role in an extraction process. This is because it serves as a vehicle by which compounds are removed from the plant matrix. The removal of compounds from the plant tissues into the extracting solvent is dependent on the polarity of the compound to create an affinity with the solvent, necessitating the movement of the compound from the plant tissue to the solvent (Azmir et al. 2013). Some of the commonly used solvents for extractions are water and ethanol. Water, being a universal solvent, has been used for centuries for the extraction of compounds from medicinal plants for the treatment of infection. These plants are infused in cold or warm water for a duration of

time; or boiled in water for a duration of time to enhance the extraction of the plant components (Sasidharan et al. 2011). Ethanol has been effectively used by researchers in extracting active components from plant material for research purposes (Kampf et al. 2002). It is also important to note that ethanol demonstrates antimicrobial properties and is often included as an ingredient in disinfectants and biocides (alcohol wipes/gels/sanitisers) (Kampf et al. 2002). Consequently, there is a need to reduce or completely remove the ethanolic variable (content) of an extract prior to any antimicrobial assay so as not to interfere with the result. The method used in reducing the ethanol content in this current study was to evaporate the ethanol from the solution. Mostly, 50% ethanol was used to re-suspend the dried extract, and then heated to evaporate the ethanol out. Ethanol has a boiling point of approximately 78^{o} C, which is lower than the boiling point of water, therefore, the ethanol evaporates leaving behind the water when a mixture of ethanol and water is subjected to such temperatures.

Several extraction methods are used for the extraction of plant compounds, some of which are: maceration method; Soxhlet method; and ultrasound method. Maceration is a commonly used method for extracting plant compounds due to its simplicity. The process of maceration involves the grinding of plant material into smaller particles to increase the surface area for ease of diffusion. The solvent is then added to the ground material in a closed vessel for a given period, after which the resultant mixture is filtered. The filtrate is stored for use while the residual plant debris is disposed (Azmir et al. 2017). Soxhlet extraction has been widely used for extracting plant compounds (Carranza et al. 2015; Moorthy et al. 2015). Due to the high yield of extract obtained from this extraction method, Soxhlet extraction is widely accepted and also used in large scale extractions (Sasidharan et al. 2011). The process of Soxhlet extraction involves the use of a Soxhlet apparatus (Figure 3.1). Generally, an amount of dry plant material is placed in a thimble,

which is then is placed in the Soxhlet extractor. Heat is applied to the round bottom flask holding the solvent of choice, which facilitates the evaporation of the solvent into the Soxhlet extractor. On reaching to an overflow level, the solution in the Soxhlet extractor is aspirated by a siphon. The solution which contains the extracted solute is then unloaded by the siphon back into the round bottom flask. The solute remains in the round bottom flask while the solvent is being evaporated to the Soxhlet extractor. This process continues until extraction is completed (Azmir et al. 2013).

Figure 3.1 Diagram of a Soxhlet apparatus (Redfern et al. 2014)

Ultrasound is sound which is above the range of human hearing. As sound energy passes through a liquid medium the frequencies of sonication are able to form cavities (or microbubbles) within the solvent as a result of successive compression and rarefaction cycles. As these cycles continue, the cavities/bubbles eventually collapse, resulting in high stresses within the solvent, thereby agitating cells, and disrupting the cell membranes. This then leads to the lysing of cell and the movement of phytochemicals (biologically active compounds found in plants) from the plant tissues into the extraction solvent (Briars and Paniwnyk 2013). Furthermore, ultrasonic sound frequency (>20 kHz)

and low temperatures between 20° C – 30° C, are best used for optimal extraction of plant constituents using ultrasound extraction, thus preventing thermal degradation of many active plant materials. In addition, an anionic surfactant (washing up liquid) is often used in the ultra-sonic water bath to reduce the water tension, in order to encourage more effective cavitation (Briars and Paniwnyk 2013).

After obtaining the crude extracts from the extraction processes, phytochemical study of the extracts is important for determining the presence or absence of phytochemical compounds. Plant phytochemicals are responsible for the plant's physiological composition, which also influences the biological activity of the plant as explained previously (Section 1.6.0). Phytochemical analysis can be: qualitative, to determine the presence of a phytochemical group; or quantitative, to determine the amount of phytochemical present in the extracts.

As previously shown (Section 2.3.0), *P. africana* and *U. chamae* ethanolic crude extracts demonstrated the greatest antimicrobial activity when compared to other selected plant extracts, which made them potential candidates for further study. Consequently, the aim of this current experiment is to determine the extraction efficiency of different extraction methods on *P. africana* and *U. chamae* and to qualitatively determine the presence of phytochemicals in the extracts yielded.

3.2.0 MATERIALS AND METHODS

Materials

Metal grater, 0.22 µm Millipore filter (Sartorius 16534K), Soxhlet glass wares (Sigma-Aldrich Z556203-1EA), Electrothermal electromantle (EM0500/CE), extraction thimbles (Fisher 11754043), glass wool, anti-bumping granules (Fisher A/7770/50).

Reagents

Ethanol (Sigma-Aldrich), aqueous $FeCl_3$ (aq) (Sigma-Aldrich), NaOH (aq) (Sigma-Aldrich), chloroform (aq) (Sigma-Aldrich), H_2SO_4 (aq) (Sigma-Aldrich), Fehlings A (aq) (Sigma-Aldrich), Fehlings B (aq) (Sigma-Aldrich), ammonium hydroxide (aq) (Sigma-Aldrich), potassium iodide (aq) (Sigma-Aldrich), iodine solution (Sigma-Aldrich), glacial acetic acid (aq) (Sigma-Aldrich), $CuSO_4$ (aq) (Sigma-Aldrich).

Plant preparation:

The plant samples, *Uvaria chamae* (dried stem) and *Prosopis africana* (dried stem), were grated into fine powder using a metal grater. This was done in order to provide a larger surface area to enable optimal extraction of the plants' components.

3.2.1 Maceration extraction

Aqueous extraction

Aqueous extraction was performed as described by Hayouni et al. (2007). Ten grams of the ground plant material were placed into a conical flask, and 100 ml of distilled water was added to the conical flask (10% w/v). The mixture was left to stand for 24 h at room temperature. After 24 h of extraction, the plant extract was centrifuged at 1500 rpm for 5 min in order to separate the liquid extract from the plant debris. Filter sterilisation was performed afterwards using the 0.22 µm millipore filter (Sartorius 16534K). The extract was stored at a temperature of 4^{o} C for subsequent use.

Ethanol extraction

Ethanol extraction was performed as described by Hayouni et al. (2007). A 100 ml of ethanol solution (Sigma-Aldrich) was added to 10 g of the plant material in a conical flask (10 % w/v) and mixed by swirling. A glass stopper was used to seal the conical flask, and the mixture was left at room temperature for 24 h. The plant extract was poured into Universal bottles (20 ml aliquots) and centrifuged at 1500 rpm for 5 min to pellet the debris from the extract. Afterwards, the extract was filtered sterilised using 0.22 µm millipore filter to remove debris and possible bacterial contamination (0.45 µm millipore filter is small enough to remove bacterial contaminants). To avoid any interference of the antimicrobial effect of the extract, the solvent (100 % ethanol) was removed from the filtered extract using a rotary evaporator (BUCHI waterbath B-480) (Figure 3.2b). The extract left after the removal of the solvent was air-dried and removed from the round bottom flask and weighed. Storage of the extract was performed at 4^{o} C for subsequent use.

For subsequent analysis, extracts were re-solubilised in 50 % aqueous ethanol and left for 3 h in a 75^{o} C water bath to evaporate the ethanol content. Extract was re-sterilised using a 0.22 µm millipore filter. A negative control was prepared to ensure the removal of ethanol and also ensure that no remnant of ethanol interferes with subsequent results. For the preparation of the negative control, 50 % aqueous ethanol (same volume to the one used in re-suspending the extract) was left for 3 h in a 75^{o} C water bath to evaporate the ethanol content (usually, the volume of the final solution is approximately half its original volume).

3.2.2 Ultrasound extraction (sonication)

Ultrasound extraction was performed as described by Briars and Paniwnyk (2013). Ten grams of the plant material was placed into a conical flask, and 100 ml of ethanol solution was added to the conical flask and mixed by swirling. The concentration of the mixture before extraction of the plant powder in the solution was 0.1 g/ml (w/v). The conical flask was submerged in a 40 kHz ultra-sonic water bath at 30^{o} C for 90 min (Figure 3.2a). After the extraction, the plant extract was poured from the conical flask into four universal containers, and centrifuged using a benchtop centrifuge at 1500 rpm for 5 min to separate the liquid extract from the plant debris. The extract was then sterilised by filtration through a 0.22 µm millipore filter.

Figure 3.2 (a) Extraction of plant sample using the ultrasound water bath at a frequency of 40 kHz and a temperature of 30^{o} C ; (b) Removal of ethanol (solvent) from a crude extract using the rotary evaporator (BUCHI waterbath B-480)

The solvent (ethanol) was removed from the filtered extract using a rotary evaporator (BUCHI water bath B-480) (Figure 3.2b). After the removal of the solvent, the residual extract was left to air-dry (Figure 3.3a), after which the dry extract was removed from the round bottom flask using a metal spatula (Figure 3.3b), weighed (Figure 3.3c) and stored at 4^{o} C for subsequent use. For subsequent analysis, extracts were

re-suspended in 50 % aqueous ethanol and left for 3 h in a 75^{o} C water bath to evaporate the ethanol content. Extract was re-sterilised using a 0.22 µm Millipore filter.

Figure 3.3 (a) Air dried extract after solvent removal; (b) a metal spatula was used in removing the dried extract from the round bottom flask; (c) dried extract weighed after removal from the flask.

3.2.3 Soxhlet extraction

Soxhlet extraction was performed as described by Carranza et al. (2015). The Soxhlet glassware and apparatus (Figure 3.1) were set up for the commencement of the extraction process (Figure 3.4a). Ten grams of the plant powder was placed in an extraction thimble, and 150 ml of ethanol solution was added to a round bottom flask and a small amount of anti-bumping granules was added to the solvent so as to avoid flash boiling during heating. The extraction thimble containing the plant material was covered with glass wool to prevent the escape of the solid plant material into the solvent during the extraction. The thimble containing the plant material was placed into the extraction chamber of the Soxhlet apparatus.

The extraction was carried out for 4 h and 8 h for *P. africana* and *U. chamae* plant materials respectively at a temperature of 80^{o} C. After the extraction, the extract was filter-sterilised using a 0.22 µm millipore filter to remove any debris and possible bacterial contamination (Figure 3.4b). The solvent was removed from the extract under a reduced pressure, at a temperature of 35^{o} C, using the rotary evaporator. The extract left

after the removal of the solvent was air-dried and removed from the round bottom flask and weighed. Storage of the extract was performed at 4^{o} C for subsequent use.

Figure 3.4 (a) Plant material undergoing a soxhlet extraction at a temperature of 80^{o} C; (b) Filter-sterilisation of the crude extract using a 0.22 µM millipore filter to obtain a sterile extract; (c) exposed allowed to air-dry after removal of solvent using the rotary evaporator.

For subsequent analysis, extracts were re-solubilised in 50 % aqueous ethanol and left for 3 h in a 75^{o} C water bath to evaporate the ethanol content. Extract was re-sterilised using a 0.22 µm millipore filter. A negative control was prepared to ensure the removal of ethanol and also ensure that no remnant of ethanol interfered with subsequent results. For the preparation of the negative control, 50 % aqueous ethanol (same volume to the one used in re-suspending the extract) was left for 3 h in a 75^{o} C water bath to evaporate the ethanol content (usually, the volume of the final solution is approximately half its original volume).

A concentration of 15 mg/ml was made for the following extracts: *Uvaria chamae* Soxhlet (UCS) extract; *Uvaria chamae* ultrasound (UCU) extract; *Prosopis africana* Soxhlet (PAS) extract; and *Prosopis africana* ultrasound (PAU) extract for further analysis.

3.2.4.0 Phytochemical screening of crude extracts

Phytochemical screening of crude extracts was performed as described by Harbone (1998).

3.2.4.1 Test for Tannins

One millilitre of aqueous $FeCl_3$ (aq) (Sigma-Aldrich) was added to 1ml of each crude extract (*Uvaria chamae* Soxhlet (UCS), *Uvaria chamae* ultrasound (UCU), *Prosopis africana* Soxhlet (PAS) and *Prosopis africana* (PAU) ultrasound extracts) and mixed. There was no incubation condition as the colour development is instantaneous. A visual check for the development of a blue-green colour indicated the presence of tannins.

3.2.4.2 Test for Saponins

Two millilitres of distilled water was added to 1 ml of each crude extract and shaken thoroughly for 30 seconds. The formation of foam indicated the presence of Saponins.

3.2.4.3 Test for Quinones

To 1 ml of each type of crude extract, 1 ml of 1 % NaOH (aq) (Sigma-Aldrich) was added and mixed for 1 min. No incubation condition as the colour development is instantaneous. A visual check observing the development of a red colouration indicated the presence of quinones.

3.2.4.4 Test for Steroids

To 1 ml of each type of crude extract, 1 ml of chloroform (aq) (Sigma-Aldrich) was added and mixed. Subsequently, 1 ml of concentrated H_2SO_4 (aq) (Sigma-Aldrich) was added and mixed for 1 min. A visual check observing the development of a red colouration in the chloroform layer indicated the presence of steroids.

3.2.4.5 Test for reducing sugars

To 1 ml of each type of crude extract, 1 ml of a mixture of Fehlings A (aq) (Sigma-Aldrich) and Fehlings B (aq) (Sigma-Aldrich) (1:1) solutions was added and mixed for 1 min. There was no incubation condition due to the instantaneous colour development. A visual check for the development of a red colouration indicated the presence of reducing sugars.

3.2.4.6 Test for Flavonoids

To 1 ml of each type of crude extract, 1 ml of diluted ammonia (aq) (Sigma-Aldrich) was added and mixed after which 1 ml of concentrated H_2SO_4 (aq) (Sigma-Aldrich) was subsequently added. There was no incubation condition due to the instantaneous colour development. A visual check for the development of a yellow colouration indicated the presence of flavonoids.

3.2.4.7 Test for Alkaloids

To 1 ml of each type of crude extract, 1 ml of 10 % potassium iodide (aq) (Sigma-Aldrich) was first added and mixed. Immediately, 2 drops of iodine solution (Sigma-Aldrich) were added to each and mixed for 1 min. A visual check for the development of a red or brown colouration indicated the presence of alkaloids.

3.2.4.8 Test for Terpenoids

To 1 ml of each type of crude extract, 1 ml of concentrated H_2SO_4 (aq) (Sigma-Aldrich) was added and heated to 70^o C for 2 min. A visual check for the development of a grey colouration indicated the presence of Terpenoids.

3.2.4.9 Test for Cardiac glycosides

To 5 ml of each type of crude extract, 2 ml of chloroform and 3 ml of glacial acetic acid (aq) (Sigma-Aldrich), containing one drop of $FeCl_3$ (aq), was added and mixed. Immediately, 1 ml of concentrated H_2SO_4 (aq) (Sigma-Aldrich) was added and mixed for

1 min. There was no incubation condition due to the instantaneous colour development. A visual check for the development of a brown colouration indicated the presence of cardiac glycosides.

3.2.4.10 Test for Proteins

To 2 ml of each type of crude extract, 1 ml of 40 % NaOH (aq) (Sigma-Aldrich) and 2 drops of 1 % $CuSO_4$ (aq) (Sigma-Aldrich) were added and mixed for 1 min. There was no incubation condition due to the instantaneous colour development. A visual check for the development of a violet colouration indicated the presence of proteins.

3.2.5 Statistical analyses

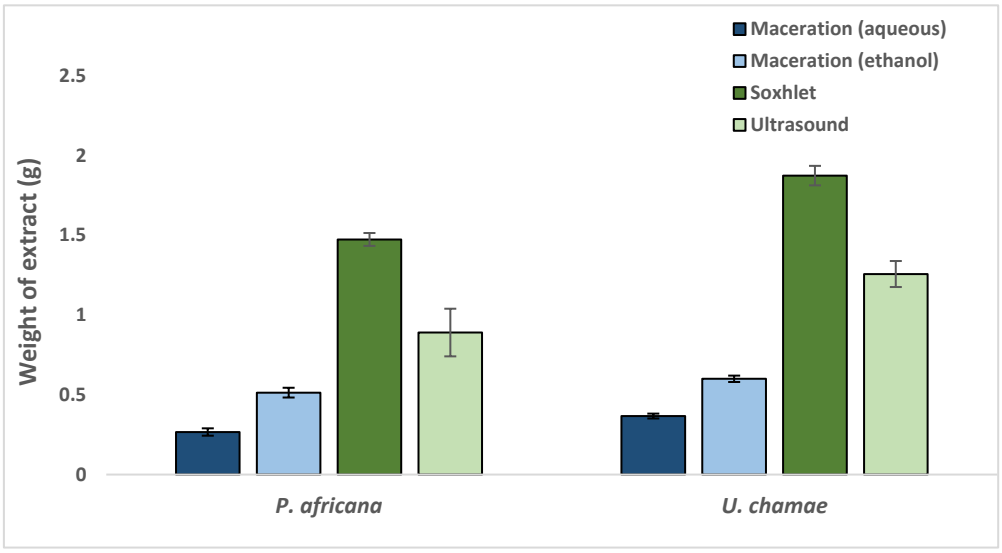
Each of the experiments described above was performed three times. Means and standard deviations of the data were obtained using SPSS version 25. Analysis of variance (ANOVA) was initially used to compare the data, with the *post-hoc* Tukey test used to identify specific differences within each data group. A *P*-value of less than 0.05 was considered as statistically significant for the purposes of this work.

3.3.0 RESULTS

3.3.1 Extraction yield of *Prosopis africana* and *Uvaria chamae*

The air-dried crude extracts of *P. africana* obtained from each of the extraction processes were observed to be a brown-coloured powdery material. The mean weights of the crude extracts obtained from 20 g of powdered *P. africana* stem/bark using maceration (aqueous), maceration (ethanol), Soxhlet and ultrasound extraction methods are shown in Figure 3.5. The amount of extract obtained from *P. africana* using the Soxhlet method was significantly greater than the amount obtained using all the other

extraction methods ($P < 0.05$). Furthermore, a significant difference was observed in the extract yield when all the extraction methods were compared to each other (Appendix 2).



Data- Mean ± SD, n=10. Significant difference was observed when all the extraction methods were compared to each other ($P < 0.05$) (Appendix 2)

Figure 3.5 Graph showing the weight of crude extracts after extraction of *Prosopis africana* and *Uvaria chamae* using Maceration, Soxhlet and ultrasound extraction methods.

The air-dried crude extracts of *Uvaria chamae* obtained from each of the extraction methods were observed to be a dark brown powdery material. However, crude extracts prepared using the Soxhlet process yielded an extract with a somewhat more oily or greasy consistency than the other three extraction methods. The mean weights of the crude extracts obtained from 20 g of powdered *U. chamae* stem/bark using maceration (aqueous), maceration (ethanol), Soxhlet and ultrasound methods are shown in Figure 3.5. The amount of extract obtained from *U. chamae* using the Soxhlet method was significantly greater than the amount obtained using all the other extraction methods ($P < 0.05$). Furthermore, a significant difference was observed in the extract yields when all the extraction methods were compared to each other (Appendix 2).

3.3.2 Phytochemical screening of *P. africana* and *U. chamae* extracts

The presence of selected phytochemicals with biological effects in plants were determined qualitatively (Section 3.2) the results presented in Table 3.1.

The presence of tannins, phenols, saponins, alkaloids, flavonoids, proteins and cardiac glycosides was observed in all extracts (Table 3.1). Furthermore, quinones and reducing sugars were only present in UCM (aq), UCM (eth), UCS extract and UCU extract. Steroids and terpenoids were absent in all extracts.

Table 3.1 Phytochemical assay of *Prosopis africana* and *Uvaria chamae* extracts

Test	PAM(aq)	PAM(eth)	PAS	PAU	UCM(aq)	UCM(eth)	UCS	UCU
Tannins	+	+	+	+	+	+	+	+
Phenols	+	+	+	+	+	+	+	+
Saponins	+	+	+	+	+	+	+	+
Quinones	-	-	-	-	+	+	+	+
Steroids	-	-	-	-	-	-	-	-
Reducing Sugars	-	-	-	-	+	+	+	+
Alkaloids	+	+	+	+	+	+	+	+
Flavonoids	+	+	+	+	+	+	+	+
Terpenoids	-	-	-	-	-	-	-	-
Proteins	+	+	+	+	+	+	+	+
Cardiac glycosides	+	+	+	+	+	+	+	+

(+) = Present, (-) = absent, aq – aqueous, eth – ethanol, PAS - *Prosopis africana* Soxhlet extract, PAU - *Prosopis africana* ultrasound extract, UCS - *Uvaria chamae* Soxhlet extract, UCU - *Uvaria chamae* ultrasound extract.

3.4.0 DISCUSSION

3.4.1 Extraction yield of *Prosopis africana* and *Uvaria chamae* when using different extraction methods

In order to explore both conventional and non-conventional methods of extraction in this research, conventional maceration, Soxhlet and ultrasound extraction techniques were used in this study to determine the optimum method for extracting bioactive compounds from both *P. africana* and *U. chamae.*

Maceration was one of the earliest and is one of the commonly used methods for extracting phytochemicals before the development of other extraction methods like Soxhlet and ultrasound (Chua 2013). For the treatment of infections in poor and remote communities where medical facilities are lacking, patients macerate medicinal plants in hot water to extract the active components for: oral intake; inhalation of vapour; and application on wound dressings (Chua 2013). In this current study, this process was simulated by macerating the herbal material into water and ethanol separately, and then determined the extract yield in this. It was observed here that maceration extraction using ethanol yielded a greater mass of extract than maceration extraction using water (Figure 3.5). The difference in extract yield between the two solvents is likely due to the differing solubility of the various phytochemicals present in the extraction solvents. Singh (2008) explained that the polarity of an extracting solvent is one of the main factors affecting the extraction of active compounds from plants. Water and ethanol are both polar solvents, which means that they both possess positively and negatively charged regions to their molecules, but ethanol is less polar than water. This gives ethanol the ability to dissolve both polar and non-polar molecules, to a greater extent than water alone (Franks and Ives 1966). When compared to other extraction methods, as shown in Figure 3.5, the maceration method yielded the least mass of extract from either *P. africana* or *U. chamae*.

This may be due to other factors playing key roles in the extraction process of other methods which is absent in the maceration methods (Azwanida 2015). Factors such as ultrasound frequency, heating and condensation (as explained subsequently) are absent in the maceration method.

Ultrasound extraction, a non-conventional extraction method, gave a greater extract yield from both *P. africana* and *U. chamae* when compared with the maceration extraction method (Figure 3.5). As noted previously, the time taken to obtain an extract using the ultrasound method was less than the time taken to obtain an extraction yield using the maceration extraction and Soxhlet extraction method. A study by Briars and Paniwnyk in 2013 showed a greater yield in the extraction of artemisinin from *Artemisia annua* using the ultrasound method than the maceration method. A similar study performed by Petigny et al. in 2013, also showed that ultrasound extraction yielded a greater amount of boldo extract at a shorter extraction time than a maceration extraction method. The greater yield of extract using the ultrasound extraction was attributed to the force generated by the ultrasound waves (Petigny et al. 2013). As explained by Leonelli and Mason (2010), the force generated by the ultrasound wave forms cavitation bubbles which is powerful enough to destroy the plant cell wall thereby aiding solvent travel and the release of plant components. Therefore, the findings in this current study correlate with previous studies which showed ultrasound extraction demonstrating greater yield in the amount of extracts compared to maceration extraction. This means that ultrasound extraction is a better extraction method than maceration method.

The Soxhlet method of extraction gave the greatest mass of extract from both *P. africana* and *U. chamae* when compared with either maceration or ultrasound extraction. Singh (2008) suggested that the degree of heat applied to the solvent at reflux at the appropriate temperature over the plant material at repeated cycles, might be a factor

contributing to the higher yield in Soxhlet extraction compared to other methods. Due to the recycling process of this method, large amounts of extract yield is obtained using a small amount of solvent, which makes it the choice of extraction method for most studies (Ebi et al. 1999; Ogbulie et al. 2007; and Singh 2008). Another factor that is likely to influence the yield of extract in a Soxhlet extraction is the solubility of the bioactive compounds in the extraction solvent chosen (Singh 2008). Similar to the present study, Soxhlet extraction has been used by other studies to extract bioactive compounds from *U. chamae* and *P. africana* (Ebi et al.1999; Ogbulie et al. 2007; Okwu and Iroabuchi 2009; Ayanwuyi et al. 2010; Taha et al. 2011). Commonly, these studies also showed the extract yield using Soxhlet extraction was more than the maceration method. In this current study, Soxhlet extraction demonstrated a greater yield of extract compared to both ultrasound and maceration methods.

3.4.2 Phytochemical screening of *Prosopis africana* and *Uvaria chamae* extracts.

It is believed that many of the physiological effects and antimicrobial activities of plant therapies can be attributed to the presence of the secondary metabolites that are used by these plants as a defence mechanism (Demain and Fang 2000; Carranza et al. 2015). In this study, the impact of using different extracting methods on the presence of specific phytochemicals in the crude extracts was observed.

The presence of tannins, phenols, saponins, alkaloids, flavonoids, proteins and cardiac glycosides were observed in all *U. chamae* and *P. africana* extracts obtained from maceration, Soxhlet and ultrasound extraction, although quinones and reducing sugars were only found in the *U. chamae* extracts obtained from the three extaction methods (Table 3.1). This suggests that the extraction methods used have no influence on the presence of phytochemicals in the crude extract since all the extracts from different

extraction methods demonstrated the same result (Table 3.1). Briars and Paniwnyk (2013) suggested using different extraction methods has no impact on the presence of phytochemicals, although it might influence the purity and concentration of the phytochemical. In their study, they demonstrated that ultrasound extraction produced a significantly greater concentration of artemisinin (terpenoid derivative) with higher purity from *Artemisia annua* than Soxhlet extraction or maceration extraction, although all the extracts from the three extractions showed the presence of artemesinin.

A previous study looking into the phytochemical composition of *U. chamae* showed the presence of alkaloids, flavonoids, tannins, phenols and saponins (Okwu and Iroabuchi 2009), which correlates to the findings in this current study. Ebi et al. (1999) also found a similar range of phytochemicals except that alkaloids were absent from the methanolic extract of *U. chamae*. The presence of a given phytochemical in an extract is influenced by its solubility in the extraction solvent (Atawodi and Ogunbusola 2004). For example, flavonoids were present in methanolic extracts but were absent in both chloroform and petroleum ether extracts. Although *U. chamae* was used in this current study and the study by Ebi et al. (1999), the absence of alkaloids in the latter might be attributed to the extraction solvent used, since methanol was used by Ebi et al. (1999) and ethanol was used in this current study.

Similarly, tannins, phenols, saponins, alkaloids, flavonoids, proteins and cardiac glycosides were also observed in the *P. africana* extract (Table 3.1), which correlates with previous studies (Atawodi and Ogunbusola 2004; Kolapo et al. 2009; Ajiboye et al. 2013). These groups of studies suggested that the presence of the mentioned phytochemicals might be responsible for the biological activities of *P. africana*. This current study demonstrated the presence of flavonoids, saponins and other compounds in *P. africana* and *U. chamae* extracts (Table 3.1). Since *P. africana* and *U. chamae* are used

as herbal remedies for the traditional treatment of bacterial infection, the presence of these phytochemicals in the plant extracts might be responsible for antimicrobial potentials. Additionally, the effect of these different metabolites on the bacteria might occur through different mechanisms of action thereby reducing the chances in viability of resistant bacterial strains (Carranza et al. 2015).

3.4.3 Conclusion

Extraction of *P. africana* and *U. chamae* using different methods, generated the following extracts: *P. africana* maceration (aqueous) (PAM aq); *P. africana* maceration (ethanol) (PAM); *P. africana* Soxhlet (PAS); *P. africana* ultrasound (PAU); *U. chamae* maceration (aqueous)(UCM aq); *U. chamae* maceration (ethanol) (UCM); *U. chamae* Soxhlet (UCS); and *U. chamae* ultrasound (UCU). Interestingly, Soxhlet extraction yielded the greatest mass of extract from *P. africana* and *U. chamae* plant parts than other extraction methods while maceration extraction (aqueous) gave the least yield of extracts in both plants when compared to other extraction methods. Consequently, due to the low extract yield, PAM aq and UCM aq were not selected for further studies. In addition, the extraction methods used had no impact on the presence of phytochemical groups in the crude extracts. Consequently, a range of phytochemical groups like flavonoids, quinones, alkaloids, saponins, tanins and phenols, which were previously reported to exhibit biological activities, were present in all the extracts in this current study.

CHAPTER FOUR

4.0 ANTIMICROBIAL ACTIVITIES OF *PROSOPIS AFRICANA* AND *UVARIA CHAMAE* EXTRACTS.

4.1.0 INTRODUCTION

With the increase in prevalence of antimicrobial resistance, a pursuit for the search of new antimicrobial compounds, particularly from plant materials, has also increased (Redfern et al. 2014). A variety of plants have been reported to possess antimicrobial activity against a range of bacteria, thereby justifying their use in the traditional treatment of bacterial infections (Ncube et al. 2008). *Prosopis africana* and *U. chamae* as previously discussed (Section 1.4.0) are examples of plants that have been used for the traditional treatment of bacterial infections. Also, *P. africana* and *U. chamae* crude extracts have previously (Chapter 2) demonstrated the greatest antimicrobial activity against *P. aeruginosa, S. aureus* and *E. coli* compared to other plant extracts, making them suitable for further antimicrobial study. Consequently, more bacterial species which were found to be resistant (Section 2.3.1) to some of the conventional antibiotics were used to assess the antimicrobial activity of *P. africana* and *U. chamae* extracts.

Pathogenic bacteria like *P. aeruginosa*, *S. aureus*, *S. epidermidis*, *E. coli* amongst others, are responsible for a wide range of infections. For example, *P. aeruginosa*, a Gram-negative, causes pulmonary, urinary tract, wound and burn infections. Gram-positive bacteria like *S. aureus* and *S. epidermidis* are responsible for a range of infections, from superficial skin infections to systemic infections like septicemia and endocarditis (Kavanaugh and Ribbeck 2012). *Clostridium difficile* infection (CDI) is a toxin-mediated infection caused by *C. difficile* strains which primarily infects the

gastrointestinal tract. The treatment of CDI has been found to be difficult recently, due to the emergence of resistance to antibiotics by *C. difficile* (Ethapa et al. 2013). *Propionibacterium acnes* is a Gram-positive anaerobic bacterium which is commonly associated with skin acne infections, although other chronic infections like blepharitis, endophthalmitis and lumbar disc herniation are caused by this bacterium (Kemal et al. 2005; Haidar et al. 2010; Coenye et al. 2012). Some of these bacteria have developed resistance to antibiotics, making treatment of infections difficult. Therefore, exploring new antimicrobial therapies to fight this resistance is important. Determining the antimicrobial activity of medicinal plant extracts is an important aspect of exploring new antimicrobial compounds (Redfern et al. 2014).

In this experiment, some factors which were previously used, like *E. coli* NCTC 8164 (Section 2.2.2); and aqueous extracts (PAMaq and UCMaq) were excluded. Due to time constraint, it was not possible to use a wide range of bacterial species for further analyses. Therefore, a random selection of Gram-negative bacteria was made which resulted in the exclusion of the *E. coli* strain. The rationale for excluding the aqueous extracts was because of their low extract yield which resulted in the limited supply of extracts.

Consequently, the aim of this experiment was to determine the antimicrobial activity of *Prosopis africana* maceration (PAM) extract, *Prosopis africana* Soxhlet (PAS) extract, *Prosopis africana* ultrasound (PAU) extract, *Uvaria chamae* maceration (UCM) extract, *Uvaria chamae* Soxhlet (UCS) extract and *Uvaria chamae* ultrasound (UCU) extract against a selection of aerobes and anaerobes. These extracts were obtained using ethanol as solvent and different extraction methods (Section 3.0). Therefore, comparing their antimicrobial activity can help with the choice of extraction method for extracting antimicrobial compounds from plant materials in the future.

4.2.0 MATERIALS AND METHODS

4.2.1.0 Bacterial Strains and Growth Conditions

Bacterial strains and growth conditions used in Section 2.2.2 were also used in this experiment with the exception of *E. coli* NCTC 8164 which was excluded based on the rationale given in Section 4.1.0.

4.2.2.0 Antimicrobial assay

4.2.2.1 Agar-well Diffusion Assay

Agar-well diffusion assay was performed using the *P. africana* extracts (PAM, PAS and PAU) and *U. chamae* extracts (UCM, UCS and UCU) against the selected bacterial strains highlighted in Section 2.2.2. The protocol as described in Section 2.2.3.2 was followed.

4.2.2.2 Broth microdilution assay

Minimum inhibitory Concentration (MIC)

Minimum inhibitory concentration assay was performed using the *P. africana* extracts (PAM, PAS and PAU) and *U. chamae* extracts (UCM, UCS and UCU) against the selected bacterial strains highlighted in Section 2.2.2. The protocol as described in Section 2.2.3.3 was followed.

Minimum Bactericidal Concentration (MBC)

Minimum bactericidal concentration assay was performed using the *P. africana* extracts (PAM, PAS and PAU) and *U. chamae* extracts (UCM, UCS and UCU) against the selected bacterial strains highlighted in Section 2.2.2. The protocol as described in Section 2.2.3.3 was followed.

4.2.3 Statistical analyses

Each of the experiments described above was performed three times. Means and standard deviations of the data were obtained using SPSS version 25. Analysis of variance (ANOVA) was initially used to compare the data, with the *post-hoc* Tukey test used to identify specific differences within each data group. A *P*-value of less than 0.05 was considered as statistically significant for the purposes of this work.

4.3.0 RESULTS

4.3.1 Agar-well diffusion assay

The antimicrobial activities of *Prosopis africana* maceration (PAM) extract, *Prosopis africana* Soxhlet (PAS) extract, *Prosopis africana* ultrasound (PAU) extract, *Uvaria chamae* maceration (UCM) extract, *Uvaria chamae* Soxhlet (UCS) extract, and *Uvaria chamae* ultrasound (UCU) extract were analysed for their antimicrobial activity by agar well diffusion against a panel of aerobic and anaerobic bacteria, as described in Section 4.2.2.1. All of the extracts demonstrated zones of inhibition against aerobic bacteria (*K. pneumoniae*, MRSA, *S. epidermidis*, *P. aeruginosa*, and *S. aureus*) ranging from 12 mm ± 1.2 to 20.20 mm ± 0.8 (Table 4.1 and 4.2).

For Gram positive organisms (MRSA, *S. epidermidis* and *S. aureus*), UCM, UCS and UCU led to significantly larger zones of inhibition than the negative control ($P < 0.05$) and in descending order, the zones were largest for *S. aureus*, then *S. epidermidis* and then MRSA. Although there were no significant differences ($P > 0.05$) between the zones of inhibition caused by *Uvaria chamae* extracts against the Gram-positive aerobes, UCS demonstrated the largest zone of inhibition followed by UCU, then UCM (Table 4.1).

Furthermore, none of the *Uvaria chamae* extracts showed activity against either of the Gram-negative bacteria (*K. pneumoniae* and *P. aeruginosa*). For the anaerobic bacteria, UCM, UCS and UCU led to a significantly larger zone of inhibition than the negative control ($P < 0.05$) and in descending order, the zones of inhibition were the largest for *P. acnes* isolates followed by the *C. difficile* isolates. In contrast, UCM did not produce a zone of inhibition with *C. difficile* isolates. Although there were no significant differences ($P > 0.05$) between the zones of inhibition caused by the different types *Uvaria chamae* extracts against *P. acnes* isolates, UCS demonstrated the largest zone of inhibition followed by UCU, then UCM.

Table 4.1 Antimicrobial activity of *Uvaria chamae* extracts against selected aerobes and anaerobes analysed using the agar-well diffusion assay as described in Section 4.2.2.1.

	Bacterial Strains			
	Diameter of Zone of inhibition (mm)			
Bacteria	**UCM**	**UCS**	**UCU**	**SCNTL**
K. pneumoniae	-	-	-	-
MRSA	13.2±0.8	18.7±0.4	15.6±0.2	-
S. epidermidis	13.8±0.9	20.0±0.3	17.8±0.7	-
P. aeruginosa	-	-	-	-
S. aureus	13.4±0.6	20.2±0.8	15.9±0.5	-
***P. acnes* NCTC 737**	13.7±0.7	30.6±1.7	24.7±1.0	-
***P. acnes* LI77**	12.7±0.3	32.5±2.6	27.4±1.6	-
***P. acnes* LI88**	11.4±0.6	33.2±4.1	28.0±3.9	-
***P. acnes* SI5**	12.1±1.0	26.9±0.7	22.8±2.5	-
***P. acnes* SI8**	12.3±3.0	25.5±0.4	21.2±0.9	-
***C. difficile* NCTC**	-	13.6±0.5	11.0±1.9	-
***C. difficile* 001**	-	13.9±0.2	10.4±0.5	-
***C. difficile* 002**	-	13.6±0.4	11.4±0.5	-
C. difficile 005	-	13.9±0.2	11.0±0.1	-

Data represented as mean ± Standard deviation (SD); (-) represents no observable zone of inhibition; n = 3; Treatment concentration = 15 mg/ml; UCM - *Uvaria chamae maceration*, UCS - *Uvaria chamae* Soxhlet extract, UCU - *Uvaria chamae* ultrasound extract, SCNTRL – Solvent Control. There was a significant difference between the extract treatments and the control for bacteria that showed zones of inhibition ($P < 0.05$)

The zones of inhibition produced by each of the *P. africana* extracts, with the exception of PAM, when tested against selected aerobic Gram-positive bacteria (MRSA, *S. epidermidis* and *S. aureus*) were significantly larger than for the negative control ($P < 0.05$). Furthermore, all *U. chamae* extracts produced zones of inhibition against *S. aureus*, but no zone of inhibition was produced against *P. aeruginosa* (Table 4.1).

The *P. africana* maceration (PAM) extract did not inhibit any of the bacteria used. On the other hand, PAS extract yielded larger zones of inhibition than PAU when used against *S. aureus, S. epidermidis* and MRSA, although this difference was not statistically significant (Table 4.2).

Table 4.2 Antimicrobial activity of *Prosopis africana* extracts against selected aerobes and anaerobes analysed using the agar-well diffusion assay as described in Section 4.2.2.1.

	Treatments			
	Diameter of Zone of inhibition (mm)			
Bacteria	**PAM**	**PAS**	**PAU**	**SCNTL**
K. pneumoniae	-	12.3±1.8	11.6±1.2	-
MRSA	-	12.0±1.2	13.2±1.0	-
S. epidermidis	-	15.1±0.4	14.6±0.5	-
P. aeruginosa	-	-	-	-
S. aureus	-	13.1±1.5	13.0±1.3	-
***P. acnes* NCTC 737**	-	18.0±2.1	20.8±2.9	-
P. acnes* LI*77	-	17.7±1.3	23.9±5.7	-
P. acnes* LI*88	-	22.0±2.3	24.0±1.1	-
P. acnes SI5	-	16.2±0.8	16.6±1.9	-
P. acnes SI8	-	16.2±0.9	17.4±1.1	-
***C. difficile* NCTC**	-	-	-	-
***C. difficile* 001**	-	-	-	-
***C. difficile* 002**	-	-	-	-
C. difficile 005	-	-	-	-

Data represented as mean ± Standard deviation (SD); (-) represents no observable zone of inhibition; n = 3; Treatment concentration = 15 mg/ml; PAM – *Prosopis africana maceration*, PAS - *Prosopis africana* Soxhlet extract, PAU - *Prosopis africana* ultrasound extract, SCNTRL – Solvent Control. There was a significant difference between the extract treatments and the control for bacteria that showed zones of inhibition ($P < 0.05$)

When used against *K. pneumoniae* both the PAS and PAU extracts yielded significantly larger zones of clearing than the negative control, although neither of these extracts was observed to have any activity against *P. aeruginosa*. Similarly, when tested against the anaerobic bacteria, significantly larger zones of clearing were observed in lawns of the *P. acnes* isolates treated with PAS and PAU compared to the negative control. However, this was not the case for the *C. difficile* isolate, where there was no significant difference between the extracts and the negative control.

All *P. africana* extracts except PAM, produced zones of inhibition against *S. aureus*, but no zone of inhibition was produced against *P. aeruginosa* (Table 4.2).

4.3.2 Minimum inhibitory concentration (MIC)

Uvaria chamae extracts (UCS and UCU) demonstrated significant ($P < 0.05$) inhibition of aerobic Gram-positive bacteria (MRSA, *S. epidermidis* and *S. aureus*) at a concentration of 0.015 mg/ml, compared to the solvent control which showed no inhibitory effect. However, UCM demonstrated significantly lower inhibition but at variable concentrations (0.166 mg/ml - 0.781 mg/ml) when compared to UCS and UCU each with an MIC value of 0.015 mg/ml. On the other hand, for the Gram-negative species used – *P. aeruginosa* and *K. pneumoniae* – UCM, UCS and UCU demonstrated significant inhibitory effect at an MIC value ranging from 0.312 mg/ml – 2.50 mg/ml, compared to the solvent control ($P < 0.05$) which showed no inhibitory effect (Table 4.3). Furthermore, *U. chamae* extracts (UCM, UCS and UCU) demonstrated significantly lower MIC values against *S. aureus* compared to *P. aeruginosa* ($P < 0.05$) (Table 4.3).

For the *P. acnes* and *C. difficile* isolates, which are both anaerobic bacteria, UCS and UCU demonstrated a significant inhibitory effect at an MIC value ranging from 0.015 mg/ml – 0.020 mg/ml compared to the solvent control ($P < 0.05$) which showed no

inhibitory effect. Although there was no significant difference ($P > 0.05$) when the inhibition activity between UCS and UCU against all bacteria were compared, UCM showed a significantly higher MIC value ($P < 0.05$) when compared to either of the other two *Uvaria chamae* extracts (Table 4.3).

Table 4.3 Minimum inhibitory concentration of *Uvaria chamae* extracts against selected aerobes and anaerobes analysed using the micro-dilution assay as described in Section 4.2.2.2.

	Extract treatment			
	Minimum inhibitory concentration (mg/ml)			
Bacteria	**UCM**	**UCS**	**UCU**	**SCNTRL**
K. pneumoniae	1.875±0.00	0.469±0.00	0.781±0.27	-
MRSA	0.166±0.26	0.015±0.00	0.015±0.00	-
S. epidermidis	0.781±0.27	0.015±0.00	0.015±0.00	-
P. aeruginosa	2.500±1.08	0.312±0.14	0.391±0.14	-
S. aureus	0.781±0.27	0.015±0.00	0.015 ±0.00	-
***P. acnes* NCTC 737**	0.781±0.11	0.015±0.00	0.020±0.01	-
P. acnes* LI*77	0.244±0.21	0.015±0.00	0.015±0.00	-
P. acnes* LI*88	0.364±0.05	0.015±0.00	0.015±0.00	-
P. acnes SI5	1.875±0.00	0.015±0.00	0.015±0.00	-
P. acnes SI8	1.34±0.02	0.015±0.00	0.015±0.00	-
***C. difficile* NCTC 11204**	0.64±0.05	0.015±0.00	0.015±0.00	-
***C. difficile* 001**	0.729±0.05	0.015±0.00	0.015±0.00	-
***C. difficile* 002**	0.934±0.03	0.020±0.00	0.015±0.00	-
C. difficile 005	0.820±0.00	0.015±0.00	0.015±0.00	-

Data represented as mean ± Standard deviation (SD); (-) represents no observable activity; n = 3; Starting concentration = 15 mg/ml; UCM – *Uvaria chamae* maceration, UCS - *Uvaria chamae* Soxhlet extract, UCU - *Uvaria chamae* ultrasound extract, SCNTRL – Solvent Control. Note: There was a significant difference between the extract treatments and the control for all the bacteria ($P < 0.05$)

Prosopis africana extracts (PAM, PAS and PAU) demonstrated a significant inhibitory effect against MRSA, *S. epidermidis* and *S. aureus* at MIC values ranging from 0.121 mg/ml – 1.875 mg/ml compared to the solvent control ($P < 0.05$) which showed no inhibitory effect. For *P. aeruginosa* and *K. pneumoniae*, PAM, PAS and PAU demonstrated a significant inhibitory effect at MIC values ranging from 0.312 mg/ml – 2.500 mg/ml compared to the solvent control ($P < 0.05$) which showed no inhibitory

effect. Furthermore, *P. africana* extracts (PAM, PAS and PAU) demonstrated significantly lower MIC values against *S. aureus* compared to *P. aeruginosa* ($P < 0.05$) (Table 4.4).

The PAM, PAS and PAU extracts demonstrated significant inhibitory activity against *P. acnes* and *C. difficile* isolates, at an MIC value ranging from 0.015 mg/ml – 0.851 mg/ml, when compared to the solvent control ($P < 0.05$) which showed no inhibitory effect. Although there was no significant difference ($P > 0.05$) between the inhibitory activities of PAS and PAU against all of the bacteria tested, PAM did show a significant difference ($P < 0.05$) by having a higher MIC when compared to PAS and PAU (Table 4.4).

Table 4.4 Minimum inhibitory concentration of *Prosopis africana* extracts against selected aerobes and anaerobes analysed using the micro-dilution assay as described in Section 4.2.2.2.

	Extract treatment			
	Minimum inhibitory concentration (mg/ml)			
Bacteria	**PAM**	**PAS**	**PAU**	**SCNTRL**
K. pneumoniae	1.563±0.54	0.312±0.14	0.391±0.14	-
MRSA	1.875±0.00	0.254±0.21	0.244±0.22	-
S. epidermidis	0.625±0.27	0.195±0.24	0.176±0.25	-
P. aeruginosa	2.500±1.08	0.469±0.00	0.391±0.14	-
S. aureus	1.69±0.62	0.254±0.20	0.121±0.02	-
***P. acnes* NCTC 737**	0.781±0.01	0.015±0.00	0.122±0.11	-
P. acnes* LI*77	0.615±0.12	0.034±0.02	0.244±0.01	-
P. acnes* LI*88	0.515±0.13	0.015±0.00	0.064±0.05	-
P. acnes SI5	0.175±0.05	0.020±0.01	0.122±0.11	-
P. acnes SI8	0.453±0.20	0.020±0.01	0.034±0.02	-
***C. difficile* NCTC 11204**	0.135±0.08	0.064±0.05	0.064±0.05	-
***C. difficile* 001**	0.825±0.43	0.059±0.05	0.029±0.05	-
***C. difficile* 002**	0.430±0.70	0.044±0.03	0.034±0.03	-
C. difficile 005	0.851±0.54	0.015±0.00	0.020±0.00	-

Data represented as mean ± Standard deviation (SD); (-) represents no observable activity; n = 3; Starting concentration = 15 mg/ml; PAM – *Prosopis africana* maceration, PAS - *Prosopis africana* Soxhlet extract, PAU - *Prosopis africana* ultrasound extract, SCNTRL – Solvent Control. Note: There was a significant difference between the extract treatments and the control for all the bacteria ($P < 0.05$)

4.3.3 Minimum bactericidal concentration (MBC)

Uvaria chamae extracts (UCS and UCU) demonstrated significant bactericidal effect against aerobic Gram-positive bacteria (MRSA, *S. epidermidis* and *S. aureus*) at an MBC value ranging from 0.015 mg/ml – 0.781 mg/ml, compared to the solvent control ($P < 0.05$) which showed no bactericidal effect. On the other hand, UCM was significantly less bactericidal than either of the other two *Uvaria chamae* extracts ($P < 0.05$) (Table 4.5).

For *P. aeruginosa* and *K. pneumonia*, UCM, UCS and UCU demonstrated a significant bactericidal effect at an MBC value ranging from 0.625 mg/ml – 2.500 mg/ml, compared to the solvent control ($P < 0.05$) which showed no bactericidal effect. Furthermore, the bactericidal activity of UCM, UCS and UCU was significantly greater in the Gram-positive aerobic bacteria than the Gram-negative aerobic bacteria ($P < 0.05$) (Table 4.5). *Uvaria chamae* extracts (UCM, UCS and UCU) demonstrated significantly lower MBC values against *S. aureus* compared to *P. aeruginosa* ($P < 0.05$) (Table 4.5).

Table 4.5 Minimum bactericidal concentration of *Uvaria chamae* extracts against selected aerobes and aerobes.

	Extract treatment			
	Minimum bactericidal concentration (mg/ml)			
Bacteria	**UCM**	**UCS**	**UCU**	**SCNTRL**
K. pneumoniae	1.875±0.00	0.938±0.00	0.781±0.27	-
MRSA	1.875±0.26	0.078±0.03	0.015±0.00	-
S. epidermidis	0.781±0.27	0.015±0.00	0.015±0.00	-
P. aeruginosa	2.500±1.08	0.625±0.27	0.391±0.14	-
S. aureus	0.781±0.27	0.015 ±0.00	0.015 ±0.00	-
***P. acnes* NCTC 737**	0.781±0.11	0.015±0.00	0.020±0.01	-
P. acnes* LI*77	0.244±0.21	0.015±0.00	0.015±0.00	-
P. acnes* LI*88	0.364±0.05	0.015±0.00	0.015±0.00	-
P. acnes SI5	1.875±0.00	0.015±0.00	0.015±0.00	-
P. acnes SI8	1.34±0.02	0.015±0.00	0.015±0.00	-
***C. difficile* NCTC 11204**	0.64±0.05	0.015±0.00	0.015±0.00	-
***C. difficile* 001**	0.729±0.05	0.015±0.00	0.015±0.00	-
***C. difficile* 002**	0.934±0.03	0.020±0.00	0.015±0.00	-
C. difficile 005	0.820±0.00	0.015±0.00	0.015±0.00	-

Data represented as mean ± Standard deviation (SD); (-) represents no observable activity; n = 3; Treatment concentration = 15 mg/ml; UCM - *Uvaria chamae* maceration extract, UCS - *Uvaria chamae* Soxhlet extract, UCU - *Uvaria chamae* ultrasound extract, SCNTRL – Solvent Control. Note: There was a significant difference between the extract treatments and the control for all the bacteria ($P < 0.05$)

For the *P. acnes* and *C. difficile* isolates UCM, UCS and UCU demonstrated a significant bactericidal effect, with an MBC value ranging from 0.015 mg/ml – 1.875 mg/ml, compared to the solvent control ($P < 0.05$) which showed no bactericidal effect. Although there was no significant difference in the bactericidal activity of UCS and UCU against any of the bacterial species tested ($P > 0.05$), UCM was significantly less bactericidal (in other words it had a higher MBC) than either of the other two *Uvaria chamae* extracts ($P < 0.05$) (Table 4.5).

Prosopis africana extracts (PAS and PAU) demonstrated a significant bactericidal effect against MRSA, *S. epidermidis* and *S. aureus* at an MBC value ranging from 0.215 mg/ml – 1.875 mg/ml compared to the solvent control ($P < 0.05$) which showed no bactericidal effect. On the other hand, PAM gave a significantly higher MBC than either

PAS or PAU ($P < 0.05$) (Table 4.6). Furthermore, *P. africana* extracts (PAM, PAS and PAU) demonstrated significantly lower MBC values against *S. aureus* compared to *P. aeruginosa* ($P < 0.05$) (Table 4.6).

Table 4.6 Minimum bactericidal concentration of *Prosopis africana* extracts against selected aerobes and anaerobes.

	Extract Treatment			
	Minimum bactericidal concentration (mg/ml)			
BACTERIA	**PAM**	**PAS**	**PAU**	**CNTRL**
K. pneumoniae	1.563±0.54	0.312±0.14	0.391±0.14	-
MRSA	1.875±0.00	0.312±0.14	0.312±0.14	-
S. epidermidis	0.625±0.27	0.273±0.18	0.215±0.22	-
P. aeruginosa	2.500±1.08	0.625±0.27	0.625±0.27	-
S. aureus	1.69±0.62	0.312±0.14	0.323±0.10	-
***P. acnes* NCTC 737**	0.781±0.01	0.015±0.00	0.122±0.11	-
P. acnes* LI*77	0.615±0.12	0.034±0.02	0.244±0.01	-
P. acnes* LI*88	0.515±0.13	0.015±0.00	0.064±0.05	-
P. acnes SI5	0.175±0.05	0.039±0.02	0.322±0.25	-
P. acnes SI8	0.453±0.20	0.020±0.01	0.034±0.02	-
***C. difficile* NCTC 11204**	0.135±0.08	0.083±0.05	0.205±0.05	-
***C. difficile* 001**	0.825±0.43	0.088±0.05	0.068±0.05	-
***C. difficile* 002**	0.430±0.70	0.137±0.09	0.068±0.02	-
C. difficile 005	0.851±0.54	0.024±0.01	0.030±0.01	-

Data represented as mean ± Standard deviation (SD); (-) represents no observable activity; n = 3; Treatment concentration = 15 mg/ml; PAM – *Prosopis africana* maceration extract, PAS - *Prosopis africana* Soxhlet extract, PAU - *Prosopis africana* ultrasound extract, SCNTRL – Solvent Control. Note: There was a significant difference between the extract treatments and the control for all the bacteria ($P < 0.05$)

For *P. aeruginosa* and *K. pneumoniae*, PAM, PAS and PAU demonstrated a significant bactericidal effect, with an MBC value ranging from 0.312 mg/ml – 2.500 mg/ml, when compared to the solvent control ($P < 0.05$) which showed no bactericidal effect. The PAM, PAS and PAU extracts demonstrated significant bactericidal activity against the *P. acnes* and *C. difficile* isolates, at an MBC value ranging from 0.015 mg/ml and 0.851 mg/ml, when compared to the solvent control (Table 4.6). Although there was no significant difference in the bactericidal activity of PAS and PAU against any of the

bacteria tested ($P > 0.05$), PAM gave a significantly higher MBC than either of the other *Prosopis africana* extracts ($P < 0.05$) (Table 4.6).

4.4.0 DISCUSSION

4.4.1 Antimicrobial activity of *Uvaria chamae* extracts

The *U. chamae* crude extracts demonstrated greater antimicrobial activity against the Gram-positive bacteria than the Gram-negative bacteria. This may be an indication that the antimicrobial activity of *U. chamae* extracts is more specific to Gram-positive bacteria than Gram-negative bacteria. The only exception to this was the *C. difficile* strains, which did not show particular sensitivity to this extract in the agar well diffusion assay but showed sensitivity to *U. chamae* extracts in the broth micro-dilution assay. Carranza et al. (2015) performed similar studies on the antimicrobial activity of *Rhamnus californica* and *Umbellularia californica* extracts against a range of Gram-positive and Gram-negative bacteria. This group's results demonstrated that the antimicrobial activity of both *R. californica* and *U. californica* were greater against Gram-positive than Gram-negative bacteria (Carranza et al. 2015). They attributed this to the presence of phytochemicals such as flavonoids, alkaloids, phenols and tannins, which might be responsible for the destruction of the Gram-positive bacterial cell wall. It is possible that the relatively simple structure of the Gram-positive cell wall – comprising largely teichoic acids and peptidoglycan – makes these bacteria more susceptible to a wide range of antimicrobial agents (Coyle et al. 2005). On the other hand, the cell wall of Gram-negative bacteria is relatively more complex, since it consists of an outer membrane, porins, lipopolysaccharides, lipoproteins, a peptidoglycan layer and the periplasmic space, all of which combined can make it more difficult for antimicrobial

agents to penetrate and act (Coyle et al. 2005). Furthermore, the antimicrobial variability of *U. chamae* and *P. africana* against *S. aureus* and *P. aeruginosa* (Table 2.3, 4.3, 4.4, 4.5 and 4.6), may also apply to other plants in Section 2.2.0 which were excluded from further study. Besides, the presence of phytochemicals such as flavonoids and saponins have been shown to correlate with the antimicrobial activity of plant extracts. For example, the flavonoid content of some Chinese medicinal plants has been previously found to correlate with their antimicrobial activity, and so it has been proposed that the flavonoids should be further characterised, isolated and studied (Zhang et al. 2013). Other workers have also reported that flavonoids contribute substantially to the antimicrobial activity of plant extracts (Cushnie and Lamb 2005; Daglia 2012). Furthermore, saponins have also been reported to possess antimicrobial properties against fungi, such as *Aspergillus ochraceous* and *Curvularia lunata,* and bacteria, such as *Bacillus megaterium*, *Salmonella* Typhimurium and *Pseudomonas aeruginosa* (Mandal et al. 2005). Linking these suggestions to this current study, the presence of flavonoids, saponins and other phytochemicals might be responsible for the antimicrobial activity of *U. chamae* as previously explained (Section 1.5.0).

The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of UCS and UCU extracts against each of the bacterial strains used fell within the 0.015 mg/ml – 0.781 mg/ml range (Tables 4.3 and 4.5). These MIC and MBC values fell below the cut-off concentration of 1mg/ml that has been suggested as the level at which plant materials can be considered to be antimicrobial (Carranza et al. 2015). Consequently, UCS and UCU extracts can be considered to be effective against the *K. pneumoniae*, MRSA, *S. epidermidis*, *P. aeruginosa*, *S. aureus*, *P. acnes*, and *C. difficile* strains tested. *Uvaria chamae* maceration extract (UCM), on the other hand, had an MIC and MBC range of 0.166 mg/ml – 2.50 mg/ml against the bacterial strains used

(Tables 4.3 and 4.5). This suggests that it cannot be considered to be an effective antimicrobial agent against the bacterial strains tested, in contrast to UCS and UCU extracts (Carranza et al. 2015). Consequently, it is likely that, in the current study, the maceration extraction method yielded a lesser concentration of the active secondary metabolites required for *U. chamae* antimicrobial activity compared with Soxhlet and ultrasound extracts of this plant. This finding correlates with previous studies. For example, against a selection of spoilage and pathogenic bacteria, it has been demonstrated previously that extracts of *Quercus coccifera* and *Juniperus phoenicea* obtained using the Soxhlet method were more strongly antimicrobial than extracts obtained using a maceration method (Hayouni et al. 2007). This group further suggested that the yield, concentration and purity of the secondary metabolite in the extracts obtained from the Soxhlet method might be greater than that found in extracts obtained from the maceration method, which might be responsible for the greater antimicrobial activity in the Soxhlet extract (Hayouni et al. 2007).

Furthermore, a study by Oluremi et al. (2009) showed the inhibitory effect of *U. chamae* extracts on MRSA, *S. aureus, P. aeruginosa, E. coli, K. pneumoniae* and *Proteus* spp. But interestingly, the antimicrobial activity of *U. chamae* extract on the bacterial species found in their study, was less than the antimicrobial activity of *U. chamae* observed in this current study (Oluremi et al. 2009). Several reasons might explain the disparity between the two studies. Reasons such as: extraction conditions as previously discussed; location on plant material; strains of bacteria used; and storage conditions may be responsible for the difference in antimicrobial activity of the extracts (Rios and Recio 2005). To further expound on the effect of storage conditions on the antimicrobial activity of a plant material, a change in the storage conditions such as pH and temperature of the plant material may affect the antimicrobial activity of plant extract (Mau et al.

2001). Furthermore, during a pilot study for the current research, it was found that poorly stored *U. chamae* plant parts, possessed weaker antimicrobial activity (data not included) when compared with the properly stored *U. chamae* plant parts. Consequently, comparing the findings from the pilot study and the findings from the current study, shows that although the same plant (*U. chamae*) was used for both studies, the storage condition affected the antimicrobial activity of the plant.

4.4.2 Antimicrobial activity of *Prosopis africana* extracts

Prosopis africana extracts (PAS and PAU) demonstrated inhibitory activity against all of the bacterial strains tested, except for the *P. aeruginosa* and *C. difficile* strains, in the agar well diffusion assay used (Table 4.2). There were no significant differences between the antimicrobial activity of PAS compared with PAU against the *K. pneumonia,* MRSA, *S. epidermidis, S. aureus* and *P. acnes* strains used.

Prosopis africana maceration (PAM) extract, on the other hand, did not demonstrate significant activity against any of the bacteria used in the well diffusion assay. The extraction method used and the extraction conditions can influence the amount and concentration of the active compounds responsible for the antimicrobial activity of the extract (Hayouni et al. 2007). Again, this might help to explain the poorer activity of the PAM extract as compared to the PAS and PAU extracts (Briars and Paniwnyk 2012).

The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of the PAS and PAU extracts against all of the bacteria used fell within the range of 0.015 mg/ml – 0.625 mg/ml (Tables 4.4 and 4.6). Again, since these MIC and MBC values are below the 1 mg/ml upper limit suggested by Carranza et al. (2015), it is reasonable to conclude that the PAS and PAU extracts are effective antimicrobials against at least the bacterial strains used, some of which are significant

pathogens. *Prosopis africana* maceration (PAM) extract, on the other hand, demonstrated an MIC and MBC against all of the bacteria tested in the range of 0.135 mg/ml – 2.50 mg/ml (Tables 4.4 and 4.6). Consequently, it is not reasonable to conclude that PAM extract is an effective antimicrobial, in contrast to PAS and PAU (Carranza et al. 2015). The implication of this is that PAM demonstrated the least antimicrobial activity which again might be attributed to the extraction method used as previously discussed.

Prosopis africana is an important plant to study based on its widespread medicinal uses, particularly in remote parts of Africa were modern healthcare is poor (Ajiboye et al. 2013). Despite the widespread use of *P. africana* as a traditional medicine, only a few studies have been performed to analyse its antimicrobial activity. In one study of its antimicrobial activity, *P. africana* extracts were found to have MICs in the range of 1.562 mg/ml – 12.5 mg/ml against strains of *K. pneumoniae*, MRSA, *P. aeruginosa*, *S. aureus*, and *E. coli.* These MIC values were higher than the MIC values obtained in the current study (Table 4.4). Again, the reason for the difference in antimicrobial activity might be attributed to the extraction method and conditions used (Hayouni et al. 2007) or other factors such as; storage conditions (Mau et al 2001), location and season of plant sample collection and differences in the strains of bacteria used for the analysis (Rios and Recio 2005). Using maceration method of extraction, Ajiboye et al. (2013) used 50% methanol as the extracting solvent, which was different from the extracting solvent used in this present study. Ethanol was the choice of solvent in this present study due to recommendations from previous solvent comparative studies (Mauricio et al. 2003; Pinelo et al. 2004; Xu and Chang 2007). All the solvent comparative studies showed that ethanol was the most favourable for extracting active secondary metabolites in natural products, mainly due to its polarity and ability to dissolve both polar and non-polar compounds (Mauricio et al. 2003; Pinelo et al. 2004; Xu and Chang 2007). Although

limited literatures have studied the antibacterial activities of *P. africana*, other interesting properties such as analgesic, anti-inflammatory, anti-fungal, and anti-trypanosonal have been documented (Atawodi and Ogunbusola 2009; Anyawuyi et al. 2010; Zarafi and Ayuba 2012).

4.4.3 Correlation between the assay type and the antimicrobial activity observed in this study

In this current study, *P. africana* and *U. chamae* extracts demonstrated antimicrobial activity against the strains of bacteria tested. There were variations in the antimicrobial activity as observed from the assays used. For example, *U. chamae* extracts demonstrated antimicrobial activity against *K. pneumoniae* and *P. aeruginosa* after performing a broth dilution assay but was inactive after the agar-well diffusion method was used. The reason for the contradiction in the antimicrobial activity of the extracts between the experimental assays may be attributed to the sensitivity of the assay. Interestingly, the sensitivity of the assays used, might be a reason for the contradictory antimicrobial activity of the extracts. For example, the broth dilution assay is seen as more sensitive and reliable in determining the antimicrobial activity of natural materials and products than the agar-well diffusion assay (Balouiri et al. 2016). Although agar diffusion assays have been generally accepted as a method for analysing antimicrobial activity, several issues concerning its effectiveness have been raised (Marsh and Goode 2008). Agar diffusion assay works best with defined microbial inhibitors (Hewitt and Vincent 1989), but when analysing natural materials with unknown compounds, factors which lead to false positive or false negative results usually occur (Eloff 1998). These factors could be attributed to agar type, salt concentration, temperature, and molecular size or concentration of antimicrobial compounds (Marsh and Goode 2008). Furthermore, the

agar diffusion assay is considered as a qualitative method that cannot determine the bactericidal and bacteriostatic effect of the material to be tested (Eloff 1998). All these factors influencing the results of an assay have been eliminated in the broth dilution assay, making it the most sensitive and reliable method of analysing antimicrobial activity of natural materials and products (Eloff 1998; Marsh and Goode 2008). Consequently, in this current study, the findings in the broth dilution assay showing the antimicrobial activity of *P. africana* and *U. chamae* extracts against the strains of bacteria tested was selected by the author as the most reliable.

4.4.4 Conclusion

Uvaria chamae Soxhlet extract (UCS) and *Uvaria chamae* ultrasound extract (UCU) demonstrated greater antimicrobial activity against all the bacterial strains tested than *Uvaria chamae* maceration (UCM). Furthermore, *Prosopis africana* Soxhlet extract (PAS) and *Prosopis africana* ultrasound extract (PAU) demonstrated greater antimicrobial activity against all of the bacterial strains tested than did *Prosopis africana* maceration extract (PAM). Therefore, UCS, UCU, PAS, and PAU were chosen as candidates for the determination of antibiofilm activity based on their antimicrobial activities, which was significantly better than the excluded extracts. This means that the extracts are effective antimicrobials with potential as novel therapeutic agents.

CHAPTER FIVE

5.0 ANTIBIOFILM ACTIVITIES OF *PROSOPIS AFRICANA* AND *UVARIA CHAMAE EXTRACTS*.

5.1.0 INTRODUCTION

Microbial biofilms present a huge challenge in clinical settings. They provide a source of infectious organisms and retard the effectiveness of many antimicrobial treatment regimens. The ability to form biofilms is one of the factors responsible for antimicrobial resistance and also serves as an important virulence factor for many pathogens (Thenmozhi et al. 2009). As explained in Section 1.2.3, bacteria within a biofilm are more resistant to antimicrobial agents than planktonic bacteria (Niyonsaba et al. 2006). Bacteria like *P. aeruginosa, S. aureus*, *S. epidermidis*, *P. acnes* and *C. difficile* are known to readily form biofilms (Thenmozhi et al. 2009; Kavanaugh and Ribbeck 2012; Vuotto et al. 2018). These bacteria were therefore selected for antimicrobial analysis in this present study based on this rationale. Biofilms formed by Gram-negative bacteria, such as *P. aeruginosa*, are able to evade conventional antibiotic treatments and cause complications through the infection of pulmonary and urinary tracts, cystic fibrosis, wounds and burns (Kavanaugh and Ribbeck 2012). Gram-positive bacterial biofilms, such as *S. aureus* and *S. epidermidis* biofilms, have been associated with a range of diseases including caries, gingivitis, periodontitis, gastrointestinal infections, and wound infections (Thenmozhi et al. 2009). Some severe skin infections, and severe forms of inflammatory acne are associated with *P. acnes* biofilms (Coenye et al. 2012). *Clostridium difficile* infection (CDI) occurs when *C. difficile* colonises and forms biofilms

in the gastrointestinal tract. Treatment of CDI is commonly complicated by the resistance of *C. difficile* biofilm against antibiotics (Vuotto et al. 2018).

As previously discussed (Section 1.4.0), *P. africana* and *U. chamae* have been used as traditional alternatives for the treatment of infections caused by the bacterial species stated above. Therefore, determining the antibiofilm activity of these plants will be of importance in the development of new antimicrobial compounds against these bacteria. Two methods: semi-quantitative antibiofilm assay; and quantitative antibiofilm assay, are utilised for assessing the antibiofilm activity of plant extracts. Semi-quantitative antibiofilm assay involves the use of crystal violet staining to assess the effect of plant extracts on the biofilm biomass with the aid of a spectrophotometer (Sandasi et al. 2009). A complication here is that some of these plant extracts are naturally coloured and so can interfere with the absorbance reading, particularly when the solvent control is colourless. To eliminate this effect, background wells containing the plant extract with no bacterial biofilm, are included in the analysis in addition to the test wells containing the plant extract and the bacterial biofilm (Figure 5.1).

Quantitative antibiofilm assay involves colony counting, to assess the effect of the plant extract on biofilm viable cells. The quantitative antibiofilm assay does not utilise absorbance reading as a way of assessing the antibiofilm activity of a plant extract, therefore, background wells are not needed. Instead, the use of ultrasound to detach the biofilm from the walls of the plate after a certain duration is vital for this assay. Therefore, a preliminary study to determine the appropriate sonication time for this assay was performed (Section 5.2.4).

The aim of this experiment was to determine the antibiofilm activities of *Prosopis africana* and *Uvaria chamae* plant extracts against *Pseudomonas aeruginosa*, *Staphylococcus aureus, Staphylococcus epidermidis, Propionibacterium acnes,* and

Clostridium difficile strains using the semi-quantitative and quantitative antibiofilm assays.

5.2.0 MATERIALS AND METHODS

Media preparation: Media preparation used in Section 2.2.0 was used in this experiment.

5.2.1 Bacterial Strains and Growth Conditions

Bacterial strains and growth conditions used in Section 2.2.2 were used in this experiment with the exception of *Klebsiella pneumoniae* NCTC 13865, Methicillin-resistant *Staphylococcus aureus* (MRSA) NCTC 43300 and *E. coli* NCTC 8164. These bacteria were excluded due to time constraint and moreover, the selected bacteria readily form biofilm as reported in previous studies (Section 5.1.0).

5.2.2 Bacterial biofilm development:

Bacterial biofilm development was performed as described by Sandasi et al. (2009). All bacterial cultures were adjusted to 0.5 McFarland scale (absorbance of 0.085 at 625nm) as previously described by Balouiri et al (2016). One hundred microlitre aliquots of overnight bacterial cultures were inoculated into the wells of the first row of a tissue culture microtitre plate (Sarstedt 83.3924). The first row of wells served as the test wells, where the antibiofilm activity of the extracts was assessed (Figure 5.1). One hundred microlitre aliquots of sterile Brain Heart Infusion (BHI) broth was added to the next row of wells in the microtitre plate, to act as the background test wells (to show the background levels of stain absorbed by the well without any bacterial culture). The solvent (vehicle) control (bacteria + BHI broth + control material) and its background (BHI broth + control material) were also included by adding 100 µl of the ten-fold diluted bacterial culture to each well of a separate row of wells. The next row of wells contained

100 µl of BHI broth, which served as the background for the solvent control. Inoculated plates were incubated, aerobically or anaerobically as appropriate, at 37ºC for 48 h for optimal biofilm formation for all bacterial strains except those of *P. acnes,* which were incubated for 72 h. Figure 5.1, gives a schematic representation of how the wells of the microtitre plate were loaded.

In this first biofilm development phase, it is important to note that each extract has its separate test row and background row. Each bacterial species to be analysed also has its own control row and background row. To avoid cross-contamination between the various bacterial strains used, each experiment with an individual strain was done in a separate 96-well microtitre tissue culture plate.

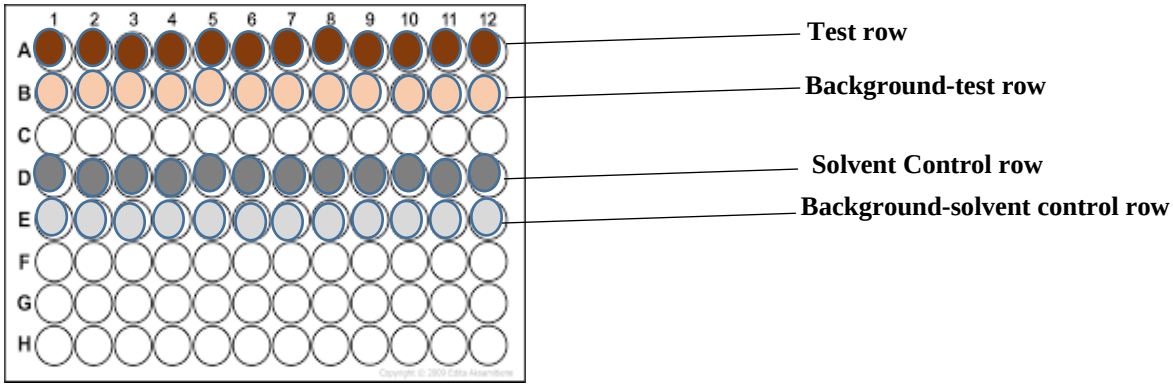


Figure 5.1 An example of the experimental layout for the semi-quantitative antibiofilm assay on a microtitre plate

5.2.3 Semi-quantitative antibiofilm assay

Semi quantitative antibiofilm assay was performed as described by Sandasi et al. (2009). After biofilm development, the broth containing any planktonic cells was carefully removed from each well and then the wells were gently rinsed using sterile phosphate buffered saline (PBS) (Sigma-Aldrich P3813), using the following method. The incubated culture and sterile broth were carefully removed from the wells using a multichannel pipette in order not to disrupt the formed biofilm. Two hundred microlitres

of sterile PBS were carefully added to each well, removed and discarded. This process was performed twice to ensure that all the loose, non biofilm-associated bacterial cells were rinsed away. The rinsed microtitre plate containing the bacterial biofilm was dabbed 3 times on a piece of clean tissue paper to blot away excess water.

One hundred microlitres of double-strength sterile BHI broth was then added to all the wells. The treatments were next added to the wells. Aliquots of 100 µl of the plant extract were added to the wells in the test row and background row (Figure 5.1). The concentration of each extract used in the antibiofilm studies was the Minimum Bactericidal Concentration (MBC) of each extract for each strain of bacterium as determined in Section 4.3.3. For example, 0.625 mg/ml of UCS extract was the MBC against *P. aeruginosa.* Therefore 0.625 mg/ml was the concentration of UCS extract added to the test wells for the *P. aeruginosa* biofilm assay. The actual concentration of extract used for each assay is shown in Chapter 4, Tables 4.5 and 4.6. One hundred microlitres of the solvent control (50 % ethanol evaporated at 75^{o} C for 3 h) was added to the wells of the solvent control and the background-solvent control row. Inoculated plates were incubated, aerobically or anaerobically as appropriate, at 37^{o} C for 24 h for all bacteria except *P. acnes* which was incubated for 72 h.

After incubation, the liquid in the microtitre plate was carefully removed from the wells using a multichannel pipette in order not to disrupt the biofilm. Two hundred microlitres of sterile PBS was carefully added to each well, removed and discarded using a multichannel pipette. This process was performed twice to ensure that all the loose, non biofilm-associated bacterial cells were removed. The rinsed microtitre plate containing the bacterial biofilm was dabbed 3 times on a piece of clean paper to blot away excess water.

The wells were then stained with 200 µl of 0.5 % crystal violet solution. This was added to each of the wells and the plate was incubated for 15 min at room temperature on the bench. The crystal violet was removed from the wells after 15 min and these were then rinsed 3 times using PBS. The rinsed plate was dabbed onto tissue 3 times to blot away excess water. Decolourisation of the stained wells was performed by adding 200 µl of 95 % ethanol and then allowing them to stand for 15 min at room temperature. The 95 % ethanol was then transferred from the treated round-bottom microtitre plate to a non-sterile flat-bottom microtitre plate (Sarstedt 82.1581) to be analysed on the microplate reader (BioTek ELx808). The absorbance of each well in the plate was measured at a wavelength of 595 nm.

For the analysis of the data obtained, the mean absorbance and standard deviation of the test wells, background-test wells, control wells, and background control wells were calculated in Microsoft Excel 2016. To determine the absorbance of the biofilm, the mean absorbance of the background wells was subtracted from the mean absorbance of the test wells. The effect of the crude extract on the bacterial biofilms was determined as a percentage reduction in biofilm density, as reported by the intensity of crystal violet staining, which was calculated using the equation below (Equation 5.1). Each test and background row consisting of 12 wells containing bacterial biofilm was treated with a single extract, subsequent rows were treated with different extracts.

Equation 5.1

Effect of extract treatment:

$$[(\bar{x}\ ODt - \bar{x}\ ODbt) / (\bar{x}\ ODc - \bar{x}\ ODbc)] * 100 = \%\ biofilm$$

$$\%\ Biofilm\ reduction = 100 - \%\ biofilm$$

Where,

$\bar{x}$ ODt = Mean Absorbance of test wells, $\bar{x}$ ODbt = Mean Absorbance of background test wells,

$\bar{x}$ ODc = Mean absorbance of control wells, $\bar{x}$ ODbc = Mean absorbance of background control wells.

5.2.4 Preliminary quantitative antibiofilm study and optimisation of sonication retrieval process

The aim of this optimisation was to assess the conditions for each step in the development of the quantitative antibiofilm protocol. Crude extracts from *Prosopis africana* Soxhlet extraction (PAS), *Prosopis africana* ultrasound extraction (PAU), *Uvaria chamae* Soxhlet extraction (UCS) and *Uvaria chamae* ultrasound extraction (UCU) and the solvent control were used as treatments against preformed biofilm of *S. aureus* and *P. aeruginosa.*

First of all, development of bacterial biofilm for *S. aureus* and *P. aeruginosa* was performed as described in section 5.2.2 but with modifications. In this experiment, the extract colouration has no effect on this analysis because the focus of this experiment was on viable cell counts and not absorbance. Therefore, a background well was not necessary for these experiments. One hundred microlitre aliquots of overnight bacterial culture e.g. *S. aureus* diluted tenfold to yield approximately 10^6 - 10^8 CFU/ml, was inoculated into the wells; A1 and A2 of a 96-well tissue culture micro-titre plate, to serve as the PAS test wells; B1 and B2 to serve as PAU test wells; C1 and C2 to serve as UCS test wells; D1 and D2 to serve as UCU test wells and; E1 and E2 to serve as control test wells (Figure 5.2). For the same bacterial culture, this procedure was performed in three different 96-well tissue culture plates for either 3 min, 5 min of 15 min sonication. Inoculated plates were aerobically incubated, at 37^o C for 48 hours, for optimal biofilm formation by each bacterial strain prior to sonication.

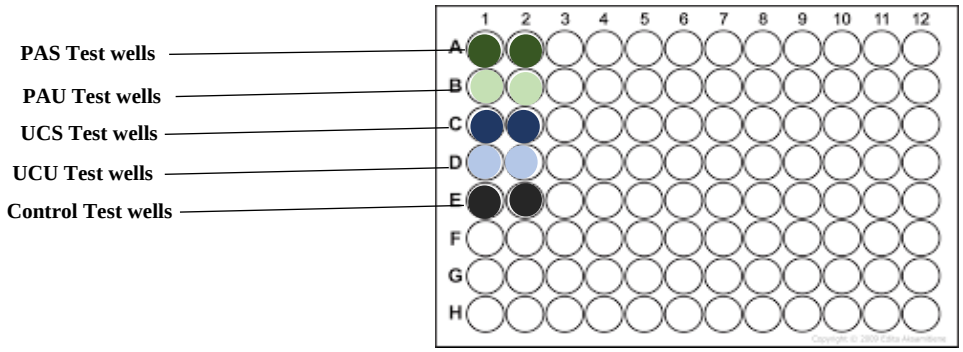


Figure 5.2 Experimental layout of *S. aureus* bacterial inoculation for biofilm development on a 96-well tissue culture treated microtitre plate (quantitative antibiofilm assay)

After the 48 h of incubation, 100 µl of the inoculum each from the duplicate control test wells (all inoculated wells are assumed to be the same since no treatments were introduced) were inoculated onto a fresh BHI agar and incubated for 24 hrs at 37^{o} C to obtain the planktonic bacterial cell count. From the 48 h incubated plate, the inoculum was carefully removed and discarded using a pipette. To ensure that the loose, non-biofilm-associated bacterial cells (planktonic bacterial cells) were rinsed off, the 48 h incubated plate was rinsed twice. The first wash was performed by carefully adding 200 µl of sterile phosphate buffered saline (PBS) to each assigned well (Figure 5.2), after which it was removed and discarded. To check for the presence of planktonic cells, 100 µl of PBS from the first wash was inoculated onto duplicate fresh BHI agar plates and incubated for 24 h at 37^{o} C. After the first wash, the 48 h incubated microtitre plate was carefully dabbed 3 times on a piece of clean tissue paper to blot away excess PBS. The second wash was also performed by carefully adding 200 µl of sterile phosphate buffered saline (PBS) to each assigned well (Figure 5.2) and removed. To check for the presence of planktonic cells, 100 µl of PBS from the second wash, after extraction from the well,

was inoculated onto duplicate fresh BHI agar plates and incubated for 24 h at 37^{o} C. After the second wash, the 48 h incubated microtitre plate was carefully dabbed 3 times on a piece of clean tissue paper to blot away excess PBS from the second wash.

After the first and second washes, 100 µl of sterile BHI broth was added to all wells. Aliquots of 100 µl of the crude extracts were added to their assigned wells in the test row (illustrated in Figure 5.2). The concentration of each extract used in the antibiofilm studies was the Minimum Bactericidal Concentration (MBC) of each extract for each strain of bacterium (Tables 4.5 and 4.6) as determined in Section 4.3.3. One hundred microlitres of the control (50 % ethanol evaporated at 75^{o}C for 3 h) was added to the assigned control wells of the control row (Figure 5.2). Inoculated plates were incubated at 37^{o}C for 24 h for both *S. aureus* and *P. aeruginosa*.

After the 24 h incubation, 100 µl of the inoculum from each of the wells was inoculated onto a fresh BHI agar plate to observe for planktonic bacterial cell count. The extract and control treatments were carefully removed from the wells. To ensure that the loose, non-biofilm-associated bacterial cells (planktonic bacterial cells) were rinsed off, the incubated plate was rinsed twice by carefully adding 200 µl of sterile phosphate buffered saline (PBS) to each assigned well, after which it was removed and discarded. After each wash, 100 µl of the added PBS from each well was inoculated onto a fresh BHI agar, to observe for planktonic bacterial count as done previously, followed by incubation at 37^{o}C for 24 h. The rinsed microtitre plate containing the bacterial biofilm was dabbed 3 times on a piece of clean paper to blot away residual PBS solution.

After the plates had been rinsed, 200 µl of sterile BHI broth was added to both the test and control wells. The plate was covered and sealed with an adhesive lid. For the detachment of the biofilm-resident bacterial cells from the surface of the wells, sonication was carried out in an ultrasound water bath at a frequency of 40 kHz at 30^{o}C for periods

of 3 min, 5 min and 15 min. The purpose of using these varying time-periods was to determine the optimum sonication time that had little or no effect on the viability of the biofilm-resident bacterial cells. After sonication, for each of the time-periods, the contents of each well were ten-fold serially-diluted down to 10^{-6} with PBS and then all of the dilutions were plated (50 µl) onto duplicate fresh BHI agar plates using the Miles and Misra method (Miles, Misra and Irwin 1938). Inoculated plates were incubated, aerobically at 37^{o} C for 24 h for both *S. aureus* and *P. aeruginosa*.

5.2.5 Quantitative antibiofilm assay

After biofilm development as described in Section 5.2.4, the broth containing planktonic cells was carefully removed from each well and then each well was gently rinsed using sterile phosphate buffered saline (PBS) solution, using the following method. The incubated culture and sterile broth were carefully removed from the wells using a multichannel pipette in order not to disrupt the biofilm that had formed. Two hundred microlitres of sterile PBS solution was carefully added to each well, and then removed and discarded. This process was done twice to ensure that all the loose, non biofilm-associated bacterial cells were rinsed away. The rinsed microtitre plate containing the bacterial biofilm was dabbed 3 times on a piece of clean tissue paper to blot away excess PBS solution.

One hundred microlitres of sterile BHI broth was added to each well. The treatments were next added to the wells. Aliquots of 100 µl of the plant extract were added to the wells in the test row (illustrated in Figure 5.2). The concentration of each extract used in the antibiofilm studies was the minimum bactericidal concentration (MBC) of each extract for each strain of bacterium as determined in Section 4.3.3. One hundred microlitres of the control (50 % ethanol evaporated at 80^{o} C for 3 h) was added to the

wells of the control row. Inoculated plates were incubated, aerobically or anaerobically as appropriate, at 37^{o} C for 24 h for all bacterial strains except *P. acnes* which was incubated for 72 h at 37^{o} C.

After incubation, the extract and control treatments were carefully removed from the wells which were then rinsed gently using sterile phosphate buffered saline (PBS) as follows. The treatments were carefully removed from the wells using a multichannel pipette in order not to disrupt the biofilm. Two hundred microlitres of sterile PBS was carefully added to each well, removed and discarded. This process was done twice to ensure that all the loose, non biofilm-associated bacterial cells were removed. The rinsed microtiter plate containing the bacterial biofilm was dabbed 3 times on a piece of clean paper to blot away excess water.

After the plates had been rinsed, 200 µl of sterile BHI broth was added to both the test and control wells. The plate was covered and sealed with an adhesive lid. Sonication was carried out in an ultrasound water bath at a frequency of 40 kHz at 30^{o} C for 5 min to detach the biofilm-resident bacterial cells from the plastic surfaces of the wells. After sonication, the contents of each well were ten-fold serially-diluted down to 10^{-6} with PBS and then all of the dilutions were plated onto duplicate fresh BHI agar plates using the Miles and Misra method (Miles, Misra and Irwin 1938). Inoculated plates were incubated, aerobically or anaerobically as appropriate, at 37^{o} C for 24 h for all bacteria except *P. acnes* which was incubated for 72 h at 37^{o} C. After incubation, the viable counts (CFU/ml) for the test and the control wells were calculated from the colony counts. To analyse the effect of the extract treatment on the bacterial biofilm formed, the log reduction of the CFU from the test with respect to the control was determined.

5.2.6 Statistical analyses

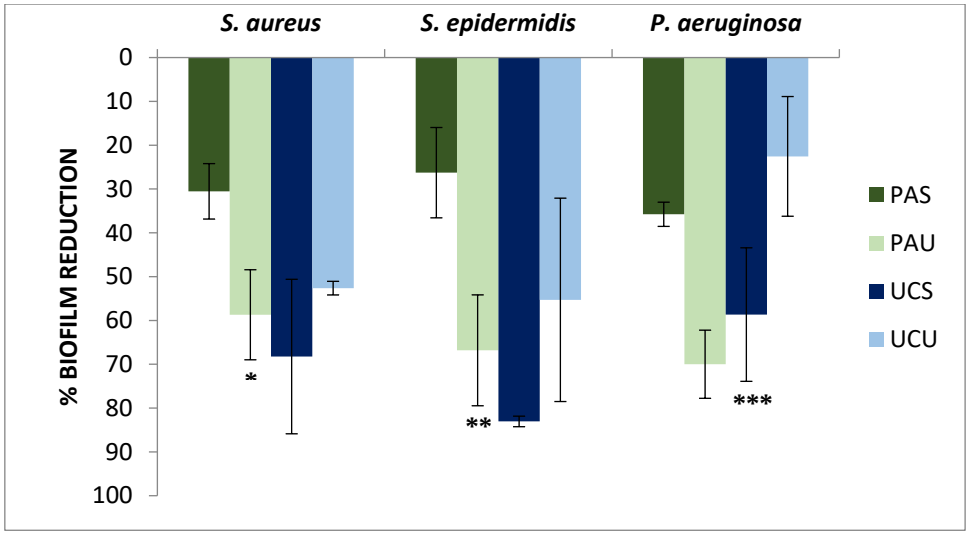
Each of the experiments described above was performed three times. Means and standard deviations of the data were obtained using SPSS version 25. Analysis of variance (ANOVA) was initially used to compare the data, with the *post-hoc* Tukey test used to identify specific differences within each data group. A *P*-value of less than 0.05 was considered as statistically significant for the purposes of this work.

5.3.0 RESULTS

5.3.1 Semi-quantitative biofilm assay

Addition of the *U. chamae* Soxhlet (UCS) and *U. chamae* ultrasound (UCU) extracts resulted in a reduction in the density of preformed biofilm of *Staphylococcus aureus, Staphylococcus epidermidis, Pseudomonas aeruginosa*, *Clostridium difficile* and *Propionibacterium acnes* strains as determined using the semi-quantitative biofilm assay.

Uvaria chamae Soxhlet (UCS) extract significantly reduced the density of preformed biofilms of both *S. aureus* and *S. epidermidis* when compared to their respective controls ($P < 0.05$). With a 68 ± 17.6 % and an 83 ± 1.2 % reduction in the density of preformed *S. aureus* and *S. epidermidis* biofilm respectively, UCS demonstrated significantly greater anti-biofilm activity than UCU or the other extracts ($P < 0.05$).

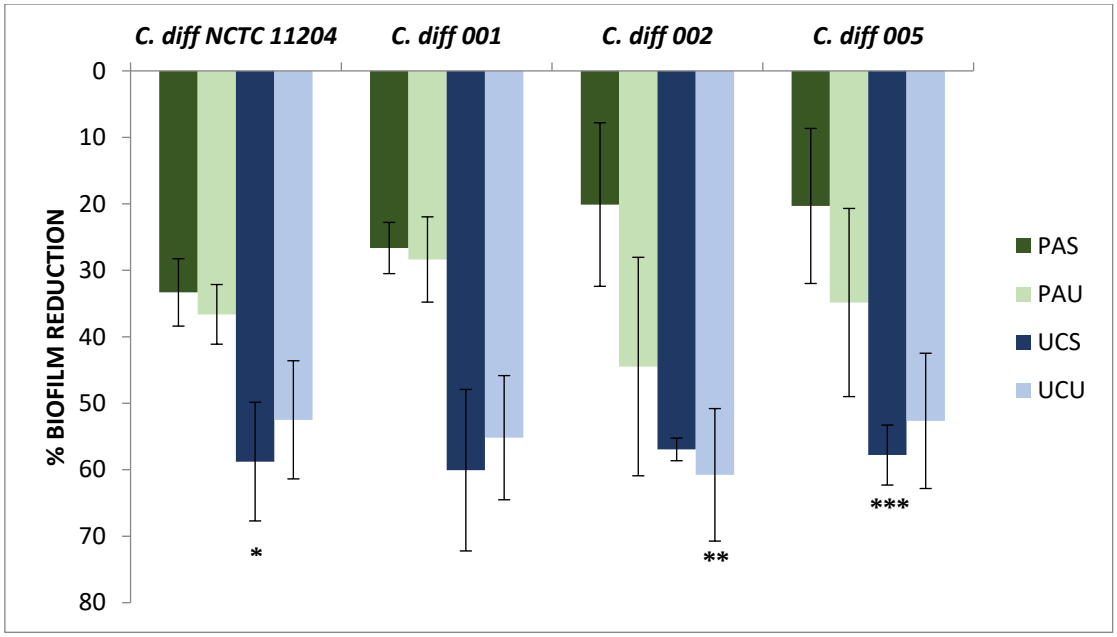


N=3; Bar=Mean; Error bar = ± SD; * =P < 0.05 vs PAS *S.aureus*; ** = P < 0.05 vs PAS *S. epidermidis*; *** = P < 0.05 vs UCU *P. aeruginosa*. Solvent control = 0% biofilm reduction. Note: There was a significant difference between the extract treatments and the control for all the bacteria (P < 0.05)

Figure 5.3 The reduction in preformed biofilm density of *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* after treatment with *Prosopis africana* Soxhlet (PAS) extract, *Prosopis africana* ultrasound (PAU) extract, *Uvaria chamae* Soxhlet (UCS) extract, *Uvaria chamae* ultrasound (UCU) extract relative to the control normalised as 0%.

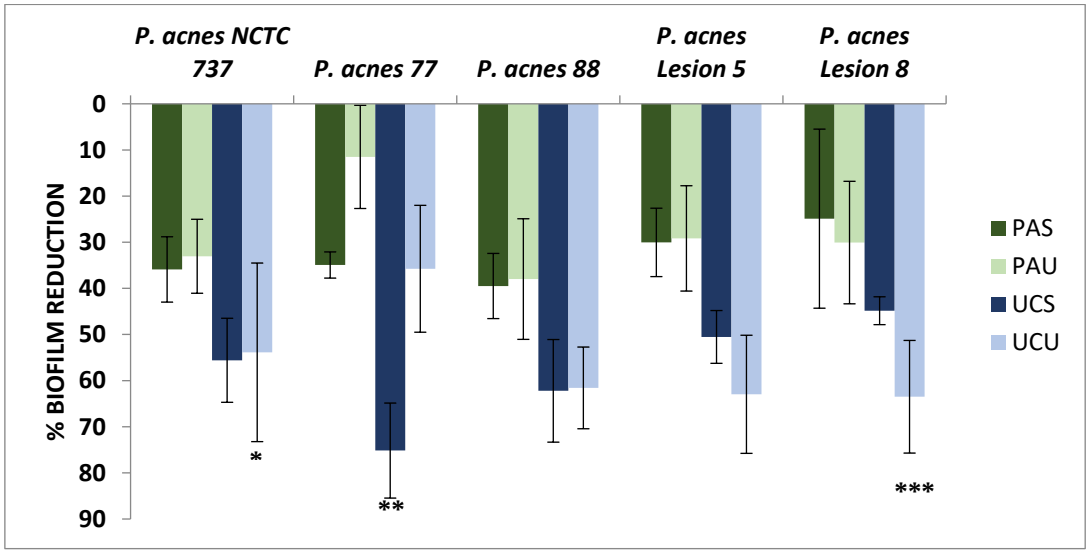
The UCS and UCU extracts significantly reduced the density of preformed *P. aeruginosa* biofilms by 58.7 ± 15.2 % and 22.6 ± 13.7 %, respectively (P < 0.05) (Figure 5.3). *Uvaria chamae* Soxhlet (UCS) demonstrated significantly greater anti-biofilm activity against *P. aeruginosa* when compared to UCU (P < 0.05).

The UCS and UCU extracts demonstrated broadly similar percentage reductions in the preformed biofilm density when the different *C. difficile* isolates were compared (P < 0.05) (Figure 5.4). The density of the preformed *P. acnes* NCTC 737 and Lumbar disc isolate (*P. acnes* 77 and *P. acnes* 88) biofilms were reduced by greater than 50 % by the UCS and UCU extracts (when compared to the control). However, UCU reduced the density of the *P. acnes* 77 biofilm by only 35.8 ± 13.7 % (Figure 5.5). The UCU extract reduced the density of biofilms formed by the *P. acnes* skin lesion isolates (*P. acnes* lesion5 and *P. acnes* lesion8) by greater than 60 %, which was a significant reduction when compared to the control treatment (P < 0.05).



N=3; Bar= Mean; Error bar = ± SD; * =P < 0.05 vs PAS *C. difficile* NCTC 737; ** = P < 0.05 vs PAS *C. difficile 002*; *** = P < 0.05 vs PAS *C. difficile 005*. Solvent control = 0% biofilm reduction, Note: There was a significant difference between the extract treatments and the control for all the bacteria (P < 0.05)

Figure 5.4 The reduction in preformed biofilm density of *Clostridium difficile* isolates after treatment with *Prosopis africana* Soxhlet (PAS) extract, *Prosopis africana* ultrasound (PAU) extract, *Uvaria chamae* Soxhlet (UCS) extract, *Uvaria chamae* ultrasound (UCU) extract relative to the control normalised as 0%.



N=3; Bar= mean; Error bar = ± SD; * =P < 0.05 vs PAU *P. acnes NCTC 737*; ** = P < 0.05 vs PAU *P. acnes 77*; *** = P < 0.05 vs PAS *P. acnes* legion 8, Solvent control = 0% biofilm reduction. Note: There was a significant difference between the extract treatments and the control for all the bacteria (P < 0.05)

Figure 5.5 The reduction in preformed biofilm density of *Propionibacterium acnes* isolates after treatment with *Prosopis africana* Soxhlet (PAS) extract, *Prosopis africana* ultrasound (PAU) extract, *Uvaria chamae* Soxhlet (UCS) extract, *Uvaria chamae* ultrasound (UCU) extract relative to the control normalised as 0%.

Prosopis africana Soxhlet (PAS) and *Prosopis africana* ultrasound (PAU) extracts significantly reduced the density of preformed biofilms of *S. aureus, S. epidermidis, P. aeruginosa, C. difficile* isolates and *P. acnes* isolate ($P < 0.05$). The PAS and PAU extracts significantly reduced the density of preformed *S. aureus* biofilms by 30.5 ± 6.3 % and 58.7 ± 10.2 % respectively ($P < 0.05$) (Figure 5.3). Similarly, PAS and PAU extracts also significantly reduced preformed biofilms of *S. epidermidis* by 26.3 ± 10.3 % and 66.8 ± 12.7 % respectively ($P < 0.05$) (Figure 5.3). Furthermore, treatment with the PAS and PAU extracts brought about a 35.8 ± 2.8 % and a 70.0 ± 7.8 % reduction in preformed biofilm of *P. aeruginosa*, respectively. Overall, the PAU extract demonstrated significantly greater anti-biofilm activity against preformed biofilms of *S. aureus, S. epidermidis* and *P. aeruginosa* than the PAS extract did ($P < 0.05$).

The PAU and PAS extracts had approximately equal effects on biofilms of the *C. difficile* strains ($P > 0.05$) (Figure 5.4). There was no significant difference between the activity of PAU and PAS extracts against *C. difficile* 11204 and *C. difficile* 001 biofilms ($P > 0.05$), although their activity was greater than the control ($P < 0.05$) (Figure 5.4). On the other hand, the PAU extract exhibited significantly greater antibiofilm activity against *C. difficile* strains 002 and 005 than did PAS extract ($P < 0.05$). Similarly, the PAU and PAS extracts had approximately equal effects on biofilms of the *P. acnes* strains except *P. acnes 77* ($P > 0.05$) (Figure 5.5). Furthermore, there was no significant difference between the activities of PAU and PAS against all *P. acnes* strains except *P. acnes* 77 where PAS showed more activity than PAU ($P < 0.05$).

5.3.2 Preliminary quantitative antibiofilm study and optimisation of sonication retrieval process

After 48 h of incubation in the microtitre plate for *S. aureus* biofilm development, the mean viable count for the unattached cells of *S. aureus* was 9.37 $\log_{10}$ CFU/ml ± 0.34.

After 48 h of incubation, the culture medium and unattached cells were aspirated from the microtitre plate wells, as described in the methods in Section 5.2.4, and the wells rinsed with PBS. After having been rinsed two times with PBS, the mean viable count for the unattached cells of *S. aureus* was reduced to 7.99 ± 0.28 $\log_{10}$ CFU/ml in the aspirated PBS after the first rinse, whilst after the second rinse no planktonic cells could be cultured from the aspirated PBS (Table 5.1).

To retrieve the bacterial cells from the rinsed and treated biofilms, the wells were sonicated with 200 µl PBS, as described in Section 5.2.4. In the pilot study, the plates were sonicated for 3 min, 5 min or 15 min, and it was noted that 15 min gave significantly lower viable count retrieval of 7.87 ± 1.01 $\log_{10}$ CFU/ml from the biofilm than 3 min sonication and 5 min sonication ($P < 0.05$) (Table 5.1). Furthermore, there was no significant difference in viable count retrieval from *S. aureus* biofilm after 3 min sonication and 5 min sonication with viable counts of 10.03 ± 0.34 $\log_{10}$ CFU/ml and 10.33 ± 0.57 $\log_{10}$ CFU/ml respectively.

Table 5.1 Viable bacterial count after each process of the quantitative antibiofilm assay optimisation.

VIABLE BACTERIAL COUNT CFU/ML (LOG_{10})		
***After 48hrs incubation* (Biofilm development)**	***S. aureus***	***P. aeruginosa***
Unattached cells	9.37 ± 0.34	9.81 ± 0.86
First rinse	7.99 ± 0.28	7.54 ± 0.34
Second rinse	0.00 ± 0.00	0.00 ± 0.00
After Sonication		
3 min Sonication	10.03 ± 0.34*	8.83 ± 0.27#
5 min sonication	10.33 ± 0.57*	8.88 ± 0.43#
15 min sonication	7.87 ± 1.01	6.75 ± 0.17

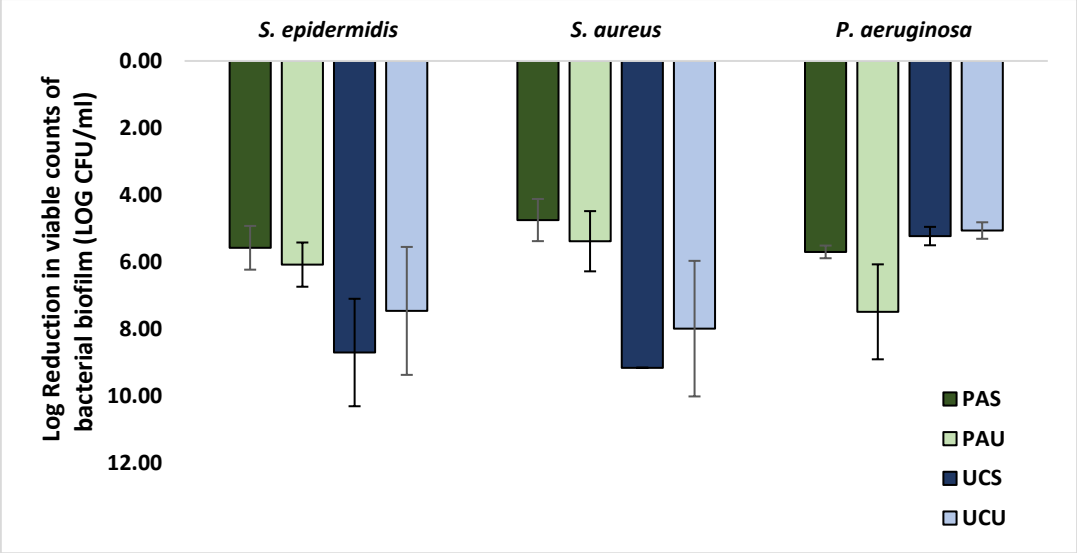
Data represented as mean ± Standard deviation (SD); n = 3; * =P < 0.05 vs 15 min sonication (*S. aureus*), # = P < 0.05 vs 15 min sonication (*P. aeruginosa*).

For *P. aeruginosa*, after 48 h of incubation in the microtitre plate for biofilm development, the mean viable count for the unattached cells of *P. aeruginosa* was 9.81 ± 0.86 $\log_{10}$ CFU/ml. After 48 h of incubation, the culture medium and unattached cells were aspirated from the microtitre plate wells, as described in the methods in Section 5.2.4, and the wells rinsed with PBS. After having been rinsed two times with PBS, the mean viable count for the unattached cells of *P. aeruginosa* was reduced to 7.54 ± 0.34 $\log_{10}$ CFU/ml in the aspirated PBS after the first rinse, whilst after the second rinse no planktonic cells could be cultured from the aspirated PBS (Table 5.1).

To retrieve the bacterial cells from the rinsed and treated biofilms, the wells were sonicated with 200 µl PBS, as described in Section 5.2.4. The plates were sonicated for 3 min, 5 min or 15 min, and it was noted that 15 min gave significantly lower viable count retrieval of 6.75 ± 0.17 $\log_{10}$ CFU/ml from the biofilm than 3 min sonication and 5 min sonication ($P < 0.05$) (Table 5.1). Furthermore, there was again no significant difference in viable count retrieval from *P. aeruginosa* biofilm after 3 min sonication and 5 min sonication with viable counts of 8.83 ± 0.27 $\log_{10}$ CFU/ml and 8.88 ± 0.43 $\log_{10}$ CFU/ml respectively ($P > 0.05$). Consequently, in all subsequent experiments, the microtitre plates were sonicated for 5 min at 40 kHz.

5.3.3 Quantitative antibiofilm assay

There was a statistically significant reduction in the viable counts retrieved from preformed biofilm of *S. epidermidis, S. aureus* and *P. aeruginosa* that had been exposed to *Uvaria chamae* Soxhlet (UCS) extract when compared to the solvent control ($P < 0.05$) (Figure 5.6).

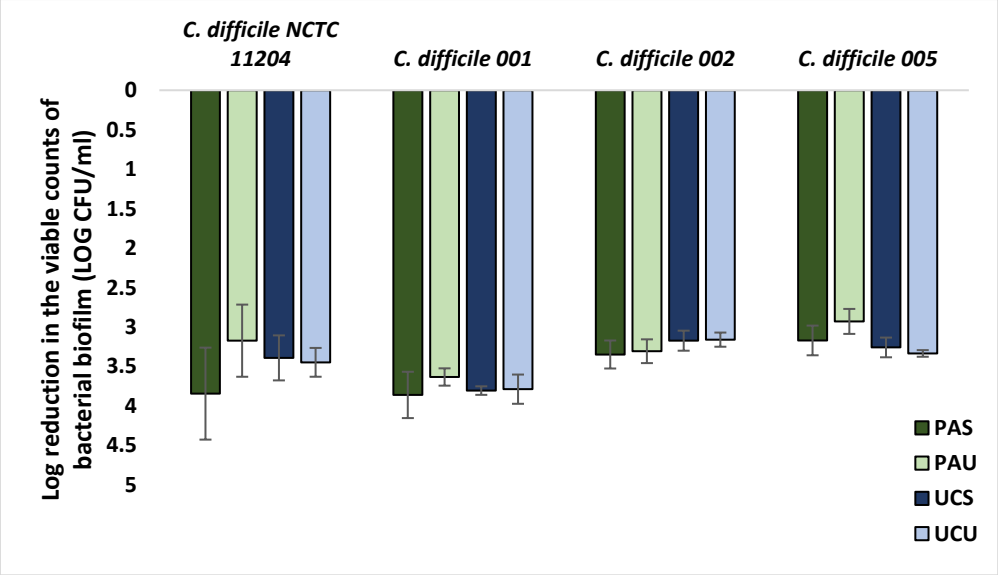


N=3; Error bar = ± SD; Control data were normalised to 0 CFU/ml, Note; A significant difference between extract treatments and control was observed at $P < 0.05$.

Figure 5.6 Log reduction in the viable counts of *Staphylococcus epidermidis, Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilms after treatment with *Prosopis africana* Soxhlet (PAS) extract, *Prosopis africana* ultrasound (PAU) extract, *Uvaria chamae* Soxhlet (UCS) extract, *Uvaria chamae* ultrasound (UCU) extract for 24 h relative to the negative control normalised as 0 CFU/ml.

The *S. aureus* and *S. epidermidis* biofilm-associated viable counts were reduced to below the minimum detection limit when treated with UCS, which was significantly lower than the solvent control ($P < 0.05$) (Appendix 3). Although there was no significant difference in the activity of UCS extract and UCU extract ($P > 0.05$) against *S. epidermidis* and *S. aureus* preformed biofilm, UCS showed more reduction in biofilm – associated viable counts than UCU extract (Figure 5.6).

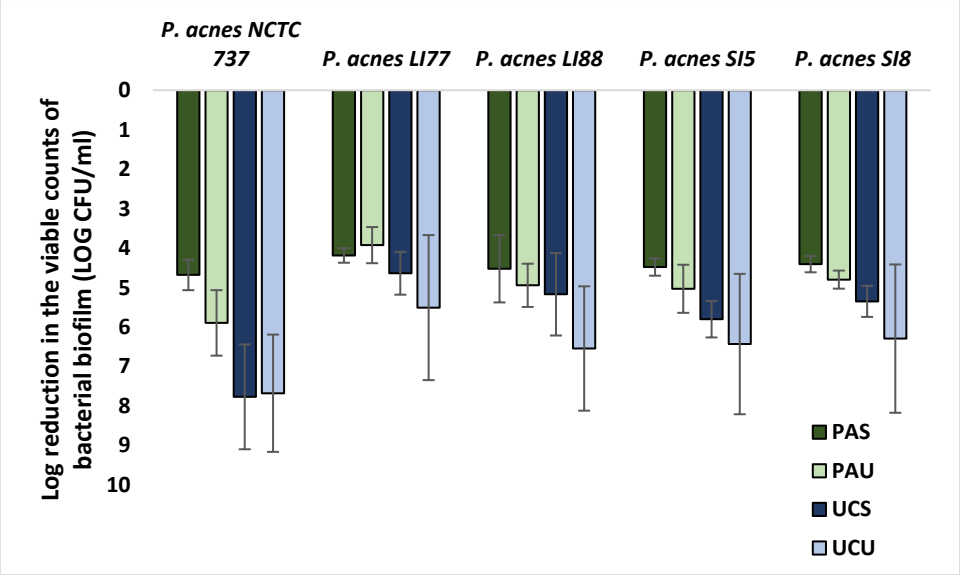
The UCS and UCU extracts were also demonstrated to have activity against preformed biofilms of *C. difficile* clinical isolates (Figure 5.7). It was found that treatment with both UCS and UCU led to a greater than three-log significant reduction in the viable counts of *C. difficile* strains when compared to the control ($P < 0.05$). In contrast, there was no significant difference between the effects of the different extracts upon many of the *C. difficile* strains tested ($P < 0.05$) (Figure 5.7).



N=3; Error bar = ± SD; Control data were normalised to 0 CFU/ml. A significant difference between extract treatments and control was observed at $P < 0.05$.

Figure 5.7 Log reduction in the viable counts of *Clostridium difficile* strains biofilms after treatment with *Prosopis africana* Soxhlet (PAS) extract, *Prosopis africana* ultrasound (PAU) extract, *Uvaria chamae* Soxhlet (UCS) extract, *Uvaria chamae* ultrasound (UCU) extract for 24 h relative to the negative control normalised as 0 CFU/ml.

The UCS and UCU extracts had the greatest activity against preformed *P. acnes* NCTC 737 biofilms. Treatment with the UCS and UCU extracts on the biofilm of *P. acnes* NCTC 737 demonstrated a retrieved biofilm-associated viable count of less than log 1 CFU/ml (Appendix 3), with both yielding up to a seven-log reduction in the viable counts (Figure 5.8). In contrast, treatment with the UCS and UCU extracts on the biofilm of other *P. acnes* isolates apart from *P. acnes* NCTC 737 demonstrated a consistent retrieved biofilm-associated viable count of at least log 2 CFU/ml (Appendix 3), leading to significant reductions, of up to four-log, in the viable counts retrieved from preformed biofilms of the other *P. acnes* isolates in comparison with the solvent control ($P < 0.05$).



N=3; Error bar = ± SD; Control data were normalised to 0 CFU/ml. Significant difference between extract treatments and control was observed at P < 0.05.

Figure 5.8 Log reduction in the viable counts of *Propionibacterium acnes* strains biofilms after treatment with *Prosopis africana* Soxhlet (PAS) extract, *Prosopis africana* ultrasound (PAU) extract, *Uvaria chamae* Soxhlet (UCS) extract, *Uvaria chamae* ultrasound (UCU) extract for 24 h relative to the negative control normalised as 0 CFU/ml.

Treatment with PAS and PAU extract resulted in a statistically significant reduction, of at least six-log, in the viable counts retrieved from preformed biofilms of *Pseudomonas aeruginosa* as compared to the control (P < 0.05) (Appendix 3). Although there was no significant difference in the antibiofilm activity between PAS and PAU against *P. aeruginosa* (P > 0.05), PAU demonstrated more antibiofilm activity with a 7.49 log reduction in *P. aeruginosa* biofilm-associated viable count than PAS with 5.7 log reduction (Figure 5.6). Treatment with the PAS and PAU extracts led to a statistically significant, 5-log reduction in the viable counts of preformed biofilms of both *S. aureus* and *S. epidermidis* in comparison with the solvent control (Appendix 3). In contrast, there was no significant difference between the activity of either PAS or PAU against preformed biofilms of *S. aureus* and *S. epidermidis* (P < 0.05).

Treatment with the PAS and PAU extracts on the biofilm of *C. difficile* isolates demonstrated a consistent retrieved biofilm-associated viable count of at least 3 $\log_{10}$ CFU/ml (Appendix 3), leading to consistent four-fold significant log reductions in the viable counts retrieved from preformed biofilms of the *C. difficile* isolates as compared to the control ($P < 0.05$, ANOVA Tukey) (Figure 5.7).

Treatment with the PAS and PAU extracts on the biofilm of *P. acnes* isolates demonstrated a consistent retrieved biofilm-associated viable count of at least 3 $\log_{10}$ CFU/ml (Figure 5.8), leading to at least a four-fold statistically significant log-reduction in the viable counts retrieved from preformed biofilms of the *P. acnes* strains as compared to the control ($P < 0.05$) (Figure 5.8). Overall, there was no significant difference between the effects of treatment with the extracts. However, PAS had the least effect on preformed biofilms of the *P. acnes* strains, except for *P. acnes* LI77 against which the PAU extract was the least effective (Appendix 3).

5.4.0 DISCUSSION

The formation of microbial biofilms is one of the factors responsible for the emergence of reduced susceptibility of bacteria to antimicrobials which can further develop into antimicrobial resistance. Factors attributed to resistance in biofilms include: the presence of extracellular polymeric substances (EPS) also known as the glycocalyx - which encloses cells in a biofilm and acts as a physical barrier; this restricts molecules from penetrating into the cells by charge attraction due to the negative charge it posseses (Hugo and Russell 2004; Sandasi et al. 2010). At the time, this is the first study to demonstrate the antibiofilm activity against preformed biofilms of *P. aeruginosa*, *S. aureus*, *S. epidermidis*, *P. acnes* and *C. difficile* strains by extracts from *Uvaria chamae* and *Prosopis africana* obtained using Soxhlet extraction and ultrasound extraction.

The preliminary semi-quantitative biofilm assays showed (Figures 5.3, 5.4 and 5.5) that all of the extracts were able to reduce the density of a preformed biofilm, using test strains of *Clostridium difficile, P. aeruginosa, P. acnes, S. aureus* and *S. epidermidis*. The subsequent quantitative biofilm assay results (Appendix 3, Figures 5.6, 5.7 and 5.8) corroborated the bactericidal effect of the extracts on preformed biofilms, yielding log reductions in the viable counts present.

5.4.1 Preliminary quantitative antibiofilm study and optimization of the sonication retrieval process, used to retrieve bacteria from the biofilms (Protocol development).

This study was performed (Section 5.2.4) as a measure to assess the accuracy of the quantitative antibiofilm assay. Results from the preliminary study (Table 5.1) show ed that rinsing the microtititre plate once after biofilm development was not adequate to remove all the unattached cell. After the second rinse, no viable cells of *S. aureus* and *P. aeruginosa* were recovered from the plate. This prevented any interference or skewedness in subsequent results caused by leftovers of the unattached cells. Interestingly, this matches the experimental method used by Kanthawong et al. (2012) when studying the antibiofilm activity of the peptide LL-37 against *Burkholderia pseudomallei* biofilm.

Sonication retrieval of viable cells from the biofilms was optimised as shown in Section 5.3.2. To enable the detachment of the cells from the biofilm and the walls of the plate, the microtitre plate containing the biofilm was subjected to an ultrasonic frequency of 40 kHz for different time periods. Ultrasound has been shown to inhibit and kill bacteria through the action of cavitation bubbles. The collapse of cavitation bubbles generates a mechanical effect powerful enough to disrupt the bacterial cell wall leading to cell death (Gao et al. 2014). Studies suggest that Gram-negative bacteria are

marginally more susceptible to sonication than Gram-positives. For example, Monsen et al. (2009) reported that sonication of *E. coli* for 5 min at 35º C led to a 40% reduction in its viable counts, whilst it took 7 minutes for a similar reduction in *S. aureus* and *S. epidermidis* viable counts. In the current study, there was a greater than 80% reduction in the viable counts of both the Gram-negative *P. aeruginosa* and Gram-positive *S. aureus* after 15 minutes of sonication. In contrast, there was no significant reduction in the viable counts of any of the bacteria after 3 or 5 minutes of sonication. This disparity between the current data and the previous study might be down to strain differences, but it is more likely to be due to the synergistic effects of sonication and temperature since the current study was performed at 30º C rather than 35º C (Monsen et al. 2009). In addition, other studies suggested that a 5 min sonication of a bacterial biofilm at 35 kHz – 45 kHz has little or no effect on the viability of bacterial cells (Kanthowang et al. 2012), while other studies demonstrated that a longer duration of sonication on bacterial biofilm may have an effect on bacterial cell viability. For example, the killing of *P. aeruginosa* and *E. coli* in their respective biofilms after a 30 min sonication might be due to the synergistic effect of the sonication and the nitric oxide-releasing silica nanoparticles as observed in a study by Hetrick et al. (2009). Consequently, since there was little or no effect on *P. aeruginosa* and *S. aureus* after the 5 min sonication in this current study, the 5 min sonication was selected for subsequent sonication retrieval process because it has a higher bacterial count than the 3 min sonication (Table 5.1).

5.4.2 Antibiofilm activity of *Uvaria chamae* against a selection of aerobes and anaerobes.

Uvaria chamae extracts demonstrated antibiofilm activity against the aerobes as observed from results obtained from the semi-quantitative and quantitative assay (Figures

5.3 and 5.6). There was a reduction in excess of 8 log in the biofilm-associated viable counts of cultures of both the *S. aureus* and *S. epidermidis* strains after treatment with the *U. chamae* extracts. Similarly, the semi-quantitative biofilm assay demonstrated that the *U. chamae* extracts had the greatest antibiofilm activity against the Gram-positive aerobes tested (Figure 5.3). Previous studies using other medicinal plants have demonstrated the inhibitory effects of phytochemicals like the phenols, flavonoids, and alkaloids against Gram-positive microorganisms in biofilms including *S. aureus* and *S. epidermidis.* (Ferrazzano et al. 2009; Prabhakar et al. 2010; Rane et al. 2012; Riihinen et al. 2014). Similarly, the antibiofilm activity of *U. chamae* might be attributed to the presence of phytochemicals like flavonoids, alkaloids and phenols. To the knowledge of the author, there was no study on the biofilm activity of plants belonging to Annonaceae plant family to which *U. chamae* belongs, although plants belonging to the Eupomatiaceae plant family like *Jatropha mutabilis*, which are closely related to the Annonaceae, have been studied for their biofilm activity (Chatrou et al. 2012). For example, Trentin et al. (2011) reported that the antibiofilm activities of *J. mutabilis* against *S. epidermidis* biofilm came from impairing bacterial adhesion and reduced formation of the biofilm. The workers described the mechanism of action of the crude extract from *J. mutabilis* against *S. epidermidis* as bacteriostatic instead of bactericidal (Trentin et al. 2011). The findings in this current study demonstrate the action of *U. chamae* extracts against preformed biofilm of the aerobes *P. aeruginosa*, *S. epidermidis*, *S. aureus* as mainly bactericidal as observed from the log reduction in bacterial count. This might have occurred through the disintegration of bacterial adhesion and lysis of bacteria (Kavanaugh and Ribbeck, 2012), which contradicts the findings reported by Trentin et al. (2011). Although the plants used in both studies are from the same plant order (Magnoliales) and thus closely related, the variation in the antibiofilm activity might be due to differences in the presence of

phytochemicals in both plants (Harbone 1998). Interestingly, Quave et al. (2008) demonstrated the antibiofilm activity of crude extracts from *Castanea sativa, Juglans regia, Ballota nigra,* and *Rosmarinus officinalis* by reducing *S. aureus* biofilm biomass at a concentration of 32 µg/ml, which was similar to the concentration at which *U. chamae* extracts reduced *S. aureus* biofilm biomass in this current study. The finding that *U. chamae* extracts are effective against *S. aureus* and *S. epidermidis* biofilms indicates that these plants could provide a source of antibiofilm compounds that can be utilised in the development of treatments for biofilm-associated infections caused by these bacteria.

In this study, the *U. chamae* extracts were also shown to possess antibiofilm activity against the anaerobic species used. The *U. chamae* extracts, UCS and UCU, were shown to reduce the density of preformed biofilms of *P. acnes* strains in both the semi-quantitative and quantitative antibiofilm assays (Figures 5.5 and 5.8). In recent years, the antimicrobial resistance of anaerobes such as *P. acnes* has drastically increased, making it difficult to treat infections by these organisms with existing antibiotics (Schmidt et al. 2015). Antimicrobial resistance in *Propionibacterium acnes* has limited the effectiveness of antibiotic treatment for skin infections due to this organism (Schmidt et al. 2015). Due to the increasing resistance of *P. acnes* against current antibiotics, novel antimicrobials are at a premium. There have only been limited studies on the eradication of *P. acnes* biofilms. To the best of the author's knowledge, there has been only one study into the antibiofilm activity of phytochemicals against *P. acnes*, using a selection of Chinese medicinal plants (Coenye et al. 2012). Significantly, the study by Coenye et al. in 2012 demonstrated the ability of the plant extracts to prevent *P. acnes* biofilm formation. In contrast, the current study analysed the ability of *U. chamae* extracts to reduce the density of preformed *P. acnes* biofilm, which is a curative measure.

Similarly, the *U. chamae* extracts, UCS and UCU, reduced the density of preformed biofilms of the *C. difficile* strains tested in both the semi-quantitative and the quantitative biofilm assays (Figures 5.4 and 5.7). *Clostridium difficile* infection (CDI), a gastrointestinal infection caused by *C. difficile* has been associated with long-term treatment with almost all classes of antimicrobials; in particular clindamycin, third-generation cephalosporins and penicillin treatment pose greatest risk of developing CDI (Owens et al. 2008). It is believed that CDI develops due to disruption of the native gut microflora by long-term antibiotic therapy. This permits the growth of toxigenic *C. difficile* (Owens et al. 2008). In addition, the possession of mobile genetic elements contributes to the increased antimicrobial resistance and virulence of *C. difficile*, making it harder to treat (Sebaihia et al. 2006). Despite the growing resistance of *C. difficile* against antibiotics, to this author's knowledge, there have been no studies into the potential of phytochemicals to treat infections. Consequently, that the current study has demonstrated the activity of *U. chamae* against *C. difficile* biofilms is potentially of great medical significance.

5.4.3 Antibiofilm activity of *Prosopis africana* against a selection of aerobes and anaerobes.

In the current study, the *P. africana* extracts (PAS and PAU) reduced the density of preformed biofilms of the *P. aeruginosa*, *S. aureus*, *S. epidermidis*, *P. acnes* and *C. difficile* strains in both the semi-quantitative and the quantitative biofilm assay. The *P. africana* ultrasound extract (PAU) was particularly effective against the *P. aeruginosa* preformed biofilm in both the semi-quantitative and quantitative biofilm assays (Figures 5.3 and 5.6); although it had a significant effect on all of the biofilms.

It has been demonstrated that biofilm formation by *P. aeruginosa* significantly increases the antibiotic resistance of this bacterium, so making treatment difficult (Kavanaugh and Ribbeck 2012). Although eradication of *P. aeruginosa* biofilm is difficult, a limited number of studies demonstrated the use of plant extracts in reducing preformed biofilm or inhibiting biofilm formation of *P. aeruginosa*. For example, Kavanaugh and Ribbeck in 2012 demonstrated that essential oils from a variety of herbs could eradicate *P. aeruginosa* preformed biofilms. Also, Parkiavathy et al. in 2011 demonstrated the antibiofilm activity of *Cuminum cyminum* methanolic extract by inhibiting the formation of *P. aeruginosa* biofilm. In addition, another study used a selection of South Florida plant extracts to inhibit the production of quorum sensing-controlled virulence factors in *P. aeruginosa* biofilm (Adonizio et al. 2008). However, to the best of this author's knowledge, the current study is the first to report on the antibiofilm activity of *P. africana*.

Prosopis africana has been used traditionally in dental treatment and hygiene to eradicate plaques and tooth aches (Ajiboye et al. 2013) and also in wound management (Inngjerdingen et al. 2004), all of which are influenced by bacterial biofilms (Palombo 2011; Cooper et al. 2014). The effectiveness of *P. africana* extract against biofilms of slectected bacteria in the current study, provides evidence to support the traditional use of *P. africana* in the management of infections (Ayanwuyi et al. 2010), making *P. africana* a suitable prospect for the development of novel antibiofilm therapy.

5.4.4 Conclusion

The current study has demonstrated the antibiofilm activity of both *Uvaria chamae* and *Prosopis africana* extracts against both the aerobic and anaerobic strains used. This substantiates the use of these plants in traditional medicine to treat infections.

Moreover, the effectiveness of extracts from these plants against biofilms of a range of Gram-positive and Gram-negative species, including some clinically – significant pathogens, suggests that they could offer novel antibiofilm therapies and warrant further study.

CHAPTER SIX

6.0 FRACTIONATION OF *PROSOPIS AFRICANA* AND *UVARIA CHAMAE* EXTRACTS.

6.1.0 INTRODUCTION

Identification, characterisation and isolation of active compounds from plant materials are important stages in the discovery of new antimicrobial compounds. Several processes are involved to achieve these. Firstly, plant extracts are assessed qualitatively by thin layer chromatography (TLC) or other chromatographic techniques for the separation of compounds in plant (Rates 2001). Due to the presence of many compounds in a plant extract, it is difficult to separate these plant compounds using the TLC method alone. Therefore, other chromatographic methods like column chromatography, high performance liquid chromatography (HPLC) and gas chromatography (GC) are utilised for the purification and separation of plant compounds (Harbone 1998). Although the separation of compounds from plant extract using TLC is difficult, TLC can also be utilised for selecting an appropriate mobile phase (solvent system) for column chromatography (fractionation) or HPLC process by assessing the movement rates of extracts over a stationary phase (for example silica gel), using different solvent systems (Harbone 1998).

Column chromatography is a method used to purify and isolate active plant compounds by sequentially fractionating the plant extract (Rates 2001). Column chromatography has a similar principle with the TLC, where fractions are being eluted based on differences in the rate of movement of the compounds over a stationary phase (for example silica) using an appropriate solvent system (mobile phase) and is performed

in a column or tube (Harbone 1998). Interestingly, several studies have used this technique to identify antimicrobial compounds from plant extracts (Awouafack et al. 2013; Luis et al. 2016; Cunha et al. 2017; Khalil et al. 2017). Fractions obtained from fractionation are then subjected to further assays like antimicrobial analysis, spectrophotometric analysis, and phytochemical screening.

The aim of this experiment is to: fractionate *Uvaria chamae* Soxhlet (UCS) extract, *Uvaria chamae* ultrasound (UCU) extract, *Prosopis africana* Soxhlet (PAS) extract, and *Prosopis africana* ultrasound (PAU) extract; analyse the antimicrobial activity of the fractions obtained; analyse the presence of selected phytochemicals; and determine the wavelength at which each eluted fraction demonstrated maximal absorption, thus providing more information on the structure and characteristics of the chemical contents in the fractions.

6.2.0 MATERIALS AND METHODS

Materials: *Uvaria chamae* Soxhlet (UCS) extract, *Uvaria chamae* ultrasound (UCU) extract, *Prosopis africana* Soxhlet (PAS) extract, *Prosopis africana* ultrasound (PAU) extract and Solvent control.

Sterile 96-well round-bottomed microtitre plates (Fisher, 11399153), thin-layer chromatography (TLC) chamber (Sigma-Aldrich, Z204226), TLC glass plate 10 cm × 10 cm (silica gel matrix, with fluorescent indicator 254 nm silica) (Sigma-Aldrich, Z292990-1PAK), microcapillary tube (Sigma-Aldrich, P0549-1PAK), UV light (CAMAG UV lamp 4), Ace chromatographic column (Sigma-Aldrich, Z163961-1EA), Biowave II spectrophotometer (Biochrom).

Media and reagent preparation: Mueller-Hinton Agar (Sigma-Aldrich, 70192-500G), Mueller-Hinton Broth (Sigma-Aldrich, 70191-500G), Ninhydrin reagent (0.2 %

ninhydrin in ethanol, Sigma-Aldrich, 151173), iodine solution (0.5 mol/l) (Sigma-Aldrich, 326143), silica powder (Acros Organics, 7631-86-9), dimethyl sulfoxide (DMSO) (Sigma-Aldrich, 472301), hexane (Sigma-Aldrich, 296090-250ML), ethyl acetate (Sigma-Aldrich, 270989-250ML), methanol (Sigma-Aldrich, 34860), ethanol (Sigma-Aldrich, 51976). All media and reagents were prepared following the manufacturers' instructions. All media were autoclaved and sterilised at 121^{o} C, 15 psi pressure for 15 min.

6.2.1 Bacterial Strains and Growth Conditions

Two bacteria (Gram-positive and Gram-negative bacteria) were selected for antimicrobial analysis in this section due to time constraint. *Staphylococcus aureus* NCTC 6591 and *Pseudomonas aeruginosa* NCTC 8295 were provided by the Health and Life Sciences (HLS) laboratory (Coventry University, UK). They were cultured in Mueller-Hinton Broth (MHB) (Sigma-Aldrich UK), incubated at 37^{o} C for 24 h and maintained on Mueller-Hinton Agar (MHA) (Sigma-Aldrich UK) at a temperature of 4^{o} C.

6.2.2 Thin layer chromatography

Thin layer chromatography was performed as previously described by Harbone (1998).

Preparation of the developing container

Different solvent systems were first tested to ascertain the system that gave the best separation. The solvent systems (v/v) used were: ethanol and DMSO in the ratios 1:1, 2:1 and 3:1; ethanol and hexane in the ratios 1:1, 2:1 and 3:1; hexane and DMSO in the ratios 1:1, 2:1 and 3:1; ethyl acetate and methanol in the ratio 6:4; ethanol alone; DMSO alone; and hexane alone.

The thin-layer chromatography (TLC) chamber was saturated with the solvent vapour by firstly pouring the solvent system of choice (as listed above) into the TLC container to a depth of 0.5 cm above the base of the chamber. The TLC chamber was then covered, gently swirled, and allowed to stand for 10 min at room temperature to allow for the chamber to become saturated with vapour.

Preparation and spotting of the TLC plate

Using a pencil, a straight line was drawn across the width of a 10 cm x 10 cm TLC plate with silica as the stationary phase 1 cm above its base to form the origin. A 5 µl aliquot of the crude extract to be separated (at a concentration of 5 mg/ml) was spotted onto the origin line using a microcapillary tube.

Running of the TLC plate

The TLC plate was placed vertically in the developing chamber containing 10 ml of the chosen solvent system, with the origin towards the bottom, so that it was sat on the base of the chamber with the origin line just above the level of the solvent. The solvent was allowed to run up the TLC plate by capillary action, until it reached a level 1 cm below the top edge of the TLC plate. The plate was removed from the chamber and the line of the solvent front was immediately marked with a pencil before it evaporated.

Visualisation and development of TLC plate

After running the TLC plate in the developing chamber, TLC plates were viewed under UV light (CAMAG UV lamp 4) at a wavelength of 254 nm. Spots visualised under UV light were marked by drawing a ring around each one with a pencil. In addition, a photograph was taken of each TLC plate whilst illuminated with UV light. Further development of the TLC plate was performed to observe the presence of compounds which might not be visualised under the UV light. Ninhydrin reagent (0.2 % ninhydrin in

ethanol, Sigma-Aldrich) was sprayed onto the TLC plate to observe the presence of amino acids by the formation of a coloured spot. Also, iodine solution (0.5 mol/l) (Sigma-Aldrich) was sprayed to observe the presence of unsaturated and aromatic compounds evident by the formation of coloured spots.

TLC Analysis

The ideal solvent system was determined based on the recommendations of Hegge et al. (1991) and Sherma and Fried (2005). The mean retention factor (Rf) of the spotted samples (extracts) for each solvent system was calculated (Equation 5.2) and then, the distance travelled by the sample on the TLC plate was visualised as described previously. The retention factor (Rf) for each spot was then calculated using the equation below:

Equation 5.2

RF = Distance travelled by the compound ÷ Distance travelled by solvent front

6.2.3 Column chromatography

Column chromatography was performed as previously described by Harbone (1998).

Preparation and packing of the column

The chromatographic column was firmly and securely clamped to a retort stand. A silica gel slurry was made in a glass beaker by dissolving 25 g of silica powder (Acros Organics) in 200 ml of the solvent system (ethanol : DMSO in the ratio of 3 : 1). The slurry was then poured into the chromatographic column and allowed to stand for at least 10 min to enable the packing of the silica gel within the column. A glass beaker was placed under the column and the pinch clamp on the column was opened to gradually drain out the solvent. The solvent that drained out was gently poured back into the column

containing the silica gel and eluted out again to help ensure proper packing of the silica gel. After the packing of the silica gel in the column (Figure 6.1a), excess solvent was drained out until there was only a few centimetres of solvent head above the packed silica gel. Care was taken to protect the silica gel from drying out by ensuring that a few centimetres of solvent always remained above the level of the packed silica gel (Figure 6.1b). This was done to prevent the packed silica gel from developing cracks, which might have interfered with the fractionation process.

Figure 6.1 Preliminary stage for column Chromatography; (a) Packing of the Silica gel in the glass column; (b) To avoid the drying up of the gel, approximately 2 centimetres of solvent are left above the packed Silica gel (c) Loading of the extract sample

Loading of the Sample

One hundred and fifty millilitres of the crude extract samples to be fractionated were concentrated to a volume of 5 ml using the rotary evaporator. Five millilitres of the concentrated extract was loaded unto the packed silica gel using a Pasteur pipette by touching the pipette tip to the inner wall of the glass column and then letting the extract gently trickle down the wall onto the surface of the packed silica (Figure 6.1c). This was done to avoid the disruption of the packed silica gel. After dispensing the crude extract on to the packed silica gel, further addition of the solvent system (ethanol : DMSO) to the

top of the gel was done so as to undertake the elution process. Addition of the solvent system was continuous during the elution process until the pigment from the extract sample had completely run through the column.

Elution of the fractions from the extract

The eluting solvent was gently added to the column (Figure 6.2a) and the pinch clamp was opened to allow the elution of the extract into the beaker at the bottom of the column. The eluting solvent was allowed to drain out and was continuously replaced until the extract sample ran into the Silica gel bed. Subsequently, fractions of the extract, as it eluted, were collected in universal bottles every 2 min 50 sec, which amounted to a 15 ml fraction of the eluted extract in each bottle (Figure 6.2b and 6.2c). Collection of the fractions was stopped when the contents of the fractions became clear (colourless), thus signifying the completion of extract elusion.

Figure 6.2 Column chromatography; (a) Filling of the column with the eluting solvent to aid the movement of the sample down the gel; (b) Collection of the fraction in universal bottles; (c) an example of the fractions collected.

6.2.4 Analysis of the eluted fractions

The eluted fractions obtained were analysed using the following techniques: broth micro-dilution assay to determine the antimicrobial activity of each in the form of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC); phytochemical analysis of the fractions; and spectrophotometry analysis of the fractions.

Antimicrobial activity of fractions by broth microdilution assay

The antimicrobial activity (MIC and MBC) of each of the eluted fractions was determined against *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The protocol as described in Section 2.2.3.3 was followed.

Phytochemical analysis of fractions

Qualitative phytochemical analyses performed on each of the eluted fractions to determine the presence of saponins, alkaloids, quinones and flavonoids. The protocols for performing these analyses as described in Section 3.2.4 were followed.

Spectrophotometry analysis of fractions

The wavelength at which each eluted fraction demonstrated maximal absorption was determined using a Biowave II spectrophotometer (Biochrom) with a scanning wavelength range of 190 – 1100 nm. The solvent system (3:1 ethanol : DMSO v/v) was used as the blank and the wavelength at which the peak absorbance for each fraction occurred was recorded.

6.2.5 Statistical analyses

Each of the experiments described above was performed three times. Means and standard deviations of the data were obtained using SPSS version 25. Analysis of variance (ANOVA) was initially used to compare the data, with the *post-hoc* Tukey test used to

identify specific differences within each data group. A *P*-value of less than 0.05 was considered as statistically significant for the purposes of this work.

6.3.0 RESULTS

6.3.1 Thin layer chromatography

On visualising the developed silica gel plates using an ultraviolet (UV) lamp, it was observed that all four extracts, *Prosopis africana* Soxhlet (PAU), *Prosopis africana* ultrasound (PAU), *Uvaria chamae* Soxhlet (UCS), *Uvaria chamae* ultrasound (UCU) travelled from the origin at relatively the same rate with the aid of the solvent mixtures used. No significant difference in the retention factor (Rf) values was seen between the extracts for each solvent system ($P > 0.05$)

Using a silica gel plate and a mixture of ethanol and dimethyl sulfoxide (DMSO) (1:1) as the development solvent system, Rf values ranging from $0.90 \pm 0.23 – 0.95 \pm 0.51$ were recorded for the four extracts (Table 6.1) and poor extract separation (faint bands) for each extract was observed (Figure 6.3d). For ethanol and DMSO (2:1) as the developing solvent, Rf values ranging from $0.80 \pm 0.27 – 0.85 \pm 0.40$ were recorded for the four extracts (Table 6.1), faint bands of the extracts (poor extract separation) were observed. For ethanol and DMSO (3:1) as the developing solvent, Rf values ranging from $0.56 \pm 0.13 – 0.58 \pm 0.09$ were recorded for the four extracts (Table 6.1) and a good separation (visible bands) for each extract was observed (Figure 6.3a). For ethanol and hexane (1:1) as the developing solvent system, Rf values ranging from of $0.61 \pm 0.13 – 0.66 \pm 0.87$ were recorded for the four extracts and a poor separation (faint bands) for each extract was observed. For ethanol and hexane (2:1) as the developing solvent system,

Rf values ranging from 0.58 ± 0.74 – 0.61 ± 0.13 were recorded for the four extracts, a poor separation (faint bands) for each extract was observed.

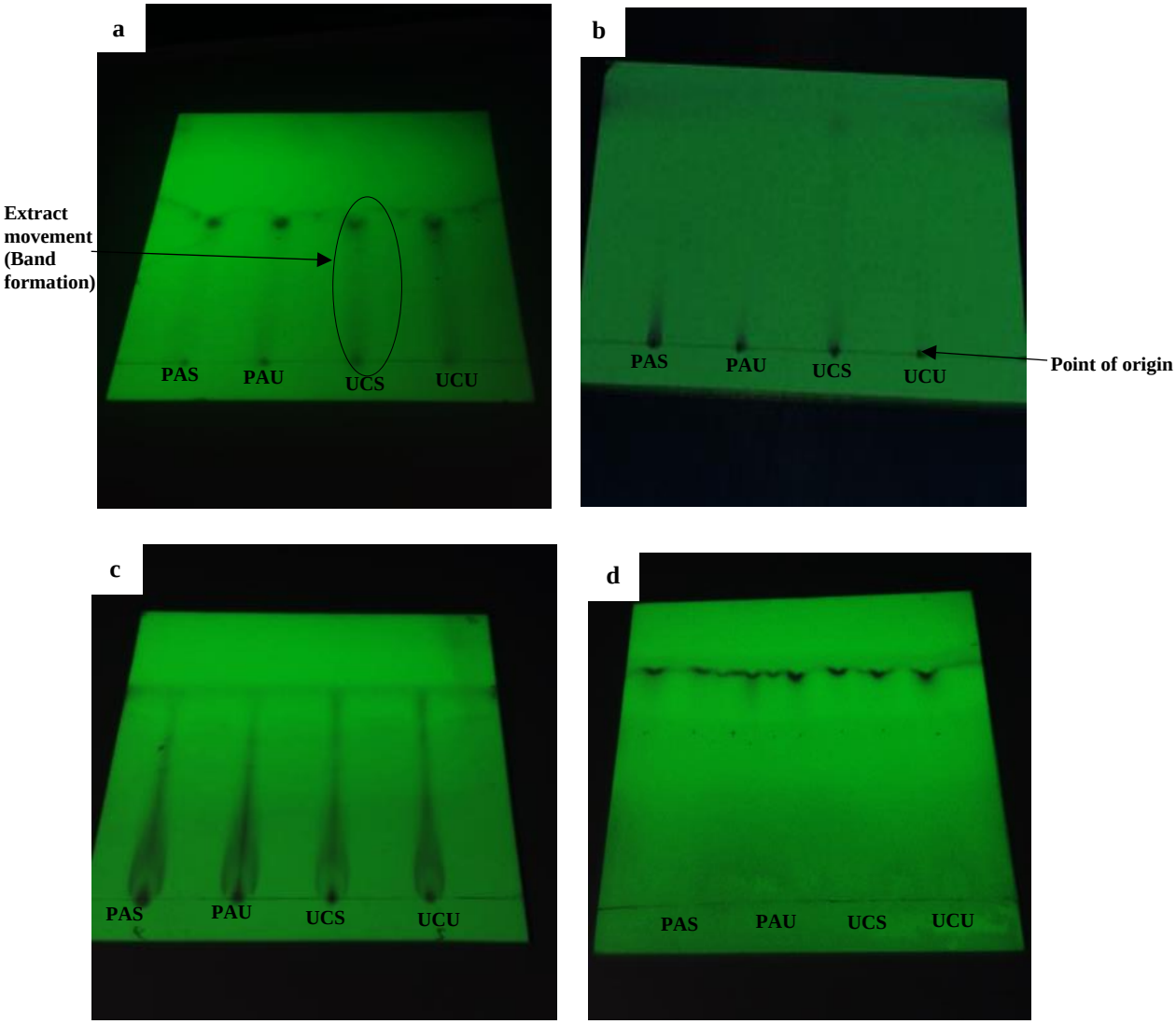


Figure 6.3 Examples of visualisation result of silica gel plates after development to analyse the movement of extracts with the aid of various solvent systems. (a) development with ethanol and DMSO (3:1) (b) development with hexane (c) development with Hexane and DMSO (2:1) (d) development with Ethanol and DMSO (1:1)

For ethanol and hexane (3:1) as the developing solvent system, Rf values ranging from 0.42 ± 0.09 – 0.44 ± 0.16 were recorded for the four extracts, a poor separation (faint band) for each extract was observed. Similarly, for hexane and DMSO (1:1) as the developing solvent system, Rf values ranging from 0.87 ± 0.43 – 0.96 ± 0.51 were recorded for the four extracts and no separation for the extract was observed. For hexane

and DMSO (2:1) as the developing solvent system, Rf values ranging from 0.81 ± 0.12 – 0.88 ± 0.32 were recorded for the four extracts and a poor separation for each extract was observed (Figure 6.3c). For hexane and DMSO (3:1) as the developing solvent system, Rf values ranging from 0.51 ± 0.10 – 0.58 ± 0.91 were recorded for the four extracts, a poor separation (faint band) for each extract was also observed. For ethyl acetate and methanol (3:2) as the developing solvent system, the Rf value for all the extracts was 0.00 ± 0.00 and no movement or separation for each extract was observed (Table 6.1).

Table 6.1 Results showing retention factor (Rf) value and separation of extracts on a silica gel plate with the aid of different solvent systems as developing solvents using thin layer chromatography.

SOLVENT SYSTEM	SEPARATION	RF VALUES			
		PAS	PAU	UCS	UCU
Ethanol : DMSO 1:1	-	0.94 ± 0.23	0.96 ± 0.51	0.87 ± 0.43	0.90 ± 0.11
Ethanol : DMSO 2:1	+	0.82 ± 0.0	0.80 ± 0.27	0.85 ± 0.40	0.83 ± 0.33
Ethanol : DMSO 3:1	++	0.57 ± 0.45	0.56 ± 0.13	0.58 ± 0.09	0.57 ± 0.43
Ethanol : Hexane 1:1	+	0.65 ± 0.08	0.62 ± 0.47	0.61 ± 0.13	0.66 ± 0.87
Ethanol : Hexane 2:1	+	0.60 ± 0.32	0.61 ± 0.13	0.58 ± 0.74	0.59 ± 0.46
Ethanol : Hexane 3:1	+	0.43 ± 0.06	0.42 ± 0.09	0.42 ± 0.23	0.44 ± 0.16
Hexane : DMSO 1:1	-	0.92 ± 0.23	0.93 ± 0.14	0.92 ± 0.09	0.91 ± 0.11
Hexane : DMSO 2:1	+	0.88 ± 0.32	0.86 ± 0.34	0.82 ± 0.62	0.81 ± 0.12
Hexane : DMSO 3:1	+	0.52 ±0.13	0.54 ±0.55	0.51 ±0.10	0.58 ±0.91
Ethyl acetate : Methanol 3:2	-	0.00 ±0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Hexane	+	0.28 ± 0.04	0.11 ± 0.12	0.18 ± 0.89	0.16 ± 0.54
DMSO	-	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00

Data represented as mean ± Standard deviation (SD); (-) represents no separation; (++) represents good separation; (+) represents poor separation n = 3; PAS – *Prosopis africana* Soxhlet extract; PAU – *Prosopis africana* ultrasound extract; UCS – *Uvaria chamae* Soxhlet extract; UCU – *Uvaria chamae* ultrasound extract. No significant difference between the extracts was observed ($P > 0.05$).

For hexane as the developing solvent system, Rf values ranging from 0.11 ± 0.12 – 0.28 ± 0.04 were recorded for the four extracts and a poor separation (faint band) for each extract was also observed (Figure 6.3b). For DMSO as the developing solvent

system, the Rf value for all the extracts was 1.0 ± 0.0 was recorded and no separation for each extract was observed (Table 6.1).

6.3.2.0 Column Chromatography

6.3.2.1 *Fraction collection*

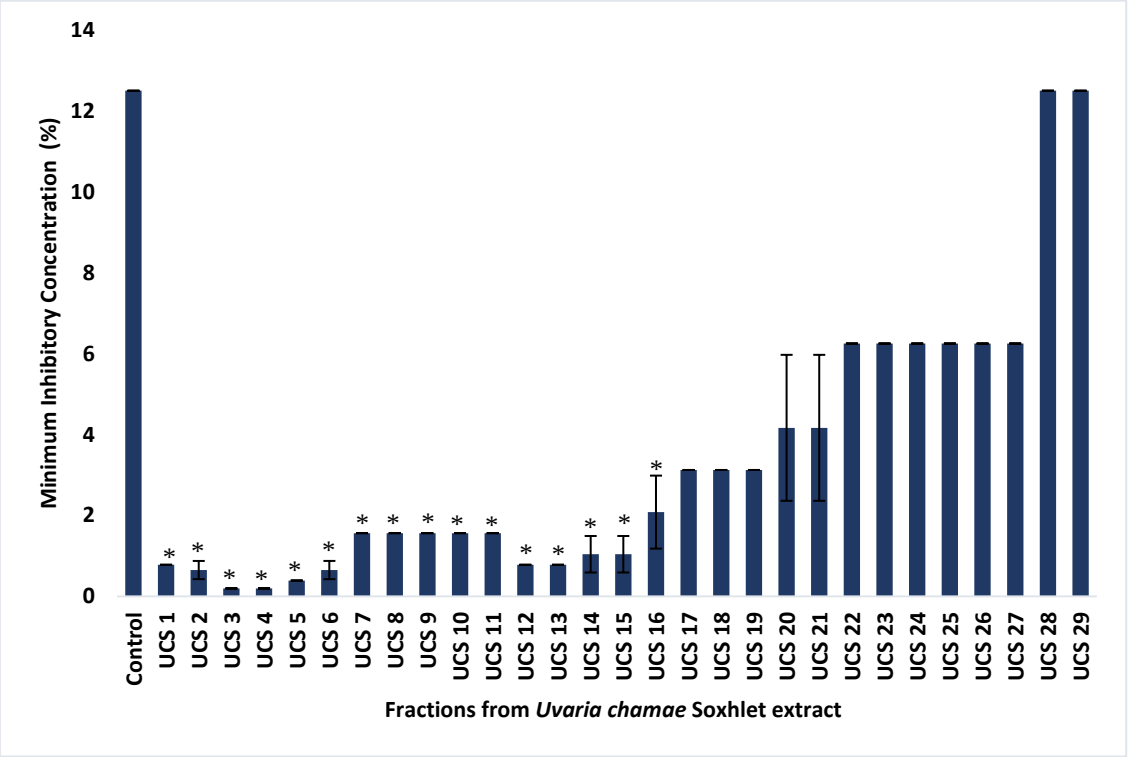
Fractions from four extracts were collected after running the extracts onto a silica-gel packed column. Results from this analysis showed that 29 fractions were obtained from *Uvaria chamae* Soxhlet (UCS) extract while 17 fractions were obtained from *Uvaria chamae* ultrasound extract. On the other hand, *Prosopis africana* Soxhlet extract generated 28 fractions after fractionation while *Prosopis africana* ultrasound extract generated 24 fractions.

6.3.3 Antimicrobial activities of fractions against *P. aeruginosa* and *S. aureus*

6.3.3.1 *Uvaria chamae* extracts (Minimum inhibitory concentration of fractions) against *P. aeruginosa* and *S. aureus*

The control used in this assay did not give a 100 % MIC value because it demonstrated antimicrobial activity with an MIC values of 12.5 % and 6.25 % against *S. aureus* and *P. aeruginosa* respectively.

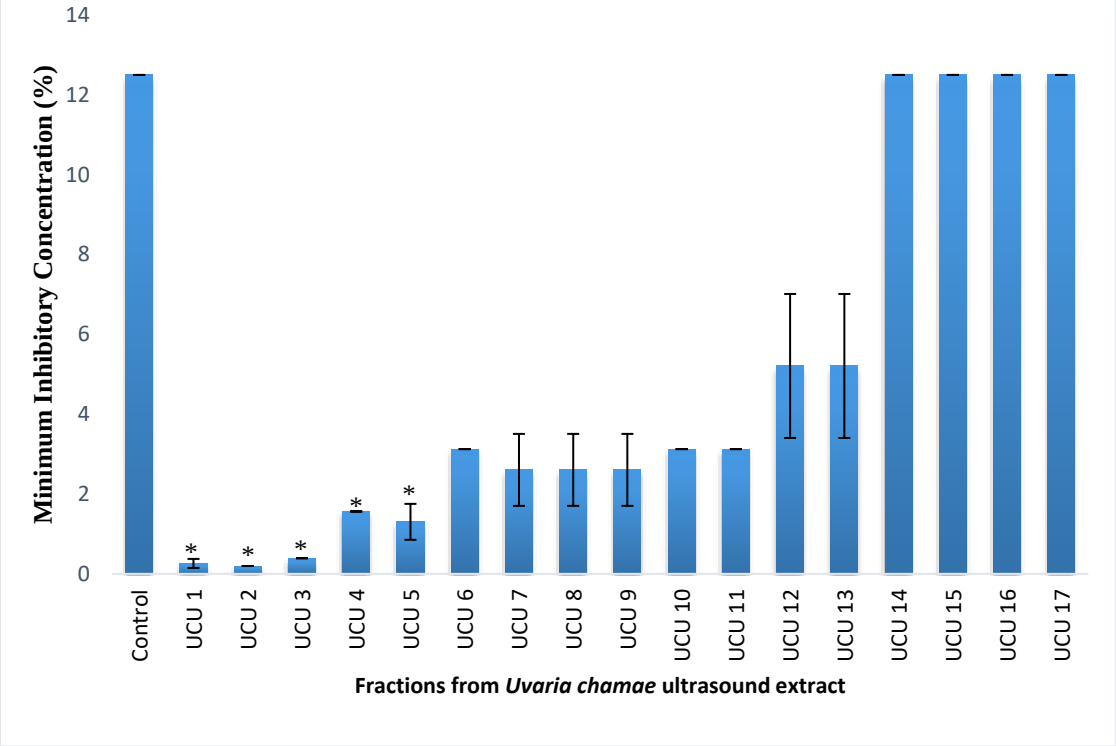
For *S. aureus*, fractions UCS 1 – 16 demonstrated a significant antimicrobial inhibitory effect with an MIC value range of 0.195 % - 2.08% (starting concentration before dilution was 100 %) compared to the solvent control ($P < 0.05$) (Appendix 4). Fractions UCS 17 – 21 and fractions UCS 22 – 27 demonstrated inhibitory effect against *S. aureus* with MIC values of 3.215 % - 4.17 % and 6.25% respectively even though no significant difference was observed when compared to the control ($P > 0.05$). Fractions UCS 28 and 29 demonstrated no inhibitory effect against *S. aureus* (Figure 6.4).



N=3; Bar= mean; Error bar = ± SD; starting concentration of fractions and control = 100%; Control = Ethanol : DMSO (3:1); UCS No. = *Uvaria chamae* Soxhlet fraction number; * = P < 0.05 vs Control

Figure 6.4 Antimicrobial activity of the fractions from *Uvaria chamae* Soxhlet extract, showing the minimum inhibitory concentration of the fractions against *Staphylococcus aureus* using the micro-dilution assay.

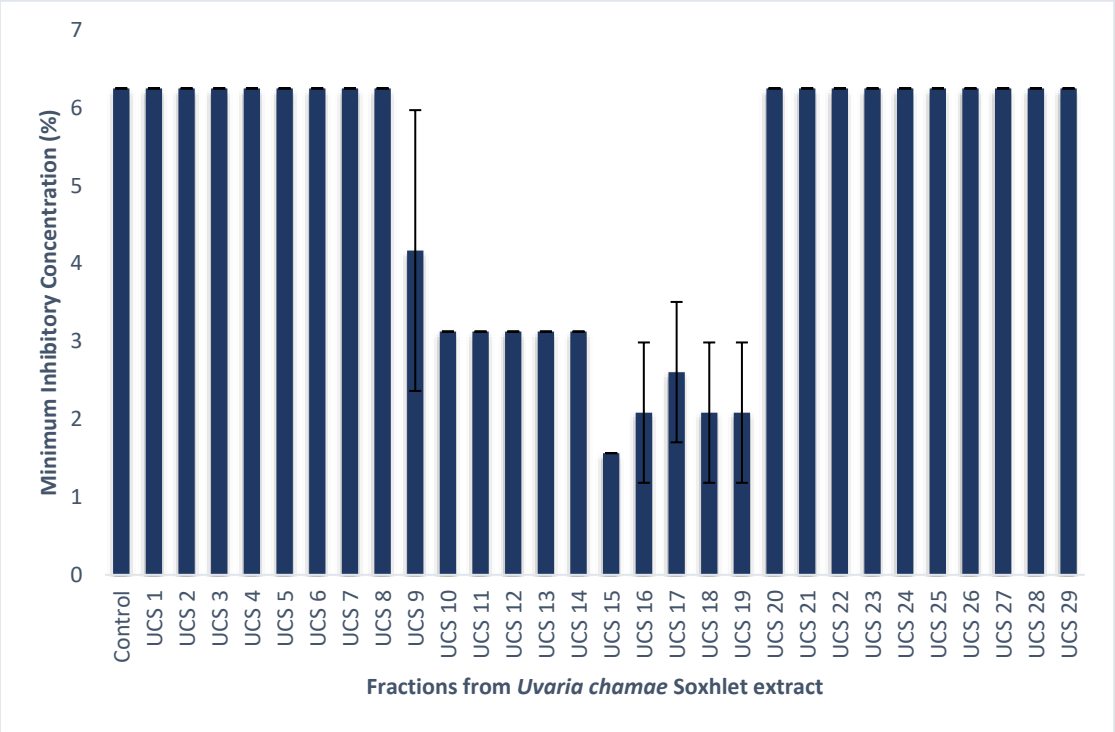
For *S. aureus,* some of the fractions obtained from *U. chamae* ultrasound extract demonstrated antimicrobial inhibitory activity (Figure 6.5 and Appendix 4). Fractions UCU 1 – 5 demonstrated a significant inhibitory effect against *S. aureus* with an MIC value range of 0.195 % - 1.56 % (starting concentration for all the fractions and solvent control was at 100%) compared to the solvent control ($P < 0.05$). Furthermore, fractions UCU 6 – 13 demonstrated an inhibitory effect against *S. aureus* with MIC value range of 2.60 % - 5.21 %, although, no significant difference was observed when compared to the solvent control ($P > 0.05$) (Figure 6.5 and Appendix 4). Meanwhile, fractions UCU 14 – 17 demonstrated no inhibitory effect on *S. aureus*.



N=3; Bar= mean; Error bar = ± SD; starting concentration of fractions and control = 100%; Control = Ethanol : DMSO (3:1); UCU No. = *Uvaria chamae* ultrasound fraction number; * = P < 0.05 vs Control

Figure 6.5 Antimicrobial activity of the fractions from *Uvaria chamae* ultrasound extract, showing the minimum inhibitory concentration of the fractions against *Staphylococcus aureus* using the micro-dilution assay.

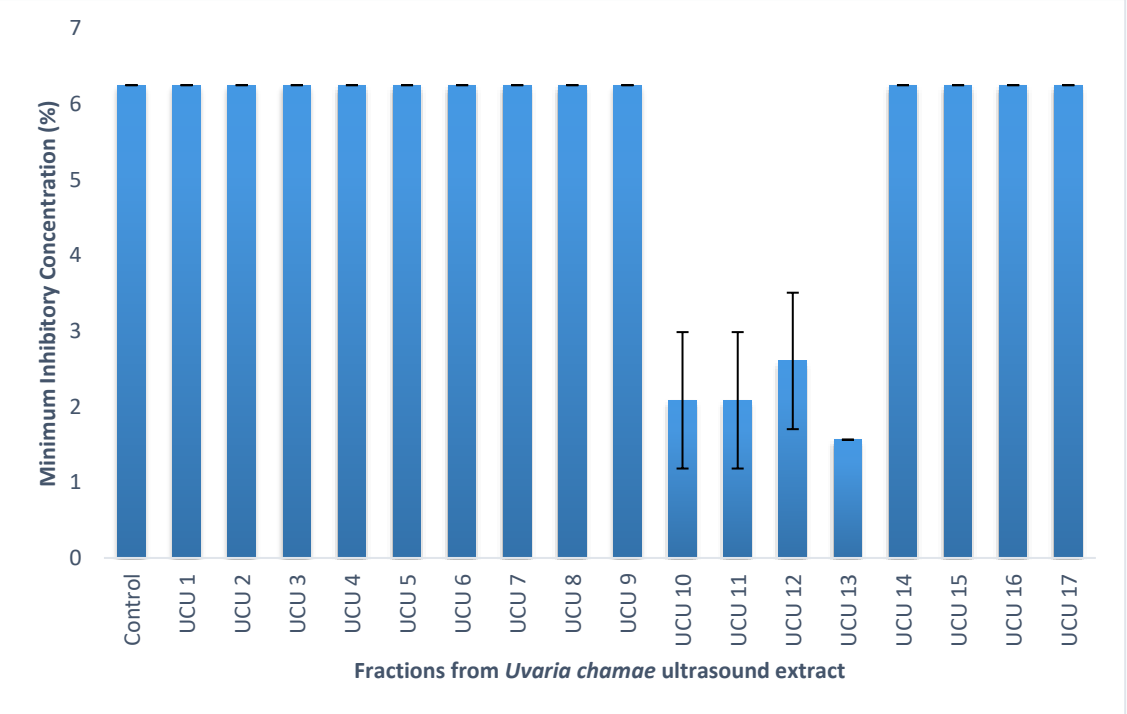
For *P. aeruginosa*, *Uvaria chamae* Soxhlet (UCS) extracts demonstrated antimicrobial inhibitory activities (Figure 6.6 and Appendix 4). Fractions UCS 15 –19 demonstrated the most antimicrobial inhibitory effect with an MIC value range of 1.563 % - 2.604% (starting concentration before dilution was 100 %) although no significant difference was observed when compared to the solvent control and other UCS fractions ($P > 0.05$) (Figure 6.6). Also, fraction 9 and fractions UCS 10 – 14 demonstrated inhibitory effect against *P. aeruginosa* with MIC values of 4.17% and 3.125 % respectively (starting concentration at 100%), although no significant difference was observed when compared to the solvent control ($P > 0.05$). Fractions UCS 1 – 8 and fractions UCS 20 – 29 demonstrated no inhibitory effect against *P. aeruginosa* ($P > 0.05$) (Figure 6.6).



N=3; Bar= mean; Error bar = ± SD; starting concentration of fractions and control = 100%; Control = Ethanol : DMSO (3:1); UCS No. = *Uvaria chamae* Soxhlet fraction number; No significant difference between fractions and Control (P > 0.05).

Figure 6.6 Antimicrobial activity of the fractions from *Uvaria chamae* Soxhlet extract, showing the minimum inhibitory concentration of the fractions against *Pseudomonas aeruginosa* using the micro-dilution assay.

For *P. aeruginosa*, some of the fractions obtained from *U. chamae* ultrasound (UCU) extract demonstrated antimicrobial inhibitory activity (Figure 6.7 and Appendix 4). Fraction UCU 10 – 13 demonstrated inhibitory effect against *P. aeruginosa* with an MIC value ranging from 1.563 % - 2.6 % (starting concentration for all the fractions and solvent control was at 100%) although there was no significant difference when compared to the solvent control and other UCU fractions (P > 0.05) (Figure 6.7). Meanwhile, UCU 1 – 9 and UCU 14 – 17 demonstrated no inhibitory effect against *P. aeruginosa* relative to the control.



N=3; Bar= mean; Error bar = ± SD; starting concentration of fractions and control = 100%; Control = Ethanol : DMSO (3:1); UCU No. = *Uvaria chamae* ultrasound fraction number; No significant difference between fractions and Control (P > 0.05).

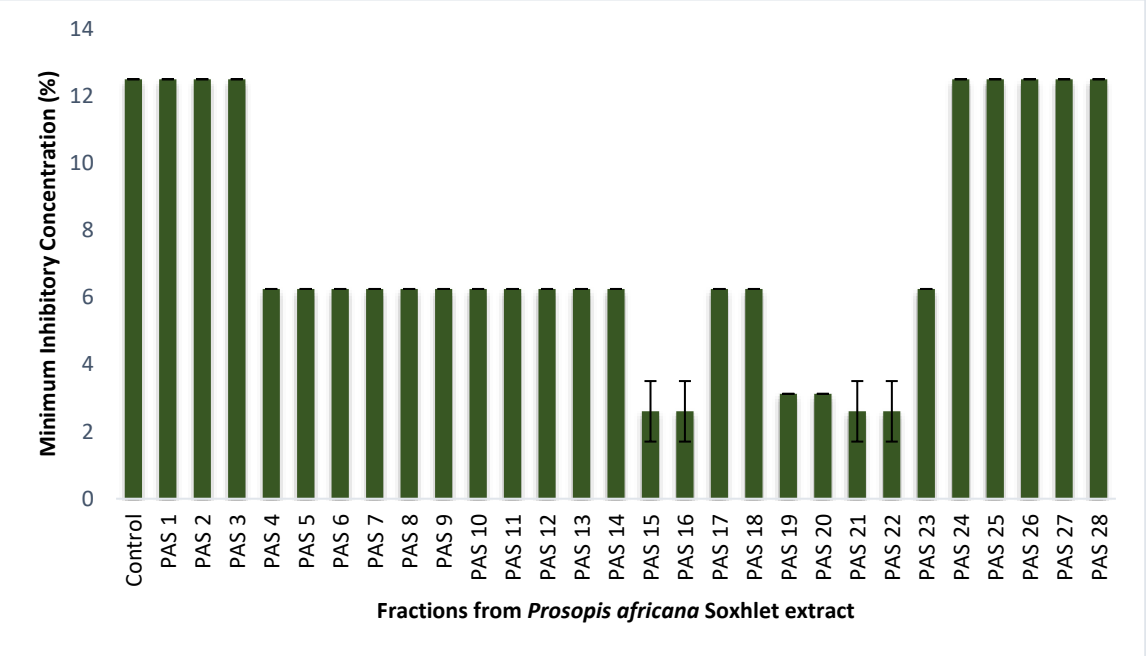
Figure 6.7 Antimicrobial activity of the fractions from *Uvaria chamae* ultrasound extract, showing the minimum inhibitory concentration of the fractions against *Pseudomonas aeruginosa* using the micro-dilution assay.

6.3.3.2 *Prosopis africana* extracts (minimum inhibitory concentration of fractions) against *P. aeruginosa* and *S. aureus*.

The control used in this assay did not give a 100 % MIC value because it demonstrated antimicrobial activity with an MIC values of 12.5 % and 6.25 % against *S. aureus* and *P. aeruginosa* respectively.

For *S. aureus*, some fractions from *Prosopis africana* Soxhlet (PAS) extract demonstrated antimicrobial inhibitory activity (Figure 6.8 and Appendix 5). Fractions 15 – 16 and PAS 19 – 22 demonstrated the greatest inhibitory effect against *S. aureus* with an MIC value range of 2.604 % - 3.125 % although, no significant difference was observed when compared to the solvent control (P > 0.05). Furthermore, fractions PAS 4 – 14, 17, 18 and 23 all demonstrated inhibitory effect against *S. aureus* with an MIC value

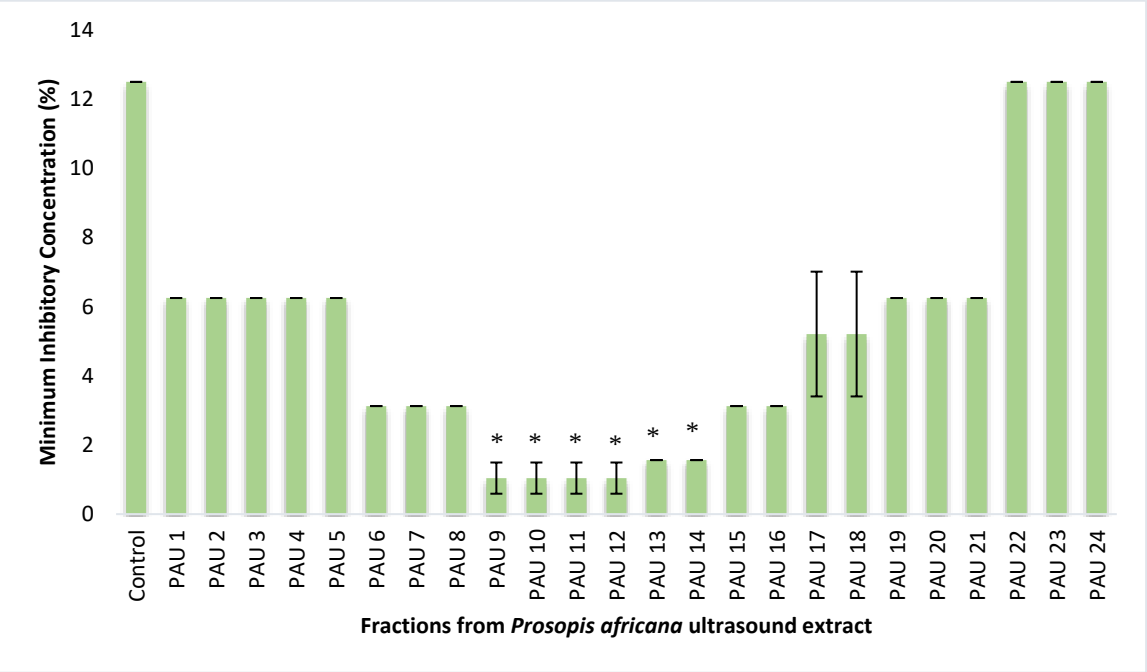
of 6.25 % although no significant difference was observed when compared to the solvent control ($P > 0.05$). Meanwhile, fractions PAS 1 – 3 and 24 – 28 demonstrated no inhibitory effect against *S. aureus* compared to the control (Figure 6.8).



N=3; Bar= mean; Error bar = ± SD; starting concentration of fractions and control = 100%; Control = Ethanol : DMSO (3:1); PAS No. = *Prosopis africana* Soxhlet fraction number; No significant difference between fractions and Control ($P > 0.05$, ANOVA Tukey).

Figure 6.8 Antimicrobial activity of the fractions from *Prosopis africana* Soxhlet extract, showing the minimum inhibitory concentration of the fractions against *Staphylococcus aureus* using the micro-dilution assay.

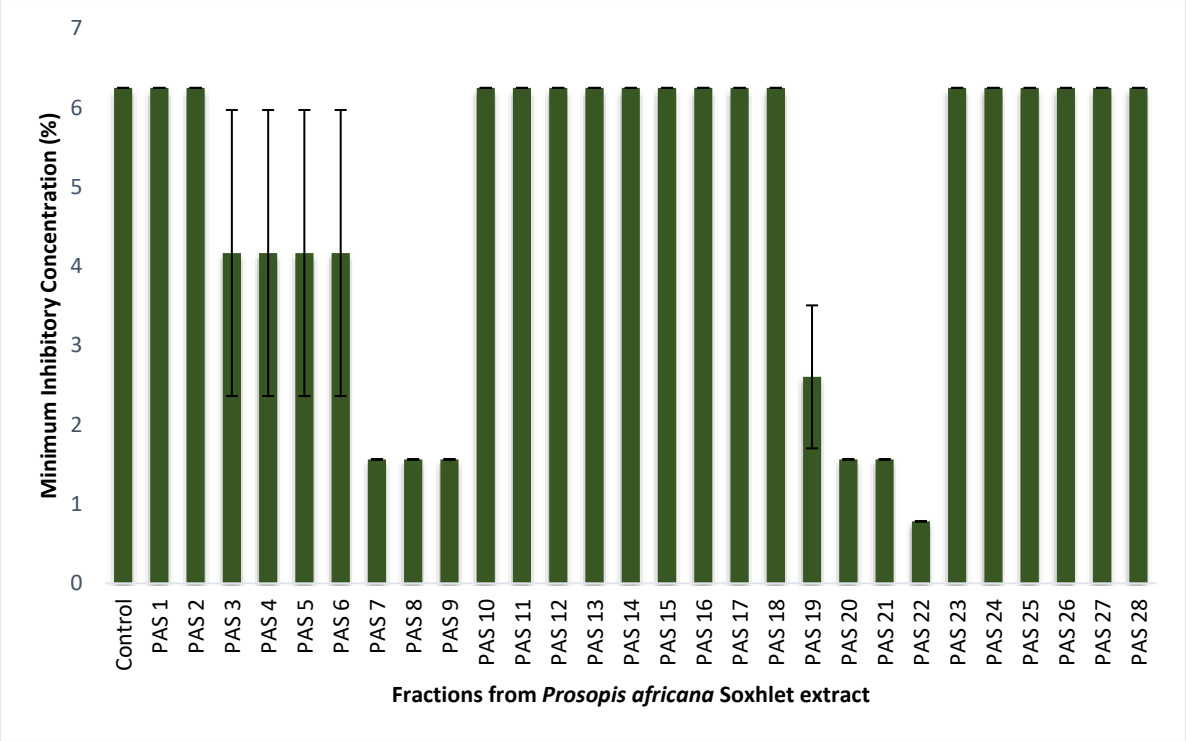
For *S. aureus*, some fractions from *Prosopis africana* ultrasound (PAU) extract demonstrated antimicrobial inhibitory activity (Figure 6.9 and Appendix 5). Fractions PAU 1 - 21 demonstrated inhibitory effect against *S. aureus* with an MIC value ranging from 3.125% - 6.25 %, except for fractions PAU 9 – PAU 14 with an MIC value range of 1.04 % – 1.56 % which was significantly lower than the MIC value of the solvent control ($P < 0.05$). Meanwhile, fractions PAU 22 – 24 demonstrated no inhibitory effect against *S. aureus* relative to the control (Figure 6.9).



N=3; Bar= mean; Error bar = ± SD; starting concentration of fractions and control = 100%; Control = Ethanol : DMSO (3:1); PAU No. = *Prosopis africana* ultrasound fraction number; * = P < 0.05 vs Control (ANOVA Tukey).

Figure 6.9 Antimicrobial activity of the fractions from *Prosopis africana* ultrasound extract, showing the minimum inhibitory concentration of the fractions against *Staphylococcus aureus* using the micro-dilution assay.

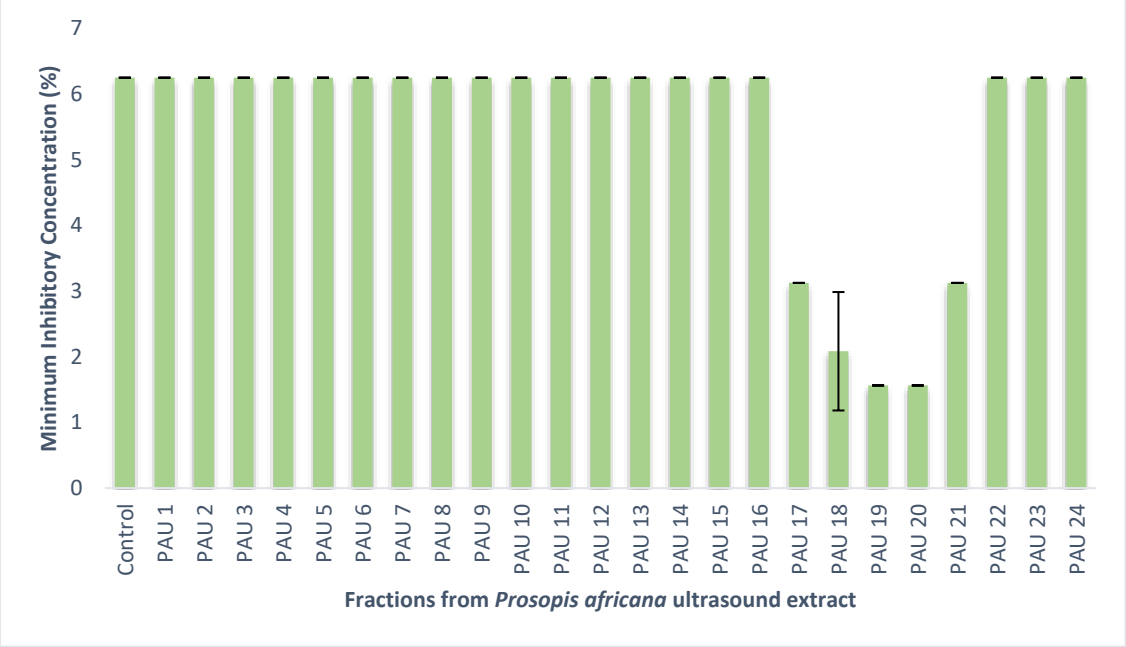
For *P. aeruginosa*, some fractions from *Prosopis africana* Soxhlet (PAS) extract demonstrated antimicrobial activity (Figure 6.10 and Appendix 5). Fractions PAS 3 – 9 and 19 – 22 demonstrated inhibitory effect against *P. aeruginosa* with an MIC value ranging from 0.78 % - 4.17 % although no significant difference was observed when compared to the solvent control (P > 0.05) (Figure 6.10). Meanwhile, fractions PAS 1 – 3, PAS 10 – 18 and PAS 23 – 28 demonstrated no inhibitory effect against *P. aeruginosa*.



N=3; Bar= mean; Error bar = ± SD; starting concentration of fractions and control = 100%; Control = Ethanol : DMSO (3:1); PAS No. = *Prosopis africana* Soxhlet fraction number; No significant difference between fractions and Control (P > 0.05, ANOVA Tukey).

Figure 6.10 Antimicrobial activity of the fractions from *Prosopis africana* Soxhlet extract, showing the minimum inhibitory concentration of the fractions against *Pseudomonas aeruginosa* using the micro-dilution assay.

For *P. aeruginosa*, some fractions from *Prosopis africana* ultrasound (PAU) extract demonstrated antimicrobial activity (Figure 6.11 and Appendix 5). Fractions PAU 17 – 21 demonstrated inhibitory effect against *P. aeruginosa* with an MIC value ranging from 1.563 % - 3.125 % although no significant difference was observed when compared to the solvent control and other PAU fractions (P > 0.05) (Figure 6.11). Fractions PAU 1 – 16 and 22 – 24 demonstrated no inhibitory effect against *P. aeruginosa*.



N=3; Bar= mean; Error bar = ± SD; starting concentration of fractions and control = 100%; Control = Ethanol : DMSO (3:1); PAU No. = *Prosopis africana* ultrasound fraction number; No significant difference between fractions and Control (P > 0.05, ANOVA Tukey).

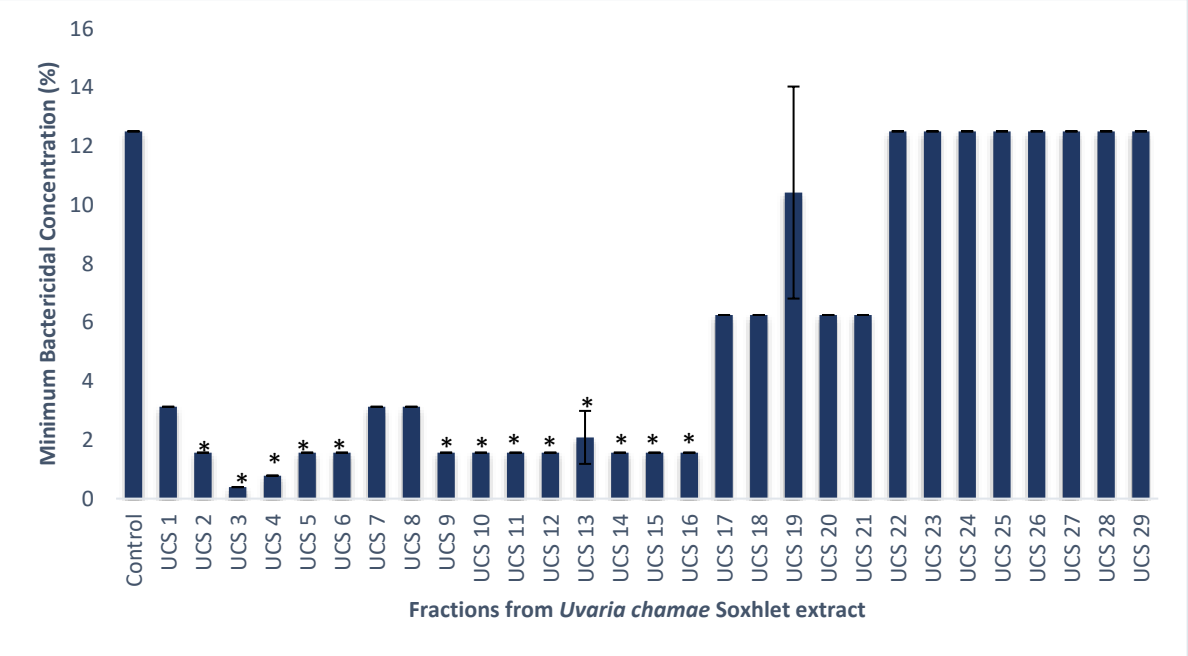
Figure 6.11 Antimicrobial activity of the fractions from *Prosopis africana* ultrasound extract, showing the minimum inhibitory concentration of the fractions against *Pseudomonas aeruginosa* using the micro-dilution assay.

6.3.3.3 *Uvaria chamae* extracts (minimum bactericidal concentration of fractions) against *P. aeruginosa* and *S. aureus*.

The minimum bactericidal concentration (MBC) of the fractions against *Staphylococcus aureus* and *Pseudomonas aeruginosa* was analysed after performing the micro-dilution broth assay. The control used in this assay did not give a 100 % MBC value because it demonstrated antimicrobial activity with an MBC values of 12.5 % against *S. aureus* and *P. aeruginosa*.

For *S. aureus*, some fractions from *Uvaria chamae* Soxhlet (UCS) extract demonstrated antimicrobial bactericidal activity (Appendix 6). Fractions 2 - 16 demonstrated significant bactericidal effect against *S. aureus* with MBC value range of 0.391 % - 2.08 % respectively (starting concentration before dilution was 100 %) compared to the solvent control ($P < 0.05$) except fractions UCS 7 and 8. Furthermore,

fractions UCS 1, 7 and 8 demonstrated bactericidal effect against *S. aureus* with MBC value of 3.125 %. Also, fractions 17 – 21 demonstrated bactericidal effect against *S. aureus* with MBC values ranging from 6.25 % - 10.42 %. Meanwhile, fractions UCS 22 - 29 demonstrated no bactericidal effect against *S. aureus* compared to the control (Figure 6.12).

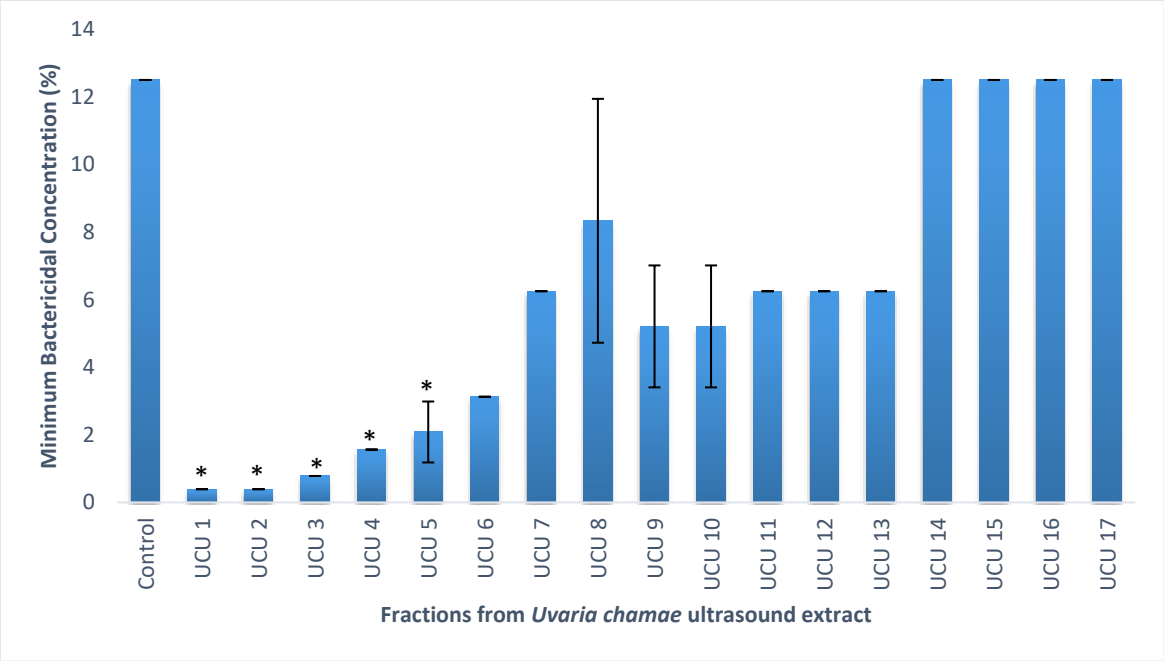


N=3; Bar= mean; Error bar = ± SD; starting concentration of fractions and control = 100%; Control = Ethanol : DMSO (3:1); UCS No. = *Uvaria chamae* Soxhlet fraction number; * = P < 0.05 vs Control

Figure 6.12 Antimicrobial activity of the fractions from *Uvaria chamae* Soxhlet extract, showing the minimum bactericidal concentration of the fractions against *Staphylococcus aureus* after performing the micro-dilution assay.

For *S. aureus*, some of the fractions obtained from *U. chamae* ultrasound extract demonstrated antimicrobial bactericidal activity (Figure 6.13 and Appendix 6). Fractions UCU 1 – 5 demonstrated significant bactericidal effect against *S. aureus* with an MBC value range of 0.391 % - 2.08 % compared to the solvent control (P < 0.05). Furthermore, fractions UCU 6 - 13 demonstrated bactericidal effect against *S. aureus* with an MBC value range of 3.125 % - 8.33 %, although no significant difference was observed when

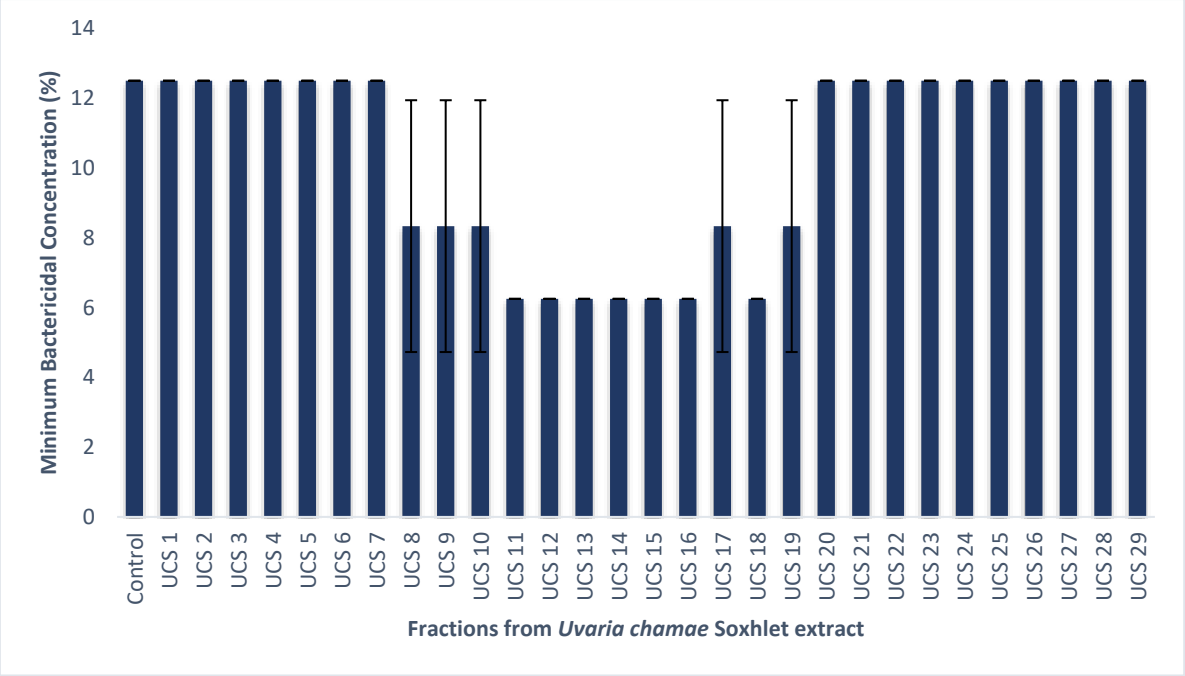
compared to the solvent control (P > 0.05). Meanwhile, fractions UCU 14 – 17 demonstrated no bactericidal effect against *S. aureus* (Figure 6.13).



N=3; Bar= mean; Error bar = ± SD; starting concentration of fractions and control = 100%; Control = Ethanol : DMSO (3:1); UCU No. = *Uvaria chamae* ultrasound fraction number; * = P < 0.05 vs Control

Figure 6.13 Antimicrobial activity of the fractions from *Uvaria chamae* ultrasound extract, showing the minimum bactericidal concentration of the fractions against *Staphylococcus aureus* after performing the micro-dilution assay.

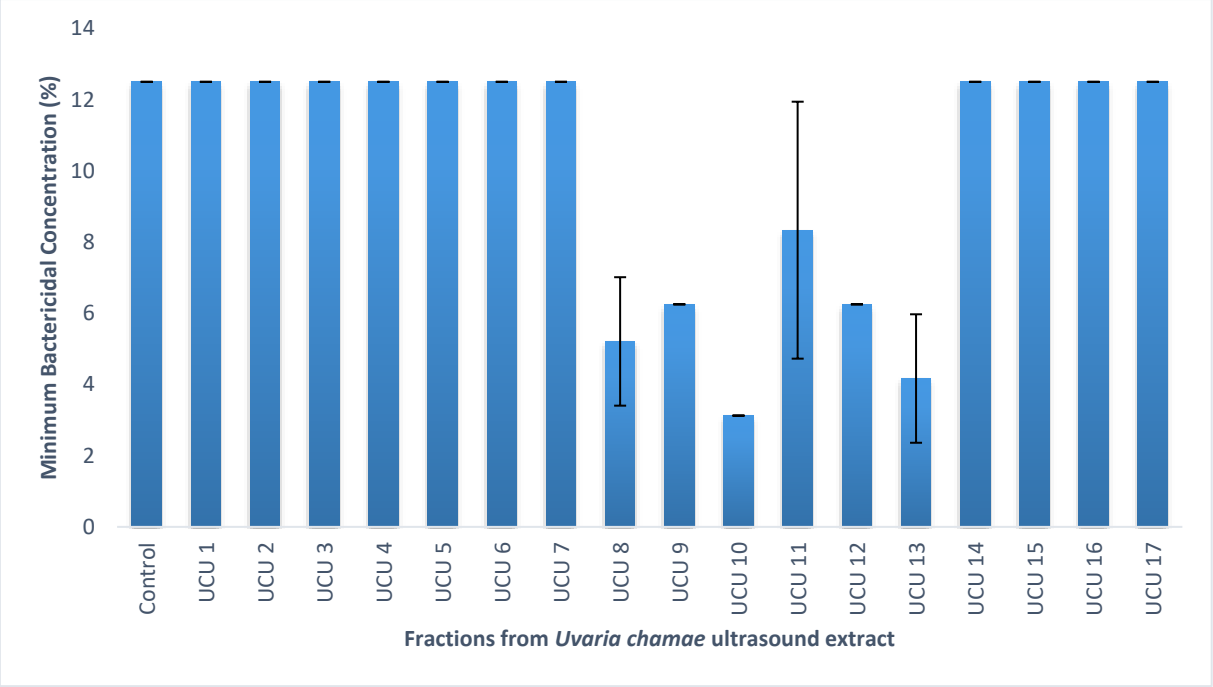
For *P. aeruginosa*, some fractions from *Uvaria chamae* Soxhlet (UCS) extracts demonstrated antimicrobial bactericidal activity (Appendix 6). Fractions UCS 8 – 19 demonstrated bactericidal effect against *P. aeruginosa* with an MBC value range of 6.25 % - 8.33 % although no significant difference was observed when compared to the solvent control and other UCS fractions (P > 0.05). Meanwhile, fractions UCS 1 – 7 and UCS 20 – 29 demonstrated no bactericidal effect against *P. aeruginosa* (Figure 6.14).



N=3; Bar= mean; Error bar = ± SD; starting concentration of fractions and control = 100%; Control = Ethanol : DMSO (3:1); UCS No. = *Uvaria chamae* Soxhlet fraction number; No significant difference between fractions and Control (P > 0.05).

Figure 6.14 Antimicrobial activity of the fractions from *Uvaria chamae* Soxhlet extract, showing the minimum bactericidal concentration of the fractions against *Pseudomonas aeruginosa* after performing the micro-dilution assay.

For *P. aeruginosa,* some of the fractions obtained from *U. chamae* ultrasound (UCU) extract demonstrated antimicrobial bactericidal activity (Appendix 6). Fractions UCU 8 - 13 demonstrated bactericidal effect against *P. aeruginosa* with an MBC value between 3.125 % - 8.17 % respectively (starting concentration for all the fractions and solvent control was at 100%) although there was no significantly difference when compared to the solvent control and other UCU fractions (P > 0.05). Meanwhile, fractions UCU 1 – 7 and 14 – 17 demonstrated no bactericidal effect against *P. aeruginosa* (Figure 6.15).



N=3; Bar= mean; Error bar = ± SD; starting concentration of fractions and control = 100%; Control = Ethanol : DMSO (3:1); UCU No. = *Uvaria chamae* ultrasound fraction number; No significant difference between fractions and Control (P > 0.05, ANOVA Tukey).

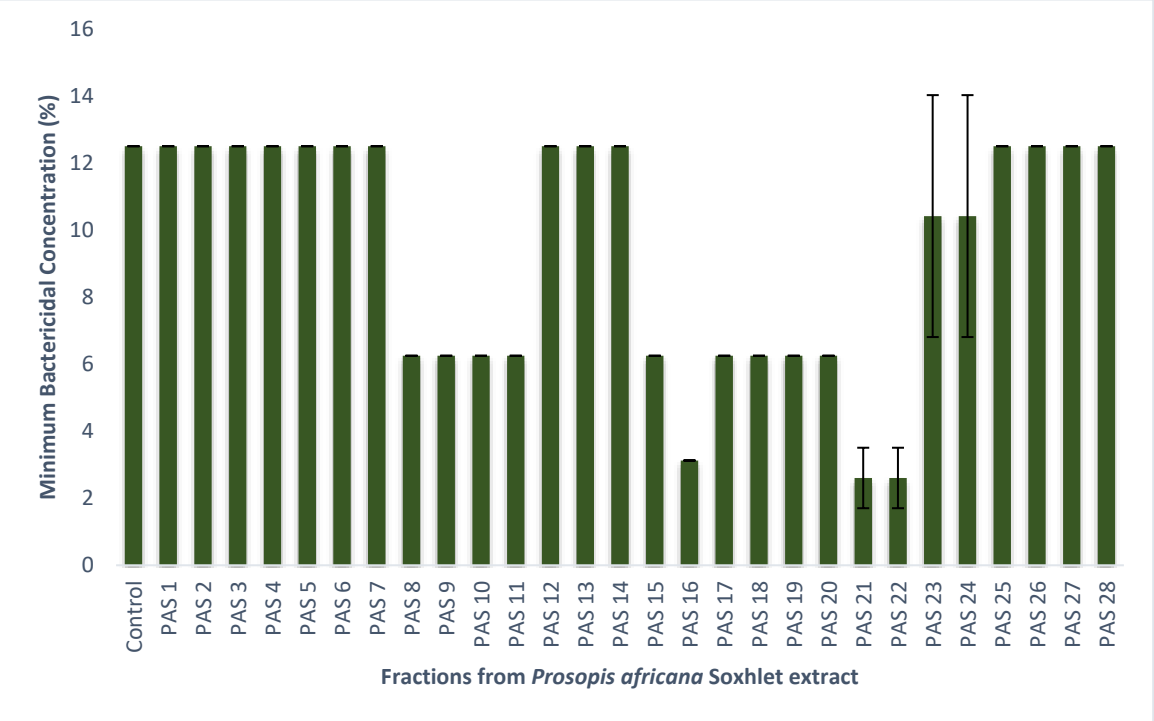
Figure 6.15 Antimicrobial activity of the fractions from *Uvaria chamae* ultrasound extract, showing the minimum bactericidal concentration of the fractions against *Pseudomonas aeruginosa* after performing the micro-dilution assay.

6.3.3.4 *Prosopis africana* extracts (minimum bactericidal concentration of fractions) against *P. aeruginosa* and *S. aureus*.

The control used in this assay did not give a 100 % MBC value because it demonstrated antimicrobial activity with an MBC values of 12.5 % against *S. aureus* and *P. aeruginosa*.

For *S. aureus,* some fractions from *Prosopis africana* Soxhlet (PAS) extract demonstrated antimicrobial bactericidal activity (Appendix 7). Fractions PAS 16, 21 and 22 and demonstrated the greatest bactericidal effect against *S. aureus* with an MBC value of 3.125 % with no significant difference compared to the solvent control and the other PAS fractions (P > 0.05). Similarly, fractions PAS 8 – 11, 15 – 20 and 23 – 24 demonstrated bactericidal effect against *S. aureus* with an MBC value range of 6.25 % -

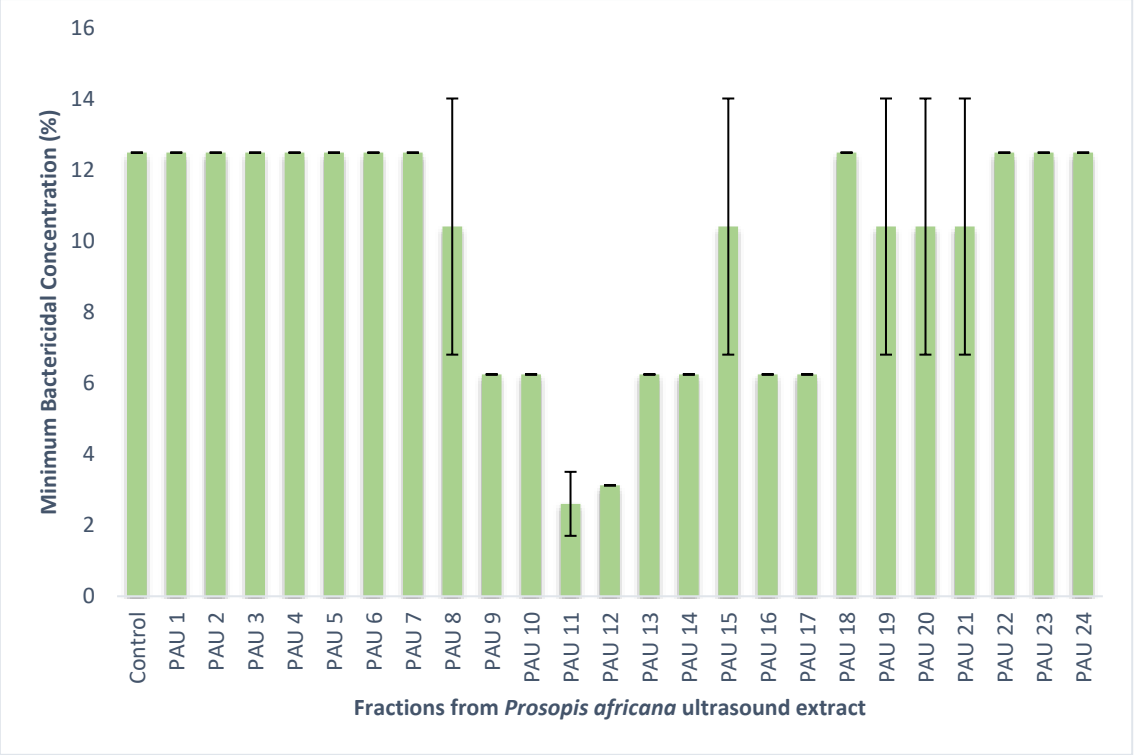
10.41 %, with no significant difference compared to the solvent control. Meanwhile, fractions PAS 1 – 7, 12 -14, and 25 – 28 demonstrated no bactericidal effect against *S. aureus* (Figure 6.16).



N=3; Bar= mean; Error bar = ± SD; starting concentration of fractions and control = 100%; Control = Ethanol : DMSO (3:1); PAS No. = *Prosopis africana* Soxhlet fraction number; No significant difference between fractions and Control (P > 0.05).

Figure 6.16 Antimicrobial activity of the fractions from *Prosopis africana* Soxhlet extract, showing the minimum bactericidal concentration of the fractions against *Staphylococcus aureus* after performing the micro-dilution assay.

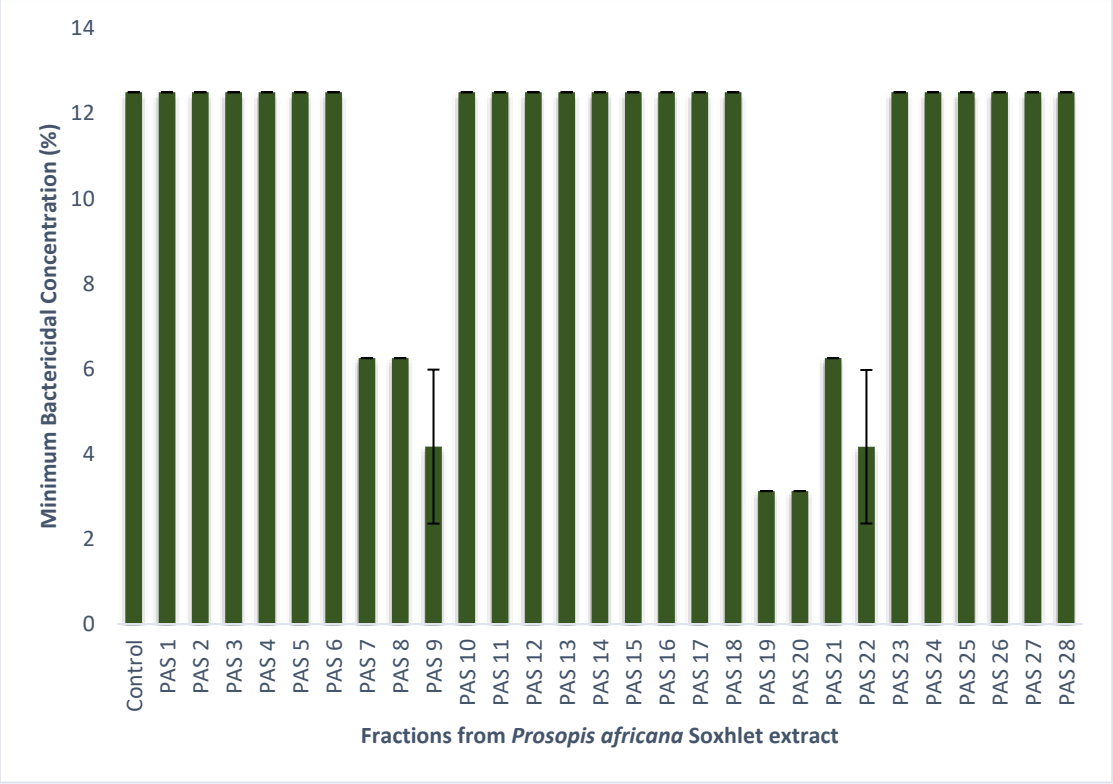
For *S. aureus,* some fractions from *Prosopis africana* ultrasound (PAU) extract demonstrated antimicrobial bactericidal activity (Figure 6.17 and Appendix 7). Fractions PAU 8 – 17 and 19 – 21 demonstrated bactericidal effect against *S. aureus* with MBC values ranging from 3.125 % - 10.42 %, although, no significant difference was observed when compared to the solvent control and other PAU fractions (P > 0.05). Meanwhile, fractions PAU 1 – 7, 18 and 22 – 24 demonstrated no bactericidal effect against *S. aureus* (Figure 6.17).



N=3; Bar= mean; Error bar = ± SD; starting concentration of fractions and control = 100%; Control = Ethanol : DMSO (3:1); PAU No. = *Prosopis africana* ultrasound fraction number; No significant difference between fractions and Control ($P < 0.05$).

Figure 6.17 Antimicrobial activity of the fractions from *Prosopis africana* ultrasound extract, showing the minimum bactericidal concentration of the fractions against *Staphylococcus aureus* after performing the micro-dilution assay.

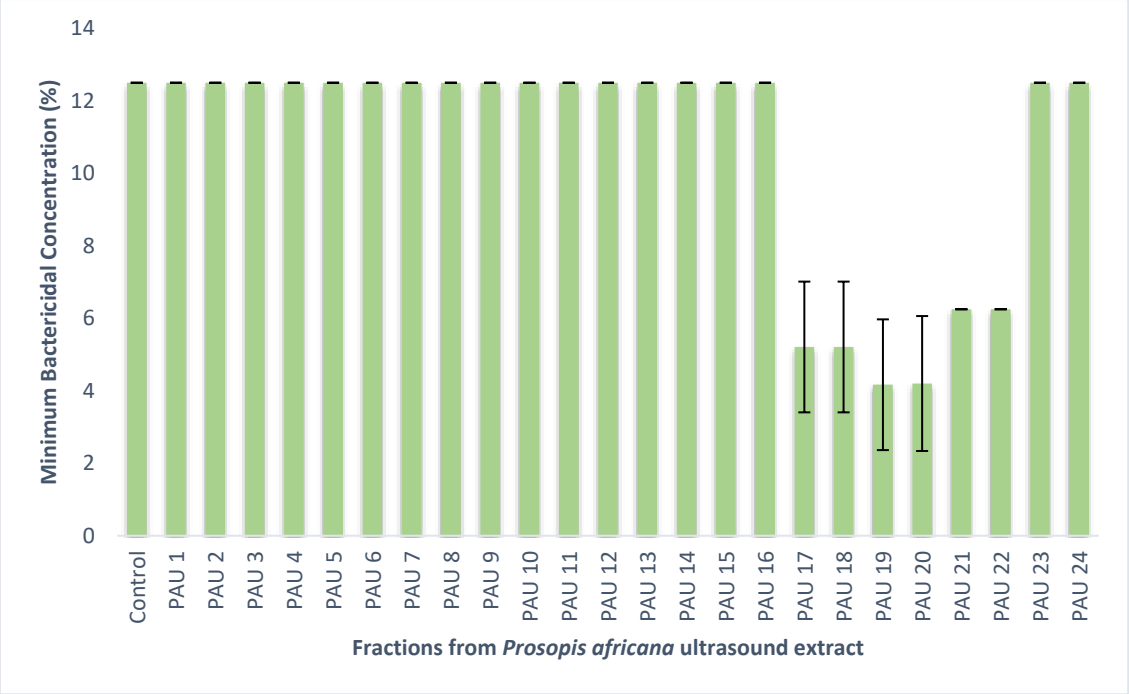
For *P. aeruginosa*, fractions from *Prosopis africana* Soxhlet (PAS) extract demonstrated antimicrobial bactericidal activity (Appendix 7). Fractions PAS 7 – 9 and 19 – 22 demonstrated bactericidal effect against *P. aeruginosa* with an MBC value range of 3.125 % - 6.25 % although, no significant difference was observed when compared to the solvent control and other PAS fractions ($P > 0.05$). Meanwhile, fractions PAS 1 - 6, 10 -18 and 23 - 28 demonstrated no bactericidal effect against *P. aeruginosa* (Figure 6.18).



N=3; Bar= mean; Error bar = ± SD; starting concentration of fractions and control = 100%; Control = Ethanol : DMSO (3:1); PAS No. = *Prosopis africana* Soxhlet fraction number; No significant difference between fractions and Control (P > 0.05).

Figure 6.18 Antimicrobial activity of the fractions from *Prosopis africana* Soxhlet extract, showing the minimum bactericidal concentration of the fractions against *Pseudomonas aeruginosa* after performing the micro-dilution assay.

For *P. aeruginosa*, fractions from *Prosopis africana* ultrasound (PAU) extract demonstrated antimicrobial bactericidal activity (Figure 6.19 and Appendix 7). Fractions PAU 17 – 22 demonstrated bactericidal effect against *P. aeruginosa* with an MBC values between 4.17 % - 6.25 %, although no significant difference was observed when compared to the solvent control and other PAU fractions (P > 0.05). Meanwhile, fractions PAU 1 - 16, 23 and 24 demonstrated no bactericidal effect against *P. aeruginosa* (Figure 6.19).



N=3; Bar= mean; Error bar = ± SD; starting concentration of fractions and control = 100%; Control = Ethanol : DMSO (3:1); PAU No. = *Prosopis africana* ultrasound fraction number; No significant difference between fractions and Control (P > 0.05, ANOVA, Tukey).

Figure 6.19 Antimicrobial activity of the fractions from *Prosopis africana* ultrasound extract, showing the minimum bactericidal concentration of the fractions against *Pseudomonas aeruginosa* after performing the micro-dilution assay.

6.3.4 Phytochemical qualitative analysis of fractions

Phytochemical qualitative analysis was performed on the fractions to analyse the presence of phytochemicals which may be responsible for their antimicrobial properties.

Results from this assay (Table 6.2) show that for *Uvaria chamae* Soxhlet (UCS) extract, saponin was present in fractions UCS 1 – UCS 17 and was absent in fractions UCS 18 – 29 (Table 6.2). Quinone was present in fractions UCS 5 – UCS 18 and was absent in fractions UCS 1 – UCS 4 and fractions UCS 19 – UCS 29 (Table 6.2). Alkaloid was present in fractions UCS 1 – UCS 20 but was absent in fractions UCS 21 – UCS 29. Flavonoid was present in fractions UCS 1 – UCS 10 and UCS 13 – UCS 27 but was absent in fractions UCS 11, 12, 28 and 29 (Table 6.2). No phytochemical was detected in the solvent control.

Table 6.2 Results from the phytochemical and spectrophotometric assay of fractions from *Uvaria chamae* Soxhlet (UCS) extract.

	Phytochemical Assay				Spectrophotometric Study
	Saponin	Quinone	Alkaloid	Flavonoid	Peak Wavelength (nm)
Fractions					
Control	-	-	-	-	-
UCS 1	+	-	+	+	236
UCS 2	+	-	+	+	226
UCS 3	+	-	+	+	274
UCS 4	+	-	+	+	257
UCS 5	+	+	+	+	262
UCS 6	+	+	+	+	240
UCS 7	+	+	+	+	226
UCS 8	+	+	+	+	262
UCS 9*	+	+	+	+	208
UCS 10*	+	+	+	+	272
UCS 11*	+	+	+	-	253
UCS 12*	+	+	+	-	254
UCS 13*	+	+	+	+	251
UCS 14*	+	+	+	+	295
UCS 15*	+	+	+	+	295
UCS 16*	+	+	+	+	333
UCS 17	+	+	+	+	295
UCS 18	-	+	+	+	294
UCS 19	-	-	+	+	294
UCS 20	-	-	+	+	291
UCS 21	-	-	-	+	210
UCS 22	-	-	-	+	209
UCS 23	-	-	-	+	211
UCS 24	-	-	-	+	211
UCS 25	-	-	-	+	210
UCS 26	-	-	-	+	214
UCS 27	-	-	-	+	211
UCS 28	-	-	-	-	211
UCS 29	-	-	-	-	212

(-) not present (+) present (*) demonstrated antimicrobial activity against *P. aeruginosa* and *S. aureus*

Results from this assay (Table 6.3) show that for *Uvaria chamae* ultrasound (UCU) extract, saponin was present in fractions UCU 1 – UCU 14 and was absent in fractions UCU 15 – UCU 17 (Table 6.3). Quinone was present in fractions UCU 4 – UCU 13 and was absent in fractions UCU 1 – UCU 3 and fractions UCU 14 – UCU 17 (Table

6.3). Alkaloid was present in fractions UCU 1 – UCU 13 but was absent in fractions UCU 14 – UCU 17. Flavonoid was present in fractions UCU 1 – UCU 13 but was absent in fractions UCU 14 – UCU 17 (Table 6.3).

Table 6.3 Results from the phytochemical and spectrophotometric assay of fractions from *Uvaria chamae* ultrasound (UCU) extract.

	Phytochemical Assay				Spectrophotometry Study
Fractions	**Saponin**	**Quinone**	**Alkaloid**	**Flavonoid**	**Peak Wavelength (nm)**
Control	-	-	-	-	-
UCU 1	+	-	+	+	255
UCU 2	+	-	+	+	207
UCU 3	+	-	+	+	273
UCU 4	+	+	+	+	261
UCU 5	+	+	+	+	207
UCU 6	+	+	+	+	248
UCU 7	+	+	+	+	295
UCU 8	+	+	+	+	294
UCU 9	+	+	+	+	333
UCU 10*	+	+	+	+	335
UCU 11*	+	+	+	+	289
UCU 12*	+	+	+	+	291
UCU 13*	+	+	+	-	290
UCU 14	+	-	-	-	290
UCU 15	-	-	-	-	209
UCU 16	-	-	-	-	209
UCU 17	-	-	-	-	215

(-) not present (+) present (*) demonstrated antimicrobial activity against *P. aeruginosa* and *S. aureus*

Results from this assay (Table 6.4) shows that for *Prosopis africana* Soxhlet (PAS) extract, saponin was present in fractions PAS 4 – PAS 8 and PAS 13 – PAS 22 but was absent in fractions PAS 1 – PAS 3, PAS 9 – PAS 12 and PAS 23 – PAS 28 (Table 6.4). For PAS extract, quinone was absent in all the fractions (Table 6.4). Also, for PAS extract, alkaloid was present in fractions PAS 4 – PAS 9 and PAS 13 – PAS 23 but was absent in fractions PAS 1 – PAS 3, PAS 10 – PAS 12 and PAS 24 – PAS 28. For PAS

extract, flavonoid was present in fractions PAS 4 – PAS 23 but was absent in fractions PAS 1 – PAS 3 and PAS 24 – PAS 28 (Table 6.4).

Table 6.4 Results from the phytochemical and spectrophotometric assay of fractions from *Prosopis africana* Soxhlet (PAS) extract.

	Phytochemical Assay				Spectrophotometry Study
Fraction	**Saponin**	**Quinone**	**Alkaloid**	**Flavonoid**	**Peak Wavelength (nm)**
(Control)	-	-	-	-	-
PAS 1	-	-	-	-	290
PAS 2	-	-	-	-	236
PAS 3	-	-	-	-	264
PAS 4*	+	-	+	+	246
PAS 5*	+	-	+	+	247
PAS 6*	+	-	+	+	274
PAS 7*	+	-	+	+	261
PAS 8*	+	-	+	+	252
PAS 9*	-	-	+	+	276
PAS 10	-	-	-	+	262
PAS 11	-	-	-	+	236
PAS 12	-	-	-	+	221
PAS 13	+	-	+	+	273
PAS 14	+	-	+	+	272
PAS 15	+	-	+	+	296
PAS 16	+	-	+	+	296
PAS 17	+	-	+	+	296
PAS 18	+	-	+	+	295
PAS 19*	+	-	+	+	294
PAS 20*	+	-	+	+	294
PAS 21*	+	-	+	+	294
PAS 22*	+	-	+	+	294
PAS 23	-	-	+	+	294
PAS 24	-	-	-	-	294
PAS 25	-	-	-	-	239
PAS 26	-	-	-	-	237
PAS 27	-	-	-	-	248
PAS 28	-	-	-	-	239

(-) not present (+) present (*) demonstrated antimicrobial activity against *P. aeruginosa* and *S. aureus*

Results from this assay (Table 6.5) shows that for *Prosopis africana* ultrasound (PAU) extract, saponin was present in fractions PAU 1 – PAU 21 and was absent in fractions PAU 22 – PAU 24 (Table 6.5). For PAU extract, quinone was absent in all the fractions

(Table 6.5). Also for PAU extract, alkaloid was present in fractions PAU 9 – PAU 21 but was absent in fractions PAU 1 – PAU 8 and PAU 22 – PAU 24 (Table 6.5). For PAU extract, flavonoid was present in fractions PAU 6 – PAU 12 and PAU 17 – PAU 21 but was absent in fractions PAU 1 – PAU 5, PAU 13 – PAU 16 and PAU 22 – PAU 24 (Table 6.5).

Table 6.5 Results from the phytochemical and spectrophotometric assay of fractions from *Prosopis africana* ultrasound (PAU) extract.

	Phytochemical Assay				Spectrophotometry Study
Fractions	**Saponin**	**Quinone**	**Alkaloid**	**Flavonoid**	**Peak Wavelength (nm)**
Control	-	-	-	-	-
PAU 1	+	-	-	-	245
PAU 2	+	-	-	-	292
PAU 3	+	-	-	-	297
PAU 4	+	-	-	-	249
PAU 5	+	-	-	-	247
PAU 6	+	-	-	+	252
PAU 7	+	-	-	+	231
PAU 8	+	-	-	+	238
PAU 9	+	-	+	+	256
PAU 10	+	-	+	+	262
PAU 11	+	-	+	+	296
PAU 12	+	-	+	+	297
PAU 13	+	-	+	-	296
PAU 14	+	-	+	-	296
PAU 15	+	-	+	-	285
PAU 16	+	-	+	-	274
PAU 17*	+	-	+	+	280
PAU 18*	+	-	+	+	297
PAU 19*	+	-	+	+	296
PAU 20*	+	-	+	+	295
PAU 21*	+	-	+	+	234
PAU 22	-	-	-	-	232
PAU 23	-	-	-	-	232
PAU 24	-	-	-	-	234

(-) not present (+) present (*) demonstrated antimicrobial activity against *P. aeruginosa* and *S. aureus*

6.3.5 Spectrophotometric assay of fractions

A spectrophotometric assay was performed to determine the peak wavelength of each fraction. From the results of this study, it was observed that fractions from *Uvaria chamae* Soxhlet (UCS) extracts had a range of peak wavelengths from 208 nm – 333 nm with fraction UCS 9 showing the lowest wavelength of 208 nm and fraction UCS 16 showing the higest wavelength of 333 nm (Table 6.2). Regarding fractions from *Uvaria chamae* ultrasound (UCU) extract, the peak wavelengths ranges from 207 nm – 335 nm with fractions UCU 2 and UCU 5 having the lowest wavelength of 207 nm and fraction UCU 10 having the highest wavelength of 333 nm (Table 6.3). For fractions from *Prosopis africana* Soxhlet (PAS) extract, the range of the peak wavelengths was recorded at 221 nm – 296 nm with fractions PAS 12 showing the lowest wavelength and fractions PAS 15, PAS 16 and PAS 17 showing the highest wavelength (Table 6.4). Regarding fractions from *Prosopis africana* ultrasound (PAU) extract, the peak wavelength range was found to be 231 nm – 297 nm with fraction PAU 7 showing the lowest wavelength of 231 nm and fractions PAU 3, PAU 12 and PAU 18 showing the higest wavelength of 297 nm (Table 6.5).

6.4.0 DISCUSSION

6.4.1 Thin layer chromatography

Medicinal plants are composed of an array of different phytochemicals that vary from one plant species to another (Muthu et al. 2006). Therefore, the optimum solvent systems used to separate and purify the active components from different plants is also likely to vary. To help identify the optimum solvent system to use in the fractionation of crude extracts by column chromatography, thin layer chromatography was first performed.

Because it is difficult to separate individual compounds from an unpurified plant extract due to numerous compounds present using TLC, choosing the right solvent system for the separation of compounds in plant extract is also difficult. Therefore, Harbone (1998), recommended the visualisation of TLC plates under UV light and the use of the retention factor (Rf) to increase the chances of identifying solvent systems with good separating potential. Since the Rf values are dependent on the nature of the adsorbent and the solvent system, a constant stationary phase (silica gel) was used because, the emphasis was on the choice of a solvent system. According to Sherma and Fried (2005), when choosing solvents for separating unknown compounds, the ideal solvent system is one which moves the components to TLC Rf values of 0.15 – 0.7 (0.3 optimal). Consequently, in this current study, the ideal solvent system, selected for fractionating *P. africana* and *U. chamae* extracts by column chromatography that met the requirement was identified by TLC to be ethanol : DMSO (3:1) (Table 6.1). An example of a study that used a similar approach to determine the ideal solvent system for separation was a study by Pyka and Dolowy (2003). The workers used scanned chromatogram, Rf value, and resolution factors (Rs) to choose n-heptane-ethylacetate-acetic acid (25 : 20 : 8) as the best solvent system for the separation of bile acids using TLC.

6.4.2 Fractionation of *Prosopis africana* and *Uvaria chamae* extracts

Using a 3:1 ethanol : DMSO mix as the mobile phase in the column chromatography (Section 6.2.3), in the *Uvaria chamae* Soxhlet (UCS) extract yielded a greater number of fractions than the *Uvaria chamae* ultrasound (UCU) extract. Likewise, the *Prosopis africana* Soxhlet (PAS) extract yielded a greater number of fractions than the *Prosopis africana* ultrasound (PAU) extract (Section 6.3.1). The difference in the

number of fractions obtained from each extract might be attributable to the extraction methods used, as previously discussed in Section 3.4.1.

6.4.3 Effect of phytochemicals on the antimicrobial activity of the *Uvaria chamae* extract fractions against *Pseudomonas aeruginosa* and *Staphylococcus aureus*

6.4.3.1 Antimicrobial activity of *U. chamae* extract fractions

Despite the numerous plant antimicrobial studies over the years, only a few analysed the antimicrobial activity of individual fractions from plant extracts. One of such studies was performed by Park et al. (2016) who determined the antimicrobial activities of ethanol and butanol fractions of rose petal extract. They reported that WRPE-EtOH (White rose petal ethanol) and WRPE-BuOH (White rose buthanol) fractions of the rose petal extract, inhibited the growth of a selection of bacteria including *S. aureus*, and effectively killed *Helicobacter pylori*, while other fractions were inactive against the selection of bacteria (Park et al. 2016). The workers suggested that the compounds responsible for the antimicrobial activity might be compartmentalised in the fractions that demonstrated the most activity against the tested bacteria (Park et al. 2016). In this current study, fractions from *U. chamae* extracts (UCS and UCU) demonstrated both inhibitory and bactericidal effect on the *S. aureus* strain. Furthermore, the early fractions of *U. chamae* extracts demonstrated a significantly greater antimicrobial activity than the later fractions against *S. aureus* strain. This pattern suggests that the active antimicrobial compounds against *S. aureus* might be compartmentalised in the early fractions towards the middle fractions of *U. chamae* extracts where the activity is the greatest (Park et al. 2016). Similarly, fractions from *U. chamae* extracts demonstrated both inhibitory and bactericidal effect on *P. aeruginosa* strain. Interestingly, the pattern of antimicrobial

activity of fractions from *U. chamae* extracts on *P. aeruginosa* strain was different from the one observed on *S. aureus* as explained previously. On *P. aeruginosa*, the middle fractions from *U. chamae* extract demonstrated the greatest antimicrobial activity in comparison to the earlier and later fractions of *U. chamae*. This might also suggest that the compounds responsible for this activity against *P. aeruginosa* might be compartmentalised in the middle fractions of *U. chamae* extracts (Park et al. 2016). Furthermore, *S. aureus* strain was observed to be more susceptible to fractions from *U. chamae* extract than *P. aeruginosa* strain. The reason for this disparity in the pattern of antimicrobial activity between *S. aureus* and *P. aeruginosa* might be due to the difference in cellular structure and composition of both bacteria.

Since the cell wall structures and composition of *S. aureus* and *P. aeruginosa* are different (Coyle 2005), it is reasonable to suggest that compounds which are likely to be effective antimicrobials against these species will be different. Consequently, a better understanding of both the antimicrobial activity and the chemical composition of the fractions is important. For example, the outer membrane of *P. aeruginosa*, which acts as a strong permeability barrier, is punctuated by hydrophilic porins, whilst *S. aureus* does not have any porins in its cell wall. Consequently, polar and non-polar compounds in the extract could interact differently with the two bacterial strains (Luis et al. 2016). Therefore, this might help to explain why fractions of *U. chamae* extract showed greater activity against *S. aureus* compared with *P. aeruginosa*. It will be vital to the development of novel therapeutic compounds, against these bacteria, to identify in detail the phytochemicals present in each fraction.

Interestingly, it was observed in this study that the individual fractions of the *U. chamae* extracts (UCS and UCU) possessed poorer antimicrobial activity against the strains of both *P. aeruginosa* and *S. aureus* used here than the unfractionated *U. chamae*

crude extracts (UCS and UCU) (Figure 6.4, 6.5, 6.6, 6.7 and Table 4.3). One study by Luis et al. (2016) showed that the unfractionated crude extract of *Eucalyptus globulus* demonstrated greater antimicrobial activity against a selection of bacterial and yeast strains than fractions obtained from the crude extract, therefore concluding that the synergistic interactions of the constituents in the extract were responsible for the greater antimicrobial activity. This might be due to synergism between the different compounds in the complex unfractionated crude leading to greater activity than in the individual fractions (Awouafack et al. 2013). It is important to note that during fractionation, the compounds in the fractions produced in this current study were eluted based on their size and difference in movement across the stationary phase (silica gel) thereby generating an uneven distribution of active antimicrobial compounds in these fractions. To the best of this author's knowledge, this is the first time that the antimicrobial activity of *U. chamae* extract fractions against *P. aeruginosa* and *S. aureus* has been analysed.

6.4.3.2 Correlation between the presence of phytochemicals and the antimicrobial activities of *U. chamae* fractions.

Certain groups of phytochemicals like quinones, saponins, alkaloids and flavonoids found in plant materials have been reported to have distinct antimicrobial properties (Holfels et al. 1994; Cushnie and Lamb 2005; Mandal et al. 2005; Özçelik et al. 2011). In addition, other phytochemical groups such as tannins and phenolics might also contribute to the antimicrobial activity of plant materials (Harbone 1998). Considering this, the phytochemical profiles of the fractions were analysed (Section 6.3.4). Interestingly, UCS and UCU fractions that demonstrated the strongest antimicrobial activity against *S. aureus* and *P. aeruginosa* were found to contain at least three, if not all of, the phytochemical groups tested for (Figures 6.20 and 6.21). On the

other hand, those *U. chamae* (UCS and UCU) fractions that showed little or no activity against *S. aureus* and *P. aeruginosa*, were found to contain at most one or even no phytochemical groups (Figures 6.20 and 6.21). This was especially true of the final fractions to be eluted from the columns. Consequently, this shows that the synergistic effect of the phytochemicals in the active fractions with three or more phytochemicals may be responsible for the greater antimicrobial activity when compared to fractions with at most one phytochemical. Furthermore, these phytochemicals have different mechanisms of actions on the bacterial cell as explained in section 1.5.0; therefore, their combined effect might increase the antimicrobial activity of that extract. To support of this notion, Luis et al. (2016) used GC-MS analysis to reveal the compounds in the fractions from the methanolic extract of *Eucalyptus globulus* that were responsible for its antimicrobial activity. The group suggested that synergistic effect of gallic (tannin derivative), ellagic (phenolic derivative), citramalic (flavonoid derivative) and citric acids (flavonol derivative) contributed significantly to the antimicrobial activity of the fractions against *S. aureus, P. aeruginosa, E. coli, K. pneumoniae, B. cereus, Candida albicans* and *Candida tropicalis*.

6.4.4 Effect of phytochemicals on the antimicrobial activity of the *Prosopis africana* extract fractions against *Pseudomonas aeruginosa* and *Staphylococcus aureus.*

6.4.4.1 Antimicrobial activity of *P. africana* extract fractions

In this current study, fractions from *P. africana* extracts (PAS and PAU) demonstrated both inhibitory and bactericidal effect on the *S. aureus* strain. Furthermore, the middle fractions of *P. africana* extracts demonstrated a significantly greater antimicrobial activity than the latter fractions against *S. aureus* strain. As previously

suggested, the active antimicrobial compounds against *S. aureus* might be compartmentalised in the middle fractions of *P. africana* extracts where the activity is the greatest (Park et al. 2016). Similarly, fractions from *P. africana* extracts demonstrated both inhibitory and bactericidal effect on *P. aeruginosa* strain. Interestingly, the pattern of antimicrobial activity of fractions from *P. africana* extracts on *P. aeruginosa* strain was different from the one observed on *S. aureus*. On *P. aeruginosa*, the latter fractions from *P. africana* extract demonstrated the greatest antimicrobial activity when compared to the earlier and middle fractions of *P. africana* extracts. This might also suggest that the compounds responsible for this activity against *P. aeruginosa* might be compartmentalised in the latter fractions of *P. africana* extracts (Park et al. 2016). Again, *S. aureus* strain was observed to be more susceptible to fractions from *P. africana* extract than *P. aeruginosa* strain. The reason for this as explained earlier, might be due to the difference in cellular structure and composition of both bacteria. Few studies have identified active compounds by determining the antimicrobial activity of fractions from plant extracts. One of such study was performed by Cunha et al. in 2017 who identified and isolated an active compound (1, 8-dihydroxy-anthra quinone-3 -carboxylic acid) from fractions (obtained from *Cassia bakeriana* ethanolic extract) that demonstrated antimicrobial activity against a selection of aerobes and anaerobes. Consequently, this current study demonstrated the antimicrobial activity of fractions from *P. africana* extracts against *S. aureus* and *P. aeruginosa*, which can be valuable for the identification and isolation of active compounds for the development of novel antimicrobial therapies.

Interestingly, it was also observed in this study that individual fractions of *P. africana* extracts (PAS and PAU) demonstrated a lower antimicrobial activity against *P. aeruginosa* and *S. aureus* (Figure 6.8, 6.9, 6.10 and 6.11) than the unfractionated *P. africana* crude extracts (UCS and UCU) (Table 4.4). As explained previously in Section

6.4.3.1, the synergistic interaction between different compounds in the complex unfractionated crude extract might be responsible for the greater antimicrobial activity than in individual fractions, which might contain less or no synergism (Awouafack et al. 2013). To further support this, Delaquis et al. (2002) in their study, reported that mixing different fractions from a plant extract resulted in an increased antimicrobial activity against the tested bacteria than individual fractions, because of the synergistic effect from the mixture. To the best of this author's knowledge, this is the first time that the antimicrobial activity of fractions from *P. africana* extract were demonstrated against *P. aeruginosa* and *S. aureus*.

6.4.4.2 Correlation between the presence of phytochemicals and the antimicrobial activities of *P. africana* fractions

Phytochemicals have been associated with the antimicrobial activity of plants against bacterial species (Holfels et al. 1994; Mandal et al. 2005). Due to this fact, the phytochemical profiles of the fractions were analysed (Section 6.3.4). Fractions from *P. africana* extracts that demonstrated antimicrobial activity against either *S. aureus* or *P. aeruginosa* showed the presence of at least three if not all of the phytochemical groups tested for (Figures 6.22 and 6.23). On the other hand, those *P. africana* (PAS and PAU) fractions that showed little or no activity against *S. aureus* or *P. aeruginosa* were found to have at most one or even no phytochemical group (Figures 6.22 and 6.23). Again, as explained earlier, the synergistic effect of the phytochemicals in the active fractions with three or more phytochemicals may be responsible for the greater antimicrobial activity when compared to fractions with at most one phytochemical. Interestingly, these phytochemicals have different mechanisms of actions on the bacterial cell as explained in Section 1.5.0. For instance, flavonoids and alkaloids have been reported to be strong

antimicrobial agents which act against bacterial cells by inhibiting nucleic acid synthesis (Mori and Nishizawa 1987); inhibiting cytoplasmic membrane functions (Tsuchiya and Iinuma 2000); and inhibiting energy metabolism (Haraguchi et al. 1998); and Saponins have been reported to destroy the bacterial cellular membrane integrity (Morrissey and Osbourn, 1999). Therefore, it is logical to conclude that the combination of two or more phytochemicals with different modes of action might lead to a greater antimicrobial activity than a single phytochemical with a single mode of action.

6.4.5 Spectrophotometric analysis of the *Prosopis africana* and *Uvaria chamae* extract fractions

Although the phytochemical screening performed in this current study qualitatively identified the presence of phytochemical groups in active fractions, the quantitative measure characterisation of these groups is still unknown. Therefore, this assay was meant to give an idea of the most prominent phytochemical group in the active fractions. Nevertheless, the identification of compounds from active fractions of *P. africana* and *U. chamae* extracts will require further analysis. The use of more advanced and sensitive analytical tools such as high-performance liquid chromatography (HPLC), gas chromatography - mass spectrometry (GC-MS) and nuclear magnetic resonance (NMR) spectroscopy, to determine the structural, chemical and physical characteristics of the compounds present in the active fractions will help in the hunt for novel antimicrobials (Cunha et al. 2017).

In the current research, a preliminary spectrophotometric analysis of the fractions was performed (Section 6.2.4). There is a paucity of literature regarding the phytochemical composition of *U. chamae* and *P. africana*. However, Harbone (1998)

suggested that the peak wavelength range of 240 nm – 333 nm, which matched that of the fractions obtained in this study might indicate the presence of flavonoid compounds present in the active fractions (Appendix 8). Working from the information given by Harbone (1998) (Appendix 8), the spectra of the UCS and UCU fractions (Table 6.6 and 6.7) with the greatest antimicrobial activity against *S. aureus* and *P. aeruginosa*, the presence of chalcones, aurones, flavones, flavanones, flavonols and isoflavones which are all classes of flavonoids, is likely. Interestingly, Hufford and Lasswell (1976) reported on the isolation of uvaretin and isouvaretin, which are two C-benzylflavanones, from *U. chamae* using fractionation and NMR. However, these workers did not report on the antimicrobial activities of these two C-benzylflavanones that they had isolated. Subsequently, Hufford et al. (1979) reported on the isolation of uvarinol, which is a tribenzylated flavanone, from *U. chamae* using column chromatography, x-ray diffraction and NMR. Uvarinol was reported to have antimicrobial activity against *S. aureus*, *B. subtilis*, and *Mycobacterium smegmatis* (Hufford et al. 1979). Furthermore, Ichimaru et al. (2004) used a combination of column chromatography, HPLC, and NMR to isolate two new C-benzylated dihydrochalcones (isochamuvaritin and acumitin), which are derivatives of chalcone, from *Uvaria acuminata*. However, these workers also did not report on the antimicrobial activities of these newly isolated C-benzylated dihydrochalcones. Although this current study used a different identification technique from the studies previously described, the findings in this current study correlates with those studies by identifying flavonoid or its derivatives as a prominent phytochemical in the extract fractions. Therefore, fractions with greater antimicrobial activities against *S. aureus* and *P. aeruginosa* might contain flavonoid-related compound in this current study.

Similarly, from the information given by Harbone (1998) (Appendix 8), the spectra of the PAS and PAU fractions (Table 6.4 and 6.5) with the greatest antimicrobial activity against *S. aureus* and *P. aeruginosa*, the presence of chalcones, aurones, flavones, flavanones, flavonols and isoflavones, which are all classes of flavonoids, is also likely. Further study utilising analytical techniques such as x-ray diffraction and NMR, is needed to help properly identify the prominent antimicrobial compounds in the extract that could be used in novel antimicrobial treatments. To the best of this author's knowledge, there have as yet been no studies published regarding the analysis of fractions made from *P. africana* extracts.

6.4.6 Conclusion

Fractions obtained from both *U. chamae* extracts (UCS and UCU) and *P. africana* extracts (PAS and PAU) were analysed using the broth microdilution assay, phytochemical screening and spectrophotometric analysis. Antimicrobial activity against *P. aeruginosa* and *S. aureus* was observed in some of the fractions whilst other fractions (especially those that eluted later) were not. Those *U. chamae* (UCS and UCU) fractions and *P. africana* (PAS and PAU) fractions that showed the greatest antimicrobial activity against the strains of *P. aeruginosa* and *S. aureus* tested, were found to contain three or more of the phytochemical groups (saponins, alkaloids, flavonoids and quinones) that have previously been shown by others to be antimicrobial. Absorption spectrum analysis also suggest that the fractions of *U. chamae* and *P. africana* with the greatest antimicrobial activity might contain flavonoid-related compounds as a major active component. It is therefore suggested that these phytochemical compounds in particular be further studied as potential novel antimicrobials.

CHAPTER SEVEN

7.0 GENERAL CONCLUSION

The problem of antimicrobial resistance has become a global threat to healthcare systems, necessitating prompt action to fight it. The search for new therapies to fight antimicrobial resistance is ongoing. It was hypothesised here that since plant materials are being used in different parts of the world as traditional alternative treatments for infections, they could represent a potentially significant weapon in our arsenal in the fight against antimicrobial resistance. In view of this, the current study aimed to analyse the antimicrobial and antibiofilm effects of traditional herbal remedies that could form the basis for novel therapies.

Crude extracts of a selection of traditional medicinal plants, obtained by using maceration, were analysed for their antimicrobial activities against *P. aeruginosa*, *S. aureus* and *E. coli* using agar-well diffusion and broth micro-dilution assays in a pilot study. These bacteria were chosen since they are pathogens that frequently cause medically-significant infections. Amongst the various plants that were tested in the pilot study, *Prosopis africana* crude extract was found to possess the greatest antimicrobial activity against the Gram-negative bacteria *P. aeruginosa* and *E. coli*. On the other hand, *Uvaria chamae* crude extract demonstrated the greatest antimicrobial activity against the Gram-positive bacterium *S. aureus*. This corroborates their use in traditional medicine as herbal remedies for the treatment of infections, at least against those strains tested. Furthermore, this suggested that *P. africana* and *U. chamae* were good candidates for more in-depth antimicrobial and antibiofilm study in this work.

After selecting *P. africana* and *U. chamae* based on their antimicrobial activity profiles in the pilot study, different extraction methods like maceration, Soxhlet and

ultrasound extractions were used to obtain crude extracts from *P. africana* and *U. chamae*. The crude extracts generated from the extraction methods were: *P. africana* maceration (aqueous) (PAM aq); *P. africana* maceration (PAM); *P. africana* Soxhlet (PAS); *P. africana* ultrasound (PAU); *U. chamae* maceration (aqueous)(UCM aq); *U. chamae* maceration (UCM); *U. chamae* Soxhlet (UCS); and *U. chamae* ultrasound (UCU) (in all cases, the solvent used was ethanol, unless otherwise stated). Consequently, due to low yield, PAM aq and UCM aq extracts were not prepared for the main study.

To observe the impact of the extraction methods used on the presence of phytochemicals in the crude extracts, a simple phytochemical analysis was performed. A range of phytochemical groups like flavonoids, quinones, alkaloids, saponins, tannins and phenols, which have all previously been reported to exhibit biological activities by other workers, were found to be present in all of the extracts prepared in this current study. This suggests that the extraction methods used in this study, had no influence on the presence of phytochemicals in the crude extracts, although other workers suggested that using different extraction methods might influence the concentration of phytochemicals in the extracts. Furthermore, the presence of these phytochemical groups might be significant to the antimicrobial activity of these plant extracts.

The ethanolic crude extracts of *P. africana* and *U. chamae*, obtained from the maceration, Soxhlet and ultrasound extraction procedures, were next analysed for their antimicrobial activity using agar-well diffusion, and broth micro-dilution assays, against a wider selection of aerobic and anaerobic bacterial species than was used in the pilot studies. The findings from the antimicrobial analysis of the selected extracts against clinically–significant pathogens show that UCS and UCU extracts possess greater antimicrobial activity against all of the bacterial strains tested than does UCM extract. Similarly, the PAS and PAU extracts also demonstrated greater antimicrobial activity

than did the PAM extract. Furthermore, the current study showed that the Gram-positive bacterial strains tested are more susceptible to the *U. chamae* extracts (UCS and UCU) whilst the Gram-negative bacterial strains tested are more susceptible to the *P. africana* extracts (PAS and PAU). Consequently, this indicates that PAS, PAU, UCS and UCU extracts could be utilized as novel antimicrobial agents for the treatment of bacterial infections, particularly those involving the strains tested. As a result of their clear antimicrobial activity, it was decided to next test the PAS, PAU, UCS and UCU extracts for their antibiofilm activity.

The formation of, or residence in, a biofilm by bacterial species is a major contributor to their antimicrobial resistance, and this can have a significant negative impact on the prognosis for treatment of bacterial infections. For this reason, the antibiofilm activity of PAS, PAU, UCS and UCU was tested against a selection of medically-significant anaerobic and aerobic bacterial species using both semi-quantitative and quantitative antibiofilm assays. This study demonstrated significant antibiofilm activity for the PAS, PAU, UCS and UCU extracts by reducing the biomass of preformed biofilms. Consequently, this suggests that these extracts could also be candidates for novel antibiofilm therapies.

Fractionation of the UCS, UCU, PAS and PAU extracts yielded fractions that were each analysed for the antimicrobial activity using the broth microdilution assay, and also subjected to simple phytochemical screening and spectrophotometric analysis. Antimicrobial activity against *P. aeruginosa* and *Sp. aureus* was observed in some of the fractions whilst other fractions were inactive. Those *U. chamae* (UCS and UCU) fractions and *P. africana (*PAS and PAU) fractions that showed the greatest antimicrobial activity against the strains of *P. aeruginosa* and *S. aureus* tested were found to contain three or more of the phytochemical groups (saponins, alkaloids, flavonoids and quinones)

that have previously been shown to be antimicrobial by other workers. Absorption spectrum analysis also indicated that the fractions of *U. chamae* and *P. africana* with the greatest antimicrobial activity likely contained flavonoid-related compounds as a major active component. It is therefore suggested that these phytochemical compounds be further studied for their antimicrobial activity.

In summary, the current study has demonstrated that both *P. africana* and *U. chamae* extracts are effective antimicrobial and antibiofilm agents. Furthermore, the data from the current study indicates that extracts from these plants could habour novel antimicrobial compounds which could be further developed and tested to aid in the fight against antimicrobial resistance.

7.1 Research limitations and recommendations for future work

Although both *P. africana* and *U. chamae*, which were used in this study, are of importance in traditional medicine, there is a paucity of published information regarding the antibiofilm activity of these plants. This was a limitation because there was no published data to compare with the current data. However, there are published research data regarding closely related plants, and so these have been used to compare with the current data. Similarly, the use of a wide range of clinically significant organisms in published plant-antibiofilm studies are limited. For example, there was no published information regarding the antibiofilm activity of plant extracts against *Clostridium difficile* strain. Therefore, there were no published data to compare with the data in this current study. It is recommended that the antimicrobial and antibiofilm activities of *P. africana* and *U. chamae* be assessed against a wider range of clinically significant organisms to determine their breadth of activity in the future.

In addition, the cytotoxicity of *P. africana* and *U. chamae* extracts needs to be assessed to determine whether they are realistic targets for use as therapeutic agents. To aid in future drug development activities, it would be interesting to determine the molecular mechanisms by which the active compounds in these plants exert their antimicrobial and antibiofilm effect. By determining the molecular mechanisms by which these plants act, it might be possible to develop synthetic or semi-synthetic compounds that could be mass-produced more easily and safely than it is possible to purify plant extracts.

When extracting the phytochemicals using the different methods, it was not possible to use constant conditions (for example, temperature or extraction time). The different working principles for each of the extraction methods meant that different reaction conditions were required. As a consequence of this, there may have been unforeseen effects on the phytochemicals of the extraction method. For example, the greater heat employed in the Soxhlet extraction method could have led to degradation of heat-labile phytochemicals, or the heat used could have led to secondary chemical reactions such as the Maillard reaction. This means that the phytochemical profile of the extract prepared by each method could have been different and led to some of the differences observed in the results. In future, it would be interesting to perform a more detailed chemical analysis, for example by using HPLC-UV spectrophotometry or GC-MS, to determine in greater detail the phytochemical profile of each type of extract, to help to elucidate some of the differences observed between the extracts.

In this study, the crude extracts used comprised too complex a mixture of compounds to permit clear resolution of individual spots using simple chromatographic techniques like the thin-layer chromatography (TLC). Consequently, it was not possible to identify, characterise and isolate individual compounds from these extracts. In the

future, identification, characterisation and isolation of the active compounds in these plant extracts using sophisticated equipment is recommended. Sophisticated assays like nuclear magnetic resonance (NMR), x-ray diffraction (XRD) and gas chromatography mass spectrophotometry (GC-MS) have previously been used by other workers to identify active compounds in different plant extracts. These assays can be valuable in identifying, characterising and isolating active compounds with antimicrobial properties from *P. africana* and *U. chamae* extracts.

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APPENDICES

Appendix 1 Performance standards supplemental table for antimicrobial susceptibility testing (Disc diffusion) (CLSI 2017).

Antimicrobial agent	CLSI Zone of inhibition breakpoint (mm)		
	S	**I**	**R**
Erythromycin	>23	13 - 22	<12
Gentamicin	>15	13 - 14	<12
Colistin	>11	-	<10
Penicillin	>25	12 - 14	< 15
Vancomycin	>17	15 - 16	< 14
Ampicillin	>25	16 - 24	< 15

S – Susceptible; I – Intermediate; R – Resistance, CLSI -Clinical and Laboratory Standards Institute

Appendix 2 Post-hoc test (Tukey HSD), comparing the extract yield using four extraction methods.

Multiple Comparisons

Dependent Variable: Extract_yield

Tukey HSD

(I) Extraction_method	(J) Extraction_method	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	Upper Bound
Maceration (aqueous)	Maceration (ethanol)	-.2400*	.03894	.000	-.3514	-.1286
	Soxhlet	-1.3567*	.03894	.000	-1.4681	-1.2452
	Ultrasound	-.7567*	.03894	.000	-.8681	-.6452
Maceration (ethanol)	Maceration (aqueous)	.2400*	.03894	.000	.1286	.3514
	Soxhlet	-1.1167*	.03894	.000	-1.2281	-1.0052
	Ultrasound	-.5167*	.03894	.000	-.6281	-.4052
Soxhlet	Maceration (aqueous)	1.3567*	.03894	.000	1.2452	1.4681
	Maceration (ethanol)	1.1167*	.03894	.000	1.0052	1.2281
	Ultrasound	.6000*	.03894	.000	.4886	.7114
Ultrasound	Maceration (aqueous)	.7567*	.03894	.000	.6452	.8681
	Maceration (ethanol)	.5167*	.03894	.000	.4052	.6281
	Soxhlet	-.6000*	.03894	.000	-.7114	-.4886

Based on observed means; The error term is Mean Square (Error) = .005.

*. The mean difference is significant at the .05 level.

Appendix 3 The effect of treatment with the *Prosopis africana* Soxhlet (PAS), *Prosopis africana* ultrasound (PAU), *Uvaria chamae* Soxhlet (UCS), *Uvaria chamae* ultrasound (UCU) extracts and the solvent control, on the biofilm-associated viable counts of the strains tested.

	VIABLE BACTERIAL COUNT LOG_{10} CFU/ml				
Bacteria	**PAS**	**PAU**	**UCS**	**UCU**	**CNTRL**
S. epidermidis	4.05±0.65#	3.55±0.66#	0.93±1.60	2.17±1.91	9.63±0.69#
P. aeruginosa	3.37±0.19	1.58±1.42	3.84±0.27	4.00±0.25	9.07±0.32
S. aureus	4.41±0.63*	3.78±0.89*	0.00±0.00	1.17±2.02*	9.16±0.48*
***P. acnes* NCTC 737**	3.86±0.39$	2.64±0.83$	0.77±1.33	0.86±1.49	8.53±0.73$
P. acnes* LI*77	3.44±0.18	3.70±0.46	2.99±0.54	2.12±1.84	7.63±0.61
P. acnes* LI*88	3.77±0.85	3.35±0.55	3.12±1.04	1.75±1.57	8.29±0.62
P. acnes SI5	3.85±0.22	3.30±0.61	2.53±0.46	1.90±1.78	8.33±0.49
P. acnes SI8	3.80±0.21	3.41±0.23	2.86±0.39	1.92±1.88	8.21±0.63
***C. difficile* NCTC**	3.22±0.58	3.89±0.46	3.67±0.28	3.61±0.18	7.06±0.47
***C. difficile* 001**	3.65±0.29	3.88±0.11	3.71±0.05	3.72±0.19	7.51±1.21
***C.difficile* 002**	3.66±0.18	3.70±0.15	3.83±0.13	3.85±0.09	7.00±0.79
C.difficile 005	3.84±0.19	4.08±0.16	3.75±0.12	3.68±0.04	7.01±0.61

Data represented as mean ± Standard deviation (SD); n = 3; Treatment concentration = Individual MBCs of treatments against each bacterium were used. Note: Significant difference between the treatments and their respective controls for all bacteria was observed ($P < 0.05$), * = $P < 0.05$ vs UCS (*S. aureus*); # = $P < 0.05$ vs UCS (*S. epidermidis*); $ = $P < 0.05$ vs UCS (*P. acnes* NCTC 737)

Appendix 4 Summary of minimum inhibitory concentration (MIC) results of *Uvaria chamae* fractions, showing the fractions that demonstrated antimicrobial activity against *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

P. aeruginosa				*S. aureus*			
UCS fractions	**MIC (%)**	**UCU fractions**	**MIC (%)**	**UCS fractions**	**MIC (%)**	**UCU fractions**	**MIC (%)**
UCS 9	4.17	UCU 10	2.08	UCS 1 **	0.781	UCU 1 **	0.195
UCS 10	3.125	UCU 11	2.08	UCS 2 **	0.651	UCU 2 **	0.195
UCS 11	3.125	UCU 12	2.60	UCS 3 **	0.195	UCU 3 **	0.230
UCS 12	3.125	UCU 13 **	1.563	UCS 4 **	0.195	UCU 4	1.56
UCS 13	3.125			UCS 5 **	0.325	UCU 5	1.30
UCS 14	3.125			UCS 6 **	0.651	UCU 6	3.125
UCS 15 **	1.563			UCS 7	1.56	UCU 7	2.60
UCS 16 **	2.08			UCS 8	1.56	UCU 8	2.60
UCS 17	2.604			UCS 9	1.56	UCU 9	2.60
UCS 18**	2.08			UCS 10	1.56	UCU 10	3.125
UCS 19**	2.08			UCS 11	1.56	UCU 11	3.125
				UCS 12 **	0.781	UCU 12	5.21
				UCS 13 **	0.781	UCU 13	5.21
				UCS 14 **	1.04		
				UCS 15 **	1.04		
				UCS 16	2.08		
				UCS 17	3.125		
				UCS 18	3.125		
				UCS 19	3.125		
				UCS 20	4.17		
				UCS 21	4.17		
				UCS 22	6.25		
				UCS 23	6.25		
				UCS 24	6.25		
				UCS 25	6.25		
				UCS 26	6.25		
				UCS 27	6.25		

UCS- *Uvaria chamae* Soxhlet, UCU- *Uvaria chamae* ultrasound, ****** - Fractions with the most antimicrobial activity against the bacterial species used, compared to other fractions from the same extract.

Appendix 5 Summary of minimum inhibitory concentration (MIC) results of *Prosopis africana* fractions, showing the fractions that demonstrated antimicrobial activity against *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

P. aeruginosa				***S. aureus***			
PAS fractions	**MIC (%)**	**PAU fractions**	**MIC (%)**	**PAS fractions**	**MIC (%)**	**PAU fractions**	**MIC (%)**
PAS 3	4.17	PAU 17	3.125	PAS 4	6.25	PAU 1	6.25
PAS 4	4.17	PAU 18	2.08	PAS 5	6.25	PAU 2	6.25
PAS 5	4.17	PAU 19 **	1.563	PAS 6	6.25	PAU 3	6.25
PAS 6	4.17	PAU 20 **	1.563	PAS 7	6.25	PAU 4	6.25
PAS 7	1.56	PAU 21	3.125	PAS 8	6.25	PAU 5	6.25
PAS 8	1.56			PAS 9	6.25	PAU 6	3.125
PAS 9	1.56			PAS 10	6.25	PAU 7	3.125
PAS 19	2.60			PAS 11	6.25	PAU 8	3.125
PAS 20	1.56			PAS 12	6.25	PAU 9 **	1.04
PAS 21	1.56			PAS 13	6.25	PAU 10 **	1.04
PAS 22 **	0.781			PAS 14	6.25	PAU 11 **	1.04
				PAS 15 **	2.60	PAU 12 **	1.04
				PAS 16 **	2.60	PAU 13	1.56
				PAS 17	6.25	PAU 14	1.56
				PAS 18	6.25	PAU 15	3.125
				PAS 19	3.125	PAU 16	3.125
				PAS 20	3.125	PAU 17	5.21
				PAS 21 **	2.60	PAU 18	5.21
				PAS 22**	2.60	PAU 19	6.25
				PAS 23	6.25	PAU 20	6.25
						PAU 21	6.25

PAS- *Prosopis africana* Soxhlet, PAU- *Prosopis africana* ultrasound, ****** - Fractions with the most antimicrobial activity against the bacterial species used, compared to other fractions from the same extract.

Appendix 6 Summary of minimum bactericidal concentration (MBC) results of *Uvaria chamae* fractions, showing the fractions that demonstrated antimicrobial activity against *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

P. aeruginosa				*S. aureus*			
UCS fractions	**MBC (%)**	**UCU fractions**	**MBC (%)**	**UCS fractions**	**MBC (%)**	**UCU fractions**	**MBC (%)**
UCS 9	8.33	UCU 8	5.21	UCS 1	3.125	UCU 1 **	0.391
UCS 10	8.33	UCU 9	6.25	UCS 2 **	1.563	UCU 2 **	0.391
UCS 11	6.25	UCU 10	3.125	UCS 3 **	0.391	UCU 3 **	0.781
UCS 12	6.25	UCU 11	8.33	UCS 4 **	0.781	UCU 4	1.56
UCS 13	6.25	UCU 12	6.25	UCS 5 **	1.563	UCU 5	2.08
UCS 14	6.25	UCU 13	4.17	UCS 6 **	1.563	UCU 6	3.125
UCS 15	6.25			UCS 7	3.125	UCU 7	6.25
UCS 16	6.25			UCS 8	3.125	UCU 8	8.33
UCS 17	8.33			UCS 9**	1.563	UCU 9	5.21
UCS 18	6.25			UCS 10**	1.563	UCU 10	5.21
UCS 19	8.33			UCS 11**	1.563	UCU 11	6.25
				UCS 12 **	1.563	UCU 12	6.25
				UCS 13 **	2.08	UCU 13	6.25
				UCS 14 **	1.563		
				UCS 15 **	1.563		
				UCS 16**	1.563		
				UCS 17	6.25		
				UCS 18	6.25		
				UCS 19	10.42		
				UCS 20	6.25		
				UCS 21	6.25		

UCS- *Uvaria chamae* Soxhlet, UCU- *Uvaria chamae* ultrasound, ****** - Fractions with the most antimicrobial activity against the bacterial species used, compared to other fractions from the same extract.

Appendix 7 Summary of minimum bactericidal concentration (MBC) results of *Prosopis africana* fractions, showing the fractions that demonstrated antimicrobial activity against *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

P. aeruginosa				*S. aureus*			
PAS fractions	**MBC (%)**	**PAU fractions**	**MBC (%)**	**PAS fractions**	**MBC (%)**	**PAU fractions**	**MBC (%)**
PAS 7	6.25	PAU 17	5.21	PAS 8	6.25	PAU 8	10.42
PAS 8	6.25	PAU 18	5.21	PAS 9	6.25	PAU 9	6.25
PAS 9	4.17	PAU 19	4.17	PAS 10	6.25	PAU 10	6.25
PAS 19	3.125	PAU 20	4.17	PAS 11	6.25	PAU 11 **	2.60
PAS 20	3.125	PAU 21	6.25	PAS 15	6.25	PAU 12 **	3.125
PAS 21	6.25	PAU 22	6.25	PAS 16 **	3.125	PAU 13	6.25
PAS 22	4.17			PAS 17	6.25	PAU 14	6.25
				PAS 18	6.25	PAU 15	10.42
				PAS 19	6.25	PAU 16	6.25
				PAS 20	6.25	PAU 17	6.25
				PAS 21 **	2.60	PAU 19	10.42
				PAS 22**	2.60	PAU 20	10.42
				PAS 23	10.42	PAU 21	10.42
				PAS 24	10.42		

PAS- *Prosopis africana* Soxhlet, PAU- *Prosopis africana* ultrasound, ****** - Fractions with the most antimicrobial activity against the bacterial species used, compared to other fractions from the same extract.

Appendix 8 Spectral characteristics of main flavonoid classes (Harbone 1998)

Principal maxima (nm)	**Subsidiary maxima (nm) (with relative intensities)**	**Indication**
475-560	ca. 275 (55%)	anthocyanins
390-430	240-270 (32%)	aurones
365-390	240-260 (30%)	chalcones
350-390 250-270	ca. 300 (40%)	flavonols
330-350 250-270	absent	flavones and biflavonyls
275-290 ca. 225	310-330 (30%)	flavanones and flavanonols
255-265	310-330 (25%)	isoflavones