Synergistic Interactions of Selected Medicinal Plants Traditionally Used to Treat Meningitis

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DEDICATION

To Almighty God Be Glory, Honour and Praise for Ever and Ever. Amen.

I dedicate this work to

- My wife, Lucy Mhone Kamwamba, and my daughter, Alinafe
- My father, Mr Edson Kamwamba, and my mother, Mrs Fainess Magwira Kamwamba

DECLARATION

I, the undersigned, hereby declare that the research work contained in this study is my own original work, and all the sources I have used or quoted have been indicated and acknowledged by means of complete references

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ABSTRACT

The antibacterial and antifungal activities of aqueous, methanol and acetone extracts of the leaves of *Geranium incanum, Mangifera indica, Bidens pilosa, Ricinus communis, Carica papaya* and fruit pulp of *Adansonia digitata* as well as activity of their combined preparation were evaluated against selected meningitis causing pathogens. The microorganisms under study were methicillin and oxacillin resistant *Staphylococcus aureus* (ATCC 43300), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium* (ATCC 49416), *Escherichia coli* (ATCC 35218), *Candida albicans* (ATCC 10231) and *Cryptococcus neoformans* (clinical strain).

The screening of antimicrobial activities were assessed using an agar well diffusion technique for the different plant extractions. The individual and combined plant extracts that demonstrated antimicrobial effects were further studied using the INT microtitre plate technique to determine the minimum inhibitory concentration. The sum of fractional inhibitory concentration indices were calculated and used to assess the type of antimicrobial interaction.

The medicinal plants exerted antimicrobial effects independently and in combination on the bacterial and fungal organisms under study. The antimicrobial activities depended on the test organism and extracting solvents used. In the combination studies, the medicinal plants under study were combined in the ratio of 1:1. Antimicrobial interactions ranging from synergistic to antagonistic effects were noted. Methanol extracts of combinations *B. pilosa: M. indica, R. communins: M. indica* and *C. papaya: M. indica* had synergistic effects against *P. aeruginosa*. On the other hand, aqueous extracts of a mixture of *C. papaya: B. pilosa* exerted synergistic effects on *E. coli* and *S. typhimurium*. Acetone extracts of a mixture of *A. digitata: G. incanum, G. incanum: M. indica* as well as methanol extract of the combination of *C. papaya: G. incanum* exhibited synergistic effects against *C. albicans*. The aqueous extract combination of *G. incanum*: *M. indica* displayed the most noteworthy synergistic activity against *C. neoformans*. In addition, aqueous extracts of the combinations of *A. digitata: B. pilosa* and *R. communis: G. incanum* exhibited synergistic effects against *C. neoformans*. The results validate the traditional healing practices which utilise combinations of different species of plants for treatment of predisposing factors of meningitis.

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LIST OF ABBREVIATIONS

A

ABC	adenosinetriphosphate binding cassette
AFB	Acid Fast Bacilli
AIDS	Acquired immune deficiency syndrome
ATPase	adenosinetriphosphatase

B

BBB	Blood-Brain Barrier

C

CDC	Centres for Disease Control and Prevention
CrAg	Cryptococcal antigen Test
CrAgEIM	Cryptococcal antigen enzyme immunoassay
CrAgLA	Cryptococcal Antigen Lateral Assay
CrAgLFA	Cryptococcal Antigen Lateral Flow Assay
CSF	Cerebrospinal fluid

D

DAEC	Diffusely adherent *E. coli*
DCA	Deoxycholate Citrate Agar
DNA	Deoxyribonucleic acid

E

EAEC	Enteroaggregative *E. coli*
EGCg	epigallocatechin gallate
EIEC	Enteroinvasive *E. coli*
EIM	Enzyme immunoassay
EITB	Enzyme immunoelectrotransfer blot

ELISA	Enzymelinked immunosorbent assay
EMB	Eosin Methylene Blue
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
F	
FBC	Full Blood Count
FIC	Fractional inhibitory concentration
G	
GC-MS	gas chromatography coupled to mass spectrometry
Gxm	Glucoronoxymannan
H	
H_2S	Hydrogen sulphide
HIV	Human immunodeficiency virus
I	
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IM	Intramuscular
INT	Iodonitrotetrazolium
IV	Intravenous
L	
LA	Latex agglutination
LCM	Lymphocytic Choriomeningitis
LP	Lumber puncture

LPS	lipopolysaccharide
M	
MATE	multidrug and toxic compound extrusion
MDRPs	multidrug resistance pumping systems
MIC	Minimum Inhibitory Concentration
mg	milligram
mg/ml	milligram per millilitre
ml	millilitre
N	
NCCLS	National Committee for Clinical Laboratory Standards
N.m.c	*Neisseria meningitis* serogroup c
P	
PCR	Polymerase Chain Reaction
PDA	potato dextrose agar
R	
RNA	Ribonucleic acid
RND	resistance-nodualtion-cell division
rpm	revolutions per minute
RPR	Rapid Plasma Reagin
S	
SAS	Subarachnoid space
∑FIC / ∑FICI	Sum of fractional inhibitory concentration index
SDA	saboraud dextrose agar
SH	sulfhydryl
SM	secondary metabolites

SMR	small multidrug resistance family
SS	Salmonella Shigella
STEC	Shiga Toxin-producing *E. coli*
U	
UTI	Urinary Tract Infection
UV	ultraviolet
V	
VDRL	Venereal Disease Research Laboratory
W	
WBC	White blood cell
WBCs	White Blood Cells
WHO	World Health Organisation
X	
XLD	Xylose Lysine Deoxycholate
Z	
ZN	Ziehl-Neelsen

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CHAPTER 1: INTRODUCTION

1.1 A historical perspective on medicinal plant use

Historically plants have been used as therapeutic agents for infectious diseases (Van Vuuren, 2007, p.1). The struggle against many ailments and infectious diseases for many years initiated awareness of medicinal plant usage to which man strove to get treatment from barks, seeds, fruits, leaves and many other parts of plants (Petrovska, 2012). A study by Touwaide and Appetiti (as cited in Dhami & Mishra, 2015) report that numerous archaic scripts of the Hindu, Chinese, Egyptian, Mesopotamian, Biblical, Greek and many other civilisations documented the historical use of plants and other natural substances for treatment of many health disorders and diseases.

The most important work in the history of the Traditional Chinese Medicine is in the 2200 year old document, the *Shen nong ben Caojing*, meaning "the Drug treatise of the divine country man". This document includes 365 drugs, mostly of botanical origin. For each drug the following information is provided (Heinrich, Barnes, Gibbons & Williamson, 2012):

- Geographic origin
- Optimum period of collection
- Therapeutic properties
- Forms of preparation and dose

Later in the 16th Century, the first systematic treatise on herbal drugs employing scientific methods was produced. This 52 chapter treatise, *Ben Cao Gang Mu* ("Drug," by Li Shizhen, 1518-1593) contains information about 1892 drugs and more than 11,000 recipes given in an appendix. The document contains classification of drugs into 16 categories. For each drug, the following information is given (Heinrich *et al*., 2012):

- A definition of the drug
- Selected commentaries
- Classification according to the four characteristics of temperatures and the five types of taste.
- Uses (detailed information on uses according to the criteria of Chinese medicine)

- Corrections on previous mistakes
- Methods of preparing the drug
- New features
- Examples of recipes

In Egypt, hieroglyphics remains revealing the use of essential oils for anointing the sick have been found. Evidences indicating the use of frankincense oil, found in an urn were discovered in Tutankhamen's tomb. The application of essential oils for embalming processes gives the impression that their use was beneficial in warding off putrefaction (Van Vuuren, 2007, p. 2). According to Heinrich *et al*. (2012), the Egyptians documented their knowledge on *papyrus* which is a paper made from *Cyperus aquaticus,* an aquatic sedge found throughout Southern Europe and Northern Africa. The *Ebers Papyrus* is a medical handbook used to record all sorts of illnesses, therapeutic treatment and pharmaceutical plant preparations. In Glesinger's study (as cited by Petrovska, 2012) *Ebers Papyrus* was written around 1550 BC. *Ebers Papyrus* contains a collection of 800 prescriptions referring to 700 plant species and drugs used for therapy. The drugs include pomegranate, castor oil plant, aloe, senna, garlic, onion, fig, willow, coriander, juniper and many others (Petrovska, 2012). *Spirea ulmaria,* a medicinal plant from which the modern drug, aspirin derived from was already prescribed for fever and swelling in Egyptian *papyri* and recommended by the Greek Hippocrates for pain and fever (Si-Yuan, Litscher, Gao, Zhou, Yu, Chen, Zhang, Tang, Sun & Ko, 2014).

In Mesopotamia, the present day, Iraq, the oldest written evidence of medicinal plants usage for preparation of drugs has been found on a Sumerian clay slab from Nagpur, approximately 5000 years old (Petroviska, 2012). It comprised 12 recipes for drug preparation referring to over 250 various plants, among which some important alkaloids such as poppy, henbane and mandrake are revealed (Petrovska, 2012). The Lietava study (as cited in Dhami and Mishra, 2015) indicates that the retrieval of pollen grains of *Achillea*-type, *Centaurea solstitialis* L., *Ephedra altissima* Desfi., *Muscari*-type and *Senecio*-type from 60,000 BC old burials of Shanidar IV gives the historical evidence of the ancient use of plants for medicinal purposes. It is reported from the Bible and the Holly

Jewish Book, the *Talmud*, that aromatic plants such as myrtle and incense were used during various rituals accompanying treatment (Dimitrova, 1999).

Studies by Katic and Thorward (as cited in Petrovska, 2012) suggest that the most prominent writer on plant drugs was Dioscorides, "the father of pharmacognosy" who as a military physician and pharmacognosist of Nero's Army, studied medicinal plants wherever he travelled with the Roman Army. Around 77 AD, he wrote a book "*De Materia Medica*". This classical work of ancient history, translated many times, offers plenty of data on the medicinal plants constituting the basic *materia medica* until the Middle Ages and the Renaissance (Petrovska, 2012). It is during the Renaissance period when the term "pharmacognosy" was born (Van Vuuren, 2007, pp. 2-3). The word is derived from two Greek words "pharmakon" meaning drug and "gignosko" meaning to acquire knowledge. Pharmacognosy studies during this time and up to twentieth century were confined on medicinal substances originated from oils, waxes, gums and resins (Van Vuuren, 2007, pp. 3-4).

1.2 Medicinal plants as antimicrobial agents

The discovery of antibiotics during the twentieth century coupled with significant advances in antimicrobial drug development improved human health through improved treatment of infections (Upadhyay, Upadhyay, Johny & Venkitanarayanan, 2014). Apart from reduced mortality and increased lifespan, it has also led to reduced affordability in developing countries (Eloff, 2000).

Kamatou, Viljoen, Van Vuuren and Van Zyl (2006) contends that the development of antimicrobial resistance has accelerated the search of new antimicrobial agents. The emergency of antibiotic resistance in pathogenic bacteria has led to the renewed interest in exploring the potential of plant derived antimicrobials as an alternative therapeutic strategy to combat microbial infections (Upadhyay *et al*., 2014). Presently, medicinal plants have become the paramount source of drug discovery in research for treating diverse form of diseases (Kayande & Patel, 2016). Many conventional drugs are derived directly from both nature and traditional medicine distributed around the world. The practice of herbal medicine demands the use of more than 53,000 species and it is horrifying to note that a number of these are facing the threat of extinction due to

overexploitation (Si-Yuan *et al*., 2014). Studies conducted by Sosa and Njunda *et al.* (as cited in Nguedia & Shey, 2014) claim that multidrug resistance to as many as seven antibiotics namely; ampicillin, amoxicillin, cotrimoxazole, gentamicin, ceftriaxone augmentin (amoxicillin and clavulanic acid) have been discovered in many epidemiological studies in Africa. Medical experts are currently cautioning of a slide back to preantibiotic era. A recent database lists the existence of more than 20,000 potential resistance genes (r genes) of nearly 400 different types predicted in the main form from available bacterial genome sequence. Fortunately, the current number of functional resistance determinants in pathogens is so diminutive (Davies & Davies, 2010). According to the World Health Organisation (WHO), the increase of resistance to antibiotics by bacterial pathogens is a growing problem in both developed and developing countries (Ahmad, Hasan, Chishti & Ahmad, 2012). Current population based studies prophesy increased rates of morbidity and mortality all over the world because of growing number of these infections from drug resistance infectious agents (Bhandari & Bhandari, 2016).

There are many reasons for antibiotic resistance. Factors contributing to antibiotic resistance include self medication, treatment outside of recognised treatment centres, consumption of drugs without medical supervision, frequent movement of population, overcrowding which provides opportunities for the rapid spread of organisms (Nguedia & Shey, 2014), uncontrolled selling of antibiotics especially in developing countries, lack of adequate number of drug development projects and inadequate funds allocated to these projects (Bhandari & Bhandari, 2016).

Therapeutic properties of medicinal plants are well recognised at a global level. The first plant compound to possess biological activities was reported in the 1930's and currently a prethora of plant compounds are readily available from herbal suppliers and natural food stores (Joshua & Takudzwa, 2013). The WHO has emphasised usage of traditional medicine and reported that 80% of the population from developing countries rely on medicinal plants for their primary health care (Adnan, Bibi, Musarat, Tariq & Shinwari, 2014). Medicinal plants represent a rich source of antimicrobial agents (Ahmad *et al*., 2012). Different parts of plant herbs and spices have been used for many years in the prevention of infection (Ahmad *et al.,* 2012). Herbs and plants can be processed and

consumed in numerous ways and forms. For example, one can use whole herb, teas, syrups, essential oils, ointments, salves, rubs, capsules and tablets that contain a ground or powdered form of a raw herb or its dried extracts (Wachtel-Galor & Benzie, 2011, p. 3). Currently herbs are used as therapeutic agents for chronic and acute conditions for numerous ailments and diseases such as cardiovascular disease, prostate problems, depression, inflammation, immune system booster, to mention but a few (Wachtel-Galor & Benzie, 2011, p. 3).

According to Si-Yuan *et al.* (2014) there are approximately 350,000 species of existing plants among which 287,655 species have been identified as of 2004. Medicinal plants are an important aspect of daily lives of many people and are an important part of the South African cultural heritage (Van Wyk, Van Oudtshoorn & Gericke, 2009, p. 7). South Africa has well over 30,000 species of higher plants (Van Wyk *et al*., 2009, p. 7). The Cape Floral Kingdom alone has nearly 9,000 species and is the most diverse temperate flora on earth, rivalling the tropical rainforests in terms of species richness (Van Wyk *et al.,* 2009, p. 7). Russo and Hou (2012, p. 53) states that approximately 200,000 of the plants that grow in Chinese soil, more than 3,000 various plant species have been identified and studied. Among these 3,000 valuable plant species, more than 500 species have been reported to be efficacious and safe for medicinal uses for humans (Russo & Hou, 2012, p. 53). Also, it is believed that more than 8,000 plant species in South Asia carries medicinal properties of which 1,000 exists in Pakistan (Adnan *et al*., 2014). Local people use these medicinal plants for the treatment of various ailments through their indigenous knowledge (Adnan *et al*., 2014).

The knowledge of medicinal plants use, has been passed on from one generation to the next through oral histories (Zonyane, Van Vuuren & Makunga, 2013). However due to modernisation (Adnan *et al.*, 2014), colonisation and displacement of people from traditional lands as well as changes in family structures that affect passing on cultural knowledge are some of the contributing factors that have negatively affected the preservation of the practice of traditional medicine (Oliver, 2012). There is a high risk of losing this valuable knowledge which is an ever present concern (Zonyane *et al*., 2013). To record this knowledge before it is lost together with scientific validation, needs to be a priority (Zonyane *et al*., 2013).

There are many advantages of using natural medicine. When used optimally, the mode of action of a herb treats the underlying cause of a disease while a synthetic drug is often designed to mitigate the symptoms or effect without addressing the root cause of a disease (Murray, 2013, p. 257). Ruso and Hou (2012, p. 49), describe synthetic drugs as foreign to the body and they tend to interfere the peace or balance of the body. Most chemical drugs are even toxic at prescribed dosages particularly to the elderly and children. Benzie and Wachtel-Galor (2011, p. 2) has assertively reported that traditional medicines are cheap and more affordable. One fascinating fact of herbal remedies is that oral consumption allows the release of active ingredients into the bloodstream slowly and gradually (Russo & Hou, 2012, p. 6). The combination of a low concentration and slow release of medication from a herb or herb mixture provides a gentle healing, as well as a long-lasting effect accomplished in part by the presence of inert substances and active ingredients (Russo & Hou, 2012, p. 6).

The therapeutic value of synergistic interactions has been known since antiquity and many different cultural healing systems have relied on this principle in the belief that combination therapy may enhance efficacy (Van Vuuren & Viljoen, 2011). Wagner (2010) alleges that studies into plant based pharmacological interactions has become a significant key in modern years. It is a fact that more and more, it is being discovered that many diseases posses a multi-causal aetiology and a complex pathophysiology which can be treated more effectively with well chosen drug combinations than monotherapy (Wagner, 2010). African traditional healers rarely rely on a single plant for therapeutic regimens but often combine various plant parts and different species inorder to achieve optimal results (Van Vuuren & Viljoen, 2011). Most of the phytomedicines are on the drug market as whole extracts and traditional practitioners have always believed that synergistic actions are important to achieve therapeutic efficacy (Kamatou *et al*., 2006). Multi-target therapy is not only confined to traditional medicine but is also practised in allopathic medicine for treatment of cancer, HIV, infectious diseases, hypertension and rheumatism (Zonyane *et al*., 2013). The use of combination therapy in allopathic medicine is often commercialised in the treatment of various infectious diseases, for instance, augmentin (Kamatou *et al.,* 2006). The basis of using multi-drug therapy for various disorders is the acknowledgement that for each drug, more than one mechanism and

gene is identified (Yang, Zhang, Li, Ye, Li & He, 2014). Without the current combination therapy used to treat tuberculosis (isoniazid, rifampicin, pyrazinamide and ethumbatol), the mortality of infected patients could reach global epidemic proportions (Van Vuuren & Viljoen, 2011).

The medical catastrophe due to the progressive antimicrobial resistance coupled with frequent episodes of infectious diseases and increasing cost of allopathic medicine in developing countries with its prolific side effects and contraindications has necessitated numerous studies into plant based pharmacological interactions. Hence, the long history and reputation of phytocombination for therapeutic regimens need to be studied rigorously in a scientific way so that the efficacious antimicrobial combinations can be translated into clinical practice (Van Vuuren & Viljoen, 2011; Benzie & Wachtel-Galor, 2011, pp. xiv, 7).

1.3 Statement of the problem

Meningitis has been a common problem to people of all age groups and continues to contribute significantly to global mortality and morbidity (Luksic, Mulic, Falconer, Orban, Sidhu & Rudan, 2013).

Typical signs and symptoms of meningitis are stiff neck, headaches, high fever, photophobia, confusion and vomiting (WHO Number of Suspected Meningitis Cases and Death Reported, 2016). Babies and young children may have an unusual high pitched or moaning cry, grunt or breathe rapidly, present with a soft spot on their heads (fontanelle), seizures, refusal to feed. Fever is absent in babies of less than three month old (Meningitis Research Foundation, 2016). Adults with bacterial meningitis present symptoms and signs of meningeal irritation and brain parenchyma inflammation. Elderly patients present an altered mental status and focal neurological deficits than young patients while neck stiffness and headache are notably less frequent (Brouwer, Tunkel & Van de Beek, 2010). Children who suffer from meningitis may recover with at least one or more of the following complications; hearing loss and tinnitus, balance problems, hydrocephalus, sightloss, epilepsy, problems with movement and coordination (ranging from muscle weakness to paralysis), behavioural and emotional problems, loss of memory and concentration (Meningitis Research Foundation, 2016). Usually survivors of meningitis who have long

term disabling squelae have impaired quality of life and they are unable to live in companionship with others in a community. Regrettably, disabled children and adults due to meningitis are hidden from view in many societies, subjected to stigma and neglect, undercounted in national and international statistics (Edmond, Clark, Korczak, Sanderson, Griffiths & Rudan, 2010).

1.4 Research aim and objectives

The primary aim of this study is to determine the antimicrobial activity of water, methanol and acetone extracts of leaves of *Geranium incanum*, *Mangifera indica*, *Bidens pilosa*, *Ricinus communis*, *Carica papaya* and fruit pulp of *Adansonia digitata* and activity of their combined preparation against selected bacterial and fungal pathogens that cause meningitis.

The objectives of the research project are:

- To collect and identify the plant materials for the study.
- To prepare and extract plant materials separately and in a combined preparation using the various solvents.
- To prepare agar wells of plant extracts in agar plates for agar well diffusion assays.
- To determine minimum inhibitory activities of the individual and combined preparations of the plant extracts against selected strains of microbes representing Gram positive, Gram negative organisms and yeast, using the microtitre plate/iodonitrotetrazolium (INT) technique.
- To determine the fractional inhibitory concentration (FIC) of the selected plant combinations

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

The central nervous system (CNS) comprises the brain and spinal cord (Tortora & Derrickson, 2014, p. 400). Tortora and Derrickson (2014, p. 476) indicates that the CNS consist of three membranes. The meninges are pia mater, arachnoid mater and the dura mater.The cerebrospinal fluid (CSF) and the three membranes enclose and protect the brain (Silverthorn, 2014, p. 312; Martini, Nath & Bartholomew, 2014, p. 511).

The CNS is proned to numerous disorders such as trauma, degeneration, structural defects, tumours, autoimmune diseases as well as infectious diseases (Tortora & Derickson, 2014, pp. 469, 470). The most commonly infectious disease that affects the CNS is meningitis. Meningitis is the inflammation of the meninges that cover the brain and spinal cord. Meningitis may be caused by bacteria, viruses, fungi, parasites, drugs, autoimmune disorders as well as malignancy (Ward, Greenwood, Kumar, Mazza & Yale, 2009). Luksic *et al.* (2013) suggests that meningitis remains one of the significant cause of global mortality and morbidity. Besides mortality and morbidity, meningitis has been described as one of the most infectious diseases that poses threat due to its high rates of long term complications and fatality (Ward *et al*., 2009). However, plants have been used traditionally to treat meningitis and other inflammatory conditions (Zhang, Luo, Li., Yi, Wang, An, Zhang, 2008)

Plants are surrounded by numerous enemies such as herbivores, insects and microbes. Plants can neither mobilise away from nor utilise weapons when attacked by herbivores or insects (Wink, 2015). Humans may run away or use weapons in response to danger. Additionally, humans can rely on their well developed immune systems such as innate and acquired immune system when challenged by microbes (Wink, 2015). On the contrary, such immune systems do not occur in plants. In spite of all these challenges plants are able to survive when attacked by herbivores, insects and microbes. Plants synthesise a wide range of organic compounds. Most of these compounds are not directly involved in growth and development of plants (Croteau, Kutchan & Lewis, 2000, p. 1250; Wink, 2008). These compounds are called plant secondary metabolites (SM). Plant SM are toxic and the ability to synthesise these compounds have helped plants to ward off

pathogens and predators. Plant secondary metabolites exert diverse array of biological and pharmacological activities. Plant SM prevent herbivory attack by serving as bitter-tasting deterrents and toxins (Croteau *et al.,* 2000, p. 1274). In microbes, SM interacts with the main targets of the cell such as proteins, nucleic acids (DNA, RNA including related enzymes and regulatory proteins such as transcription factors) and biomembranes. This interferes with the normal functioning of the cell and consequently results in cell death (Wink, 2015; Wink, 2008). In addition, plant SM have been reported to exert multiple mechanism of action on microbial cell, and as such, they are able to reverse the mechanism of antimicrobial resistance (Wagner & Ulrich-Merzenich, 2009). Resistance mechanisms of microbes include active site modification, enzymatic degradation, reduced accumulation of the drug within the microbe cell wall, decreased membrane permeability as well as efflux pumps (Hemaiswarya, Kruthiventi & Doble, 2008; Van Vuuren & Viljoen, 2011).

Given the fact that plant SM possess biological and pharmacological properties, they are employed as therapeutic agents either alone or in a combination of various plant parts and different plant species in order to improve efficacy (Van Vuuren & Viljoen, 2011; Upadhyay *et al*., 2014). Globally, traditional healers have used medicinal plants since ancient times to treat different ailments and health disorders. Numerous studies have been undertaken on the antibacterial and antifungal activities of a single plant part and single plant species. However, traditional healers rarely rely on a single plant regimens but often combine various plant species inorder to achieve optimal results (Van Vuuren & Viljoen, 2011). Different cultural healing systems have a belief that combination therapy may improve efficacy (Van Vuuren & Viljoen, 2011). Given that it is a traditional practice to combine medicinal plants, very little have been published on antibacterial and antifungal activities involving combination of two or more than two medicinal plants (Van Vuuren & Viljoen, 2011).

Traditional healers use *A. digitata, G. incanum, R. communis, C. papaya, B. pilosa* and *M. indica* to treat predisposing factors of meningitis, manage signs and symptoms of meningitis such as headache, fever and inflammation. According to Mvere (2004, pp. 114-115*), B. pilosa* leaves are utilised as a remedy for headache and inflammation. Watt and

Brandwijk (1962, p. 430) claims that *R. communis* leaves are traditionally used to treat headache. *A. digitata* fruit pulp, *G. incanum* (Van Wyk *et al.,* p. 134), *M. indica* (Ross, 1999, p. 199) are traditionally used as a remedy for fever. Traditional healers also use *R. communis* (Jena & Gupta, 2012), *C. papaya* (Aravind, Duraivel & Harrish, 2013) and *M. indica* (Ross, 1999, p. 198) as anti-inflammatory agents.

2.2 A brief anatomy of the central nervous system (CNS)

The CNS is made up of the brain and spinal cord (Tortora & Derrickson, 2014, p. 400). When examined on a macroscopic level, the tissue of the CNS are divided into gray matter and white matter (Silverthorn, 2014, pp. 307, 309).

Gray matter consists of unmyelinated nerve cell bodies, dendrites and axon terminals. The cell bodies are assembled in an organised fashion in both the brain and spinal cord. They form layers in some parts of the brain and in other parts they cluster into groups of neurons that have similar functions. Clusters of cell bodies in the brain and spinal cord are called *nuclei* (Silverthorn, 2014, p. 309).

White matter is mostly myelinated axons and contains very few cell bodies. Its pale colour comes from the myelin sheaths that surround the axons. Bundles of axons that connect different regions of the CNS are known as tracts (Silverthorn, 2014, p. 309).

The adult brain constituting 2% of total body weight, is comprised of four major parts; brain stem, cerebellum, diencephalon and cerebrum. The brain is continuous with the spinal cord and is composed of medulla oblongata, pons and mid brain. The cerebellum is situated at the back of the brain stem and above the brain stem is the diencephalon which consists of the thalamus, hypothalamus and epithalamus. The diencephalon and the brain stem support the cerebrum which is the largest part of the brain (Tortora & Derrickson, 2014, p. 474).

The meninges of the CNS comprise three membranes that together with the cerebrospinal fluid (CSF) enclose and protect the brain. The cranial meninges bearing the same names; the dura mater, the arachnoid mater and the pia mater are continuous with the spinal meninges and have the same basic structure. The cranial dura mater has two layers while the spinal dura mater has only one. The two dural layers around the brain

are fused together except where they separate to enclose the dural venous sinuses that drain venous blood from the brain and deliver it into the internal jugular veins (Tortora & Derrickson, 2014, p. 477).

2.2.1 Formation and flow of CSF in the ventricles

Cerebrospinal fluid is a clear, colourless, salty solution that is produced by the choroid plexus, a specialised region on the walls of the ventricles (Tortora & Derrickson, 2014, pp. 477-478; Silverthorn, 2014, p. 309). The choroid plexus is comparable to kidney tissue and is comprised of capillaries and a transporting epithellium derived from the ependyma that form CSF from blood plasma through filtration and secretion (Silverthorn, 2014, p. 309). Since the epindymal cells are joined by tight junctions, materials entering CSF from choroid capillaries cannot leak between these cells, alternatively they pass through the ependymal cells (Tortora & Derrickson, 2014, p. 478).

The choroid plexus produces CSF at a rate of 500 ml per day (about 2.1 cups per day). The total volume of CSF at any moment is roughly 150 ml, thus, entire volume of CSF is restored approximately every eight hours (Martini *et al*., 2014, p. 512) . The composition of CSF is closely regulated and the rate of removal keeps pace with the rate of production regardless of this turnover (Martini *et al*., 2014, p. 512). The CSF formed in the choroid plexuses of each lateral ventricle flows into third ventricle through two narrow, oval openings, the interventricular foramina (Figure 2.1).

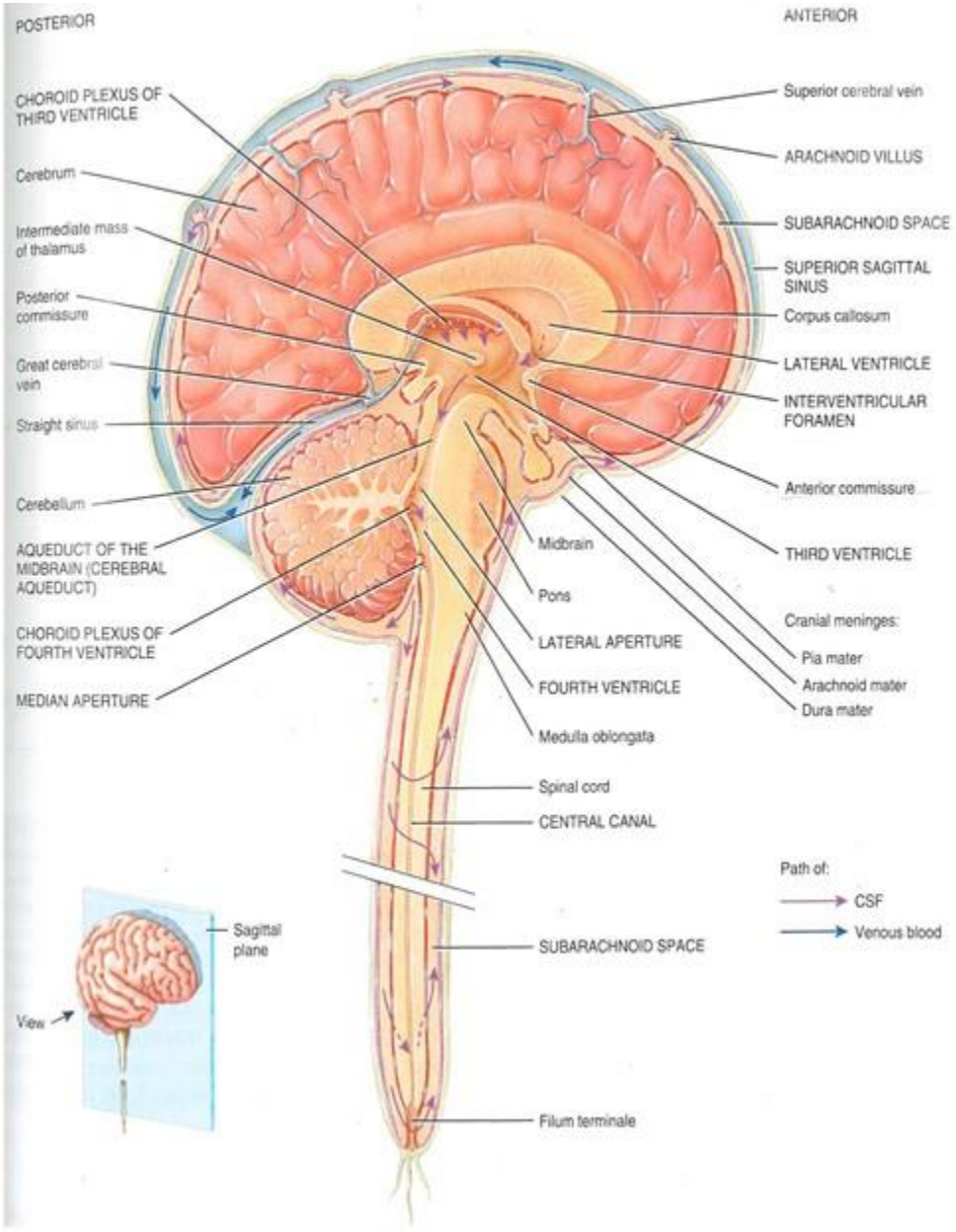


Figure 2.1: Pathways of circulating cerebrospinal fluid

(obtained from Tortora & Derrickson, 2014, p 480)

More CSF is added by the choroid plexus in the roof of the third ventricle. The CSF then flows through the aqueduct of the midbrain, which passes through the midbrain, into the fourth ventricle. The choroid plexus of the fourth ventricle adds more fluid. The CSF reaches the subarachnoid space through two lateral apertures, openings in the roof of the fourth ventricle. The CSF enters the subarachnoid space through three openings in the

roof of the fourth ventricle; a median aperture and the paired lateral aperture, one on each side.The CSF then circulates in the central canal of the spinal cord and in the subarachnoid space around the surface of the brain and spinal cord (Tortora & Derrickson, 2014, p. 479). Fingerlike projections of the arachnoid membrane called the arachnoid villi, penetrate the meningeal layer of the dura mater and extend into the superior sagittal sinus. In adults a collection of villi form a large arachnoid granulations. The CSF fluid is absorbed into the venous circulation at the arachnoid granulations (Martini *et al.,* 2014, p. 512).

The CSF renders physical and chemical protection. The CSF act as a shock-absorbing medium that protects the delicate tissue of the brain and spinal cord from jolts that would otherwise cause them to hit the bony walls of the cranium (Martini *et al*., 2014 p. 511; Silverthorn, 2014, p. 312; Tortora & Derrickson, 2014, p. 478). The brain and the spinal cord float in CSF. The CSF buoys the brain so that it floats in the cranial cavity. The buoyance of the CSF reduces the weight of the brain nearly 30-fold (Silverthorn, 2014, p. 312). A human brain weighs about 1400g in air but only approximately 50g when supported by CSF (Martini *et al*., 2014, p. 511). Lighter weight renders less pressure on the blood vessels and nerves attached to the CNS. The CSF also provides an optimal chemical environment for accurate neuronal signalling. The choroid plexus is selective about which substances it transports into the ventricles and as a result, the composition of CSF differs from that of the plasma. For instance, the normal level of glucose in CSF is 60% of the level of serum glucose (Nigrovic, Kimia, Shah & Neuman, 2012). The concentration of potassium is lower in CSF and hydrogen is higher than plasma. The concentration of sodium in CSF is similar to that in the blood (Silverthorn, 2014, p. 312). A slight change in the ionic composition of CSF within the brain can seriously interrupt production of action potentials and postsynaptic potentials (Tortora & Derrickson, 2006, p.478). The CSF also serves as a conduit for removal of waste products and entry of nutrients between the blood and the nervous tissue (Silverthorn, 2014, p. 312; Tortora & Derrickson, 2014, p. 478)

2.2.2 Brain blood flow and blood- brain barrier (BBB)

Blood flows to the brain mostly through the internal carotid artery and vertebral arteries and it goes back from the head to the heart through internal jugular vein. The adult human brain utilises about 20% of the oxygen and glucose even at rest. A brief slowing of a blood flow to the brain may cause unconsciousness. An interruption in blood flow for one or two minutes impairs neuronal function and total deprivation of oxygen for about four minutes causes permanent injury. Since no glucose is stored in the brain, the supply of glucose must be constant (Tortora & Derrickson, 2014, p. 477).

The final layer of protection for the brain is the blood-brain barrier (BBB). The presence of BBB shields brain cells from harmful substances and blood borne pathogens by preventing entry of many substances from blood into brain tissue (Tortora & Derrickson, 2014, p. 477). The BBB is composed of tight junctions (Figure 2.2) that seal together the endothelial cells of brain capillaries.

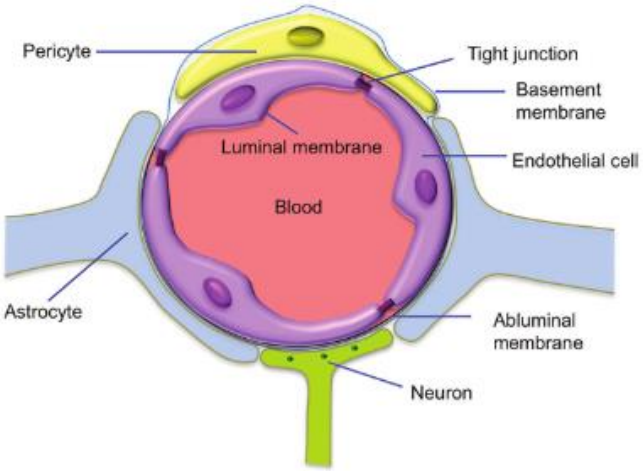


Figure 2.2: An illustration of a Blood-Brain Barrier (taken from Liu, Perlin, & Xue, 2012)

Permeability characteristics of the tight junctions is maintained by press up of the astrocytes against the capillaries through chemical secretions. A few water soluble substances such as glucose cross the BBB by active transport. Other substances such as creatinine, urea and most ions cross the BBB very slowly. Proteins and most antibiotic drugs do not pass at all from the blood into the brain tissue. Nevertheless, lipid soluble

substances such as oxygen, carbon dioxide, alcohol (Tortora & Derrickson, 2014, p. 477; Martini *et al*., 2014, p. 513), most anaesthetic agents (Tortora & Derrickson, 2014, 477) and lipids such as steroids or prostagrandins (Martini *et al.,* 2014, p. 513) easily cross the BBB.

2.3 Meningitis

Meningitis is a life threatening disease in which there is inflammation of the protective membranes that surround the brain (Donovan & Blewitt, 2009; Gonzalez, Clement & Yero, 2007).

2.3.1 Brief history of meningitis

The meningitis syndrome has been recognised for centuries. Hippocrates realised the intracranial consequences of otitis infection. Clear clinical description of meningitis has been found dating from the sixteenth century (Scheld, Marra & Whitely, 2014).

Gaspard Vieussex (1746-1814) and Andrey Matthey (1778-1842) in Geneva and Elias North (1771-1843) in Massachussets described epidemic (meningococcal) meningitis (Mandal, 2012). Meningitis outbreaks was first recorded in Geneva, Switzerland in 1805 and Gaspard Vieussex recorded the syndrome of the epidemic as “malignant purpuric fever”. This was the first clinical description of meningococcemiae with meningitis (Scheld *et al*., 2014). The pathologic features of the condition, inflammation within the subarachnoid space (SAS) was described in autopsy reports in the French literature the following year. Danielson and Mann further recorded the detailed examinations of meningococcemiae and meningitis in 1806 (Scheld *et al*., 2014).

Towards the end of nineteenth century more symptoms of the condition were reported. The symptoms of meningitis were described in 1884 by Russian physician Vladimir Kernig (1840-1917) in 1899 and by Polish physician Jozef Brudzinski (1874-1917) (Ward, *et. al.,* 2009; Mandal, 2012). The signs were thus called Kernig’s signs and Brudzinski signs in 1882 and 1909 respectively. Meningococci were first isolated in 1887 by Anton Vaykselbaum in Viena. This meningococci isolates were obtained from CSF of six patients with meningitis and were originally named “Diplococcus intracellularis meningitidis”. Heinrich Quinkle (1842-1922) initiated lumber puncture (LP) in 1891 (Mandal, 2012; Scheld *et al*., 2014) and the most changes associated with meningitis

such as pleocytosis, hypoglycorrhachia and elevated protein concentration were well identified by the end of the century. In addition, the therapy for bacterial meningitis in the early years of this century was dominated by methods of withdrawl of large volumes of CSF or direct instillation of substances, for instance, dyes, enzymes into the SAS (Scheld *et al*., 2014).

In Africa, the first outbreak was described in 1840. African epidemics became much common in the twentienth century. The first and most serious one was reported in Nigeria and Ghana in 1905-1908 (Mandal, 2012). According to early reports, multitude of people died of the disease (Mandal, 2012). By the second half of the twentienth century influenzae viruses A and B as well as adenovirus were found to be associated with meningitis (Mandal, 2012). Armstrong and Lilly isolated the virus from the CSF of patients. In 1968, A.A. Smorodintsev proved that there are more than 200 different viruses and their serotypes that may cause meningeal infections (Mandal, 2012). William Mestrezat (1883-1929) and Houston Merrit (1902-1979) compiled large series of CSF profile in meningitis (Mandal, 2012).

2.3.2 Global, regional and national burden of meningitis

According to Luksic *et al.* (2013), meningitis continues to contribute significantly to global mortality and morbidity.

Asia experienced some major epidemics of meningococcal disease in the last 30 years (China 1979 and 1980, Vietnam 1977, Mongolia 1973-1974 and 1994-1995, Saudi Arabia 1987, Yemen 1988) (WHO, 2016). Epidemics have also been occurring during the last thirty years but they have not reached the very high incidence levels of epidemics in other parts of the world (WHO, 2016). The largest and most frequently recurring meningococcal outbreaks have been in the semi arid area of Sub-Saharan Africa which is called African Meningitis Belt (Garcia-Pando, Thomson, Stanton, Diggle, Hopson, Pandya, Miller & Hugonnet, 2014). This area stretches from Senegal to Ethiopia (Figure 2.3) with an estimated total population of 300 million people (WHO, 2016).

Figure 2.3: Map of Africa showing African Meningitis Belt (obtained from Garcia-Pando *et al*., 2014)

The most recent meningococcal meningitis outbreak in the African Meningitis Belt began in the mid 1990. In 1996, almost 190,000 cases were reported to WHO in Burkina Faso, Chad, Mali, Niger, Nigeria. The response to the epidemic by these countries incapacitated the routine healthcare systems and consumed all international stocks of vaccine (WHO, 2016).

In 2001, six countries in the African Meningitis Belt; Benin, Burkina Faso, Central African Republic, Chad, Ethiopia and Niger experienced large epidemics (WHO, 2016). Although globally meningitis is a serious infectious disease, it is generally underreported. Several studies have shown a decrease in the frequency of bacterial meningitis and meningococcal sepsis in modern years although these largely reflect changes seen in children (McGill, Defres, Glennie, Kaczmarski, Davies, Solomon, Heyderman, Beeching, Gaillemin, Nadel, Miller, Michael, Borrow, Wyncoll, Thwaites, Rhodes, Cohen & Read, 2016) . According to the WHO Global, Regional and National causes of child mortality from 2000-2013, meningitis contributed 2% of global causes of child death in 2013 (Liu, Oza, Hogan, Perin, Rudan, Lawn, Cousens, Mathers & Black, 2015) (Figure 2.4).

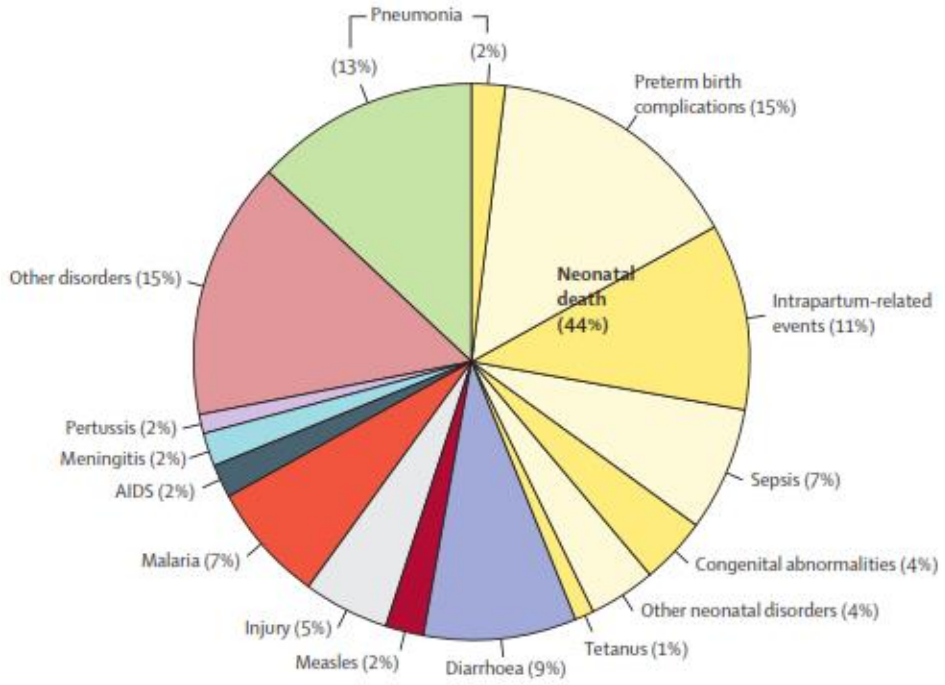


Figure 2.4: Pie chart showing global causes of child death in 2013 (taken from Liu, *et al*., 2015)

The disease has remained stable or increased in adults. A recent study in England and Wales showed an increase in the incidence of meningitis in adults between 2004 and 2011, with an increase of 3% per year in patients over 65 years of age (McGill *et al*., 2016). The incidence in adults was approximately 1.05 cases per 100,000 population

(between 2004 and 2011) with the highest incidence in the 45-64 age group (1.21 per 100,000) (McGill *et al*., 2016). The mortality rate of community acquired bacterial meningitis is high approximately 20% for all causes and up to 30% in pneumococcal meningitis, increasing with age (McGill *et al*., 2016). In 2015 an extraordinary epidemic of meningitis due to *Neisseria meningitidis* serogroup C (*N.m.*C) occurred in Niger and Nigeria with approximately 11000 cases and 800 death (WHO, 2015)

2.3.3 Aetiology and classification of meningitis

Ward *et al*. (2010) highlight that meningitis may be caused by any of a host of infectious and non-infectious agents including bacteria, viruses, fungi, parasites, drugs, autoimmune disorders or malignancy which considerably influences morbidity and mortality. According to the Centres for Disease Control and Prevention (2014), the disease is classified into bacterial meningitis, viral meningitis, fungal meningitis, parasitic meningitis and non-infectious meningitis. This classification is based upon the aetiological agent of the disease.

2.3.4 Possible pathogen in each classification of meningitis

The following are examples of possible pathogens in each classification;

2.3.4.1 Bacterial meningitis

2.3.4.1.1 Gram positive bacteria

- *Streptococcus pneumoniae*
- *Staphylococcus aureus*
- *Streptococcus agalactiae (Group B)*
- *Streptococcus pyogenes*
- *Enterococci species*
- *Listeria monocytogenes*
- *Bacillus anthracis* (McGill *et al*., 2016; Tunkel, 2014)

2.3.4.1.2 Gram negative bacteria

- *Neisseria meningitidis*
- *Haemophilus influenzae (type B)*
- *Escherichia coli*
- *Pseudomonas aeruginosa*

- *Proteus species*
- *Salmonella species*
- *Klebisiella pneumoniae*
- *Enterobacter* ((McGill *et al*., 2016; Tunkel, 2014)

2.3.4.1.3 Acid fast bacilli

- *Mycobacterium tuberculosis* (McGill *et al*., 2016; Tunkel, 2014; Cheesbrough, 2006, p. 116)

2.3.4.1.4 Spirochaetes

- *Treponema pallidum* (Cheesbrough, 2006, p. 116)

2.3.4.2 Viral meningitis

- Enteroviruses
- Arboviruses
- Mumps viruses
- Herpes viruses
- Coxsackie viruses
- Echo viruses
- Varicella zoster
- Lymphocytic choriomeningitis virus (LCM)
- Human Immunodeficiency Viruses (HIV) (Tunkel, 2014; Cheesbrough, 2006, p. 116)

2.3.4.3 Fungal meningitis

2.3.4.3.1 Yeast pathogen

- *Cryptococcus neoformans*
- *Candida albicans* (Liu *et al*., 2012)

2.3.4.3.2 Dimorphic fungi

- *Histoplasma capsulatum*
- *Coccidioides immitis*
- *Paracoccidioides brasiliensis*
- *Blastomyces dermatitidis* (Liu *et al.,* 2012)

2.3.4.3.3 Filamentous fungi

- *Aspergillus species*
- *Zygomycetes* (Liu *et al*., 2012)

2.3.4.3.4 Dematiaceous mould

- *Bipolaris spicifera*
- *Exophiala jeanselmei*
- *Cladophialophora bantiana*
- *Ochroconis gallopavum*
- *Ramichloridium mackenziei* (Liu *et al*., 2012)

2.3.4.4 Parasitic meningitis

- *Naegleria fowleri*
- *Angiostrongylus cantonensis*
- *Dirofilaria immitis*
- *Trypanosomes*
- *Toxoplasma gondii* (Cheesbrough, 2006, p. 116; Chen & Lai, 2007)

2.3.4.5 Non-infectious meningitis

2.3.4.5.1 Certain drugs

- Rofecoxib
- Metronidazole
- Amoxicillin
- Sulfamethoxazole-trimethoprime
- Ciprofloxacin
- Ranitidine (Centres for Disease Control & Prevention, 2014; Janocha-Litwin & Simon, 2013)

2.3.4.5.2 Medical conditions

- Cancers e.g Multiple myeoloma
- Skull fractures
- Brain surgery (Centres for Disease Control & Prevention, 2014)

2.3.5 Clinical features of meningitis

Classical signs and symptoms of meningitis are neck stiffness and muscle pain, headaches, high fever, photophobia, confusion and vomiting. In addition to these typical clinical features, infants may present moaning cry, respiratory distress, refusal to feed, pale or mable skin, dislike being handled (Van de Beek, Cabelles, Dzupova, Esposito, Klein, Kloek, Leib, Mourvillier, Ostergaard, Pagliano, Pfister, Read, Sipahi & Brouwer, 2016; Meningitis Research Foundation, 2015; Donovan & Blewitt, 2009). Fever is absent in neonates of less than three months. Children and adults may have fever, severe headache, nausea and vomiting, stiff neck and muscle pain, drowsy or difficult to wake, confusion, meningeal irritation and brain parenchyma inflammation (Donovan & Blewitt, 2009; Brouwer *et al*., 2010). Elderly patients may manifest altered mental status and focal neurological deficits than young patients while neck stiffness and headache are notably less frequent (McGill *et al*., 2016; Brouwer *et al*., 2010).

2.3.6 Risk factors for some of the aetiological agents of menigitis

Some of the predisposing factors of meningitis are age, migration, community settling, certain medical conditions and drugs (Centres for Disease Control & Prevention, 2014; Tunkel, 2014)

2.3.6.1 Age

Age can also be an indicator of likely causative agents of meningitis (Table 2.1).

Table 2.1: Predisposing factors for some of the aetiological agents of meningitis according to age (Centres for Disease Control & Prevention, 2014; Tunkel, 2014)

AGE GROUP	POSSIBLE AETIOLOGICAL AGENT
New born <1 month	• *Group B Streptococcus* • *Escherichia coli* • *Listeria monocytogenes* • *Viral meningitis*
Infants and children	• *.Streptococcus pneumoniae* • *Neisseria meningitidis* • *Haemophilus influenzae type b*
Adolescents and young adults	• *Neisseria meningitidis* • *Streptococcus pneumoniae* • *Viral meningitis*
Older adults	• *Streptococcus pneumoniae* • *Neisseria meningitidis* • *Listeria monoctytogenes* • *Streptococcus agalactiae*

2.3.6.2 Medical conditions

Certain underlying medical conditions are likely to induce meningitis (Table 2.2).

Table 2.2: Predisposing medical conditions for meningitis (taken from Tunkel, 2014 and Baldwin & Whiting, 2016)

AETIOLOGICAL AGENT	PREDISPOSING MEDICAL CONDITION
Streptococcus pneumoniae	• Pneumonia • Otitis media • Mastoiditis • Sinusitis • Endocarditis • Asplenia/splenectomy/splenic dysfunction
Listeria monocytogenes	• Diabetes mellitus • Liver disease • Chronic renal disease • Collagen vascular disease • Acquired immune deficiencies e.g. HIV • Alcoholisation
Neisseria meningitidis	• Deficiencies in terminal complements (C5, C6, C7, C8, C9) • Respiratory tract infections with viruses
Streptococcus agalactiae	• Diabetes mellitus • Pregnancy or postpartum • Cardiac disease • Malignancy • Hepatic failure • Renal failure • Neurogenic bladder • Decubitus ulcers • Corticosteroid therapy • Alcoholisation
***Staphylococcus* species**	• Diabetes mellitus • Chronic renal failure requiring hemodialysis • Malignancies • Head trauma or neurosurgery • Endocarditis • Sinusitis • Osteomyelitis • Pneumonia • CSF shunts • Alcoholisation

Table 2.2 Predisposing medical conditions for meningitis (Continued)

AETIOLOGICAL AGENT	PREDISPOSING MEDICAL CONDITION
Aerobic Gram Negative Bacilli (*Klebsiella pneumoniae, E. coli, P. aeruginosa, Serratia marcescens, Salmonella* species)	• Head trauma • Neurosurgery • Strongyloidiasis
C. neoformans	• Immunosuppressed most often common in HIV
C. albicans	• Immunosuppressed e.g. HIV infection • Diabetes mellitus

2.3.6.3 Migration (Travel)

Travellers to the meningitis endemic area are at high risk of getting infected with meningitis causing organisms. For instance, travellers to the meningitis belt in Sub-Saharan Africa may be at risk for meningococcal meningitis, particularly during the dry season. Also at risk for meningococcal meningitis are travellers to Mecca during the annual Hajj and Umrah Pilgrimage (Centres for Disease Control & Prevention, 2014).

2.3.6.4 Community settling

Living in certain areas may increase a risk of meningitis. For example, soil, bird and bat droppings may contain *Cryptococcus neoformans, Histoplasma, Blastomyces*, *Aspergillus* or *Coccidioides* (Baldwin & Whiting, 2016). Infectious diseases also spread more quickly where larger groups of people gather together. Students living in domitories and millitary personel are at increased risk for meningococcal meningitis (Centres for Disease Control & Prevention, 2014).

2.3.6.5 Drugs

Certain drugs have been reported to induce meningitis. Examples of such drugs are;

- Rofecoxib
- Metronidazole
- Amoxicillin
- Sulfamethoxazole-trimethoprime
- Ciprofloxacin
- Ranitidine (Centres for Disease Control & Prevention, 2014; Janocha-Litwin & Simon, 2013).

2.3.7 Complications and long-term sequelae of meningitis

Numerous complications have been reported in survivors of meningitis. According to Van de Beek *et al*. (2016), common complications observed in neonatal meningitis are shock, convulsions and hydrocephalus. Most of the children who survive from meningitis may recover with atleast one of the following long term complications; hearing loss and tinnitus balance problems, hydrocephalus, sight loss, epilepsy, problems with movement and cordination (ranging from muscle weakness to paralysis), behavioural / emotional problems, learning problems (ranging from mild difficulties to severe disability), speech and language problems (Meningitis Research Foundation, 2015).

A large number of adults who survive from meningitis may recuperate with complications ranging from systemic to neurological complications. The most common reported complications are haemorrhagic stroke, ischaemic stroke, respiratory failure, circulatory shock, toxic shock syndrome, hyponatremia (Lukas, Brouwer, Bovenkerk, Man, Van der Ende & Van de Beek, 2015) cerebral infarction, cerebral abscess, subdural empyema, hydrocephalus, hearing impairment, seizures, carvenous sinus thrombosis, sigmoid sinus thrombosis (Lukas *et al*., 2015; Van de Beek *et al*., 2016).

2.3.8 Laboratory diagnosis

Laboratory investigations help to determine the aetiology of meningitis, identify antibiotic resistant organisms, assist with prognosis and guide health management including infection control, immunisation for the patient and contacts and antibiotic prophylaxis (McGill *et al*., 2016). The following are some of the laboratory investigations that can be performed to establish the cause of meningitis; blood tests, culture and sensitivity, biochemical testing, staining and microscopy, polymerase chain reaction (PCR), immunodiagnosis and serological testing (McGill *et al*., 2016).

2.3.8.1 Blood tests

The following blood tests should be performed in suspected meningitis cases; full blood count (FBC), urea, creatinine, electrolytes, liver function tests, clotting screen (McGill *et al*., 2016).

2.3.8.2 Culture and sensitivity

According to Mc Gill *et al*.(2016), culture is the gold standard for the diagnosis of bacterial meningitis. Blood and CSF cultures should be taken in all cases of suspected meningitis. Despite whether antibiotics have been given before CSF collection and depending on the aetiological agent of meningitis, culture is diagnostic in 70-85% of cases of bacterial meningitis (McGill *et al*., 2016). It is advisable to obtain culture specimens from the nasopharynx for *N. meningitidis. Meningococci* can be isolated from nasopharygeal swabs in up to 50% of patients with meningococcal disease (McGill *et al*., 2016). Given that blood and CSF cultures are negative after administration of antibiotics, specimens from nasopharyx may still be positive even if blood and CSF cultures are negative. Sensitivity will assist in identifying antibiotic resistant organisms (McGill *et al*., 2016).

2.3.8.3 Biochemical testing

The CSF glucose, protein and lactate are important in differentiating viral, bacterial and other aetiological agents of meningitis (McGill *et al.,* 2016). The values can give valuable pointers to the likely cause of meningitis but are not conclusive because of overlap between the different diseases. The CSF glucose is lowered in bacterial meninigitis, however the concentration also varies according to blood glucose (McGill *et al*., 2016). Normal CSF glucose is about half to two thirds of plasma glucose (Cheesbrough, 2006, p. 121). In bacterial meningitis the ratio is usually significantly lower than this (McGill *et al*., 2016). The CSF glucose should be assayed within 20 minutes of collection to avoid a false low result due to glycolysis (Cheesbrough, 2006, p. 120).

Increased CSF protein occurs in all forms of meningitis except in viral meningitis. In viral meningitis total CSF protein is normal or mildly increased (Cheesebrough, 2006, p. 124; McGill *et al*., 2016). A CSF lactate has a high senstivity and specificity (93% and 96% resectively) in differentiating between bacterial and viral meningitis. A CSF lactate cut off of 35 mg/dl has been suggested to have the best sensitivity in distinguishing between bacterial and viral meningitis (McGill *et al.,* 2016) where significantly increased CSF lactate are suggestive of bacterial meningitis. If antibiotics have been administered, the sensitivity drops to less than 50%. The high negative predictive value makes it a useful

test if done prior to commmencing antibiotics to rule out bacterial meniningitis and confidence to stop or withhold antibiotics (McGill *et al.,* 2016)

2.3.8.4 Staining and microscopy

2.3.8.4.1 Gram stain

Gram stain of the CSF is a rapid method for detecting bacteria with a sensitivity of between 50-99% (dependent on organism and prior antimicrobial therapy) (McGill *et al.,* 2016). Gram stain of CSF sample may demonstrate bacterial meningitis but absence of bacteria does not exclude bacterial meningitis as visualisation of bacteria correlates with CSF concentration of bacteria (Tunkel, 2014). Gram stain are positive in 90% of meningitis cases caused by *S. pneumoniae*, 86% of cases caused by *H. influenzae,* 75% of cases caused by *N. meningitidis,* 50% of cases caused by Gram negative bacilli and about one third of patients with meningitis caused by *L. monocytogenes* (Tunkel, 2014). Although Gram stain is a rapid and accurate identification of causative bacteria, some pitfalls may arise due to observer's misinterpretaion or reagent contamination (Tunkel, 2014). However, the method is highly specific for bacterial meningitis (Tunkel, 2014) and some fungal agents that cause meningitis such as *Cryptococcal neoformans* (Coovadia, Mahomed, Dorasamy & Chang, 2015).

2.3.8.4.2 Ziehl-Neelsen (ZN) for acid fast bacilli (AFB)

Ziehl-Neelsen (ZN) stained CSF smear for AFB should be examined when tuberculous meningitis is clinically suspected. Unlike other bacterial meningitis, the identification of tuberculous organisms in CSF by specific stains is difficult because of the small population of organisms (Tunkel, 2014; Cheesbrough, 2006, p. 121). Staining the pellicle as well as layering the centrifuged sediments of large CSF volume on to a single slide may increase the chances of visualising the organisms (Tunkel, 2014). Obtaining repeated specimens may also increase the yield (Tunkel, 2014).

2.3.8.4.3 Fluorescence microscopy for acid fast bacilli

Examination of an auramine stained smear by fluorescence microscopy is a more sensitive method for identifying alcohol acid-fast bacilli (AFB) in CSF. Fluorescent staining technique has 10% higher sensitivity in comparison to regular light microscopy employed with ZN staining without compromising specificity (Minion, Pai, Ramsay, Menzies & Greenaway, 2011). Ziehl-Neelsen technique is slow (Rost, 1995, p. 168; Minion *et al.,* 2011) and require pain taking examination (Rost, 1995, p. 168) while fluorescent microscopy is faster (Rost, 1995, p. 168; Minion *et al*., 2011). In fluorescent microscopy, the AFB appear as bright stained objects against black background (Rost, 1995, p. 168).

2.3.8.4.4 Indian ink

Indian ink stain is a negative staining technique used to establish the presence of *Cryptococcal neoformans* in CSF. According to Coovadia *et al*. (2015) and Cheesbrough (2006, p. 122), in the Indian ink preparation the polysacharide capsule does not take up the stain resulting in appearance of a refractile large white circle against a dark background (Figure 2.5).

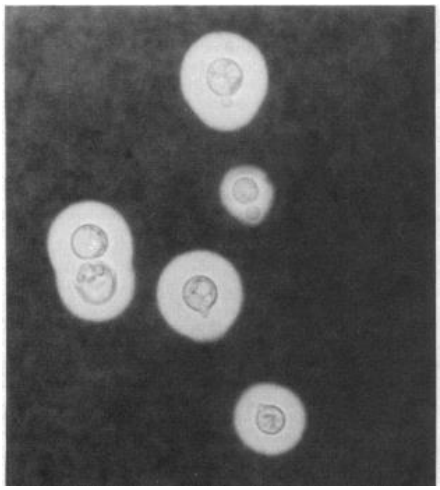


Figure 2.5: Negative staining in Indian ink (obtained from Granger, Perfect & Durack, 1985)

It has been reported that the method is positive in approximately 75% of *Cryptococcal* meningitis who have acquired immune deficiency syndrome (AIDS) but much lower in those who do not have AIDS (Coovadia *et al.,* 2015).

However, Indian ink technique has some pitfalls. Due to the inconsistent sensitivity pattern, negative samples should be re-examined with a back up method (Coovadia *et al.,* 2015). An additional confounding problem with the Indian ink is that lymphocytes may be confused with *Cryptococci* when examined by an inexperienced technologist (Coovadia *et al*., 2015). Furthermore, reports have indicated that *Cryptococci* isolated from AIDS patients have smaller capsules which may be an added limitation (Coovadia *et al*., 2015).

2.3.8.4.5 CSF cell count

The CSF cell count is an important investigation in the diagnosis of CNS infections (Bremell, Mattsson, Wallin, Henriksson, Wall, Blennow, Zetterberg & Hagberg, 2014). The total white cell count and white blood cell (WBC) differential count may be significant in attempting to distinguish different forms of meningitis (Jerrard, Hanna & Schindelheim, 2001; Cheesbrough, 2006, p. 124; McGill *et al.,* 2016), therefore CSF total WBC count and WBC differential count along with other CSF parameters should be taken into account in initiating therapy in CNS disorders (Jerrard *et al.,* 2001).

2.3.8.4.6 Wet mount

Wet mount technique is also utilised to identify some parasitic meningeal pathogens such as *N. fowleri, trypanosomes, A. cantonensis*, *D. immitis*. Wet preparations may show motile *N. fowleri* trophozoites as they are in multitude in the CSF specimen (Bursle & Robson, 2016). Demonstration of trypanosomes should be done approximately 15 minutes after CSF collection as the trypanosomes lose their motility and are rapidly lysed beyond this time (Cheesbrough, 2006, p. 122). Careful searching from the CSF preparation is required as the trypanosomes are usually scanty (Cheesbrough, 2006, p. 122).

2.3.8.5 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) in CSF has been used to amplify mirobial DNA in the CSF of patients with meningitis (Tunkel, 2014; Mascitti & Lautenbach, 2009). Primers have been developed that permit detection of common bacterial meningeal pathogens; *N. meningitidis, S. pneumoniae, H. influenzae* (Tunkel, 2014; Mascitti & Lautenbach, 2009*), S. agalactiae* and *L. monocytogenes* (Tunkel, 2014). A bacterial meningeal pathogen that cannot be identified by pathogen specific PCR, then PCR for 16S ribosomal RNA, which is present in almost all bacteria may be used (McGill *et al*., 2016). Multiplex PCR and other platforms that can detect multiple pathogens at the same time are increasingly being trialled and can reduce time and increase sensitivity (McGill *et al*., 2016).

The clinical use of PCR for the diagnosis of bacterial meningitis was examined with utility of a diverse range of primers yielding a sensitivity of 100%, specificity of 98.2%, positive predictive value of 100% (Tunkel, 2014). Hence, broad based PCR may be used as a tool for excluding the diagnosis of bacterial meningitis (Mascitti & Lautenbach, 2009; Tunkel, 2014) with the potential for influencing decisions to initiate or discontinue antimicrobial therapy (Tunkel, 2014).

Polymerase chain reaction has also exhibited optimistic results in the detection of viral meningitis especially enteroviral meningitis (Tunkel, 2014; Mascitti & Lautenbach, 2009). Additionally PCR has also been used to establish the presence of other viral meningeal pathogens such as herpes simplex virus, varicella-zoster and herpes zoster meningitis. Furthermore, HIV RNA has been detected in CSF of patients with meningitis disease (Tunkel, 2014).

The technique of PCR for identifying fragements of mycobacterial DNA in CSF specimens appears to be an equally propitious tool (Tunkel, 2014). Direct smears of CSF are positive for tuberculous meningitis in approximately 25% while PCR is positive in about 80% (Lisak, Truong, Carroll & Bhidayasiri, 2016).

2.3.8.6 Immunodiagnosis

Several immunological tests have been developed for the demonstration of aetiological agents of meningitis (Tunkel, 2014). Different techniques like enzyme linked

immunosorbent assay (ELISA), enzyme immunoelectrotransfer blot (EITB), latex agglutination (LA) assays are currently in use (Patil, Pai & Veerendrakumar, 2014). Immunodiagnostic tests use serum containing bacterial antibodies or commercially available antisera directed against the capsular polysaccharides of meningeal pathogens. Depending on the meningeal pathogen, LA has displayed good sensitivity in identifying the antigens of popular meningeal pathogens; 78-100% for *H. influenzae* type b, 67-100% for *S. pneumoniae*, 69-100% for *S. agalactiae* (Tunkel, 2014).

ELISA and EITB which utilise total antigens or partially purified antigens have been used for the detection of parasitic meningitis (Patil *et al*., 2014). Anticysticercal IgG antibodies have been identified in serum or CSF using ELISA and EITB. ELISA has been used in different studies for immunodiagnosis of neurocysticercosis and results of different sensitivities of 85%, 93%, 41% and 86.7% using serum and 71% and 90% using CSF have been reported (Patil *et al.,* 2014). Nevertheless, the EITB assay requires technical expertise and hence not so convenient for epidermiological purposes (Patil *et al*., 2014).

2.3.8.7 Serodiagnosis

A wide range of serological diagnostic tests are utilised for identification of meningeal pathogens. Currently, Venereal disease research laboratory (VDRL), Rapid plasma reagin (RPR), Cryptococcal antigen test (CrAg) and Western blot are some of the serological tests that are widely used (Baldwin & Whiting, 2016).

2.3.8.7.1 Venereal disease research laboratory (VDRL)/Rapid plasma reagin (RPR)

Since *T. pallidum* cannot be cultured, diagnosis of neurosyphilis depends on the CSF WBC Count, protein concentration or reactivity of the CSF VDRL (Ahsan & Burrascano, 2015). VDRL and RPR are the gold standards for screening syphilis (Baldwin & Whiting, 2016). However, the main restriction of nontreponemal tests are their diminished sensitivity in primary syphilis and late latent syphilis and false results due to cross reactivity (Baldwin & Whiting, 2016). The sensitivtiy of nontreponemal and treponemal tests for syphilis increases with duration of infection and varies from around 75% in the primary stage to virtually 100% in the secondary stage (Baldwin & Whiting, 2016). Whilst most serological tests are reactive in the early latency stage, the reactivity of the non-

treponemal tests declines with the increasing duration of latency. In about 30% of patients with latent syphilis, VDRL and RPR are negative (Baldwin & Whiting, 2016).

2.3.8.7.2 Cryptococcal antigen test

Laboratory diagnosis of Cryptococcal antigen test (CrAg), a constituent of the cryptococcal polysaccharide capsule glucoronoxymannan (GXM) is becoming more and more significant (Prattes, Heldt, Eigl & Hoenigl, 2016). Currently two main methods for identification of *Cryptococcus neoformans* using CrAg exist, latex agglutination (LA) and enzyme immunoassay (EIM). Cryptococcal antigen latex agglutination (CrAgLA) and Cryptococcal antigen enzyme immunoassay (CrAgEIM) are extremely sensitive with rapid results in contrast to culture or microscopy (Prattes *et al.,* 2016). However, CrAgLA as well as CrAg EIM have some pitfalls. Firstly, they need laboratory infrustructure and skilled laboratory technician. Secondly, false negative results and prozone effects have been noted (Prattes *et al.,* 2016).

The inception of Cryptococcal antigen lateral flow assay (CrAgLFA) have more advantages as compaired to CrAgLA and CrAgEIM such as low cost, excellent test performance, easy to use and rapid test results available in 10 minutes. In addition, CrAgLFA is stable at room temperature and cross reactions with other fungi are rare (Prattes *et al*., 2016).

Several studies have been conducted to evaluate the performance of CrAgLFA. In one study, sensitivity of the serum CrAgLFA of 100% and a specificity of 99.8% was reported when utilising serum LA test as gold standard (Prattes *et al.,* 2016). In the same year another study also reported a 100% sensitivity of the serum CrAgLFA which was higher than the 91% found for serum LA (Prattes *et al.,* 2016). Other studies comparing the CrAgLFA to other CrAg based tests in low income countries showed similar results, sensitivities of 95.6-100% and specificities of 96.9-99.5% when using serum specimens and sensitivities of 80-100% and specificities of 73.8-91.5% when using urine samples (also depending on diluent used) (Prattes *et al*., 2016). The reliable performance of the CrAgLFA was confirmed in studies evaluating its performance compared to culture or

composite reference standard sensitivities of 98.3-100% in serum samples, 92-98% in urine specimens and 86.1-100% in CSF samples (Prattes *et al.,* 2016).

2.3.8.7.3 Western blot

Western blot may also be employed in lyme neuroborreliosis as a confirmatory test. Diagnosis of CNS lyme disease is supported with a two tier serological test that include ELISA and Western blot (Brindusa, Rodica, Andrea & Loan, 2016). Specific antibodies (IgM, IgG) in serum and CSF are identified by ELISA test and a positive ELISA test is confirmed by a Western blot (Brindusa *et al*., 2016; Baldwin & Whiting, 2016)

Table 2.3: Summary of laboratory findings in different forms of meningitis (obtained from Cheesbrough, 2006, p. 124)

	Appearance	**Cells (WBCs)**	**Protein**	**Glucose**	**Microscopy and Other Tests**
Normal CSF	Clear and colourless	Below $5x10^6$/L, Lymph	0.15-0.40g/L (15-40mg/dl) *Pandy's:* Negative	2.5-4.0mmol/L (45-72 mg/dl)	_
Pyogenic bacterial meningitis	Purulent or cloudy	Usually many Pus cells	High *Pandy's*: Positive	Very low	Gram stain: Bacteria may be seen resembling: • *N. meningitidis* • *S. pneumoniae* • *H. influenzae* type b (children <5 years old) • *Coliforms* • *S. agalactiae*, *L. monocytogenes* etc
Viral meningitis	Clear or slightly cloudy	Raised lymphs	Normal or increased	Usually Normal	_
Tuberculous meningitis	Clear or slightly turbid	Raised lymphs	High *Pandy's*: Positive	Reduced	Ziehl-Neelsen: AFB difficult to find
Cryptococcal meningitis	Clear or slightly turbid	Raised lymphs	Usually increased *Pandy's*: Positive	Normal or reduced	
Primary amoebic meningoencephalitis	cloudy	Raised pus cells	Increased *Pandy's*: Positive	Reduced	
Trypanosomiasis encephalitis	Clear or slightly turbid	Raised lymphs	High (Igm: very high) *Pandy's:* Positive	Normal or reduced	Indian ink (CSF sediment) Encapsulated yeast
Syphilitic meningitis	Usually clear	Raised lymphs	Normal or increased *Pandy's*: Positive	Normal or increased	Wet preparation: motile amoeba, often with vacuoles

2.3.9 Treatment and prevention

2.3.9.1 Treatment of bacterial meningitis

The choice of antibiotics in patients with bacterial meningitis is a three stage process with initial empirical decisions based on clinical manifestations, modified once CSF gram stain is available and then again if the CSF culture results are positive (McGill *et al.,* 2016).

Antibiotics should be administered imperically to all patients with suspected bacterial meningitis. McGill *et al*. (2016) and Tunkel (2014) insist that third generation cephalosporins have known bactericidal activity for pneumococci, meningococci (Table 2.4) and penetrate inflammed meninges as such they are the empirical antibiotics of choice in most settlings where resistance rates are low (McGill *et al*., 2016).

Table 2.4: Empirical antibiotic treatment for bacterial meningitis (McGill *et al*. 2016)

AGE	PREFERRED CHOICE	ALTERNATIVE CHOICE
Adults < 60 years of age	Cefotaxime OR Ceftriaxone	Chloramphenicol
Adults ≤60 years of age	Cefotaxime OR Ceftriaxone AND Amoxicillin	Chloramphenicol AND Co-trimoxazole of trimethoprim component

Antimicrobial penetration into the CSF is dependent on lipid solubility, molecular size, capillary and choroid plexus efflux pumps, protein binding and the degree of inflammation of the meninges (McGill *et al*., 2016). Although there is little high quality trial evidence to guide the antibiotics used in suspected meningitis and meningococcal sepsis, the choice of empirical antibiotics is based largely on known pharmakokinetics, possible aetiological agent of meningitis and known or suspected antimicrobial resistance pattern (McGill *et al.*, 2016). A definitive antimicrobial treatment should commence depending on the microbiology results (Table 2.5).

Table 2.5: Definitive antibiotic treatment for bacterial meningitis (obtained from McGill *et al*., 2016)

AETIOLOGICAL AGENT	ANTIBIOTIC	ALTERNATIVE ANTIBIOTIC CHOICES
N. meningitidis	Cefotaxime OR Ceftriaxone	Chloramphenicol (if anaphylaxis) OR Benzylpenecillin
S. pneumoniae **(Sensitivities unknown or Penicillin resistant, Cephalosporin sensitive**)	Cefotaxime OR Ceftriaxone	Chloramphenicol
S. pneumoniae **(Penicillin sensitive** **MIC ≤ 0.06 mg/L)**	Benzylpenicillin OR Cefotaxime OR Ceftriaxone	Chloramphenicol
S. pneumoniae **(Penicillin and Cephalosporin non-susceptible,** **Penicillin MIC > 0.06 mg/ml or Cefotaxime / Ceftriaxone** **MIC >0.5 mg/ml**	Cefotaxime OR Ceftriaxone AND Vancomycin OR Rifampicin	Chloramphenicol
L. monocytogenes	Amoxicillin	Co-trimoxazole
H. influenzae	Cefotaxime OR Ceftriaxone	Moxifloxacin
Enterobacteriacea	Ceftriaxone	

2.3.9.2 Treatment of fungal meningitis

Although recent guidelines have made attempts to standardise antifungal susceptibility testing, limitations still exist as a result of the incomplete relationship between in-vitro susceptibility and clinical response to treatment (Kanafani & Perfect, 2008). Amphotericin B is the drug of choice due to its fungicidal activity (Toprak, Demir,Kadayifci, Turel, Soysal & Bakir, 2015) but some resistant strains to this drug have been reported (Kanafani & Perfect, 2008).

Meningitis caused by *C. neoformans* and *C. albicans* should be treated with Amphotericin B and flucytosine in combination followed by fluconazole (Schmiedel & Zimmerli, 2016; Toprak *et al.,* 2015). There are no widely accepted guidelines in relation to the time span of antifungal therapy for CNS fungal infection. Once antifungal treatment has commenced it should proceed until WBC count, glucose and protein levels are back within normal reference ranges, culture is sterile and the patient signs and syptoms cease to exist (Toprak *et al.*, 2015).

2.3.9.3 Treatment of syphylitic meningitis

For treatment of relapsed syphilis (primary, secondary or latent), benzathine penicillin G 2.4 units IM is administered for three weeks till CSF investigations show that neurosyphilis is absent. If CSF investigation show positive result for neurosyphilis, the recommended therapy is acqueous crystalline penicillin G (Centres for Disease Control and Prevention, 2015; Ahsan & Burrascano, 2015) 18-24 million units per day given as 3-4 million units IV every 4 hours or continuous infusion for 10-14 days (Ahsan & Burrascano, 2015).

2.3.9.4 Treatment of parasitic meningitis

The choice of drug for treatment of parasitic meningitis depends on the parasite infecting the CNS. Graeff-Teixera, Da Silva and Yoshimura (2009) highlighted that corticoids and antihelminths are used to treat parasitic meningitis. For instance, albendazole, a benzimidazole derivative, has been utilised in human angiostrongyliasis (Jitpimolmard, Sawanyawisuth, Morakote, Vejjajiva, Puntumetakul, Sanchaisuriya & Tassaneeyakul, 2006). Albendazole eliminates angiostrongyliasis by binding to parasite beta-tubulin, blocking worm polymerilisation and inhibiting glucose uptake (Jitpimolmard *et al*., 2006; Centres for Disease Control & Prevention, 2014). Eflornithine has been used to treat

West African trypanosomiasis. Melarsoprol is a more toxic alternative used to treat meningitis caused by trypanosomes (Eneh, Uwaezuoke, Edelu & Ogbuka, 2016).

2.3.9.5 Prevention of meningitis

There are a number of ways of preventing meningitis. According to the Centres for Disease Control and Prevention (2016), the most effective way to protect against certain types of bacterial meningitis is vaccination. Vaccines are available for three types of bacteria; *N. meningitidis*, *S. pneumoniae* and *H. influenzae*. Maintenance of good health habits such as not smoking and avoiding cigarette smoke, getting plenty of rest and avoiding close contact with people who are sick may also prevent from getting bacterial meningitis (Centres for Disease Control and Prevention, 2016).

Prevention of fungal meningitis involves avoiding endemic areas and maintaining one's immune system healthy (Bauman, 2014, p. 691). Given that fungal infections mostly attack immunosuppressed individuals, the underlying risk factors leading to immunosuppression need to be eradicated to acquire best results from the fungal therapy (Murray, Kobayashi, Pfaller & Rosenthal, 1994, p. 434). People who are immunosuppressed should deter birds droppings, places where birds roost at night (Bauman, 2014, p. 702; Centres for Disease Control and Prevention, 2016), cultivating in fields and other dusty related activities (Centres for Disease Control and Prevention, 2016). Patients who are on catheters must be observed frequently and lines must be changed regularly so that they do not become a source of fungal infection (Murray *et al.,* 1994, p. 434).

Public education on good hygiene practices are essential for preventing parasitic meningitis. Infection with *T. gondii* can be prevented by washing fruits and vegetables before eating, washing utensils, dishes, counters with hot soapy water after contact with raw meat, freezing meat for several days at sub-zero ($0^{o}F$) temperatures before cooking to reduce the chances of infection, wearing gloves when hoeing (Centres for Disease Control and Prevention, 2013). *T. gondii* is found in faeces of infected cat. Cats should be fed with only dried commercial food or well cooked table food. Handling of stray cats should be avoided (Centres for Disease Control and Prevention, 2013). Ingestion of undercooked meat should also be avoided to prevent infection from *A. cantonensis*

(Centres for Disease Control and Prevention, 2010) and *T. gondii* (Centres for Disease Control and Prevention, 2013). Trypanosomes which cause meningitis in man are transmitted by tsetse flies of *Glossina species.* Control measures are targeted at minimising contact with tsetse flies. Some actions that may be taken to prevent bites from tsetse flies are (Centres for Disease Control and Prevention, 2012);

- check vehicles before entering. The flies are attracted to the motion and dust from moving vehicle.
- Wear long sleeved shirts and pants of medium-weight material that mix with the background environment. Tsetse flies like bright or dark colours and they can bite through lightweight clothing.
- Keep away from bushes.

Public education on the impact of syphilis is important in the prevention of neurosyphilis, symptoms for medical attention and behavioural changes such as safer sex practices (Singh & Romanowski, 1999). Screening of syphilis is necessary for several reasons; it reduces transmission of the disease, prevents complications of syphilis and in pregnant mothers it prevents transmission of the disease to the newborn baby (Centres for Disease Control and Prevention, 2016; Singh & Romanowski, 1999).

2.4 Plant secondary metabolites

The biological activities of medicinal plants have been documented in several studies. The antimicrobial properties of medicinal plants have been associated with plant derived compounds known as secondary metabolites (SM). Mathobela (2016) and Anibijuwon and Udeze (2009) claim that there is no plant that does not have biological properties. Man has used plant SM as prophylaxis and therapeutic agents for controlling diseases (Upadhyaya *et al*., 2014). Gutzeit and Muller (2014, p. 2) highlighted that SM are synthesised during plant development as a mechanism for plants to defend themselves against herbivores, bacteria, fungi, viruses. Furthermore, secondary metabolites are also produced by plants to suppress germination and growth of other competing plants in an environment. Some SM play a symbiotic role by attracting pollinating insects and seed

dispersing animals. In addition, SM have been reported as good protectors against UV light (Gutzeit & Muller, 2014, p. 2).

2.5 Biosynthesis of secondary metabolites

According to Wink (2008) more than 100000 SM belonging to different groups have been identified. Secondary metabolites are synthesised from a wide range of precursors of primary metabolism. A precursor is a molecule which is transformed into a product by a biosynthetic enzyme. The product can serve as an intermideate in the biosynthetic pathway and in this respect it is employed as a precursor for the subsequent biosynthetic enzyme (Gutzeit & Muller, 2014, p. 11). The major precursors for the biosynthesis of SM are derived from proteins (amino acids), carbohydrates (sugars) and lipids (fatty acids). Most of the phenolics and polyphenols are synthesised in the biosynthetic pathway for aromatic amino acids (Gutzeit & Muller, 2014, p. 12). Acetyl-CoA formed from sugar metabolism and β-oxidation of fatty acids are utilised in the tricarboxylic acid cycle in the synthesis of organic acids. Furthermore Acetyl-CoA is employed in the biosynthesis of terpenes (Gutzeit & Muller, 2014, p. 12). Finally, enzymatic modification follows after the synthesis of the basic skeleton of the SM. The enzymatic modification includes oxidation, hydroxylation, reduction, acylation, methylations, prenylations and glucosylations (Gutzeit & Muller, 2014, p. 12).

2.6 Major groups of plant secondary metabolites

According to Cowan (1999) and Upadhyay *et al.* (2014) SM are classified into phenolics and polyphenols, alkaloids, terpenes, lectins and polypeptides.

2.6.1 Phenolics and polyphenols

Phenolics and polyphenols consist of a variety of aromatic SM comprising flavonoids, quinones, tannins and coumarins (Cowan, 1999; Upadhyay *et a*l., 2014).

Flavonoids consist of different groups of SM which include flavones, flavonols, anthocyanidins (Upadhyay *et al*., 2014; Croteau *et al*., 2000, p. 1304), chalcones, aurones, isoflavonoids, catechins and anthocyanins (Croteau *et al*., 2000, p. 1304).

Flavonoids promote pollination in plants by acting as chemoattractants for insects, modifying plant physiology by signalling to beneficial microbiota, defending plants against herbivores, bacteria and fungi (Upadhyay *et al*., 2014). Croteau *et al*. (2000, p. 1304) highlighted that the dietary isoflavonoids inhibit incidence of cancer. Isoflavonoids daidzein and genistein in soybeans are believed to suppress the development of breast and prostate cancers in human (Croteau *et al*., 2000, p. 1304).

Quinones are organic compounds comprising of two ketone substitution (Upadhyay *et al*., 2014; Cowan, 1999). Some quinones such as vitamin K, a complex naphthoquinone has been utilised by man as an antihaemorrhage. Hypericin, an anthraquinone has reportedly been used as an antidepressant (Cowan, 1999).

According to Upadhyay *et al*. (2014) tannins are a group of water soluble oligomeric and polymeric polyphenolic compounds with remarkable properties. The molecular weight for tannins range from 500 to 3,000 (Cowan, 1999) and they exist in most of the plant parts including bark, leaves, fruits and roots (Upadhyay *et al*., 2014; Cowan, 1999). Tannins are divided into two groups, non-hydrolysable and hydrolysable tannins (Wink, 2015; Cowan, 1999). Non-hydrolysable tannins may be formed when catechins, a special class of flavonoids, dimerise or polymerise to procyanidins and oligomeric procyanidins. The phenolic hydroxyl groups can react with proteins to yield hydrogen and ionic bonds and probably covalent bonds. These compounds may be refered to as tannins if they contain more than 10 hydroxyl groups (Wink, 2015). Hydrolysable tannins constitutes gallic acid between esters and sugar (Wink, 2015; Cowan, 1999). Tannins are used as antioxidants, anti-inflammatory, antidiarrhoeal, antiparasitic, antibacterial, antifungal and antiviral (Wink, 2015) and also in leather industry (Upadhyay *et al*., 2014; Cowan, 1999) and food industry (Upadhyay *et al*., 2014).

Coumarins are a group of phenolic benzopyrone substances made up of fused benzene and α-pyrone rings (Upadhyay *et al*., 2014; Wink, 2015). Coumarins are often found to a concentration of up to 2% in most genera of *Apiaceae*, *Fabaceae*, *Poaceae*, *Rubiaceae*. They have been used in traditional medicine as anti-inflammatory, anti-edemic and antimicrobial agents. Coumarins have also reportedly been utilised in cosmetics and in beverages (Wink, 2015).

2.6.2 Alkaloids

Alkaloids are SM comprised of one or several nitrogen atoms in a ring structure (true alkaloids). Besides true alkaloids, other alkaloids consist of nitrogen atoms in a side chain (Wink, 2015). Examples of well known alkaloids are morphine, codeine and atropine (Croteau *et al*., 2000, p. 1271). Alkaloids defend plants against herbivores, carnivores due to their bitter taste (Wink, 2015; Croteau *et al*., 2000, p. 1274) and to a lesser degree against bacteria, fungi and viruses (Wink, 2015). Alkaloids are classified into several groups depending on the ring structure.

2.6.2.1 *Amaryllidaceae* alkaloids

Classical alkaloids in this group are ambelline, galanthamine, haemanthamine, lycorine and narciclasine synthesised by several genera of the *Amaryllidaceae*. Galanthamine from *Galanthus woronowi, Leucojum aestivum*, *Narcissus pseudonarcissus* and *N. nivalis* is used as an analgesic and a therapeutic agent for Alzheimer's (Wink, 2015).

2.6.2.2 Pyrrolizidine alkaloids

Pyrrolizidine alkaloids are synthesized from almost all members of the *Boraginaceae*, a number of *Asteraceae* (*Scenecioninae*) and *Fabaceae* (*Crotalarieae*). A number of pyrrolizidine alkaloid containing plants especially *Crotalaria, Heliotropium, Petasites* and *Senecio* are used to arrest bleeding, treat diabetes and consumed as herbal tea (Wink, 2015). Additionally, pyrrolizidine alkaloids from *Symphytum officinale* and some *Boraginaceae* are utilised in wound and fracture therapies.

2.6.2.3 Pyrrolidine alkaloids

A typical example of pyrrolidine alkaloid is nicotine from *Nicotiana tabacum*. Previously nicotine was used as natural insecticide in agriculture before synthetic insecticides. Currently nicotine is utilised in cigarette manufacturing (Wink, 2015).

2.6.2.4 Steroid alkaloids

Steroid alkaloids are mostly made up of a lipophylic steroid moiety and a hydrophilic oligosaccharide chain. Steroid alkaloids are synthesised by four unrelated plant families; *Apocynaceae*, *Buxaceae*, *Liliaceae* and *Solanacea*. They are distributed in the genus *Solanum* that comprises potato and tomato (Wink, 2015). Other dietary plants have reportedly been producing steroid alkaloid of the spirosolane type; with soladulcidine and tomatidine and solanidane type with solanine and chaconine (Wink, 2015). Steroid alkaloids of *Solanum* species including *Solanium dulcamara* are used in phytomedicine as anti-inflammatory drugs. *Solanium* alkaloids have also been employed in agriculture as an insecticide. Furthermore, steroidal alkaloids such as cyclobuxine D and buxamine E from the genus *Buxus* have been utilised as purgative (Wink, 2015).

2.6.3 Terpenes

Terpenes are synthesised from C5-units building block (isoprene units) in a “head to tail” fashion. Terpenes are classified into monoterpenes (C10), sesquiterpenes (C15), diterpenes (20), triterpenes (30), tetraterpenes (C40) and polyterpenes (Croteau *et al*., 2000, p. 1252; Cowan, 1999; Wink, 2015). Steroid terpenes (C27) are synthesised from triterpenes. When additional elements such as oxygen are incorporated in the terpenes, the compound is termed as terpenoid (Cowan, 1999).

Monoterpenes have reportedly been found in *Asteraceae*, *Apiaceae*, *Burseraceae*, *Dipterocarpaceae*, *Lamiaceae*, *Myricaceae*, *Myristicaceae*, *Poaceae*, *Rutaceae*, *Verbenaceae* and resin of conifers (Wink, 2015). Monoterpenes play a significant role in plants by attracting insect pollinating flowers. Monoterpenes have been utilised in aroma therapy and ethnomedicine as a remedy for rheumatism, bacterial and fungal infections, cold, unrest, flatulence, intestinal spasms and as a stimulant for an appetite (Wink, 2015). In addition, monoterpenes possessing phenolic hydroxyl or an aldehyde functional groups are utilised as antiseptics (Wink, 2015).

According to studies conducted by Wink (2015), sesquiterpenes are widely distributed in *Asteraceae* and a few other families such as *Apiaceae*, *Magnoliaceae*, *Menispermaceae*,

Lauraceae. Sesquiterpenes possess various antimicrobial properties such as cytotoxic, antihelminthic, phytotoxic, insecticidal, antibacterial and antifungal properties. Furthermore, sesquiterpenes have also been known to possess expectorant and anti-inflammatory properties (Wink, 2015).

Diterpenes are natural compounds found in *Euphorbiaceae* and *Thymelaeceae* (Wink, 2015; Vasas & Hohmann, 2014). They possess different moities such as jatrophane, ingenane, daphnane, tigliane, lathyrane. The different moities are as a result of different macrocyclic and polycyclic skeletons. Diterpenes are regarded as significant taxonomic markers due to their limited occurrence in *Euphorbiaceae* and *Thymelaeceae* (Vasas & Hohmann, 2014). Diterpenes existing in plants of the genus *Euphorbia* are the main target of natural product drug discovery due to their broad range of biological properties (Vasas & Hohmann, 2014). Diterpenes have been used in phytomedicine due to their biological properties such as antitumour, cytotoxic, antiviral, multidrug-resistance-reversing, different vascular effects, anti-inflammatory (Vasas & Hohmann, 2014).

Triterpenes are one of the subclasses of terpenes occurring in large quantities in plant natural products (Thimmappa, Geisler, Louveau, O'Maille & Osbourne, 2014).Triterpenes are formed by cyclisation of 2,3-oxidosqualene (plants, animals and fungi) by oxidosqualene cyclases giving rise to more than 100 different triterpene scaffolds. The scaffords are further transformed by oxidation, reduction, isomerisation, acylation to impart functional properties of the triterpenoids. Currently, more than 20,000 triterpenes have been identified (Thimmappa *et al*., 2014). Simple triterpenes (sterols) have been reported to perform as signalling molecules while complex glycosylated triterpenes (saponins) shield plants from potential enemies such as bacteria, fungi and predators. Sterols such as *Bryoma* cucurbitacins have been used as a remedy for rheumatism and muscle pain (Wink, 2015). Additionally, glycosylated triterpenes have been employed as anti-inflammatory (glycyrrhizic acid from *Glycyrrhiza glabra*) agents. Furthermore, triterpenes have also been used as detergents for washing clothes (Wink, 2015). Since glycosylated triterpenes are very toxic for fish as they hinder respiration, they are traditionally used in fishing . They have also been utilised in elimination of water snails that tansmit *Schistosoma* that cause schistosomiasis (Wink, 2015).

2.6.4 Lectin and polypeptide

Lectins have been reported in seeds of several plants such as abrin in *Abrus precatorius,* phasin in *Phaseolus vulgaris*, ricin in *R. communis*. Like majority of proteins, the information for lectin biosynthesis is encoded in the genes. The mRNA transmits the information to ribosomes to synthesise polypeptides in the endoplasmic reticulum (Sharon & Lis, 2012, p. 65). The polypeptides serve as precursors for lectin biosynthesis. The polypeptides go through co- and post-translational modification to yield mature lectins. A co-translational modification involves proteolytic removal of a 20-30 amino acid signal sequence from the amino terminal end (Sharon & Lis, 2012, p. 65). Post-translational modification involves; N-glycosylation and additional modification of the carbohydrate units such as O-glycosylation of hydroxyproline (Sharon & Lis, 2012, p. 65). According to Wink (2015) lectins defend plants against herbivores. Lectins have been reported to possess a wide range of activities such as antitumor, antifungal, immunomodulatory activities, anti-insecticide activities (Lam & Ng, 2011), HIV-1 reverse transcriptase inhibitor (Lam & Ng, 2011; Cowan, 1999). A minority of lectins have shown antibacterial and antinematode activities. Lectins from seeds of *Vicia cracca* have been utilised in human ABO erythrocyte grouping (Lam & Ng, 2011).

2.7 Modes of action of plant secondary metabolites

Plants synthesise diverse compounds of small molecular weight. The majority of these molecules possess weak antimicrobial properties compared to synthetic antibiotics. However, plants are able to defend themselves against pathogens due to synergistic interactions of these low molecular weight compounds (Hemaiswarya *et al*., 2008). Wagner and Ulrich-Merzenich (2009) reported that a single component of a monoextract or multi-extract combination exerts effects on various molecular targets in a cell. The multi-target effects of SM has demonstrated the need for application of SM in treatment of various diseases and health disorders. Wink (2008) suggests that SM has three main molecular targets in prokaryotic and eukaryotic cells:

- Proteins

- DNA, RNA including related enzymes and regulatory proteins (transcription factors)
- Biomembranes

2.7.1 Proteins

2.7.1.1 Covalent modification of proteins and DNA base

Proteins play a major role in a cell which is actively involved in performing numerous functions. Some SM possess reactive functional groups such as aldehydes and sulfhydryl (SH) groups, epoxides, double bonds, triple bonds which can form convalent bonds with proteins, peptides and DNA (Wink, 2015). Under normal conditions SM with an aldehyde group can form a Schiff's base with amino and imino group of proteins, amino acid residues. Proteins may function as enzymes, substrates, receptors, ion channels, transporters (Wink, 2015; Wagner & Ulrich-Merzenich, 2009), transcription factors and cytoskeletal proteins (Wink, 2015). However, the modification of receptors and enzymes at their catalytic sites may lead to loss of binding activity in ligands and substrates. Wink (2015) indicates that the maintenance of 3 D-structure in proteins is required for identification of substrates or interaction with other proteins or Deoxyribonucleic acid (DNA). Alkylation of proteins at other sites may induce change in the 3D-structure of proteins which is vital for protein-protein recognition, binding or catalytic activity and the protein becomes non-functional (Wink, 2015). Consenquently, this may lead to cell death. Consequently, alkylation of DNA bases by reactive functional groups of SM such as aldehydes or epoxides may lead to mutation or cancer (Wink, 2015).

2.7.1.2 Non-covalent modification of proteins

A wide range of SM such as flavonoids, stilbenes, chalcones, anthocyanins and catechins may react with proteins and display non-covalent bonding (Wink, 2015). A typical feature of SM that form non-covalent bonds with proteins is that they possess hydroxyl groups which can dissociate into negatively phenolate ions under physiological conditions (Wink, 2015). The phenolate ions react with positively charged amino group of proteins (such as lysine, arginine, histidine) or with the binding or catalytic site of the protein.The non-covalent or ionic bond formed is relatively stable and fixed (Wink, 2015). Phenolics can

also modify proteins that regulate differential gene expression. This may result into loss of a structural flexibility of a protein, hence the protein becomes non-functional (Wink, 2015). Consequently, this may lead to cell death.

2.7.2 Interaction of secondary metabolites with Deoxyribonucleic acid, Ribonucleic acid and related enzymes

Nucleic acids are macromolecules composed of nucleotide chains that are important constituents for all living cells. Voet and Voet (2004, p. 1107) highlight two main classes of nucleic acids, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). Deoxyribonucleic acid is the hereditary molecule in all living cells and performs two functions (Voet & Voet, 2004, p. 1107);

- To direct its own replication during cell division
- To direct the transcription of complementary RNA molecules

Ribonucleic acid has several functions compared to DNA. The transfer of genetic information from DNA to ribosomes occur through the RNA transcripts of DNA sequences, the messenger RNA (mRNA) for synthesis of polypeptides. The biochemical process is catalysed by RNA polymerases (Voet & Voet, 2004, p. 92-99). This process is called translation. The RNA of the ribosomes comprise approximately two thirds and have both functional and structural roles. Unlike DNA, RNA serves as a hereditary molecule in most of the viruses (Voet & Voet, 2004, p. 1107). Repair enzymes ensures that the DNA integrity is maintaned by fixing translation errors or mutations attributed by spontaneous disamination, depurination, irradiation, alkylating and intercalating agents (Wink, 2008).

Numerous SM contain reactive functional groups which can intercalates, alkylates, methylate or oxidise DNA. Lipophilic and planar SM intercalates DNA bases leading to stabilisation of DNA double helix (Wink, 2008). The stabilisation of DNA double helix interferes with DNA polymerase activity. This can progress into frame shift mutations. Consenquently, frame shift mutation can lead to loss of function in the corresponding protein (Wink, 2008). Epoxides and aldehydes have been reported to alkylate substances and cause mutations, malformations and cancer. Cycasin, a glycoside isolated from the seeds of *Cycas circinalis* methylate DNA bases leading to cancer (Wink, 2009;

Weisburger, 1966). Drugs with intercalating SM have been reported to possess cytotoxic, antibacterial, antifungal and antiviral properties (Wink, 2008).

2.7.3 Interaction of SM with biomembranes

All cells are surrounded by a semipermeable biomembrane which serves as a permeation barrier preventing entry of polar and charged molecules but also leakage of cellular metabolites into the external environment (Wink, 2008). Lipophilic SM such as monoterpenoids, sesquiterpenoids and uncharged particles have an affinity for biomembranes, hence, they can cross the cellular biomembrane freely by diffusion. Biomembranes holds various proteins such as ion channels, receptors, transporters which mediate the exchange of substances with other cells or tissues (Wink, 2015; Wink, 2008).

When the lipophilic SM enter the biomembrane, they bind to the inner core of the membrane. Higher concentration of the lipohilic SM will induce membrane fluidity and increased membrane permeability (Wink, 2015; Wink, 2008). Higher concentration of lipophilic SM may also interfere with membrane protein and membrane lipid interactions which are essential for correct 3 D-structure conformation (Wink, 2008).

Saponins, glycosides of triterpenes or steroids complex with cholesterol in cellular biomembranes of animal and ergosterol in fungal membranes (Wink, 2015; Wink, 2008).The hydrophilic side chain of the saponin stays at the membrane surface and react with other sugars of glycolipids or glycoproteins. This reaction may interfere with the permeability and cause polar compounds to leak or enter the cell willfully and this can interrupt cellular metabolism and consequently cell death (Wink, 2008).

2.8 Mechanisms of resistance to antimicrobial agents

Antibiotics for today might not be effective tomorrow as microbes are tirelessly developing mechanisms of resisting antibiotics (Cashel & Raxlen, 2015; White, Duncan & Baumle, 2012, p. 75). The evolution of antimicrobial resistance in microganisms can be intrisic or acquired. Natural resistance is attained by gene mutation (Hemaiswarya *et al*., 2008) while the acquired resistance occurs through acquistion of a new DNA fragement such

as transposons, plasmids, integrons from another microorganism (Van Hoek, Mevius, Guerra, Mullany, Roberts & Aarts, 2011) The DNA fragements can be transferred within the same or different species of microorganism.

Microorganisms become resistant to antimicrobial agents through several mechanisms such as receptor or active site modification, enzymatic degradation and modification of the drug, decreased outermembrane permeability, active efflux (Van Hoek *et al*., 2011; Hemaiswarya *et al*., 2008) acquisition of alternative metabolic pathways to those inhibited by the drug, increased production of the target enzyme (Van Hoek *et al*., 2011).

2.8.1 Receptor or active site modification

For an antimicrobial agent to be effective, it should be made in a such way that it can interfere with its critical targets in the microbe. Introduction of mutation at the molecular target lessens the activity of the drug, rendering the drug ineffective (Hemaiswarya *et al*., 2009). Hemaiswarya *et al.* (2008) believe that mutations in RNA polymerase and DNA gyrase have contributed to unfavourable therapeutic results with rifamycin and quinolones. Another worrisome problem is the structural modulation of the penicillin-binding proteins (PBPs) by the penicillin resistant microbes (Hemaiswarya *et al*., 2008). Nevertheless, studies have shown that some SM such as Corilagin when combined with oxacillin and cefmetazole inhibits PBP2a production in methicillin resistant *S. aureus* (MRSA) (Hemaiswarya *et al.,* 2008).

2.8.2 Enzymatic degradation and modification of the drug

Microbes continue to pose threat by synthesising enzymes that modulate the antimicrobial agent. The modulation involves hydrolysis, redox reaction and group transfer of the active group (Hemaiswarya *et al.*, 2008).

Chemical bonds of the antimicrobial agent such as ester bonds or amide bonds are cleaved by the enzymes synthesised by the resistant microbe leading to formation of an inactive compound. Redox reaction involves oxidation and reduction of the antimicrobial agent rendering the drug impotent. Modulation of the active group include acylation,

phosphorylation, glycosylation, nucleotidylation, rebosylation by the microbe rendering the drug ineffective (Hemaiswarya *et al*., 2008).

However numerous studies have indicated SM as resistance modulating compounds (Upadhyay *et al*., 2014; Wagner & Ulrich-Merzenich, 2009). For example, a natural product Epigallocatechin gallate (EGCg) when combined with penicillin inhibits penicillinase in *S. aureus* rendering penicillin effective (Hemaiswarya *et al*., 2008).

2.8.3 Decreased outer membrane permeability

All bacteria possess cell wall except mycoplasma. The cell wall is made up of peptidoglycan which provide rigidity to the bacteria. All Gram-negative bacteria have an additional layer consisting of lipopolysaccharide (LPS) molecules which act as a permeability barrier for many hydrophobic substances such as detergents, hydrophobic dyes and antimicrobial agents (Hemaiswarya *et al*., 2008). In addition, the LPS prevent the entry of toxic host defence factors such as lysozyme, β-lysin and various leukocyte proteins. Certain Gram-negative bacteria have been reported to possess glycosphingolipids instead of LPS (Hemaiswarya *et al*., 2008).

Hemaiswarya *et al*. (2008) and Helander, Alakomi, Latva-kala, Mattila-Sandholm, Pol, Smid, Gorris and Von Wright (1998) highlight that plant SM such as thymol and carvacrol act as membrane permeabilisers enhancing the uptake of drugs and other hydrophobic antimicrobial agents.

2.8.4 Efflux of the antimicrobial agent from the cell wall

Microbes have developed multidrug resistance pumping systems (MDRPs) that inhibit antimicrobial agents from permeating the bacteria through the cell membrane or extruding the antimicrobial agents that have already penetrated into the microbe. The efflux pumping systems utilise ATP hydrolysis or ion gradient to extrude the antimicrobial agents out of the microbial cell (Hemaiswarya *et al*., 2008).

Hemaiswarya *et al*., (2008) highlights five major classes of MDRPs namely, the adenosine triphosphate (ATP)-binding cassette (ABC) superfamily, the major facilitator superfamily (MFS), the small multidrug resistance family (SMR), the resistance-

nodualtion-cell division (RND) superfamily and the multidrug and toxic compound extrusion (MATE). Typical classes of MDRPs in prokaryotic cells are RND, SMR and MATE (Hemaiswarya *et al.*, 2008).

Plant SM have been reported to block the MDRPs and potentiate the activity of certain antibiotics. For instance, two diterpenes from *Lycopus europaeus* have been reported to potentiate the activities of tetracycline and erythromycin against two strains of *S. aureus* by blocking MDRPs Tek (K) and Msr (A) (Hemaiswarya *et al*., 2008).

2.8.5 Use of alternative pathway to bypass the sequence inhibited by the agent

Resistant microbes may either utilise an alternative metabolic pathway to bypass the sequence inhibited by the agent or increase the production of the target metabolite (Akpan, Odeomena, Nwachukwu & Danladi, 2012). For example, in fungi ergosterol is the main sterol of fungi and it maintains the integrity and fluidity of cell membranes. Azole compounds inhibit cytochrome P450 enzyme which is involved in 14α-demethylation of lanosterol in *Saccharomyces cerevisiae* and 24-methylenedihydrolanosterol in majority of the fungus and thus blocking ergosterol biosynthesis (Kelly, Lamb, Kelly, Manning, Loeffler, Hebart, Schumacher & Einsele, 1997).

Some fungal strains have been reported to resist azole compounds. Inhibition of ergosterol biosynthesis leads to depletion of ergosterol and increased levels of substrates and synthesis of abnormal sterols without removal of 14α-methyl group such as 14α-methyl-3,6-diol (Kelly *et al.,* 1997). In a normal fungal cell 14α-methyl-3,6-diol is toxic and its accummulation can lead to growth arrest of a fungal cell (Kanafani & Perfect, 2008; Kelly *et a*l., 1997). The compound, 14α-methyl-3,6-diol is synthesised from14α-methyl-fecosterol in a chemical reaction catalysed by the enzyme sterol $\Delta^{5,6}$ desaturase (Kelly *et al.*, 1997). Mutation of *ERG3* gene negates the function of sterol $\Delta^{5,6}$ desaturase leading to accumulation of 14α-methyl-fecosterol. In ergosterol deficiency, 14α-methyl-fecosterol maintains the fungal membrane functional (Kanafani & Perfect, 2008; Kelly *et al.,*1997). Given that other major antifungals such as amphotericin B interrupt cell membrane through binding to ergosterol, cross resistance may occur rendering amphotericin B ineffective (Kelly *et al*., 1997).

Unlike azole compounds which has ergosterol as a molecular target (Kanafani & Perfect, 2008; Kelly *et al.,* 1997), plant SM possess multitarget activities which can be used in fungal therapy (Dhamgaye, Devaux, Vandeputte, Khandelwal, Sanglard, Mukhapadhyay & Prasad, 2014).

2.9 Background of the selected medicinal plants

2.9.1 *Adansonia digitata*

Common names: Baobab (English), Shimuwu (Tsonga), Kremetart (Afrikaans)

2.9.1.1 Botanical description

Van Wyk *et al*. (2009, p. 30) describes *A. digitata* as a relatively short tree (up to about 15 metres in height), but develops a huge, irregular folded trunk of more than 20 metres in circumference (Fig 2.6A). The bark is smooth, grey or yellowish-grey. The large leaves are split into about five to seven leaflets, all born on a single long, thin stock (Van Wyk *et al*., 2009, p. 30). Each leaflet narrows to a sharp point and is up to 150 mm long. Large, pendulous white flowers are produced in early summer (October to December). The fruits are very large, egg shaped of up to 150 mm long (Van Wyk *et al*., 2009, p. 30) (Fig 2.6B) made up of an outer shell (epicarp) (45%), fruit pulp (15%) and seeds (40%) (De Caluwe, Halamova & Van Damme, 2010). The woody pericarp or pod holds the internal fruit pulp (endorcap) which is divided into small floury, dehydrated and powdery slides that enclose multiple seeds and filamments, the red fibres that subdivide the pulp into sections (De Caluwe *et al*., 2010).

A B

Figure 2.6: *A. digitata* tree (A) and *A. digitata* fruit (B) (taken from Van Wyk *et al*., 2009, p. 31)

2.9.1.2 Plant parts used

Fruit pulp, leaves, seeds, bark (Van Wyk *et al*., 2009, p. 30; Decaluwe *et al*., 2010) stem, root barks, flowers, roots (Decaluwe *et al*., 2010) are used for medicinal purposes.

2.9.1.3 Medicinal uses

A refreshing drink made from the yellow or whitish fruit pulp which has been referred to as “cream of tarta” has been used to treat fevers, diarrhoea (Decaluwe *et al*., 2010; Van Wyk *et al.,* 2009, p. 30), dysentery liver disorders, smallpox, measles, bronchial asthma, inflammations, body pains (De Caluwe *et al*., 2010). The bark has been used as an antipyretic, prophylaxis, mouthwash for toothache and for cleansing soul (De Caluwe *et al.*, 2010). The leaves have also been reported to treat fevers, diarrhoea, inflammation, reduce perspiration (Van Wyk *et al.,* 2009, p. 30; De Caluwe *et al*., 2010), asthma, kidney and bladder diseases, respiratory disorders, insects bites, guinea worm, general fatigue (De Caluwe *et al*., 2010). *A. digitata* leaves are also used as an astringent. Young leaves are crushed into poultice for painful swelling (De Caluwe *et al*., 2010). The seeds have been used to treat diarrhoea, inflammed gums, skin complaints, hiccoughs and easing of sore teeth. The flowers have been used for treatment of respiratory disorders (De Caluwe *et al*., 2010).

2.9.1.4 Phytochemistry

A wide range of chemicals have been identified from *A. digitata*. Different phytochemical constituents belonging to the classes of terpenoids, flavonoids, steroids, vitamins, amino acids, carbohydrates and lipids have been isolated (De Caluwe *et al*., 2010). The plant contains flavonoids such as quercetin-7-O-β-D-xylopyranoside and 7-baueren-3-acetate, a triterpenoid (Van Wyk *et al*., 2009, p. 30). The fruit pulp contains citric acid, tartaric acid (Van Wyk *et al*., 2009, p. 30; De Caluwe *et al.*, 2010) malic acid, succinic acid and ascorbic acid (De Caluwe *et al*., 2010). The fruit pulp has also a very high Vitamin C content (De Caluwe *et al*., 2010). The seeds contain lysine, thiamine, calcium and iron. High proportions of linoleic and oleic acid, palmitic acid and α-linolenic acid have also been reported in *A. digitata* seeds (De Caluwe *et al*., 2010).

2.9.1.5 Geographical distribution

A. digitata is found in most of Sub-Saharan Africa's semi arid and sub-humid regions (De Caluwe *et al*., 2010) as well as Madagascar (De Caluwe *et al*., 2010; Van Wyk *et al*., 2009, p. 30). *A. digitata* has also been introduced to areas outside Africa and grown successfully (De Caluwe *et al*., 2010).

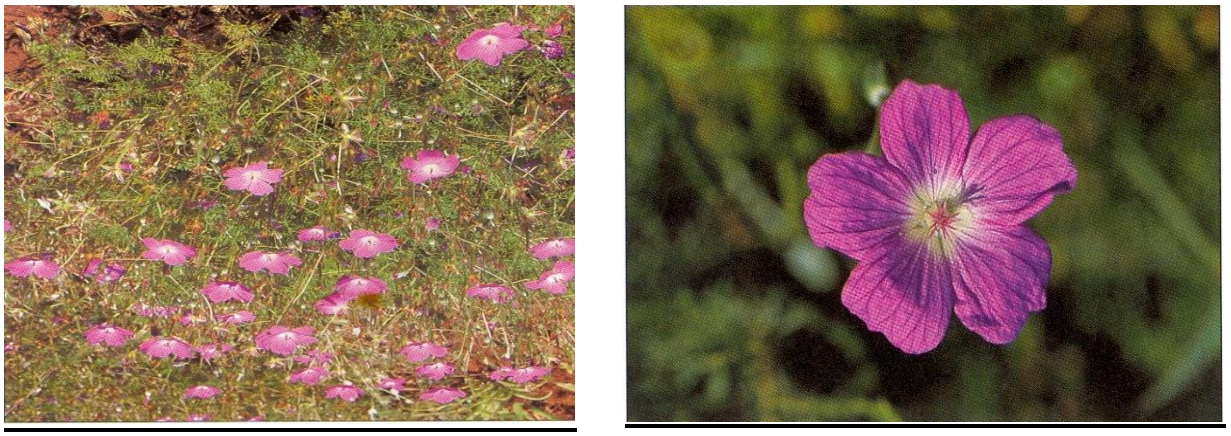
2.9.2 *Geranium incanum*

Common names: Carpet Geranium (English), Vrouebossie, Bergtee, Amarabossie (Afrikaans), Ngope-sethsoha, Tlako (Sotho)

2.9.2.1 Botanical description

Van Wyk *et al*. (2009, p. 134) describes *G. incanum* as an attractive, sprawling perennial shrublet with finely divided silvery leaves (Fig 2.7A). The white, pale pink, violet or magenta flowers (Fig 2.7B) are born on long, slim stalks , followed by a characteristically elongated fruit with an appearance like a stork's bill (Van Wyk *et al*., 2009, p. 134). The hairs on the exterior of the flowers and flowerstalks lie flat and are not spreading as in related species (Van Wyk *et al*., 2009, p. 134). Species of *Geranium* are all very similar

and have been much confused in the past. *G. incanum* has been divided into two varieties; *var. incanum* with small, white or pale pink flowers and *var. multifidum* with larger light violet to magenta pink flowers (Van Wyk *et al*., 2009, p. 134).



A B

Figure 2.7: *G. incanum plant* (A) and *G. incanum* flower (B)

(taken from Van Wyk *et al*., 2009, p. 135)

2.9.2.2 Plant parts used

The leaves and flowers are used (Roberts, 2007, p. 17). Rarely the roots or fruits are used (Van Wyk *et al*., 2009, p. 134).

2.9.2.3 Medicinal uses

Traditionally, a tea made from leaves and flowers ease diarrhoea, excessive and irregular menstruation, colic, bladder infections (Van Wyk *et al*., 2009, p. 134; Roberts, 2007, p. 17), fever, venereal diseases (Van Wyk *et al*., 2009, p. 134), flatulence (Roberts, 2007, p. 17). Tea prepared from *G. incanum* is reported to be the best tea for expelling the afterbirth, starting milk flow for the new born in women (Roberts, 2007, p. 17). *G. incanum* is also used as a lotion for washing itchy, dry skin, rinse for hair that gets oily very quickly (Roberts, 2007, p. 17). When mixed with oats it makes an excellent scalp treatment for dandruff, flaky scalp, psoriases of the scalp, soothing and softening the irritated area (Robert, 2007, p. 17).

2.9.2.4 Phytochemistry

Amabeoku (2009) contends that leaves of *G. incanum* contain tannins, saponins especially steroidal saponin and flavonoid.

2.9.2.5 Geographical distribution

Van Wyk *et al*. (2009, p. 216) and Roberts (2007, p. 17) claim that *G. incanum* ocurrs along the southern coastal areas of the Western and Eastern Cape Provinces of South Africa. It is also grown in Tanzania, Malawi, Mozambique and Zimbabwe (Bauman, 2005, pp. 62-63).

2.9.3 *Ricinus communis*

Common names: Kasterolieboom (Afrikaans), Castor oil (English), Mokhura (Sotho), Umhlakuva (Xhosa, Zulu), Mtsatsi (Chewa, Nyanja)

2.9.3.1 Botanical description

Van Wyk *et al*. (2009, p. 216) states that *R. communis* is a large shrub or small tree of up to 4 metres in height with very large, handshaped leaves on long, stout leafstalks. The flower clusters appear near the tips of the branches. Female flowers grow above the male flowers (Van Wyk *et al*., 2009, p. 216). The fruits are three lobbed capsules with spinelike projections on their surfaces. Each capsule has three seeds which are about 10 mm long, conspicuously shiny, unevenly mottled with silver, brown and black. At the tip of the seed is a hard, white, fleshy aril (Van Wyk *et al*., 2009, p. 216).

Figure 2.8: *R. communis* plant (taken from Van Wyk *et al*., 2009, p. 217)

2.9.3.2 Plant parts used

Leaves, barks, seeds, roots and oil of the plant are mostly used (Nemudzivhadi & Masoko, 2014).

2.9.3.3 Medicinal uses

Leaf infusions of *R. communis* are administered orally or as enema for stomachache. Root and leaf poultices are commonly applied to wounds, sores and boils (Van Wyk *et al*., 2009, p. 216). The plant also possesses beneficial effects such as antioxidant, antimicrobial, hepatoprotective, antifertility, anti-inflammatory, antidiabetic (Jena & Gupta, 2012; Nemudzivhadi & Masoko, 2014) antihistamic, antinociceptive, antiasthmatic, immunomodulatory, CNS stimulant, insecticidal and larvicidal (Jena & Gupta, 2012), hypoglycemic, laxative (Nemudzivhadi & Masoko, 2014). *R. communis* is also a remedy for colds, tumours (Nemudzivhadi & Masoko, 2014; Zarai, Chobba, Mansour, Bekir, Gharsallah & Kadri, 2012) and watts (Zarai *et al*., 2012).

2.9.3.4 Phytochemistry

R. communis contains steroids, saponins, alkaloids, flavonoids and glycosides (Jena & Gupta, 2012). Leaves contain two alkaloids, ricine (0.55%) and N-demethylricine (0.016%) and the following six flavones glycosides;

- Kaempferol-3-O-β-D-xylopyranoside
- Kaempferol-3-O-β-D-glucopyranoside
- Quercetin-3-O-β-D-xylopyranoside
- Quercetin-3-O-β-D-glucopyranoside
- Kaempferol-3-O-β-rutinoside
- Quercetin-3-O-β-rutinoside

(Jena & Gupta, 2012)

The leaves of *R. communis* also contain the following phenolic compounds;

- Monoterpenoids (1,8-Cineole, Camphor, α-pinene)
- Sesquiterpenoids (β-Caryophyllene)
- Gallic acid
- Gentistic acid
- Rutin
- Epicatechin
- Ellagic acid

(Jena & Gupta, 2012)

The seeds contain 45% of fixed oil which consists of ricinoleic, isoricinoleic, stearic and dihydroxystearic alkaloid, ricine. Ingredients in the seeds are ergost-5-en-3-ol, stigmasterol, Y-sitosterol, fucosterol, probucol. The stem contains ricine (Jena & Gupta, 2012).

2.9.3.5 Geographical distribution

R. communis is found in tropical and temperate regions of the world (Malook, Jamil, Naz, Shinwari, Jan, Tayyab, Idrees, Malook & Rha, 2013)

2.9.4 *Carica papaya*

Common names: Papaw (English), Papaya (Chewa, Nyanja)

2.9.4.1 Botanical description

Ross (1999, p. 88) describes papaya tree as a perennial herbaceous plant of the *Caricaceae* family with a lot of milky latex reaching to as high as 10 metres (Fig 2.9A). The stem is approximately 25 cm thick, simple or branched above the middle and roughened with leaf scars. Leaves are clustered around the apex of the stem and branches (Fig 2.9B) with nearly rounded stalks, 25 to 100 cm long (Ross, 1999 p. 88). The leaf blade has 7 to 11 main lobes with prominent veins. Leaf surface is yellow-green to dark-green above and paler beneath (Ross, 1999, p. 88). Generally male and female flowers are borne on separate plants usually with hermaphrodite flowers and a male plant may transform to a female after being beheaded (Ross, 1999, p. 88). Flowers appear singly or in clusters from the main stem among the leaves, the female short-stalked, the male with drooping peduncles 25-100 cm long (Ross, 1999, p. 88). Corolla is 1.25 cm to 2.5 cm long with five rectangular curved white petals. The fruit varies in form and size; it may be nearly round, pear-shaped, rectangular shaped or oval (Ross, 1999, p. 88). The fruit of wild papaya tree may be as small as an egg whereas in cultivation, the fruit varies from 10 cm to 60 cm in length and up to 20 cm thick (Ross, 1999, p. 88). The skin of the papaya fruit is smooth, relatively thin and deep-yellow to orange when it is ripe. The flesh of the papaya fruit is juicy, yellow to orange or salmon-red, sweet and more or less musky (Ross, 1999, p. 89).

A B

Figure 2.9: *C. papaya* tree (A) and leaves of *C. papaya* clustering around the apex of branch (B) (Photograph taken from Nguludi Village, Chiradzulu District, Malawi)

2.9.4.2 Plant parts used

Leaves, Bark, Fruits, Roots, Seeds, Milky latex are commonly used (Ross, 1999, pp. 89-92).

2.9.4.3 Medicinal uses

Papaya plant is traditionally used for treatment of numerous diseases like warts, corns, sinuses, eczema, cutaneous tubercles, glandular tumours, blood pressure, dyspepsia, constipation, amenorrhoea, general debility, worm expulsion, relief of inflammatory conditions (Aravind *et al*., 2013). The leaf of *C. papaya* is traditionally used to treat skin diseases, beriberi, kidney and bladder troubles, fever. The root is a remedy for yaws, piles, syphilis (Watt & Brandwijk, 1962, pp. 167-168) and yellow fever (Ross, 1999 p. 90). The bark is the remedy for venereal diseases (Watt & Brandwijk, 1962, p. 168). The milky latex is used for treatment of ringworm, skin rashes, ulcers, constipation and as a digestive (Ross, 1999, pp. 90-91). Hot water extracts of the dried fruit is taken orally for gallbadder and liver conditions and for disorders of fat digestion and dyspepsia. Dried seeds of a ripe fruit are taken for cough and stomachache (Ross, 1999, p. 91).

2.9.4.4 Phytochemistry

C. papaya plant contains the following phytoconstituents;

- Enzymes (Papain and Chymopapain)
- Carotenoids (B carotein and Crytoxanthin)
- Carposide
- Glucosinolates (Benzyl isothiocynate and Papaya oil)
- Minerals (Ca, K, Mg, Zn, Mn, Fe)
- Monoterpenoids (Linalool, 4-terpinol)
- Flavonoids (Myricetin, Kaemferol)
- Alkaloids (Carpinine, Carpaine, Vitamin C and E)

(Aravind *et al*., 2013)

2.9.4.5 Geographical distribution

C. papaya is grown in all tropical countries and many subtropical countries (Ross, 1999, p. 89)

2.9.5 *Bidens pilosa*

Common names: Blackjack (English), Chisoso (Chewa, Nyanja)

2.9.5.1 Botanical description

B. pilosa grows to about 1 meter high. The leaves are compound with three to five leaflets (Fig 2.10). They are petiolate, opposite, slightly hairy and sharply serrated (Roodt, 1998, p. 39). The flowerheads are about 1.5 cm in diameter and consist of an outer row of female ray flowers which are normally white or light yellow in colour and the inner disc flowers which are lemon-yellow in colour, bisexual and tubular (Roodt, 1998, p. 39). The fruits are born in a star-shaped disc and the seeds are black, narrowly ribbed with barbs on the ends (Roodt, 1998, p. 39). A single plant can produce 3000-6000 seeds (Bartolome, Villasenor & Yang, 2013).

Figure 2.10: *B. pilosa* plant (taken from www.zimbabwe(http://eflora.co.zw/speciesdata/species.php?species_id=160650)

2.9.5.2 Plant parts used

Leaves, roots, flowers, seeds, stems and roots are comonly used (Bartolome *et al*., 2013).

2.9.5.3 Medicinal uses

B. pilosa is utilised in various folk medicines as anti-inflammatory, antiseptic, liver protective, blood pressure lowering and hypoglycemic effects (Deba, Xuan & Yasuda, 2008). An infusion of the leaf and root is a common colic remedy. Infusions of the leaf are administered orally or as an enema for constipation. The powdered leaf is dissolved in water to cure diarrhoea (Roodt, 1998, p. 39). *B. pilosa* has been reportedly to be efficacious against dysentery and other gastric ailments. The plant has been taken as a major ingredient of herbal tea which is believed to prevent cancer (Deba *et al*, 2008) and has also been a remedy for laryngitis, headache and malaria (Abajo, Boffill, del Campo, Mendez, Gonzalez, Mitjans & Vinardell, 2004). Eardrops are prepared for treating earaches by warming the fresh plant sap and dropping directly into the ear (Roodt, 1998, p. 40; Watt & Brandwijk, 1962, p. 205). The warmed juice of the fresh plant is used as a styptic (to contract blood vessels on wounds) (Roodt, 1998, p. 40). An eyewash to treat eye problems is prepared by warming the juice of the fresh plant to treat conjunctivitis (Roodt, 1998, p. 40; Watt & Brandwijk, 1962, p. 205; Dimo, Azay, Tan, Pellecuer, Cros, Bopelet & Serrano, 2001). The leaves are remedy for jaundice, threatened abortion,

toothache, cough, intestinal helminthiasis and fever (Dimo *et al*., 2001). The combination of leaves and flowers is used to treat flank pains while the whole plant is used to treat fractures and febrile convulsions (Dimo *et al*., 2001).

2.9.5.4 Phytochemistry

According to Bairwa, Kumar, Sharma and Roy (2010) *B. pilosa* has a diverse classes of chemical constituents such as polyacetylenes, polyacetylenic glycosides, aurons, auron glycosides, p-coumeric acid derivatives, caffeoylquinic acid derivatives, flavonoids and flavonoid glycosides, sesquiterpenes, acetylacetone phenylheptadiynol, phenylpropanoid glucosides, pheophytins, diterpenes.

2.9.5.5 Geographical distribution

B. pilosa is a cosmopolitan weed and grows in all parts of Southern Africa (Van Wyk & Gericke, 2000, p. 68).

2.9.6 *Mangifera indica*

Common names: Mango (Chewa, Nyanja, English)

2.9.6.1 Botanical description

Mango trees are of the *Anacardiaceae* family. According to Ross (1999, p. 198) mango trees vary in sizes depending on variety and can be from 3 to 30 metres tall, typically heavy branched from a stout trank (Fig 2.11). Leaves are spirally arranged on the branches, lanceolate-elliptical, pointed at both ends, the blades mostly up to about 25 cm long and 8 cm wide, sometimes much larger, reddish and thinly flaccid when first formed (new flash) (Ross, 1999, p. 198). Inflorescences are large terminal pannicles of small polygamous, fragrant, yellow to pinkish flowers. Fruit is a drupe, variously shaped, according to the variety, from ellipsoid to obliquely reniform, 5 to 15 cm long (Ross, 1999, p. 198).

Figure 2. 11: *M. indica* tree (taken from mangoworldmagazine.blogspot.co.za/2013/06/the-power-of-singletree-meet-me.html)

2.9.6.2 Plant parts used

Leaves, bark, fruit, roots are commonly used (Ross, 1999, pp. 198-199).

2.9.6.3 Medicinal uses

Fresh leaf juice is a remedy for inflammation of the eye. Infusion of dried leaves is used for treating fever, chills, dizziness and lower abdominal pain presumed to result from insufficient rest during puerperum (Ross, 1999, p.199). Fruit is used as a laxative, diuretic, diaphoretic, astringent and refrigerant. Unripe fresh fruit pulp mixed with curd is used for indigestion and stomachache (Ross, 1999, p. 198). Decoction of the root is taken orally for malaria (Ross, 1999, p. 199). Various parts of the plant are used as remedy for diarrhoea, dysentery, anaemia, bronchitis, cough, hypertension, rheumatism, toothache, haemorrhage, leucorrhea and piles (De & Pal, 2014). All parts of the plant are used to treat abscesses, rabid dog or jackal bite, inflammation of the inner ear, blisters, wounds, liver disorders, excessive urination, tetanus and asthma (Sha, Patel, Patel & Parma, 2010). According to De and Pal (2014) *M. indica* has also been utilised as an antiseptic, vermifuge and tonic.

2.9.6.4 Phytochemistry

Sha *et al*. (2010) claim that the leaves and flowers of *M. indica* contain essential oil containing humulene, elemene, ocimene, linalool, nerol and many others. The bark is

reported to contain protocatechic acid, catechin, mangiferin, alanine, glycine, γ-aminobutyric acid, kinic acid, shikimic acid and the tetracyclic triterpernoids, phenolic antioxidants, free sugars, polyols (Sha *et al*., 2010). The flowers yield alkyl gallates such as gallic acid, ethyl gallate, methyl gallate, n-propyl gallate, n-pentyl gallate, n-octyl gallate, 4-pheny gallate, 6-phenyl-n-hexyl gallate, dihydrogallic acid. The root contains chromones; 3-hydroxy-2-(4'-methylbenzoyl)-chromone and 3-methoxy-2-(4'-methylbenzoyl)-chromone. Phenolic antioxidants, free sugars and polyols have also been reported from the bark (Sha *et al*., 2010).

2.9.6.5 Geographical distribution

M. indica was originated from India and now it is cultivated throughout the tropical and subtropical world (Alkizim, Matheka, Abdulrahman & Muriithi, 2012; Ross, 1999, p. 198)

2.10 Background of the selected pathogens

2.10.1 *Staphylococcus aureus*

Berker and Von Eiff (2011, p. 308) describes *S. aureus* as gram positive, non-motile, non-spore forming, spherical cells of 0.5 to 1.5 µm in diameter occurring in clusters but also as single cocci, in pairs, tetrads and short chains. With the exception of the anaerobic species, *S. aureus* subsp. *anaerobius*, most *S. aureus* strains grow well aerobically (Berker & Von Eiff, 2011, p. 308). Most *S. aureus* colonies are 1-3 mm in diameter within 24 hours and 3-8 mm in diameter after 72 hours of incubation in air at 34-37ºC (Berker & Von Eiff, 2011, p. 316). On routine blood agar, the classical *S. aureus* colony is pigmented (cream to yellow), smooth, entire, slightly raised and haemolytic (Berker & Von Eiff, 2011, p. 316). Mucoid colonies due to highly encapsulated strains are rarely encountered (Berker & Von Eiff, 2011, p.316). A number of isolates of *S. aureus* may have a hazy or distinct zone of β-haemolysis around the colonies ranging from weak to strong. *S. aureus* is catalase positive, coagulase positive and mannitol positive (Berker & Von Eiff, 2011, p. 308-316).

S. aureus has been implicated in meningitis (Tunkel, 2014), empyema, otitis media, stye, conjunctivitis, osteomylitis, staphylococcal food poisoning, impetigo, wound infections (Cheesbrough, 2006), pneumonia, cellulitis, bacteremia, (Kobayashi & Malachowa, 2015).

2.10.2 *Escherichia coli*

E. coli is a gram negative motile rod and can grow aerobically or anaerobically at 35ºC (Nataro, Bopp, Fields, Kapper & Strockbine, 2011, pp. 603-604). *E. coli* grow well on non-selective media usually ferment lactose and produce large red colonies on MacConkey agar (Chart, 2012, p. 280). Certain strains are haemolytic when grown on suitable erythrocyte containing media, ferment D glucose and produce gas from the fermentation of this substrate and other fermentable carbohydrates (Chart, 2012, p. 280).

E. coli is usually found in the intestines of people and animals. Most *E. coli* are normal flora in the intestinal tract. However some *E. coli* are virulent and capable of causing diarrhoea (Centres for Disease Control and Prevention, 2015). The following are the six pathogenic *E. coli* implicated in diarrhoea;

- Shiga toxin-producing *E. coli* (STEC)
- Enterotoxigenic *E. coli* (ETEC)
- Enteropathogenic *E coli* (EPEC)
- Enteroaggregative *E. coli* (EAEC)
- Enteroinvasive *E. coli* (EIEC)
- Diffusely Adherent *E. coli* (DAEC)

(Centres for Disease Control and Prevention, 2015)

E. coli is also responsible for urinary tract infections (UTI), wound infections and meningitis in neonates (Cheesbrough, 2006, pp. 24, 80, 116).

2.10.3 *Pseudomonas aeruginosa*

P. aeuruginosa are aerobic, non-spore forming, gram negative rods which are straight or slightly curved and are 0.5 to 1.0 by 1.5 to 5.0 µm. *P. aeruginosa* grow well at 25ºC and

can grow at temperatures up to 42ºC (Henry & Speert, 2011, pp. 677-680). It grows well on a standard broth and solid laboratory media such as tryptic soy agar with 5% sheep blood agar, chocolate and MacConkey agar (Henry & Speert, 2011, p. 680). Most *P. aeruginosa* organisms are easily identified on primary isolation media on the basis of characteristic colonial morphology, production of diffusible pigments and a grapelike odour (Henry & Speert, 2011, p. 680). *P. aeruginosa* is oxidase positive. Colonies are usually flat and spreading and have a serrated edge and metallic sheen that is mostly linked with autolysis of the colonies (Henry & Speert, 2011, p. 680). Numerous strains of *P. aeruginosa* can produce the blue pigment pyocyanin (Henry & Speert, 2011, p. 680). The bright green colour, a characteristic of *P. aeruginosa* is created when pyoverdin combines with the blue water soluble phenazine pigment pyocyanin (Henry & Speert, 2011, p. 680).

P. aeruginosa is one of the pathogens causing meningitis (Cheesbrough, 2006, p. 116), UTI, bacteremia, pneumonia, otitis externa, keratitis, otitis media (Gellatly & Hancock, 2013), wound infections and skin infections in burn injuries (Cheesbrough, 2006, p. 80).

2.10.4 *Salmonella typhimurium*

According to Nataro, Bopp, Fields, Kaper and Strockbine (2011, p. 616) *S. typhimurium* is a member of *Samonellae.* They belong to the family *Enterobacteriaceae* and the genus *Salmonella* (Chart, 2012, p. 265; Nataro *et al*., 2011, p. 616). These bacteria are gram negative motile facultative anaeobes (Nataro *et al*., 2011, p. 616). *Salmonella* are lactose negative, however, they ferment glucose with gas production (Bauman, 2014, p. 637). The optimal growth temperature for *Samonella* is 37ºC*. Samonella* is citrate positive, urease negative, oxidase negative and hydrogen sulfide (H_2S) positive (Nataro *et al*., 2011, p. 616). Many differential plating media varying from slightly selective are available for isolation of *Samonella* (Nataro *et al*., 2011, pp. 617-618). Eosin Methylene Blue (EMB) is an example of media of low selectivity (Nataro *et al*., 2011, p. 617). Media of intermidiate selectivity for *Samonella* include Xylose Lysine Deoxycholate (XLD), Deoxycholate Citrate agar (DCA) and *Salmonella Shigella* (SS) (Nataro *et al*., 2011, p. 618). Highly selective media include Bismuth Sulphite agar. XLD has an H_2S indicator

system which is helpful for the detection of lactose fermenting *Salmonella* strains. According to Bauernfeind, Holley, Jungwirth, Mangold, Rohnisch, Schweighart, Wilhelm, Casellas and Goldberg ,(1992) multiresistant *S. typhimurium* strains causing grievous infections in humans such as meningitis and sepsis have been reported.

2.10.5 *Cryptococcus neoformans*

C. neoformans is a gram positive (Cheesbough, 2006, p. 124), aerobic yeast cell growing at 37ºC (Howell & Hazen, 2011, p. 1797). The size of *C. neoformans* ranges from 3.5 to 8 µm or more in diameter with single budding and a narrow neck between parent and daughter cell (Howell & Hazen, 2011, p. 1796). *C. neoformans* is urease positive and indian ink positive. *C. neoformans* is germ tube negative, a non-fermenter of glucose, maltose, sucrose, lactose and galactose (Howell & Hazen, 2011, p. 1797). Large yeast cells of up to 60 µm in size have been identified and this is linked with high incubation temperatures. Sometimes a number of buds may be seen and occasionally pseudohyphae are detected (Howell & Hazen, 2011, p. 1796). *C. neoformans* is an opportunistic fungal pathogen that cause meningitis in individuals with severe immunosuppression (Coelho & Casadevall, 2016).

2.10.6 *Candida albicans*

C. albicans is a gram positive (Warnock, 2012, p. 625), monomorphic, oval budding yeast (Kobayashi, 1990, p. 757). *C. albicans* grows aerobically (Howell & Hazen, 2011, p. 1796) on Saboraud Dextrose Agar (SDA) or blood agar at 25 to 37ºC and typical yeast cells are visible within 1 to 2 days (Warnock, 2012, p. 625). On SDA plate, colonies are smooth, creamy and resemble bacterial colonies (Kobayashi, 1990, p. 757) and the older larger colonies may appear wrinkled (Howell & Hazen, 2011, p. 1796; Kobayashi, 1990, p. 757) but may change back to smooth colonies on subculture (Howell & Hazen, 2011, p. 1796). On Cornmeal agar, *C. albicans* is distinguished from other *Candida* species by the formation of chlamydospores (Kobayashi, 1990, p. 757). *C. albicans* is Indian ink negative (Howell & Hazen, 2011, p. 1797) and produces germ tube after incubation in serum at 37ºC for 1.5 to 2 hours (Warnock, 2012, p. 625; Cheesbrough, 2006, p. 243).

C. albicans is an opportunistic pathogen that causes opportunistic diseases in immunocompromised individuals. *C. albicans* is reported as an aetiological agent for vaginitis and oral candidiasis (Bauman, 2014, p. 698). Bauman (2014, p. 698-699) states that oral candidiasis occurs in only 5% of newborns and 10% of adults and close to all HIV infected individuals as soon as they develop clinical manifestation of AIDS. *C. albicans* is also implicated in meningitis (Toprak *et al*., 2015).

2.11 Previous studies

Freidberg (2009) assessed the in-vitro antimicrobial activities of *G. incanum* leaves. Aqueous, methanol and acetone extracts demonstrated antimicrobial activities against *S. aureus* and *C. albicans*. However, *P. aeruginosa* and *E. coli* resisted against aqueous extracts of *G. incanum*.

In a study undertaken by Obumselu, Okerulu, Onwukeme, Onuegbu and Eze (2011), aqueous extract of *R. communis* leaves inhibited the growth of *S. aureus, P. aeruginosa*, and *E. coli*. Furthermore essential oils from fresh leaves of *R. communis* obtained by gas chromatography coupled to mass spectrometry (GC-MS) inhibited the growth of *S. aureus, P. aerugnosa, and E. coli* (Zarai *et al.,* 2012). In a study undertaken by Khan and Yadav (2011) *E. coli* demonstrated resistance against acetone extract of *R. communis*. A study conducted by Mansoor, Shaheen, Javed, Shaheen and Iqrar (2013), *E. coli* resisted against aqueous extract of *R. communis*.

In a study undertaken by Barti (2013), acetone extract of *M. indica* leaves inhibited the growth of *S. aureus* and *P. aeruginosa*, however, *E. coli* demonstrated resistance. A study conducted by Parekh and Chanda (2008), methanol extract of *M. indica* leaves inhibited the growth of *C. albicans* but *C. neoformans* demonstrated resistance. Doughari and Manzara (2008) reported lack of activities of aqueous extract of *M. indica* leaves against *S. aureus, P. aeruginosa* and *E. coli*. In a study undertaken by Doughari (2007), aqueous, methanolic and acetone extracts of *M. indica* leaves demonstrated antimicrobial effects on *C. albicans* and *C. neoformans*.

In a study undertaken by Elashi (2015), *C. albicans* and *E. coli* demonstrated resistance against ethanolic extract of *A. digitata*. Komolafe (2014) assessed antimicrobial effects of

aqueous extract of fruit pulp of *A. digitata*. The results demonstrated lack of activity against *S. aureus* and *E. coli* (Komolafe, 2014, p. 33). In a study undertaken by Seukep, Fankam, Djeussi, Voukeng, Tankeo, Noumdem, Kuete and Kuete (2013), methanol extract of fruit pulp of *A. digitata* exhibited weak activities against *P. aeruginosa* and *E. coli.*

A study conducted by Adedapo, Jimoh and Afolayan (2013), aqueous extract of *B. pilosa* leaves inhibited the growth of *S. aureus*, however, no activities were demonstrated on *E. coli*. In a study undertaken by Motsei, Lindsey, Van Staden and Jager (2003), aqueous, ethanol, ethyl acetate and hexane extracts of *B. pilosa* leaf inhibited the growth of *C. albicans.* The plant has been reported to show antibacterial activities against numerous microorganisms including five enteric pathogens (Watt & Brandwijk, 1962, pp. 205-206). The extract of *B. pilosa* plant have been reported to have antifungal activities against *C. albicans* (Roodt, 1998 p. 40).

Aruljothi, Uma, Sivagurunathan and Bhuvaneswari (2014) investigated antimicrobial properties of *C. papaya* leaf. The results indicated growth inhibitions on *S. aureus, P. aeruginosa* and *E. coli.* In a study undertaken by Tewari, Subramanian and Gomathinayagm (2014) methanol, ethyl acetate and chloroform extracts of *C. papaya* leaf exerted antimicrobial effects on *E. coli, S. aureus* and *C. albicans*.

A study conducted by Hannan, Asghar, Naeem, Ullah, Ahmed, Aneela and Hussain (2013), acetone extract of *M. indica* leaves inhibited the growth of *S. typhimurium*. In a study undertaken by Azhagesan, Rajan, & Soranam, (2015), aqueous extract of *M. indica* seed kernel demonstrated antimicrobial effects against *S. typhimurium.* A study conducted by Shahid, Durrani, Iram, Durrani, and Khan (2013), methanol extract of *R. communis* leaves exerted antimicrobial effects against *S. typhimurium*. In a study undertaken by Lawal, Amisu, Akinyemi, Sanni, Simelane, Mosa and Opoku (2015), aqueous extract of *B. pilosa* displayed antimicrobial activities on *S. typhimurium*.

CHAPTER 3: METHODOLOGY

3.1 Introduction

The current chapter discusses the methodology and materials used in the study. The aim of the study was to investigate the antimicrobial activities of aqueous, methanol and acetone extracts of leaves of *G. incanum, B. pilosa, M. indica, C. papaya* and fruit pulp of *A. digitata* and activity of their combined preparation against selected bacterial and fungal pathogens that cause meningitis.

3.2 Method justification

3.2.1 Selected medicinal plants and pathogens

Leaves of *G. incanum*, *B. pilosa, R. communis, M. indica, C. papaya* and fruit pulp of *A. digitata* were used in this study. Selection of the leaf parts of *G. incanum, B. pilosa, R. communis, M. indica, C. papaya* and fruit pulp of *A. digitata* was based on the reports in the literature for their use in the traditional treatment of predisposing factors of meningitis such as ear infections, managing signs and symptoms of meningitis such as headache, fever and inflammation. *B. pilosa* leaves have been used for treatment of headaches and as anti-inflammatory agents (Mvere, 2004, pp. 114-115). The leaves of *R. communis* have been utilised as a remedy for headache (Watt & Brandwijk, 1962, p. 430). Traditional healers also use leaves of *R. communis* (Jena & Gupta, 2012), *C. papaya* (Aravind, 2013), *M. indica* (Ross, 1999, p. 198) as anti-inflammatory agents. *A. digitata* fruit pulp, *G. incanum* (Van Wyk *et al*., 2009, p. 134) *B. pilosa* (Watt & Brandwijk, 1962, p. 205), *M. indica* (Ross, 1999, p. 199) leaves are traditionally used to treat and manage fever.

The microorganisms under study were Methicillin resistant and oxacillin resistant *S. aureus* (ATCC 43300*), P. aeruginosa* (ATCC 27853), *E. coli* (ATCC 35218), *S. typhimurium* (ATCC 49416), *C. albicans* (ATCC 10231) and *C. neoformans* (clinical strain). The organisms were selected based on literature report on their implications in meningitis.

3.2.2 Drying of plant materials

Dried plant leaves were used in this study. According to Eloff (1998a) dried plant materials are preferred when working on the chemistry of secondary metabolites in plants for the following reasons:

- There are few challenges encountered with huge quantity extraction of dried plant material than with fresh material.
- The time delay between collecting plant material and processing it makes it hard to work with fresh material as differences in moisture content may affect solubility or subsequent separation by liquid-liquid extraction.
- Secondary metabolites plant components are moderately stable if they are mainly to be used as antimicrobial agents.
- Majority of plants are utilised in the dried form by traditional healers.

The dried leaves were pulverised because majority of medicinal plants are used in powder form by traditional healers (Ncube, Afoloyan & Okoh, 2008; Eloff, 1998a).

3.2.3 Selection of solvents for extraction

Extraction is the critical first step in the investigation of antimicrobial activities in medicinal plants as it demands the extraction of desired phytochemicals from the plant materials (Sasidharan, Chen, Saravanan, Sundram & Latha Yoga, 2011). Pandey and Tripathi (2014) believe that attributes of good solvents include low toxicity, ease of removal at low heat, promotion of rapid physiologic absorption of the extract, preservative action and inability to cause the extract to complex or dissociate.

Water, methanol and acetone were the extractants that were selected in this study. The selection was done taking into consideration that there is no perfect extractant as each one has its own shortfalls (Hughes, 2002; Mohlakoana, 2010, p. 39). Nevertheless, the following factors were taken into consideration when selecting the extractant (Pandey & Tripathi, 2014):

- Quantity of phytochemical to be extracted
- Rate of extraction

- Diversity of different compounds extracted
- Diversity of inhibitory compounds extracted
- Ease of subsequent handling of the extracts
- Toxicity of the solvents in the bioassay procedure
- Potential health hazard of the extractant

Given that most of the traditional medicinal plants are dried and prepared using water, consumed as tea, infusions, concoctions, decoctions or inhalation through steam from boiling suspensions of the parts (Eloff, 1998a; Ncube *et al*., 2008), water was selected as one of the extractant in this study. The laboratory preparation and extraction was performed in a manner that closely resemble the traditional way of medicinal herbal preparation.

Given that most of the identified antimicrobial compounds are aromatic or saturated organic compounds, they are not soluble in water (Ncube *et al*., 2008) and consequently low yields are obtained. To increase the yield of isolated antimicrobial compounds, organic extractants such as acetone and methanol were used in this study. In addition, acetone has several advantages such as volatility, miscibility with polar and non-polar solvents and has low toxicity to bioassay (Eloff, 1998a).

Masoko, Picard and Eloff (2005) investigated the antifungal activity of *Combretum* species. The extractants used comprised of hexane, dichloromethane, acetone and methanol. The results demonstrated that acetone and methanol extracted more phytochemicals from the leaves as compared to other solvents. Both acetone and methanol extracted saponins which have biological activities against microbes.

3.2.4 Selection of method for antimicrobial screening

Eloff (2004) seems inclined towards the idea that screening for biological activities in medicinal plants is performed to identify new lead compounds for drug development, to confirm herbal-based traditional practice that uses various plant materials for both preventive and therapeutic therapy as well as to develop medicinal plant use as herbal medicine. Agar well diffusion technique was the method selected for antimicrobial screening in this study. The method was employed in this study as it is rapid (Valgas, de

Souza, Smania & Smania Jr., 2007), simple, easy to reproduce, inexpensive, easy to read and interpret. In addition the method can be used in laboratories with limited resources (Magaldi, Mata-Essayag, Hartung de Capriles, Perez, Colella, Olaizola & Ontiveros, 2004).

3.2.5 Selection of method for minimum inhibitory concentration (MIC)

In this study iodonitrotetrazolium chloride (INT) microtitre plate technique was used to determine the minimum inhibitory concentration (MIC) of the individual and combined plant extracts. Mukherjee (2015, p. 501) suggests that microtitre plate technique is an appropriate method for testing MIC of phytochemicals. The INT microtitre plate technique was selected in this study as it is cheap, rapid, requires small amount of sample (Mukherjee, 2015, p. 501; Eloff, 1998b), very easy to set up (Mukherjee, 2015, p. 501), does not require high levels of skill, leaves a perpetual record, easy to duplicate the results and is as 30 times more sensitive than other methods described in the literature (Eloff, 1998b).

3.2.6 Selection of reference standards

Ciprofloxacin and Amphotericin B were the reference standards used in the present study. The selection of Ciprofloxacin and Amphotericin B as reference standards was based on NCCLS guidelines (2003) (Lahlou, 2004). Lahlou (2004) cited a number of authors in a review article on methods of determining antimicrobial activity who supported Ciprofloxacin and Amphotericin B as standard controls.

3.3 Plant collection

The medicinal plants under study were collected from South Africa and Malawi in August 2015 using simple random sampling technique. The collection of the medicinal plants from Malawi and South Africa was based in the literature on their endemism. Geographically some of the medicinal plants under study favour tropical areas whereas others are cosmopolitan. *G. incanum* was identified and collected by Professor Nanette Smith from Nelson Mandela Metropolitan University, Port Elizabeth, Eastern Cape Province of the Republic of South Africa. Port Elizabeth is found on latitude 33º 57’ 29’’

S, longitude 25º 36’ 0’’ E with an altitude of 88.74 metres above sea level. *A. digitata, M. indica, R. communis, C. papaya* and *B. pilosa* were identified and collected by Lucy Mhone Kamwamba, a nurse by profession at St Josephs Mission Hospital, Chiradzulu District, Southern part of Malawi. Chiradzulu District lies on latitude 15º 40’ 33.42’’S, longitude 35º 8’ 26.26’’E with an altitude of 1119.25 metres above sea level.

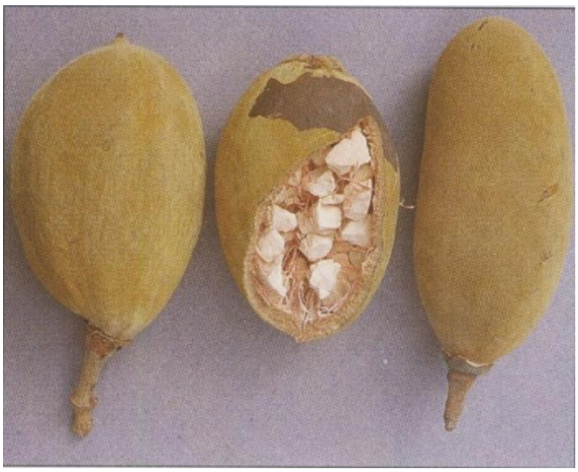
3.4 Plant preparation and extraction

3.4.1 Plant preparation

Leaves of *G. incanum*, *M. indica*, *R. communis*, *C. papaya* and *B. pilosa* were washed with sterile distilled water and allowed to air-dry on a bench at room temperature. The leaves were then grounded to powder using motor and pestle (Figure 3.1).

Figure 3.1: Air dried leaves grounded using a motor and a pestle

Fruit pulp of *A. digitata* was obtained from the fruit of *A. digitata*. Dried mature fruits of *A. digitata* were broken (Figure 3.2A) and the fruit pulp coating the seeds were separated from the seeds (Figure. 3.2 B).



(A)

(B)

Figure 3.2*:* (A) *A digitata* fruit broken and (B)*: A. digitata* fruit pulp separated from the seeds

3.4.2 Extraction procedure for the individual plant extracts

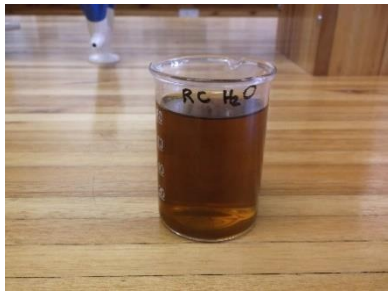
The extraction procedure was performed according to Nkosi (2013, p. 43) with some modifications on plant powder: extractant ratio and temperature settings on the waterbath.

- 10g of plant powder of *G. incanum*, *M. indica*, *B. pilosa*, *C. papaya*, *R. communis* were weighed and placed in separate sterile polypropylene centrifuge tubes. 50ml of sterile distilled water, methanol and acetone were added to each tube respectively.
- For the aqueous extracts of fruit pulp of *A. digita*ta, 5g of *A. digitata* fruit pulp powder was weighed and placed in two separate polypropylene centrifuge tubes. 40 ml of sterile distilled water was added to each tube.
- For the organic extracts of *A. digitata*, 5g of *A. digitata* fruit pulp powder was weighed and placed in separate centrifuge tubes. 40 ml of methanol and acetone (Associated Chemical Enterprises) were added to each tube respectively. To increase the yield of extraction, ten tubes comprising a mixture of *A. digitata* fruit powder and organic extractant were used.
- The mixtures were shaken vigorously and were left in a shaking incubator (LABCON) for overnight for thorough extraction of the phytochemicals. The

temperature and speed of the shaking incubator was set at 22ºC and 90 rpm respectively.

- On the following day, the mixtures were centrifuged at 1000 rpm for 5 minutes.
- The supernatants were filtered using a Whatman filter paper and placed in sterile beakers (Fig 3.3 - 3.5).
- The beakers containing the supernatants were left in waterbath set at 40ºC. The waterbath was placed in an airflow fume cabinet (IVID).
- The dried extracts were quantified by subtracting the weight of empty beaker from the weight of the beaker plus dry extracts.
- The extracts were dissolved in Dimethyl Sulfoxide (DMSO) (Associated Chemical Enterprises) as a reconstituting solvent.
- To ensure sterility, the extracts were sterilised using a membrane filter (OSMONICS) of 40 µm size and transferred to a sterile screwed cap bottles, labelled and stored under refrigerated conditions till use.

M. indica water extract



R. communis water extract

B. pilosa water extract

A. digitata water extract

C. papaya water extract

G. incanum water extract

Figure 3.3: Appearance of single water extracts

G. incanum methanol extract

B. pilosa methanol extract

M. indica methanol extract

R. communis methanol extract

C. papaya methanol extract

A. digitata methanol extract

Figure 3.4: Appearance of single methanol extracts

A. digitata acetone extract

G. incanum acetone extract

R. communis acetone *extract*

C. papaya acetone extract

B. pilosa acetone extract

M. indica acetone extract

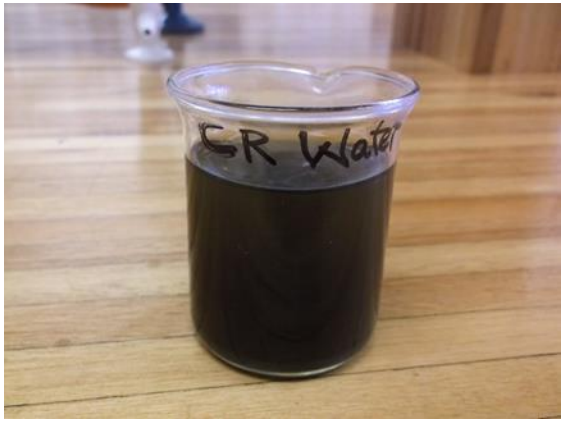
Figure 3.5: Appearance of single acetone extracts

3.4.3 Extraction procedure for the combined plant extracts

For the combined extract preparation, a total of 16 combinations for water, methanol and acetone were prepared before extraction. The combinations were as follows; *A. digitata*: *R. communis* (A:R), *A. digitata: B. pilosa* (A:B), *A. digitata: G. incanum*, (A:G), *A. digitata: M. indica* (A:M), *A. digitata: C. papaya* (A:C), *B. pilosa: G. incanum* (B:G), *B. pilosa: M. indica* (B:M*), R. communis: B. pilosa* (R:B), *R. communis: M. indica* (R:M*), R. communis*: *G: incanum* (R:G), *C. papaya: M. indica* (C:M), *G. incanum: M. indica* (G:M), *R. communis: C. papaya* (R:C), *C. papaya: B. pilosa* (C:B), *C. papaya: G. incanum* (C:G) and *A. digitata*: *R. communis*: *B. pilosa: G. incanum: M. indica: C. papaya* (A:R:B:G:M:C). The plant mixtures were blended in the ratio of 1:1. Five grams of each plant powder was blended together to obtain a uniform fine powder of total mass of 10g. The extraction procedure was performed as follows:

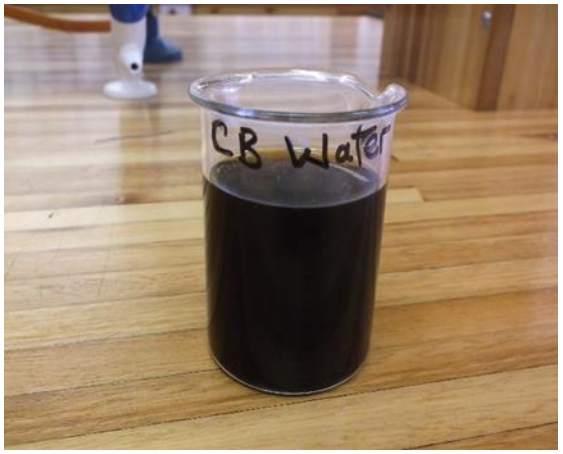
- 10g of each plant combination was placed in separate sterile polypropylene centrifuge tubes.
- 40ml of sterile distilled water, methanol and acetone were added to each tube respectively.
- The mixtures were shaken vigorously and were left in a shaking incubator (LABCON) for overnight for thorough extraction of the phytochemicals. The temperature and speed of the shaking incubator was set at 22ºC and 90 rpm respectively.
- On the following day, the mixtures were centrifuged at 1000 rpm for 5 minutes.
- The supernatants were filtered using a Whatman filter paper and placed in sterile beakers (Fig 3.6 – 3.8).
- The beakers containing the supernatants were left in waterbath set at 40ºC. The waterbath was placed in an airflow fume cabinet (IVID).
- The dried extracts were quantified by subtracting the weight of the empty beaker from the weight of the beaker plus combined extracts.
- The dried extracts were then reconstituted using DMSO (Associated Chemical Enterprises) as a reconstituting solvent.

- To ensure sterility, the extracts were sterilised using a membrane filter (OSMONICS) of 40 µm size and transferred to a sterile screwed cap bottle, labelled and stored under refrigerated conditions at 4ºC till use



C. papaya / *R. communis* combined water extracts

C. papaya / *M. indica* water extracts



C. papaya / *B. pilosa* combined water extracts

A. digitata / *B. pilosa* combined water extract

Figure 3.6: Appearance of combined water extracts

A. digitata / *R. communis* combined water extracts

R. communis / *B. pilosa* combined water extracts

A. digitata / *C. papaya* combined water extracts

R. communis / *M. indica* combined water extracts

Figure 3.6: Appearance of combined water extracts (continued)

B. pilosa / *G. incanum* combined water extracts

R. communis / *G. incanum* combined water extracts

A. digitata / *M. indica* combined water extracts

A. digitata / *G. incanum* combined water extracts

Figure 3.6: Appearance of combined water extracts (continued)

C. papaya / *G. incanum* combined water extracts

B. pilosa / *M. indica* combined water extracts

G. incanum / *M. indica* combined water extracts

G. incanum / *M. indica* / *R. communis* / *C. papaya* / *A. digitata* / *B. pilosa* combined water extracts

Figure 3.6: Appearance of combined water extracts (continued)

R. communis / *M. indica* combined methanol extracts

A. digitata / *M. indica* combined methanol extracts

G. incanum / *M. indica* combined methanol extracts

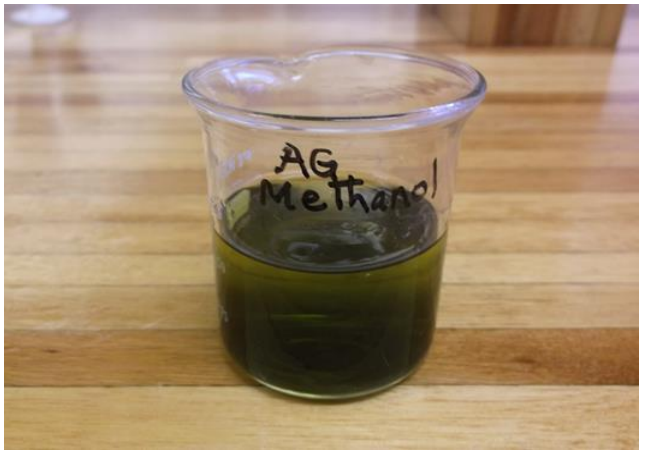
A. digitata / *R. communis* combined methanol extract

Figure 3.7: Appearance of combined methanol extracts

C. papaya / *M. indica* combined methanol extracts

C. papaya / *R. communis* combined methanol extracts

C. papaya / *G. incanum* combined methanol extracts



A. digitata / *G. incanum* combined methanol extracts

Figure 3.7: Appearance of combined methanol extracts (continued)

A. digitata / *C. papaya* combined methanol extracts

A. digitata / *B. pilosa* combined methanol extracts

R. communis / *G. incanum* combined methanol extracts

B. pilosa / *G. incanum* combined methanol extracts

Figure 3.7: Appearance of combined methanol extracts (continued)

B. pilosa / *M. indica* combined methanol extracts

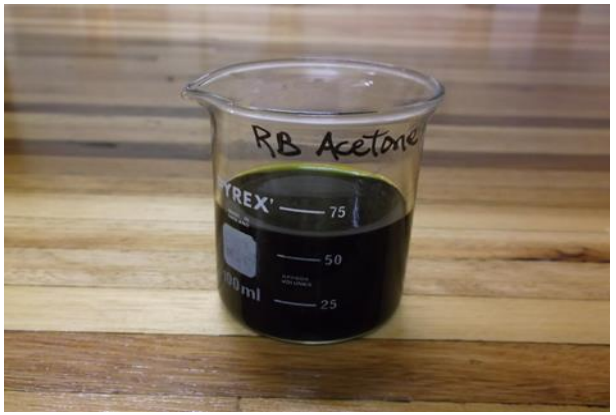
R. communis / *B. pilosa* combined methanol extracts

C. papaya / *B. pilosa* combined methanol extracts

G. incanum / *M. indica* / *R. communis* / *C. papaya* / *A. digitata* / *B. pilosa* combined methanol extracts

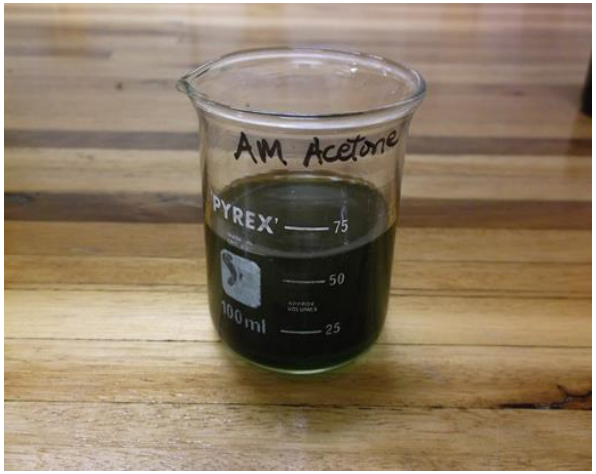
Figure 3.7: Appearance of combined methanol extracts (continued)

B. pilosa / *M. indica* combined acetone extracts



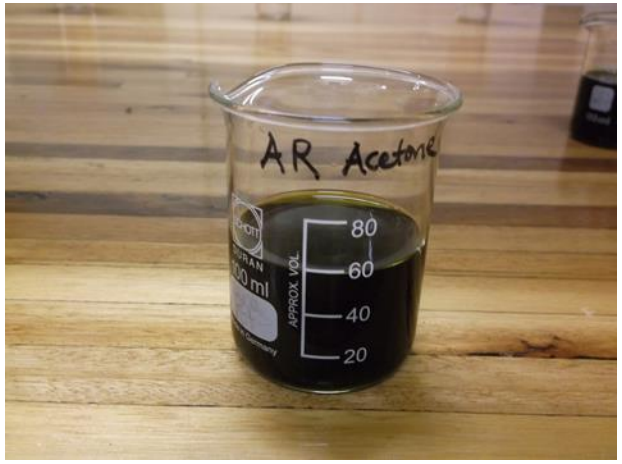
R. communis / *B. pilosa* combined acetone extracts

C. papaya / *M. indica* combined acetone extracts



A. digitata / *M. indica* combined acetone extracts

Figure 3.8: Appearance of combined acetone extracts



A. digitata / *R. communis* combined acetone extracts

A. digitata / *B. pilosa* combined acetone extracts

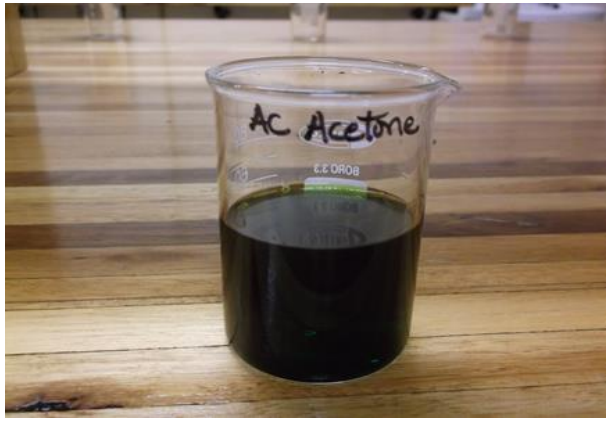
C. papaya / *B. pilosa* combined acetone extracts

B. pilosa / *G. incanum* combined acetone extracts

Figure 3.8: Appearance of combined acetone extracts (continued)

C. papaya / *G. incanum* combined acetone extracts

A. digitata / *G. incanum* combined acetone extracts



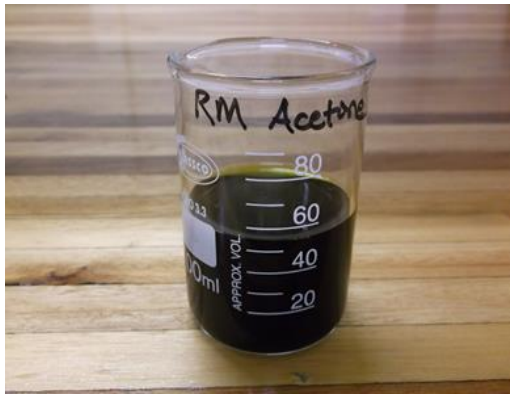
A. digitata / *C. papaya* combined acetone extracts

R. communis / *G. incanum* combined acetone extracts

Figure 3.8: Appearance of combined acetone extracts (continued)

C. papaya / *R. communis* combined acetone extracts

G. incanum / *M. indica* combined acetone extracts



R. communis / *M. indica* combined acetone extracts

G. incanum / *M. indica* / *R. communis* / *C. papaya* / *A. digitata* / *B. pilosa* combined acetone extracts

Figure 3.8: Appearance of combined acetone extracts (continued)

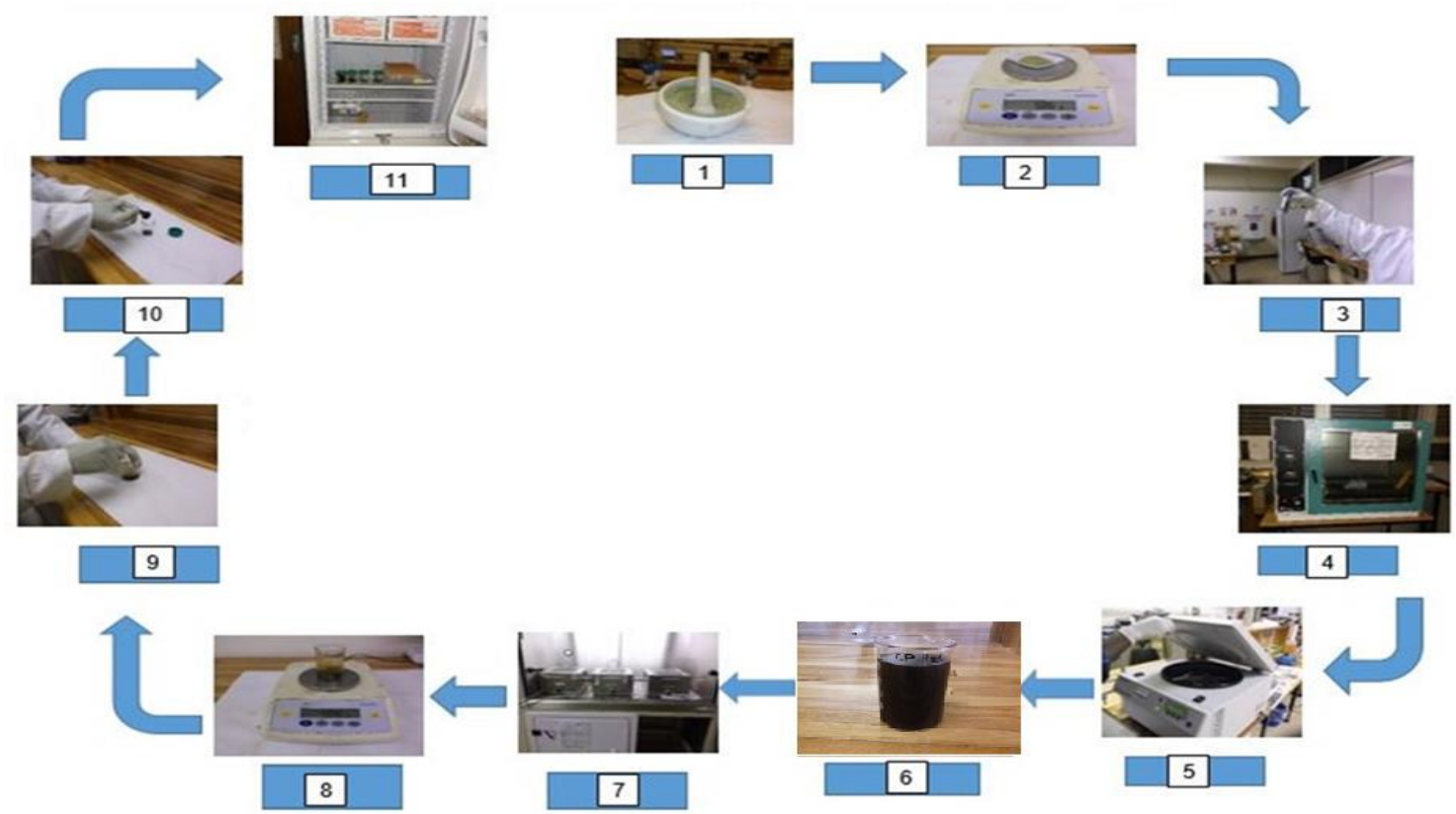


Figure 3.9: Schematic diagram showing the extraction process of the medicinal plants

Key in the schematic diagram

Step 1 Grounding of the medicinal plant leaves using a mortar and a pestle

Step 2 Weighing the grounded medicinal plant leaves on a digital scale

Step 3 Vigorous shaking of grounded leaves and extractant mixture

Step 4 Grounded leaves and extractant mixture placed in shaking incubator

Step 5 Centrifugation of the mixture after overnight shaking in a shaking incubator

Step 6 Extracts decanted in a sterile beaker after centrifugation

Step 7 Beakers with extracts placed in waterbath in a fume cabinet to remove extractant

Step 8 Weighing a beaker with extracts to quantify the extracts

Step 9 Reconstitution of extracts with DMSO

Step 10 Sterilising extracts through a membrane filter after reconstitution

Step 11 Sterilised extracts transferred in a screwed bottle and placed in a fridge

3.5 Antimicrobial screening

The microorganisms under study were methicillin and oxacillin resistant *S. aureus* (ATCC 43300*), P. aeruginosa* (ATCC 27853), *E. coli* (ATCC 35218), *S. typhimurium* (ATCC 49416), *C. albicans* (ATCC 10231) and *C. neoformans* (clinical strain). The organisms were provided by the Department of Medical Laboratory Sciences at Nelson Mandela Metropolitan University (NMMU), Port Elizabeth in South Africa. The organisms were selected because they are responsible for meningitis. To ensure viability, all bacterial cultures were subcultured on Nutrient agar (NA) and Saboraud dextrose agar (SDA) once per week.

Materials used in this study were sterilised in an autoclave (TOMY SX-700). Inoculating loops were sterilised by flaming on a gas burner. Yellow tips (200 µl size) and blue tips (1000 µl size), glass pasteur pipettes for punching holes in the plated agar plates were sterilised by autoclaving. The working benches were disinfected with 6% savlon (Johnson & Johnson) before and after each experiment.

Mueller Hinton agar (MHA) and SDA (BIOLAB-MERCK) were prepared according to manufacturer's instructions (see Appendix A). Twenty millilitres of MHA and SDA were poured into the sterile petri dishes and left to gel on a laboratory bench. To ensure sterility and purity of the culture media from each batch prepared, one plate of MHA and SDA was incubated for 24 hours and 48 hours at 37ºC. After the incubation period, the plates were examined for growth of contaminants.

Bacterial and fungal cultures growing on NA and SDA were subcultured to MHA and SDA. The culture plates were incubated at 37º for 24 hours for the bacterial pathogens and 48 hours for the fungal pathogens. After the incubation period, a single bacterial and fungal colony from streak plate of MHA and SDA were suspended in 2ml of sterile distilled water. The bacterial and fungal suspensions were diluted to 0.5 McFarland standard which corresponds to $1.5x10^8$ CFU/ml (Montalvo, Boulogne, & Suarez, 2015). The adjustments of the broth cultures were performed using sterile distilled water. The optical densities of the 0.5 McFarland suspensions were verified by comparing with the optical density of 0.5 Becton Dickinson (BD) commercial standard using sterile matched cuvettes of 1 cm path and sterile distilled water as a blank. A spectrophotometer (UNICO) was used to measure the optical densities at 620 nm.

Agar well diffusion technique described by Ncube *et al*., (2008) and Patel, Shrivastava and Kumar (2009) was employed as follows:

- MHA and SDA were plated with 0.5 McFarland suspension of bacteria and fungi under study using sterile disposable swabs.
- Wells of 6 mm in diameter were aseptically punched on plated MHA and SDA using a sterile glass pasteur pipette. The wells were 30mm apart.
- 100 µl of DMSO reconstituted extracts were carefully introduced into the wells using an Eppendorf adjustable pipette. Ciprofloxacin and Amphotericin B solutions (SIGMA-ALDRICH) were used as positive controls for bacterial and fungal microorganisms under study. Sterile distilled water, methanol, acetone and 10% DMSO were used as negative control.
- The extracts were allowed to diffuse in the MHA medium at room temperature on a laboratory bench for one hour.
- The culture plates were then incubated at an upright position at 37ºC for 24 hours for the bacteria and 48 hours for the fungal pathogens.
- Zones of inhibition were measured and recorded after 24 hours of incubation for the bacteria and 48 hours for the fungal culture plates.
- The procedure was performed in triplicate and repeated for five times.

3.6 Determination of minimum inhibitory concentration (MIC)

Saboraud dextrose broth (SDB) and Mueller Hinton broth (MHB) (SIGMA-ALDRICH) were prepared according to manufacturer's instructions (see Appendix A). Bacterial and fungal cultures growing on NA and SDA were subcultured to MHB and SDB. The broth cultures were incubated at 37º for 24 hours for the bacterial pathogens and 48 hours for the fungal pathogens. After the incubation period, the bacterial and fungal cultures were diluted to 0.5 McFarland standard which corresponds to $1.5x10^8$ CFU/ml (Montalvo *et al.,* 2015). The adjustments of the broth cultures were performed using MHB for the bacterial pathogens and SDB for the fungal pathogens. The 0.5 McFarland suspensions were verified by reading the optical densities and comparing with the readings of 0.5 BD Mc Farland commercial standard using sterile matched cuvettes of 1cm path. Sterile distilled water was used as a blank standard. A spectrophotometer (UNICO) set at 620 nm was

used to determine the absorbances of the 0.5 Mc Farland suspensions. The experiments were repeated for five times due to inconsistencies that occurred in some of the results.

Purity check of the 0.5 McFarland standard suspension of the bacteria and fungi under study was performed according to Hewitt and Vincent (1989, p. 12) by subculturing an aliquot on to NA and SDA plates. After incubation at 37ºC for 24 hours for bacteria and 48 hours for fungi, culture colony characteristics such as shape, colour, size were examined. Gram stain of a typical colony from each bacterial strain and fungal strain under study was performed and examined under oil immersion objective.

A known volume of extract was pipetted aseptically from the stock bottle and diluted to a final concentration of 100 mg/ml using MHB and SDB. The following formula was employed in the mathematical calculations (Sackheim & Lehman, 1998, p. 168);

Concentration 1 x Volume 1 = Concentration 2 x Volume 2

($C_1V_1=C_2V_2$)

Iodonitrotetrazolium microtitre plate assay reported by Eloff (1998b) was used in MIC determination as follows:

- A sterile 96 well plate was labelled
- 200 µl of the undiluted medicinal plant extracts with an initial concentration of 100 mg/ml were mixed, pipetted and dispensed into the first row (Row A) of the microtitre plate well.
- 100 µl of broth media (MHB for bacteria and SDB for fungi) were dispensed from row B-H.
- 100 µl of plant extract was pipetted from row A and dispensed into second row (Row B) containing broth media. The contents were mixed together to form homogeneous solution.
- The medicinal plant extracts were serially diluted from row B to H with a concentration range of 100 mg/ml to 0.781 mg/ml
- 100 µl of 0.5 McFarland standard of the test organisms were mixed, pipetted and dispensed in all rows (Row A-H).

- The contents were mixed together to form homogenous solution. For each run, the MIC determination for each extract was performed in triplicate (Figure 3.10).
- Ciprofloxacin and Amphotericin B antibiotic solutions (SIGMA-ALDRICH) were used as positive control for bacteria and fungi under study. Sterile distilled water, methanol, acetone and 10% DMSO were used as a negative control.
- The microplates were put in small plastic bags, sealed and incubated in moist environment at 37ºC for 24 hours for bacteria with fungi extending to 48 hours.
- 50 µl of INT chloride at a concentration of 4 mg/ml was pipetted and dispensed into microplate wells after 24 hours of incubation for bacteria and 48 hours for fungi.
- The contents were mixed thoroughly and incubated at 37ºC for 30 minutes for bacteria and for 24 hours for fungi. The INT chloride was used to indicate biological activity because the colourless compound acts as an electron acceptor and is reduced to a formazan product (pink/red colour). The pink/red colour indicates bacterial growth.
- The lowest concentration at which a decrease in the red colour is visible compared to the next dilution is taken as the MIC value (Figure 3.12).
- The procedure was repeated for five times.

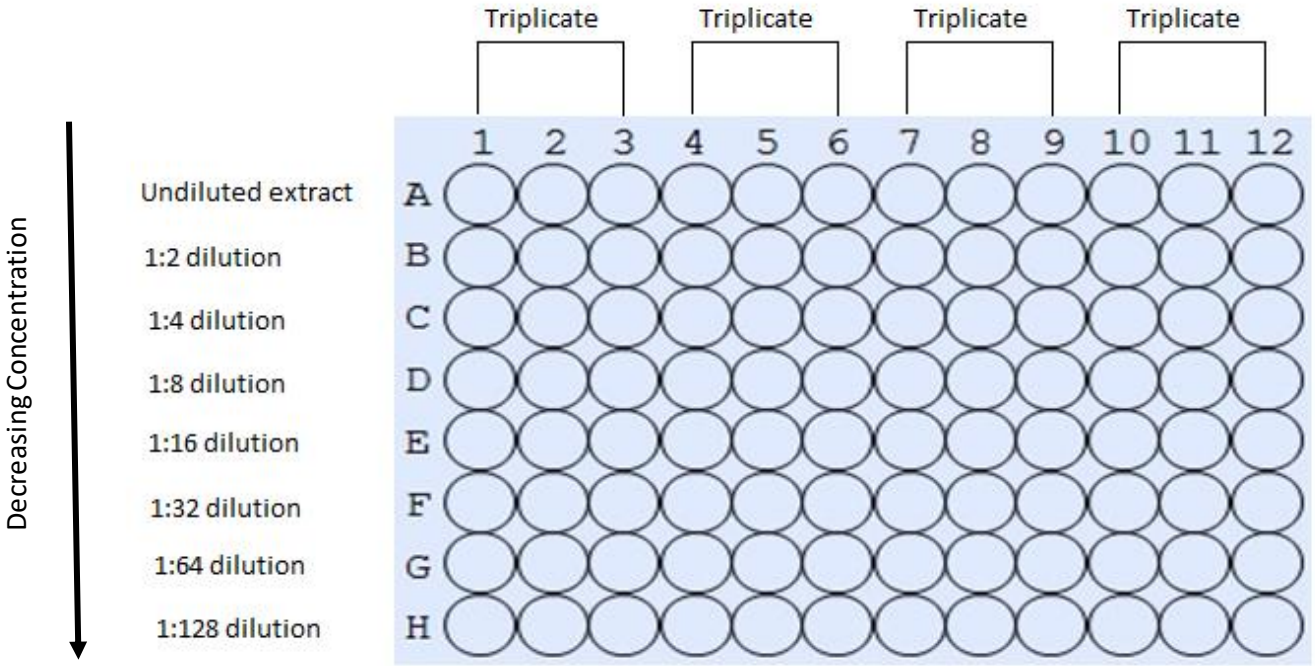


Figure 3.10: The layout of the 96-well microtitre plate for MIC determination

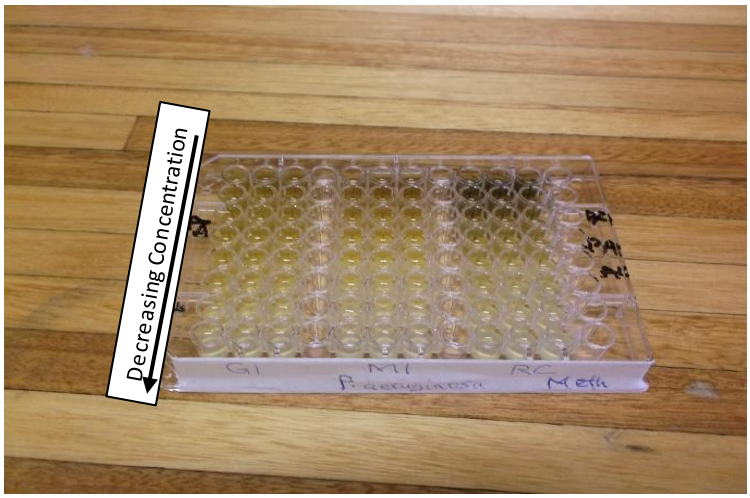


Figure 3.11: Microtitre plate before adding 50 µl INT chloride

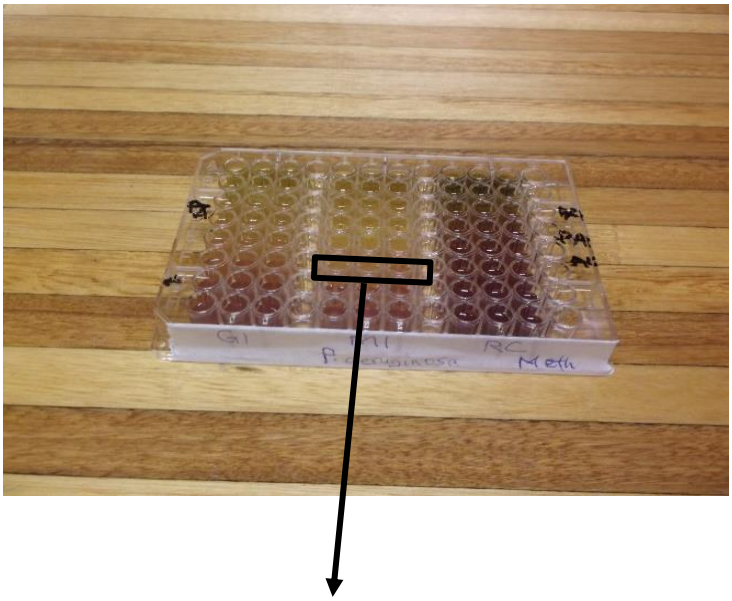


Figure 3.12: Microtitre plate after adding 50 µl of INT chloride

The sum of the fractional inhibitory concentration index (ΣFIC)

The sum of the fractional inhibitory concentration index (ΣFIC) is expressed as the interaction of two agents where the concentration of each test agent in combination is expressed as a fraction of the concentration that would produce the same effect when used independently (Van Vuuren & Viljoen, 2011). The ΣFIC was calculated for each plant extract independently as specified in the equations:

$$FIC^{(*i)} = \frac{MIC\ (a)\ in\ combination\ with\ (b)}{MIC\ (a)\ independently}$$

$$FIC^{(*ii)} = \frac{MIC\ (b)\ in\ combination\ with\ (a)}{MIC\ (b)\ independently}$$

The sum of the fractional inhibitory concentration indices (ΣFICI) were calculated to determine the effect of the plant extract combination using the following algebraic equation (Van Vuuren & Viljoen, 2011):

$$\Sigma FICI = FIC^{(*i)} + FIC^{(*ii)}$$

A range was used to categorise the antimicrobial interactions of the plant extract combinations into synergy (≤0.5), additive (>0.5-1.0), indifference (>1.0-≤4), antagonism (>4.0) (Van Vuuren & Viljoen, 2011; Mabona, Viljoen, Shikanga, Marston & Van Vuuren, 2013).

CHAPTER 4: RESULTS

4.1 Introduction

The present chapter discusses challenges faced by the study, solutions to the challenges and the results obtained in the study. The results include preparation of the medicinal plant materials, extraction of individual and combined plant materials, antimicrobial screening of single and combined plant extracts, minimum inhibitory concentration (MIC) of single and combined plant extracts and the sum of the fractional inhibitory concentration index (ΣFICI).

4.2 Challenges

4.2.1 Reconstitution of the dried extracts

One of the notable challenges faced in the study is the complete insolubility and partial solubility of the dried extracts in reconstituting solvents. In the present study methanol and acetone were used as extractants. Surprisingly, dried methanol and acetone extracts could not solubilise in methanol and acetone. Eloff and McGaw (2006, p. 103) and Eloff (2004) reported that dried extracts do not readily dissolve in a solvent even in the same extractant. This is one of the impediments in ethnopharmacological research.

Water could not even dissolve the dried methanol and acetone extracts. This might be attributed to the molecular structure of the constituents of methanol and acetone extracts of the medicinal plants under study. Ncube *et al.* (2008) highlighted that most antimicrobial active compounds that have been discovered do not solubilise in water. Sackheim and Lehman (1998, p. 148) believe that polar liquids solubilise polar compounds and non-polar liquids solubilise non-polar compounds. Given that water is a polar solvent, polar compounds and hydrophilic substances dissolve in water. Non-polar and lipophilic biologically active compounds of medicinal plants do not dissolve in water.

To overcome this challenge, the methanol and acetone extracts were first dissolved in 100% DMSO. The extracts were further diluted in fresh MHB for antibacterial screening and SDB for antifungal screening. The final concentration of the DMSO was at 10% at which DMSO does not inhibit the bioassay.

4.2.2 Contamination of medicinal plant extracts

Growths of contaminants were noted on two occasions around the agar wells of aqueous *B. pilosa* and *M. indica* extracts of bacterial and fungal culture plates. This challenge was overcome by enforcement of quality control measures and quality assurance. This include among others:

- Disinfecting working benches before and after work using 6% savlon (Johnson & Johnson). The concentration was prepared according to manufacture's instructions on the stock bottle.
- Sterilising glassware, culture media and tips used in the study by autoclaving.
- Ensuring that all aqueous extracts are prepared using sterile distilled water. Distilled water was sterilised by autoclaving and left to cool at room temperature before use.
- Performing sterility check on the prepared agar and broth media. One prepared fresh MHA plate and SDA plate were incubated at 37ºC for 24 and 48 hours and examined for growth of contaminants. Presence of colonies is an indication of media contamination. For fresh prepared broth, two separate sterile screwed tubes containing MHB and SDB were incubated at 37ºC for 24 and 48 hours and examined for contamination. Growth of contaminants is exhibited by turbidity in the broth.
- Performing purity check on organisms under study.
- Ensuring that the autoclave is cleaned, maintained and serviced regularly according to manufacture's instructions.
- Using autoclave tape to check the performance of the autoclave. Autoclave tape has diagonal markings which change colour after exposure to temperature of 121ºC maintained for 15 minutes.
- Ensuring that the reconstituted medicinal plant extract are filtered using a syringe and a 40µm membrane size filter.
- Monitoring and recording temperature readings on the refrigerator and incubator on the appropriate temperature logs and performing corrective actions on all non-conformities identified on the equipment.

- Ensuring regular cleaning of the refrigerator and incubator as specified by the manufacture's instructions.

4.2.3 Lack of biological activities in extracts due to low extraction efficiency

Lack of biological activities in the antimicrobial screening studies due to low extraction efficiency in most of the individual medicinal plants posed a great challenge. Leaves were extracted with a 10:1 solvent to dry weight powder ratio. Initially 2.5g of finely grounded leaves of *G. incanum, B. pilosa, R. communis, M. indica, C. papaya* and fruit pulp of *A. digitata* were separately dissolved in 25 ml of water, methanol and acetone. The mixture was shaken vigorously and centrifuged for 5 minutes at 1000 rpm. The extracts were decanted, filtered and let to dry in waterbath set at 40ºC in an airflow cabinet (IVID). Quantification of the dried extracts was performed and it was observed that the yield of extracts obtained were very low. Acetone and methanol extracted 0.01g of dried extracts from 2.5g of *A. digitata* powder which was the lowest yield of extracts among the individual extracts. The extracts were redissolved and screened for biological activities against the microorganisms under study. It was observed that water, methanol and acetone extracts of *G. incanum* exhibited antimicrobial activities against *S. aureus* at a concentration as low as 80 mg/ml. Bioassay of *M. indica, R. communis, B. pilosa, A. digitata* and *C. papaya* did not show biological activities against microorganisms under study.

To overcome the challenge, the extraction procedure was slightly modified as explained in section 3.4.2. The modification includes:

- Ratio of solvent to dry weight powder
- Time of extraction

4.3 Preparation of medicinal plant materials

Medicinal plants under study were collected as stated in section 3.2.1 and 100g of fresh leaves of *G. incanum, M. indica, R. communis, B. pilosa* and *C. papaya* were weighed on a digital scale. The fresh leaves were allowed to dry at room temperature on a laboratory bench. The samples were reweighed to determine the weight loss and percentage weight loss. Weighing of dry *G. incanum* leaves showed a decrease of 21.09g which represented 21.09% water loss. A reduction in weight of 68.20g was noted in dry *M. indica* leaves.

Water constituted 68.20%. A decrease in weight of 76.00g was observed in dry *C. papaya* leaves representing 76% of water loss. *R. communis* lost 67.80g. Water comprises 67.80% for *R. communis* leaves . A weight loss of 77.20g was noted in dry *B. pilosa* leaves representing 77.20% of water loss. The results on drying and weighing of the medicinal plants are summarised in Table 4.1.

Table 4.1: Summary of results on drying of medicinal plant samples

NAME OF PLANT SAMPLE	INITIAL WEIGHT	DATE WEIGHED	MODE OF DRYING	DATE SAMPLES REWEIGHED	WEIGHT OF DRIED SAMPLES	WEIGHT LOSS	% WEIGHT LOSS
G. incanum Leaves	100.00 g	20 August 2015	Room Temperature	30 August 2015	78.91 g	21.09 g	21.09
M. indica Leaves	100.00 g	27 August 2015	Room Temperature	4 September 2015	31.80 g	68.20 g	68.20
C. papaya Leaves	100.00 g	27 August 2015	Room Temperature	4 September 2015	24.00 g	76.00 g	76.00
R. communis Leaves	100.00 g	27 August 2015	Room Temperature	4 September 2015	32.20 g	67.80 g	67.80
B. pilosa Leaves	100.00 g	27 August 2015	Room Temperature	4 September 2015	22.80 g	77.20 g	77.20
A.digitata Powder	-	-	-	-	-	-	-

4.4 Results for extraction of medicinal plant samples

4.4.1 Results for extraction of individual medicinal plant samples

The extraction procedure for all individual extracts for each extractant was performed five times. Quantification of extracts was done each time extraction procedure was performed. Mean weight and mean percentage yield of extracts were calculated and recorded. Results for mean weight, mean percentage yield and mean concentration of individual extracts are summarised in appendix B.

In the present study aqueous extracts of *R. communis, C. papaya* and *B. pilosa* had the highest mean weights in comparison to methanol and acetone extracts. Aqueous extracts of *G. incanum* and *M. indica* had lower mean weight in comparison to methanolic extracts. Methanol extracted higher mean weights in *G. incanum, M. indica, R. communis, B. pilosa* and *C. papaya* compared to acetone. Acetone yielded the lowest mean weights of dry extracts in *G. incanum, M. indica, B. pilosa, R. communis* and *C. papaya* compared to water and methanol. Initially 10g of *A. digitata* dry powder yielded insufficient extracts of approximately 0.03g using methanol and acetone. Following some modifications as

explained in section 3.4.2, sufficient yields were obtained and it was noted that the mean weight and mean extraction efficiency of methanolic extract of *A. digitata* was higher in comparison to acetone extract. Approximately 3 ml to 3.5 ml of DMSO was used to redissolve completely the individual dry aqueous extracts. Around 2.5 ml to 3 ml of DMSO was required to reconstitute the individual dry organic extracts of methanol and acetone.

The mean weight for all dry individual aqueous extracts ranged from 2.13g to 4.90g while the mean percentage yield ranged from 21.3% to 49.0%. Highest yield of dry aqueous extracts was 4.90g from *C. papaya* with a mean percentage yield of 49.0%. The mean concentration of aqueous extract of *C. papaya* used for screening of antimicrobial activities on the microorganisms under study was 271 mg/ml. Water extracted a mean weight of 4.89g from a dry powder of *A. digitata* with a mean percentage yield of 48.9%. Screening of biological activities in aqueous extract of *A. digitata* using the microorganisms under study was performed at a mean concentration of 283 mg/ml. *B. pilosa* yielded mean weight of 3.40g of dry aqueous extract representing 34.0% mean percentage yield. Bioassay of aqueous extract of *B. pilosa* using the microorganisms under study was performed at a mean concentration of 307 mg/ml. Dry aqueous extract of *R. communis* had a mean weight of 3.33g with a mean extraction efficiency of 33.3%. Antimicrobial screening of *R. communis* aqueous extract was carried out at a mean concentration of 303 mg/ml. Water extracted 2.82g mean weight of *G. incanum* representing 28.2% mean percentage yield. The lowest mean weight of dry aqueous extract was 2.13g from *M. indica* with a mean percentage yield of 21.3%. Bioassay of *G. incanum* and *M. indica* aqueous extracts using the microorganisms under study was performed at mean concentrations of 208 mg/ml and 349 mg/ml respectively.

All dry individual methanolic extracts had a mean weight ranging from 1.53g to 3.52g with a mean percentage yield ranging from 15.3% to 35.2%. *G. incanum* yielded a mean weight of 3.52g and a mean percentage yield of 35.2%. Bioassay of *G. incanum* methanolic extract on the microorganisms under study was performed at a mean concentration of 315 mg/ml. Methanol extracted a mean weight of 2.30g of dry extract of *M. indica* with mean percentage yield of 23%. Screening of biological activities of methanolic extract of *M. indica* on the microorganisms under study was performed at a mean concentration of 288 mg/ml. Dry methanolic extract of *R. communis* had a mean

weight of 1.73g with a mean percentage yield of 17.3%. Sceening for antimicrobial activities of *R. communis* methanolic extract was done at a mean concentration of 348 mg/ml. *C. papaya* yielded a mean weight of 1.53g of dry methanolic extract representing a mean percentage yield of 15.3%. Screening of biological activities of methanolic extract of *C. papaya* on the microorganisms under study was performed at a mean concentration of 179 mg/ml. In the present study, methanolic extract of *B. pilosa* had a mean weight of 1.73g with a mean percentage yield of 17.3%. *A. digitata* methanol extract yielded a mean weight of 7.86g with a mean percentage yield of 15.7%. Antimicrobial screening of methanolic extracts of *B. pilosa* and *A. digitata* against the microorganisms under study was performed at mean concentrations of 225 mg/ml and 371 mg/ml respectively.

In the present study mean weight of dry acetone extracts ranged from 0.45g to 1.48g with a mean percentage range of 2.9% to 14.8%. A mean weight of 1.48g of acetone extract was obtained from G. *incanum* with a mean percentage yield of 14.8%. Assessment of antimicrobial properties of *G. incanum* acetone extract on the microorganisms under study was performed at a mean concentration of 301 mg/ml. *M*. *indica* yielded a mean weight of 1.42g with a mean percentage yield of 14.2%. Bioassay of acetone extract of *M. indica* using the microorganisms under study was undertaken at a mean concentration of 282 mg/ml. Acetone extracted a mean weight of 0.76g of *R. communis* and the mean percentage yield was 7.6%. The reconstituted acetone extract of *R. communis* was screened for antimicrobial activities against the microorganisms under study at a mean concentration of 244 mg/ml. Dry acetone extract of *C. papaya* had a mean weight and mean percentage yield of 0.67g and 6.7% respectively. The acetone extract of *C. papaya* was reconstituted at a mean concentration of 221 mg/ml for screening of biological activities against the microorganisms under study. The acetone extract of *B. pilosa* had a mean weight of 0.45g with a mean percentage yield of 4.5%. Following reconstitution, bioassay against the microorganisms under study was undertaken at a concentration of 277 mg/ml. *A. digitata* acetone extract yielded a mean weight of 1.45g with a mean percentage yield of 2.9%. Bioassay of acetone extract of *A. digitata* fruit pulp against microorganisms under study was undertaken at a mean concentration of 290 mg/ml.

4.4.2 Results for extraction of combined medicinal plant samples

For the combination studies, extraction procedure for each combined preparation and extractant was repeated five times. Quantification of extracts, calculation of mean weight and mean percentage yields were performed as in individual plant extracts. The mean weight for all dry combined aqueous extracts ranged from 0.91g to 5.69g while the mean percentage yields ranged from 9.1% to 56.9%. Dry methanolic extracts had a mean weight ranging from 0.91g to 2.40g with a mean percentage yield ranging from 9.1% to 24%. All dry acetone extracts had a mean weight ranging from 0.41g to 1.22g with a mean percentage yield ranging from 4.1% to 12.2%.

All combined aqueous extracts had a mean concentration ranging from 180 mg/ml to 393 mg/ml. The mean concentration of all combined methanolic extracts ranged from 219 mg/ml to 616 mg/ml. All combined acetone extracts had a mean concentration ranging from 171mg/ml to 354 mg/ml. Approximately 3 ml to 3.5 ml were required to dissolve completely the dry combined aqueous extracts. Around 2.5 ml to 3 ml of DMSO was required to reconstitute the dry combined methanolic and acetone extracts.

Aqueous mixture of *A. digitata*: *R. communis* yielded a mean weight and a mean extraction efficiency of 3.99g and 39.9%. The aqueous extract for *A. digitata*: *R. communis* combination was screened for antimicrobial properties against the microorganisms under study at a concentration of 343 mg/ml. Methanolic extract of *A. digitata: R. communis* combination had a mean weight of 2.4g with a mean percentage yield of 24.0% respectively. Antimicrobial screening of the combined methanolic extract of *A. digitata*: *R. communis* against the microorganisms under study was undertaken at a mean concentration of 303 mg/ml. Dry acetone extract of *A. digitata*: *R. communis* combination had a mean weight of 0.56g and a mean percentage yield of 5.6%. Bioassay of acetone extract of *A. digitata*: *R. communis* combination against the microorganisms under study was undertaken at a mean concentration of 220 mg/ml. Among the three extractants used in *A. digitata*: *R. communis* combination, water yielded the highest mean weight of extract with the highest mean percentage yield followed by methanol. Acetone extract of *A. digitata: R. communis* had the lowest mean weight and mean percentage yield.

Aqueous extract of *A. digitata*: *M. indica* combination had a mean weight of 3.38g and a mean percentage yield of 33.8%. Bioassay of aqueous extract of *A. digitata*: *M. indica* combination using the microorganisms under study was carried out at a mean concentration of 298 mg/ml. Methanol extracted a mean weight of 1.60g from *A*. *digitata: M. indica* combination with a mean percentage yield of 16%. The dry methanolic extract of *A. digitata*: *M. indica* combination was screened for biological activities using the microorganisms under study at a mean concentration of 349 mg/ml. Acetone extracted a mean weight of 0.41g from *A. digitata*: *M. indica* combination with a mean percentage yield of 4.1%. Bioassay of the combined acetone extract of *A. digitata*: *M. indica* using the bacterial and fungal pathogens under study was carried out at a mean concentration of 252 mg/ml. *A. digitata*: *M. indica* combination had a highest mean weight and mean percentage yield in aqueous extract followed by methanol extract. The lowest mean weight and mean percentage yield of *A. digitata*: *M. indica* combination was observed in acetone extract.

Water extracted a mean weight of 3.19g from the combination of *A. digitata*: *B*. *pilosa.* In this study, the mean percentage yield of aqueous extract of *A. digitata*: *B. pilosa* combination was 31.9%. The aqueous extract of *A. digitata*: *B. pilosa* was reconstituted for antimicrobial screening against the microorganisms under study at a mean concentration of 311 mg/ml. Methanol extracted a mean weight of 1.70g from *A. digitata*: *B. pilosa* combination with a mean percentage yield of 17%. Biological activities for the methanolic extract of *A. digitata*: *B. pilosa* combination against the microorganisms under study was performed at a mean concentration of 262 mg/ml. Acetone yielded a mean weight of extract of 0.51g from *A. digitata*: *B. pilosa* combination representing a mean percentage of 5.1%. The mean concentration at which antimicrobial screening of acetone extract of *A. digitata*: *B. pilosa* was undertaken was 171 mg/ml. Water demonstrated the highest mean weight of extract and mean extraction efficiency in *A. digitata*: *B. pilosa* combination in comparison to methanol and acetone. Acetone had the lowest mean weight of extract and mean extraction efficiency in comparison to water and methanol.

For the aqueous extract of *A. digitata*: *C. papaya* combination, a mean weight and a mean percentage yield of 3.29g and 32.9% were obtained. To perform antimicrobial screening against microorganisms under study, the dry extract of *A. digitata*: *C. papaya* was

solubilised at a mean concentration of 343 mg/ml. Methanol extracted a mean weight of 1.99g with a mean percentage yield of 19.9% from *A. digitata*: *C. papaya* combination. The dry methanolic extract of *A. digitata*: *C. papaya* was resuspended for biological screening against the microorganisms under study at a mean concentration of 251 mg/ml. Acetone yielded a mean weight of 0.55g and a mean percentage yield of 5.5% from a combination of *A. digitata: C. papaya*. Resuspension of dry acetone extract of *A. digitata*: *C. papaya* combination for antimicrobial screening activities against microorganisms under study was undertaken at a mean concentration of 172 mg/ml. Water exhibited a highest mean weight and mean percentage yield in *A. digitata*: *C. papaya* combination compared to methanol and acetone. Acetone proved to be the extracting solvent with lowest mean weight and mean percentage yield in *A. digitata*: *C. papaya* combination in comparison to water and methanol.

Water yielded a mean weight and a mean percentage yield of 5.69g and 56.9% from a mixture of *A. digitata*: *G. incanum*. The mean concentration of 271 mg/ml was adopted for screening of biological activities against the microorganisms under study. Methanol extracted 2.16g mean weight with a mean percentage yield of 21.6% from *A. digitata*: *G. incanum* combination. Screening of biological activities from a methanolic extract of a mixture of *A. digitata*: *G. incanum* against the microorganisms under study was performed at a mean concentration of 359 mg/ml. Acetone extracted a mean weight of 0.80g representing 8% of mean dry powder weight of a combination of *A. digitata*: *G. incanum*. Mean concentration of 231 mg/ml of *A. digitata*: *G. incanum* combination was adopted for screening of biological activities against microorganisms under study. Water extract of *A. digitata*: *G. incanum* had the highest mean weight and mean yield percentage compared to methanol and acetone extracts. Acetone extract of *A. digitata*: *G. incanum* combination demonstrated lowest mean weight and mean percentage yield compared to water and methanol extracts.

For the aqueous extract of *R. communis*: *G. incanum* combination, mean weight and mean percentage yield were 4.10g and 41% respectively. The dry aqueous extract of a mixure of *R. communis*: *G. incanum* was resuspended at a mean concentration of 254 mg/ml. Bioassay of reconstituted aqueous extract of a combination of *R. communis: G. incanum* using the microorganisms under study was undertaken at a mean concentration

of 254 mg/ml. In the present study methanolic extract of *R. communis*: *G. incanum* combination had a mean weight of 2.39g with a mean percentage yield of 23.9%. Dry methanolic extract of a mixture of *R. communis*: *G. incanum* was dissolved at a mean concentration of 301 mg/ml for biological screening against microorganisms under study. Acetone extract of *R. communis*: *G. incanum* combination had a mean weight of 1.21g representing a percentage yield of 12.1%. Bioassay of acetone extract of *R. communis*: *G. incanum* combination using the microorganisms under study was undertaken at a mean concentration of 214 mg/ml. Water extract of *R. communis*: *G. incanum* combination exhibited highest mean weight and mean percentage yield in comparison to methanol and acetone. Among the extracting solvents used in *R. communis*: *G. incanum* combination, acetone displayed the lowest mean weight and mean percentage yield.

In the present study, aqueous extract of a mixture of *B. pilosa*: *G. incanum* had a mean weight of 3.30g and a mean percentage yield of 33.0%. The screening of biological activities on aqueous extract of a mixture of *B. pilosa*: *G. incanum* against the selected bacterial and fungal pathogens under study was carried out at a mean concentration of 234 mg/ml. Methanol extracted a mean weight of 1.72g representing a mean percentage yield of 17.2%. To determine the antimicrobial activities of methanolic extract of *B. pilosa*: *G. incanum* combination against the selected bacterial and fungal pathogens under study, the extract was redissolved at a mean concentration of 261 mg/ml. Mean weight and mean percentage yield of 1.22g and 12.2% were obtained from the acetone extract of *B. pilosa*: *G. incanum* combination. Screening of antimicrobial activities from acetone extract of a combination of *B. pilosa*: *G. incanum* was employed at a mean concentration of 245 mg/ml. Water extract of *B. pilosa*: *G. incanum* demonstrated the highest mean weight and mean percentage yield followed by methanol extract. Acetone extracted the lowest mean weight and mean percentage yield from a mixture of *B. pilosa*: *G. incanum* compared to water and methanol.

Water yielded a mean weight of 1.80g from a mixture of *R. communis*: *M. indica* representing a mean percentage of 18%. Dry aqueous extract of *R. communis*: *M. indica* combination was reconstituted for screening of biological activities against the bacterial and fungal pathogens under study at a mean concentration of 180 mg/ml. The methanolic extract from a mixture of *R. communis*: *M. indica* yielded a mean weight of 1.85g

representing a mean percentage yield of 18.5%. Reconstitution of the dry methanolic extract of *R. communis*: *M. indica* combination for antimicrobial screening against the selected bacterial and fungal pathogens under study was adopted at a mean concentration of 313 mg/ml. Acetone extracted a mean weight and a mean percentage yield of 0.81g and 8.1% from a mixture of *R. communis*: *M.* indica. The extract was reconstituted for antimicrobial screening against the selected bacterial and fungal pathogens at a mean concentration of 250 mg/ml. Methanol exhibited the highest mean weight and mean percentage yield from a mixture of *R. communis*: *M. indica* compared to water and acetone. Acetone extracted the lowest mean weight and mean percentage yield from the mixture of *R. communis*: *M. indica* compared to water and methanol.

For the dry aqueous extract of the combination of *R. communis*: *B. pilosa*, a mean weight of 1.60g representing a mean percentage yield of 16% was obtained. To screen antimicrobial activities against the selected pathogens, the dry aqueous extract of *R. communis*: *B.pilosa* combination was reconstituted at a mean concentration of 239 mg/ml. In the present study, methanolic extract from a mixture of *R. communis*: *B. pilosa* had a mean weight of 1.98g with a mean percentage yield of 19.8%. The methanolic extract of *R. communis*: *B. pilosa* was screened for biological properties using the bacterial and fungal pathogens under study at a mean concentration of 230 mg/ml. Acetone extracted a mean weight and a mean percentage yield of 0.86g and 8.6% from a mixture of *R. communis*: *B. pilosa*. Acetone extract from a mixture of *R. communis*: *B. pilosa* was screened for biological properties using the bacterial and fungal pathogens under study at a concentration of 240 mg/ml. Methanol extracted highest mean weight and mean percentage yield from the mixture of *R. communis*: *B. pilosa* seconded by water. Acetone extract of *R. communis*: *B. pilosa* combination had the lowest mean weight and mean percentage yield compared to water and methanol extracts.

After extracting a mixture of *G. incanum*: *M. indica* using water, a mean weight and a mean percentage yield of 3.50g and 35% were obtained. The dry aqueous extract of *G. incanum*: *M. indica* combination was resuspended and screened for biological properties using the bacterial and fungal pathogens under study at a mean concentration of 271 mg/ml. Methanol extracted a mean weight and a mean percentage yield of 1.60g and 16% respectively. The methanolic extract of a mixture of *G. incanum*: *M. indica* was

reconstituted and screened for biological activities against the selected bacterial and fungal pathogens at a mean concentration of 616 mg/ml. Acetone extracted a mean weight of 1.05g and a mean percentage yield of 10.5% from *G. incanum*: *M. indica* combination. A mean concentration of 354 mg/ml of acetone extract of *G. incanum*: *M. indica* combination was used for the screening of antimicrobial properties against the microorganisms under study. Water extracted the highest mean weight and mean percentage yield in *G. incanum: M. indica* combination followed by methanol. Acetone extracted the lowest mean weight and mean percentage yield of extract in *G. incanum*: *M. indica* combination compared to water and methanol.

For the dry aqueous extract of *B. pilosa*: *M. indica* combination, a mean weight and a mean percentage yield of 1.02g and 10.2% were obtained. Screening of *B. pilosa*: *M. indica* combination for antimicrobial properties against the bacterial and fungal pathogens under study was performed at a mean concentration of 199 mg/ml. Extraction of a mixture of *B. pilosa*: *M. indica* using methanol yielded a mean weight of 1.14g and mean percentage yield of 11.4%. The methanol extract of a mixture of *B. pilosa*: *M. indica* was screened for biological activities against microorganisms under study at a mean concentration of 299 mg/ml. Acetone extracted a mean weight and a mean percentage yield of 0.74g and 7.4% from a mixture of *B. pilosa*: *M. indica*. Antimicrobial properties of acetone extract for the combination of *B. pilosa*: *M. indica* were screened against the bacterial and fungal pathogens under study at a mean concentration of 266 mg/ml. Methanol demonstrated the highest mean weight and mean percentage yield in *B. pilosa: M. indica* combination followed by water and acetone. Acetone extracted the lowest mean weight and mean percentage yield in *B. pilosa: M. indica* combination compared to methanol and water.

Water extract of *C. papaya*: *M. indica* combination yielded a mean weight and a mean percentage yield of 1.11g and 11.1%. A mean concentration of 293 mg/ml was prepared from a dry aqueous extract of a mixture of *C. papaya*: *M. indica* for bioassay using the microorganisms under study. Methanol extracted a mean weight of 1.42g with a mean percentage yield of 14.2%. Reconstitution of the dry methanolic extract of the mixture of *C. papaya*: *M. indica* was performed at a mean concentration of 261 mg/ml for bioassay against the selected bacterial and fungal isolates under study. Acetone extracted a mean

weight of 0.80g with a mean percentage yield of 8% from a mixture of *C. papaya*: *M. indica*. Acetone extract of *C. papaya*: *M. indica* combination was screened for biological activities against bacterial and fungal pathogens under study at a mean concentration of 278 mg/ml. Methanol extracted the highest mean weight and mean percentage yield from a mixture of *C. papaya*: *M. indica* followed by water. Acetone extract from a mixture of *C. papaya*: *M. indica* demonstrated the lowest mean weight and mean percentage yield compared to water and methanol extracts.

Aqueous extract of a mixture of *C. papaya*: *G. incanum* demonstrated a mean weight of 3.43g and a mean percentage yield of 34.3%. Screening of biological activities in extract of a mixture of *C. papaya*: *G. incanum* was undertaken at a mean concentration of 251 mg/ml. The methanolic extract of *C. papaya*: *G. incanum* exhibited a mean weight of 1.99g with a mean percentage yield of 19.9%. The dry methanol extract of a mixture of *C. papaya*: *G. incanum* was resuspended at a mean concentration of 230 mg/ml for antimicrobial screening against the microorganisms under study. Acetone extract of a mixture of *C. papaya: G. incanum* had a mean weight of 1.21g and a mean percentage yield of 12.1%. Bioassay of the acetone extract of *C. papaya*: *G. incanum* combination was undertaken using the bacterial and fungal pathogens under study at a mean concentration of 232 mg/ml. Water extract of *C. papaya: G. incanum* combination displayed a highest mean weight and mean percentage yield followed by methanol extract. A combination of *C. papaya: G. incanum* had the lowest mean weight and mean percentage yield in acetone extract compared to water and methanol extracts.

Water extracted a mean weight and a mean percentage yield of 1.71g and 17.1% from a mixture of *C. papaya*: *R. communis*. A mean concentration of 393 mg/ml of an aqueous extract of *C. papaya*: *R. communis* was adopted for screening of antimicrobial activities using the microorganisms under study. Methanol extract of *C*. *papaya*: *R. communis* had a mean weight and a mean extraction efficiency of 2.21g and 22.1% respectively. The dry methanolic extract of *C. papaya*: *R. communis* combination was reconstituted for antimicrobial screening using microorganisms under study at a mean concentration of 219 mg/ml. Acetone extract yielded a mean weight and a mean percentage yield of 0.95g and 9.5%. Resuspension of acetone extract of a mixture of *C. papaya*: *R. communis* for bioassay using the bacterial and fungal isolates under study was done at a mean

concentration of 221 mg/ml. Methanol extract from a mixture of *C. papaya*: *R. communis* demonstrated the highest mean weight and mean percentage yield compared to water and acetone extracts. Acetone extract from a mixture of *C. papaya*: *R. communis* had the lowest mean weight and mean percentage yield compared to water and methanol.

Dry aqueous extract of *C. papaya*: *B. pilosa* had a mean weight of 0.91g and a mean percentage yield of 9.1%. The aqueous extract of a mixture of *C. papaya*: *B. pilosa* was screened for biological activities against the microorganisms under study at a mean concentration of 322 mg/ml. Methanol extracted a mean weight of 1.54g representing a mean yield of 15.4% from a mixture of *C. papaya*: *B. pilosa*. The methanolic extract of a mixture of *C. papaya*: *B. pilosa* was screened for biological activities against the bacterial and fungal isolates under study at a mean concentration of 242 mg/ml. Acetone extract from a mixture of *C. papaya*: *B. pilosa* had a mean weight and a mean percentage yield of 0.88g and 8.8% respectively. Acetone extract from *C. papaya*: *B. pilosa* combination was screened for biological activities against microorganisms under study at a mean concentration of 184 mg/ml. Methanolic extract from a combination of *C. papaya*: *B. pilosa* had the highest mean weight and mean percentage yield followed by aqueous extract. Acetone extract from a mixture of *C. papaya*: *B. pilosa* had the lowest mean weight and mean percentage yield compared to aqueous and methanolic extracts.

The dry aqueous extract for combination of all six selected medicinal plants yielded 1.71g with a mean percentage yield of 17.1%. Screening of antimicrobial properties using microorganisms under study was carried out at a mean concentration of 331 mg/ml. Methanolic extract for combination of all six medicinal plants had a mean weight of 0.91g and a mean percentage yield of 9.1%. Screening for antimicrobial activities from the methanolic extract of six medicinal plant combination against microorganisms under study was undertaken at a mean concentration of 272 mg/ml. Acetone extracted a mean weight of 0.55g from a combination of all six selected medicinal plants representing a mean percentage yield of 5.5%. The acetone extract of a combination of all six selected medicinal plants was dissolved at a mean concentration of 243 mg/ml. For the combination of all six medicinal plants, water proved to be most efficient by extracting the highest mean weight and mean percentage yield followed by methanol. Acetone had the lowest mean weight and mean percentage of yield in a combination of all six medicinal

plants compared to water and methanol. The results for mean weight, mean percentage yield and mean concentration for all 16 medicinal plant combinations are summarised in figures 4.1 - 4.9.

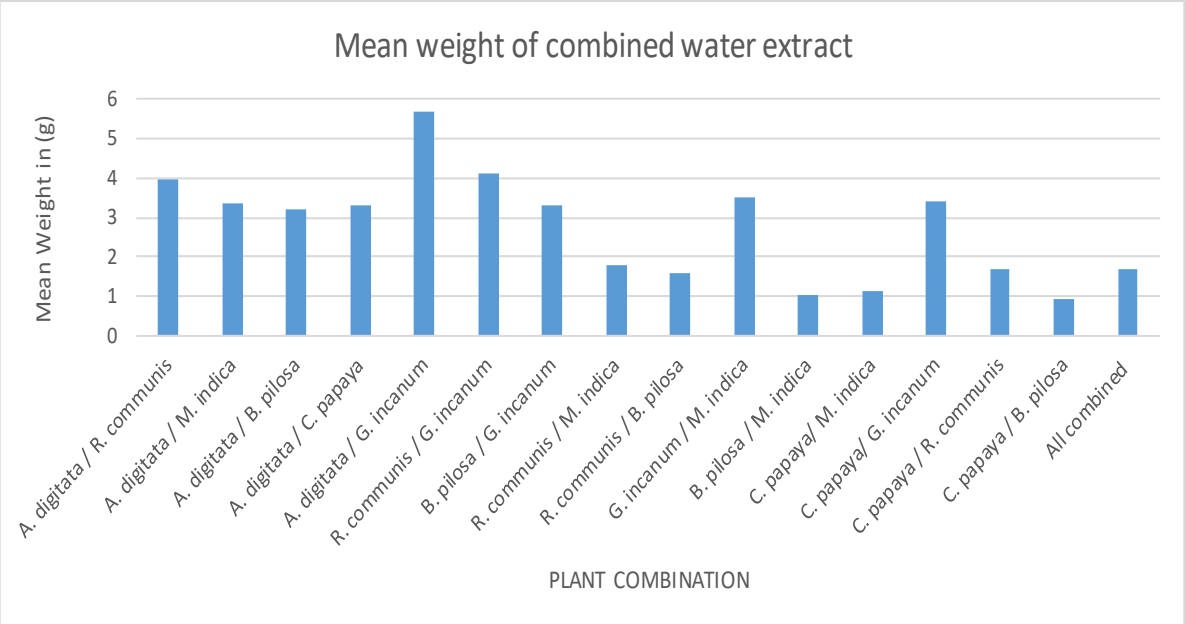


Figure 4.1: Graph showing the mean weight of combined water extracts

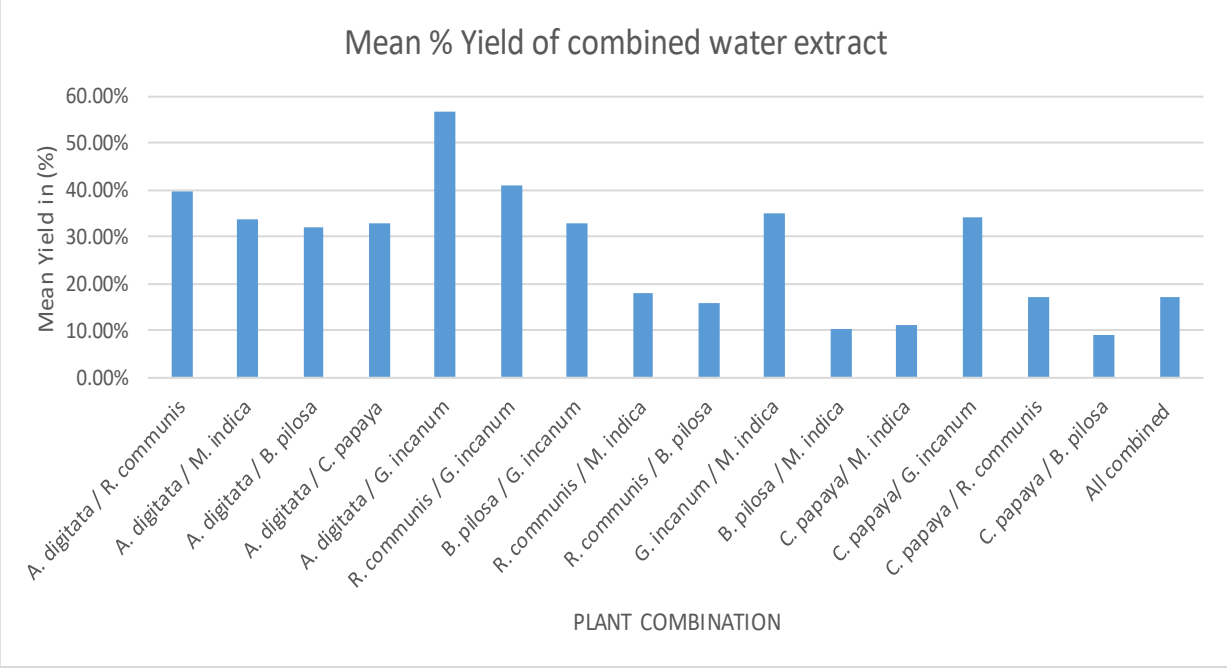


Figure 4.2: Graph showing the mean percentage yield of combined water extracts

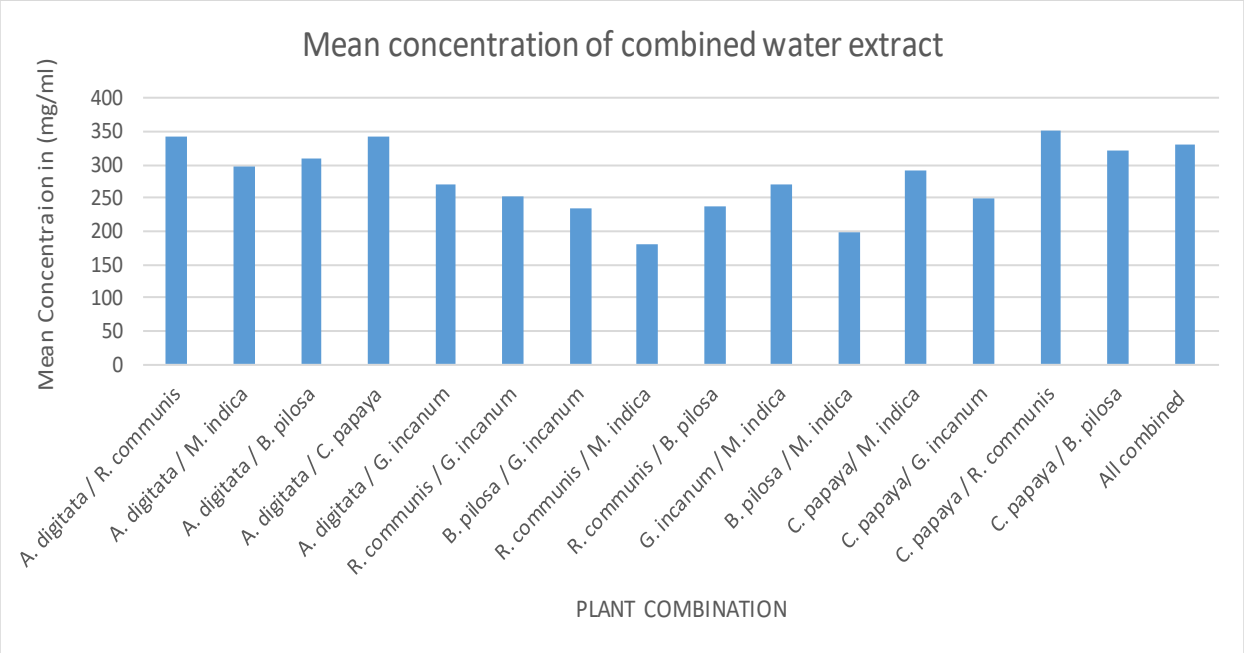


Figure 4.3: Graph showing the mean concentration of combined water extracts

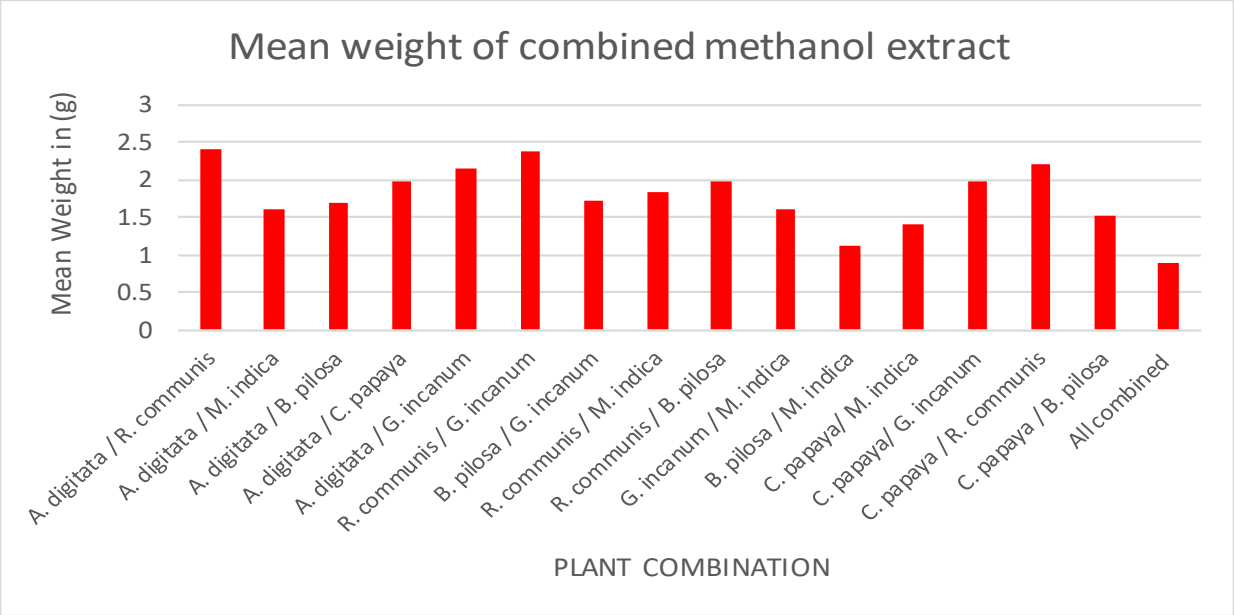


Figure 4.4: Graph showing the mean weight of combined methanol extracts

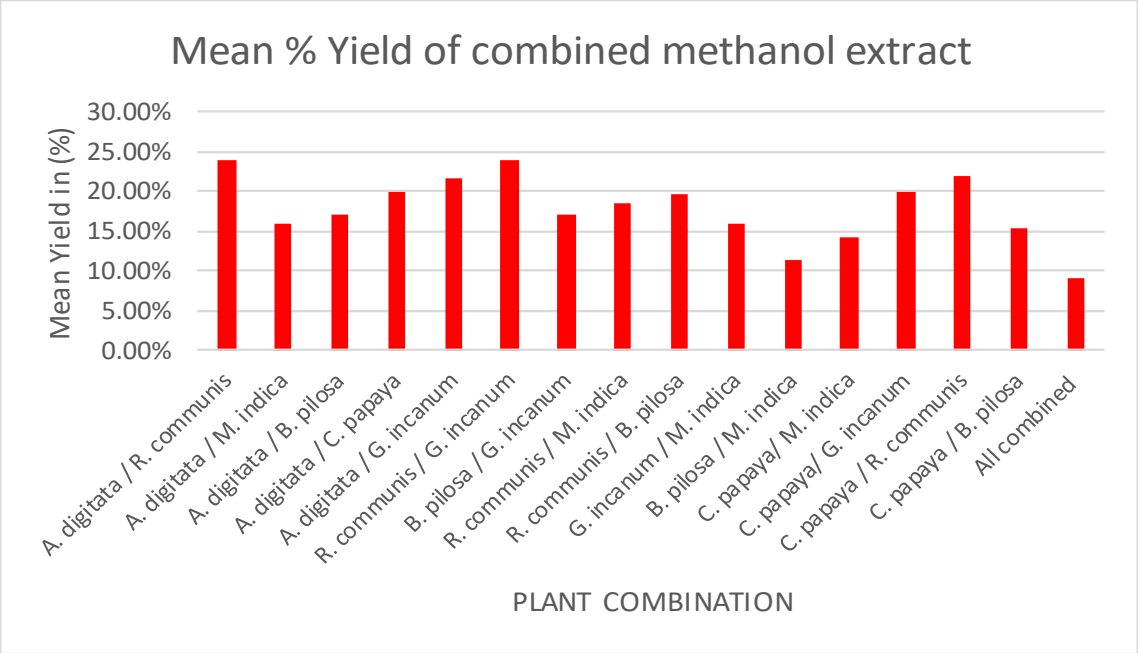


Figure 4.5: Graph showing the mean percentage yield of combined methanol extracts

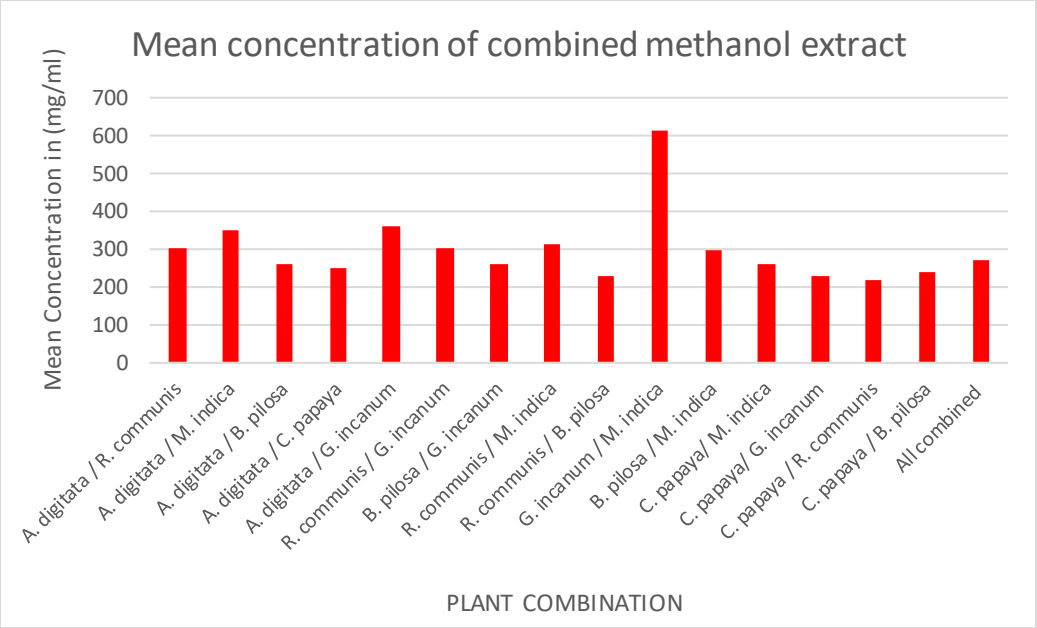


Figure 4.6: Graph showing the mean concentration of combined methanol extracts

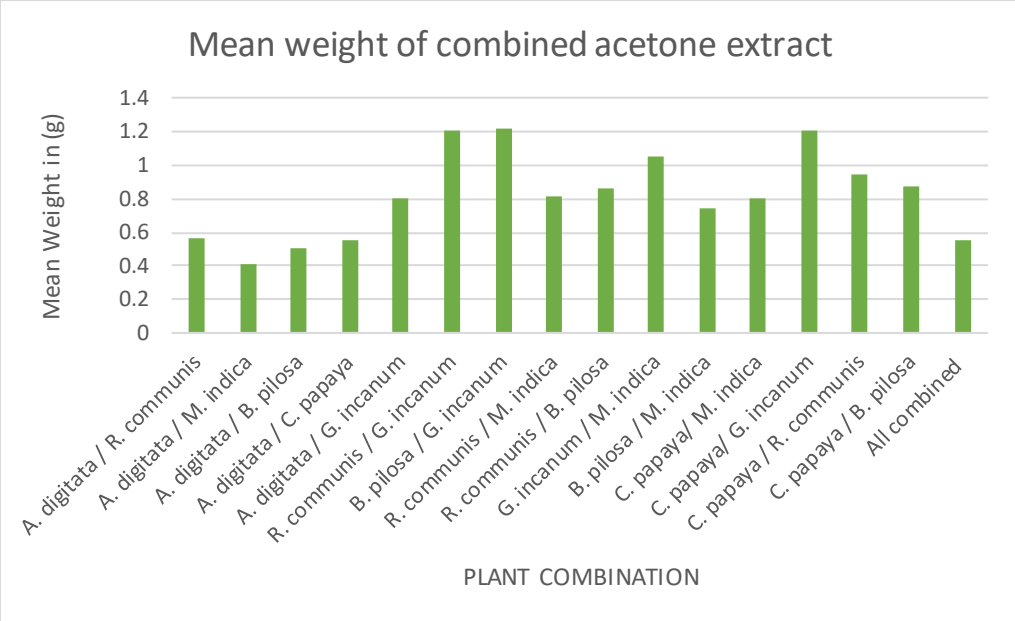


Figure 4.7: Graph showing the mean weight of combined acetone extracts

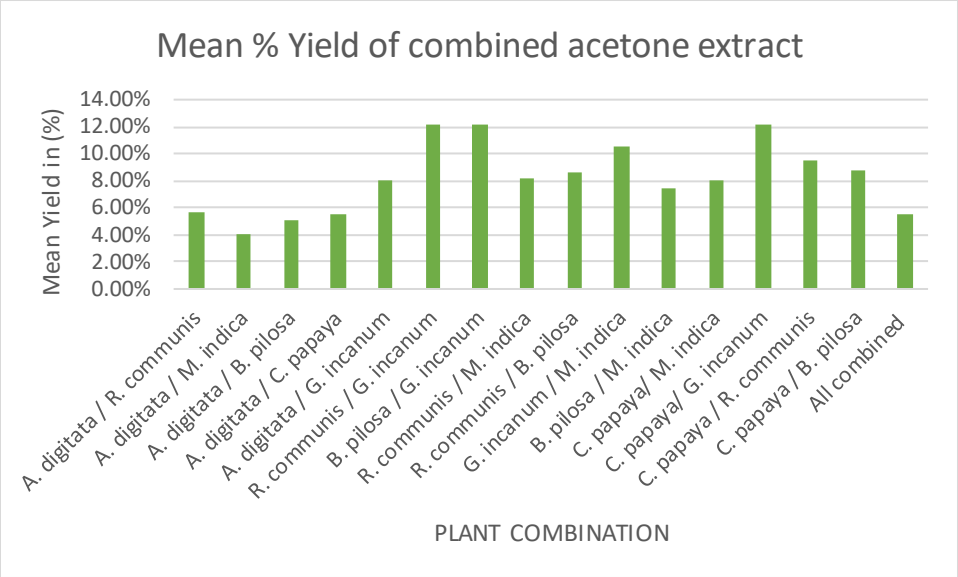


Figure 4.8: Graph showing the mean percentage yield of combined acetone extracts

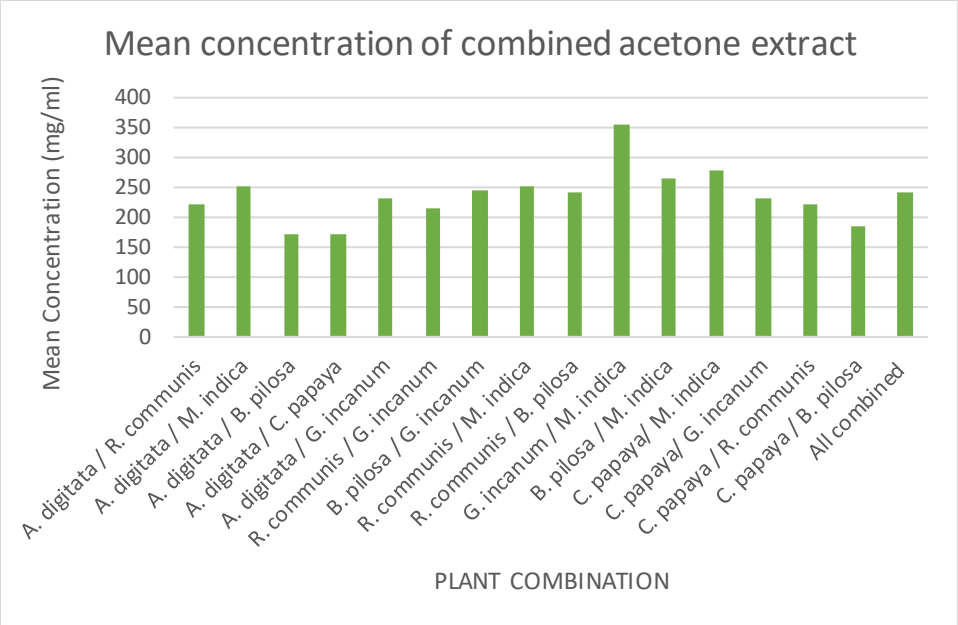


Figure 4.9: Graph showing the mean concentration of combined acetone extracts

4.5 Screening

In the present study, antibacterial screening of dry grounded leaf extracts of *G. incanum, M. indica, R.communis, B. pilosa*, *C. papaya* and *A. digitata* fruit powder was performed using four bacterial strains of methicillin and oxacillin resistant *S. aureus* (ATCC 43300), *P. aeruginosa* (ATCC 27853), *S. typhimurium* (ATCC 49416) and *E. coli* (ATCC 35218) representing Gram positive and Gram negative bacteria. One standard strain of *C. albicans* (ATCC 10231) and one clinical strain of *C. neoformans* were included to determine the antifungal activity. Antibacterial and antifungal activities in a combined preparation as discussed in section 3.4.3 was also assessed. Water, methanol and acetone were used as extractants. As Kamineth (2005) puts it, screening was performed to identify antibacterial and antifungal activities for further testing to establish the MIC's of the individual plant extracts and the combined plant extracts.

For the bacterial positive control, 100 µl of Ciprofloxacin solution (SIGMA-ALDRICH) at a concentration of 5 µg/ml displayed a strong inhibition against the bacterial strains under study. For the fungal positive control, Amphotericin B (SIGMA-ALDRICH) at a concentration of 20 µg/ml demonstrated a strong antifungal activity against the fungal strains under study. Sterile distilled water, methanol, acetone and 10% DMSO were used as negative controls. In the present study no inhibition of bacterial and fungal growth were

noted in the culture plates where negative controls were used. The DMSO at a concentration of 10% showed no growth inhibition against bacterial and fungal strains under study. Results for antimicrobial screening for individual and combined plant extracts are summarised as the average of three experiments replicated five times in appendices C and D.

4.5.1 Results for antimicrobial screening on individual plant extracts

When the plant extracts were diffusing from the agar well into the agar, round zones of colouration were noted in the agar ranging from small to very large as the agar was taking on the pigmentation of the extracts. The colouration of the zones were brown, dark brown and black depending on the colour of the medicinal plant extract. The colouration of the agar was strongest proximal to the agar well and gradually faded away as the extracts diffused distally from the agar well. In the present study, it is fascinating to observe that all extracts of *C. papaya* did not demonstrate antimicrobial activities against all microorganisms under study.

S. aureus was strongly inhibited by water, methanol and acetone extracts of *G. incanum* and *M. indica*. Aqueous and methanol extracts of *A. digitata* exhibited antimicrobial activities against *S. aureus*. Aqueous extract of *B. pilosa* displayed slight inhibition against *S. aureus*. Methanol extract of *B. pilosa* demonstrated biological activities against *S. aureus*. However, acetone extracts of *A. digitata* and *B. pilosa* demonstrated the highest antibacterial properties against *S. aureus*. Antibacterial activities of *R. communis* against *S. aureus* were exhibited in water, methanol and acetone extracts.

All extracts of *G. incanum* and *M. indica* displayed antibacterial properties against *P. aeruginosa*. However, the strongest inhibition of *G. incanum* and *M. indica* extracts against *P. aeuruginosa* were noted in methanol and acetone extracts. All extracts of *A. digitata* demonstrated antibacterial activity against *P. aeruginosa*. The strongest antibacterial activity of *A. digitata* on *P. aeruginosa* was displayed in acetone extract. Aqueous extracts of *B. pilosa* and *R. communis* displayed antibacterial activity against *P. aeuruginosa*. Nevertheless, methanol extract of *R. communis* demonstrated slight inhibition against *P. aeruginosa* with no antibacterial properties from acetone extract. No antibacterial activities of methanol and acetone extracts of *B. pilosa* were displayed against *P. aeruginosa.*

Aqueous extract of *G. incanum* demonstrated antibacterial activity against *S. typhimurium*. No antibacterial activity against *S. typhimurium* were exhibited from methanol and acetone extracts of *G. incanum*. Aqueous and Methanol extracts of *M. indica* displayed antibacterial properties against *S. typhimurium*. No antibacterial properties of acetone extract of *M. indica* were demonstrated on *S. typhimurium*. All extracts of *A. digitata* inhibited *S. typhimurium*. Aqueous extracts of *B. pilosa* and *R. communis* inhibited *S. typhimurium*. No antibacterial activities were displayed by acetone and methanol extracts of *B. pilosa* and *R. communis* against *S. typhimurium*.

All extracts of *A. digitata* displayed antibacterial activity on *E. coli*. Aqueous extracts of *G. incanum*, *M. indica*, *B. pilosa* and *R. communis* inhibited *E. coli*. No antibacterial activities were displayed on *E. coli* by methanol and acetone extracts of *G. incanum*, *M. indica*, *B. pilosa* and *R. communis*.

All extracts of *G. incanum* inhibited *C. albicans*. All extracts of *M. indica*, *A. digitata* and *R. communis* did not inhibit *C. albicans*. Aqueous and acetone extracts of *B. pilosa* showed biological properties on *C. albicans*. However, methanol extract of *B. pilosa* did not exhibit biological activities on *C. albicans*.

All extracts of *G. incanum* demonstrated biological activities on clinical strain of *C. neoformans*. All extracts of *B. pilosa* demonstrated biological activities on *C. neoformans* with strongest inhibition on acetone extract and slight inhibition on methanol extract. All extracts of *M. indica*, *A. digitata* and *R. communis* did not demonstrate inhibitory activities on *C. neoformans*.

4.5.2 Results for antimicrobial screening on combined plant extracts

In the present study screening of biological activities using combined plant extracts was perfomed on the microorganism under study. Surprisingly all methanol and acetone extracts of combined plant mixtures displayed zones of colouration of the plant extracts with no antibacterial activities on *S. typhimurium* and *E. coli*.

All extracts of a mixture of *A. digitata*: *R. communis* inhibited *S. aureus*. A combination of *A. digitata*: *R. communis* demonstrated antibacterial activities on *S. aureus* with the strongest inhibition on methanol and acetone extracts. Aqueous and methanol extracts of a mixture of *A. digitata*: *C. papaya* demonstrated slight inhibition on *S. aureus*. No

antibacterial activities were noted on acetone extract of *A. digitata*: *C. papaya*. Acetone extract of *A. digitata*: *G. incanum* combination exhibited strongest inhibition on *S. aureus*. All extracts of a mixture of *A. digitata*: *B. pilosa* displayed antibacterial activities with a strongest inhibition on acetone extract. All extracts of the mixtures of *R. communis*: *G. incanum, B. pilosa*: *G. incanum, R. communis*: *M. indica, G. incanum*: *M. indica, B. pilosa*: *M. indica, C. papaya*: *M. indica, C. papaya*: *G. incanum* demonstrated strongest inhibition on *S. aureus*. Extracts of a combination of *R. communis*: *B. pilosa* inhibited *S. aureus*. However aqueous extract of a combination of *R. communis*: *B. pilosa* showed slight inhibition on *S. aureus*. Extracts of a mixture of *C. papaya*: *B. pilosa* inhibited *S. aureus* with methanol extract demonstrating a slight inhibition. *S. aureus* was inhibited by extracts of a combination of *C. papaya*: *R. communis* with methanol and acetone extracts displaying a slight inhibition. Extracts of a mixture of all six medicinal plants inhibited *S. aureus* with methanol and acetone extracts displaying strongest inhibition.

In the present study, acetone extract of a combination of *A. digitata*: *M. indica* exhibited strong inhibition on *P. aeruginosa* with slight inhibition on aqueous and methanol extracts. Aqueous extract of a mixture of *A. digitata*: *C. papaya* showed inhibition on *P. aeruginosa*. However, no inhibition of methanol and acetone extracts of a combination of *A. digitata*: *C. papaya* was displayed on *P. aeruginosa*. All extracts of a mixture of *A. digitata*: *G. incanum* displayed antibacterial activities on *P. aeruginosa*. Nevertheless, acetone extract of a combination of *A. digitata*: *G. incanum* showed strongest inhibition on *P. aeruginosa* with water extract displaying slight inhibition. Aqueous extract of *A. digitata*: *B. pilosa* combination inhibited *P. aeruginosa* with no inhibition on methanol and acetone extracts. Aqueous extract of a mixture of *R. communis: B. pilosa* showed slight inhibition on *P. aeruginosa*. However, no inhibition of *P. aeruginosa* was displayed by methanol and acetone extracts of a combination of *R. communis: B. pilosa.* All extracts of a combination of *R. communis*: *G. incanum* displayed antibacterial activities on *P. aeuginosa* with methanol extract displaying slight inhibition. Antibacterial properties were noted on all extracts of a mixture of *R. communis: M. indica* with aqueous extract showing slight inhibition. All extracts of a combination of *B. pilosa*: *M. indica* showed inhibition of growth on *P. aeruginosa*. All extracts of a combination of *B. pilosa*: *G. incanum* demonstrated antibacterial activity on *P. aeruginosa*. Nevertheless, methanol extract of a

combination of *B. pilosa: G. incanum* exhibited slight inhibition on *P. aeruginosa*. All extracts of a mixture of *C. papaya*: *M. indica* inhibited *P. aeruginosa* with aqueous extract demonstrating slight inhibition. Aqueous extract of a mixture of *C. papaya: R*. *communis* showed slight inhibition on *P. aeruginosa*. No inhibition of *P. aeruginosa* was exhibited by methanol and acetone extracts of a mixture of *C. papaya*: *R. communis.* No antibacterial properties were noted on *P. aeruginosa* on all extracts of a mixture of *C. papaya*: *B. pilosa*. *P. aeruginosa* was inhibited by all extracts of *C. papaya: G. incanum* combination but aqueous and methanol extracts demonstrated slight inhibition. All extracts of a combination of six medicinal plants under study demonstrated antibacterial properties on *P. aeruginosa*.

In the present study it was interesting to note that methanol and acetone extracts of all plant combinations under study exhibited no antibacterial activity on *S. typhimurium* with exception of some aqueous extracts. *S. typhimurium* was inhibited by aqueous extracts of mixtures of *A. digitata: G. incanum, R. communis*: G*. incanum, B. pilosa*: *G. incanum, R. communis: M. indica, G. incanum*: *M. indica, B. pilosa*: *M. indica, C. papaya*: *M. indica, C. papaya*: *B. pilosa, C. papaya*: *R. communis, C. papaya*: *G. communis.* Aqueous extracts displaying no antibacterial activities on *S. typhimurium* were *A. digitata*: *R. communis*, *A. digitata*: *M. indica*, *A. digitata*: *C. papaya*, *A. digitata*: *B. pilosa, R. communis*: *B. pilosa.* Aqueous extract of a mixture of six medicinal plants under study exhibited antibacterial activities on *S. typhimurium*.

Extracts of plant combination exhibiting no antibacterial activity on *E. coli* were *A. digitata*: *M. indica*, *A. digitata*: *C. papaya, A. digitata: G. incanum, A. digitata: B. pilosa, R. communis*: *B. pilosa.* Aqueous extracts of medicinal plant combinations of *A. digitata: R. communis*, *R. communis*: *G. incanum, B. pilosa*: *G. incanum, R. communis*: *M. indica, G. incanum*: *M. indica, B. pilosa*: *M. indica, C. papaya*: *M. indica, C. papaya: B. pilosa, C. papaya*: *R. communis, C. papaya*: *G. incanum* demonstrated antibacterial activities against *E. coli*. However, aqueous extract of a combination of *A. digitata: R. communis* displayed a slight inhibition on *E. coli*. Aqueous extract of a combination of all six medicinal plants under study showed antibacterial activity on *E. coli* with exception of methanol and acetone extracts.

Extracts of plant combinations exhibiting no antifungal activities on *C. albicans* were *A. digitata*: *R. communis*, *A. digitata*: *M. indica, A. digitata*: *C. papaya, C*. *papaya: R. communis, R. communis: M. indica* and *C. papaya: M. indica*. Organic extracts of a combination of *A. digitata*: *G. incanum* displayed antifungal activity on *C. albicans* with methanol extract exhibiting slight inhibition. No inhibition of *C. albicans* was demonstrated by the aqueous extract of *A. digitata*: *G. incanum.* All extracts of a mixture of *A. digitata*: *B*. *pilosa* displayed antifungal activities on *C. albicans* with aqueous extract displaying the strongest activity. All extracts of a mixture of *R. communis*:*G. incanum* showed antifungal activity on *C. albicans*. However, a strongest activity of a combination of aqueous extract of *R. communis: G. incanum* was demonstrated on *C. albicans*. All extracts of a mixture of *C. papaya*: *G*. *incanum* displayed antifungal activity on *C. albicans* with a strongest activity on methanol extract. Methanol and acetone extracts of the combination of *B. pilosa*: *M. indica* inhibited *C. albicans*. Furthemore, *C. albicans* were inhibited by all extracts of a mixture of *B. pilosa*: *G*. *incanum* with a strongest inhibition on aqueous and methanol extracts. *C. albicans* demonstrated resistance against acetone and aqueous extracts of a combination of *R. communis*: *B. pilosa*. Methanol extract of a combination of *R. communis: B. pilosa* displayed antifungal activity on *C. albicans*. All extracts of a mixture of *G. incanum*: *M. indica* showed antifungal activity on *C. albicans* with acetone extract displaying a slight inhibition. Aqueous extract of *C. papaya: B. pilosa* did not exhibit antifungal activity on *C. albicans*, however, methanol and acetone extracts of *C. papaya: B. pilosa* displayed antifungal activity on *C. albicans.* All extracts of a mixture of six medicinal plants under study demonstrated antifungal activity on *C. albicans.*

In the present study aqueous and organic extracts of plant combinations demonstrating no antifungal activities on *C. neoformans* were *A. digitata: R. communis, A. digitata: M. indica, A. digitata: C. papaya, C. papaya: R. communis.* Aqueous and organic extracts of plant combinations exhibiting antifungal activities on *C. neoformans* were *A. digitata: B. pilosa, R. communis: G. incanum, B. pilosa: G. incanum, G. incanum: M. indica, C. papaya: G. incanum.* Organic extracts of *R. communis: B. pilosa* showed inhibition of growth on *C. neoformans* with acetone extract demonstrating slight inhibition. However, *C. neoformans* demonstrated resistance against aqueous extract of a mixture of *R. communis: B. pilosa.* Acetone extract of *R. communis: M. indica* exhibited slight inhibition

on *C. neoformans.* No inhibition of growth was displayed on *C. neoformans* by aqueous and methanol extracts of a mixture of *R. communis: M. indica.* Acetone and methanol extracts of *A. digitata: G. incanum* showed antifungal activities on *C. neoformans.* Nevertheless, acetone extract of a mixture of *A. digitata: G. incanum* displayed slight inhibition on *C. neoformans*. Acetone extract of a mixture of *C. papaya: M. indica* demonstrated activity on *C. neoformans.* However, no antifungal activity of aqueous and methanol extracts of a mixture of *C. papaya: M. indica* was noted on *C. neoformans.* Methanol and acetone extracts of a mixture of *C. papaya: B. pilosa* inhibited *C. neoformans.* No antifungal activity was displayed on *C. neoformans* by aqueous extract of a mixture of *C. papaya: B. pilosa.* Acetone extract of a combination of all six medicinal plants inhibited the growth of *C. neoformans*. However, *C. neoformans* demonstrated resistance against aqueous and methanol extracts of a combination of all six medicinal plants.

4.6 Minimum inhibitory concentration

Turnidge, Ferraro and Jorgensen (2011, p. 1116) believe that MIC is a basic laboratory measurement of the activity of antimicrobial compounds against microbial isolates. Minimum inhibitory concentration is defined as the lowest concentration of antimicrobial agent that would inhibit growth of a standard inoculum of microorganisms after appropriate incubation under defined in-vitro conditions (Turnidge, Ferraro & Jorgensen, 2011, p. 1116: Ochei & Kolhatkar, 2000, p. 801).

In the present study, MIC determination for individual and combined plant extracts was performed using INT microtitre plate as described by Eloff (1998b). A 96 well microtitre plate was used and 100 µl of a suspension of plant extracts at an initial concentration of 100 mg/ml were serially diluted using MHB. Ciprofloxacin (SIGMA-ALDRICH) and Amphotericin B solution (SIGMA-ALDRICH) were employed as positive controls for the bacterial and fungal strains under study. For the bacterial positive controls, 100 µl of suspension of Ciprofloxacin at an initial concentration of 72 µg/ml was serially diluted using MHB. For the fungal positive control, 100 µl of suspension of Amphotericin B at an initial concentration of 40 µg/ml was serially diluted using SDB.

4.6.1 Results for minimum inhibitory concentration for individual plant extracts

The results for MIC determination for individual extracts are summarised in table 4.2. All extracts of *M. indica* had the lowest MIC value of 0.39 mg/ml against *S. aureus*. The highest MIC value of 50 mg/ml against *S. aureus* was obtained from aqueous extract of *B. pilosa*. Methanol and acetone extracts of *G. incanum* had an MIC value of 0.781 mg/ml against *S. aureus*. The aqueous extract of *G. incanum* had an MIC value of 1.562 mg/ml against *S. aureus*. Aqueous extract of *R. communis* displayed an MIC of 0.781 mg/ml against *S. aureus*. However, methanol and acetone extracts of *R. communis* had an MIC of 3.125 mg/ml against *S. aureus*. The MIC assays of aqueous and methanol extracts of *A. digitata* exhibited an MIC value of 12.5 mg/ml against *S. aureus*. An MIC value of 6.25 mg/ml was obtained from acetone extract of *A. digitata*. All extracts of *A. digitata* and *B. pilosa* had high MIC values against *S. aureus* compared to all extracts of *G. incanum, M. indica* and *R. communis*. Acetone extracts of *A. digitata* and *B. pilosa* had lower MIC values against *S. aureus* compared to aqueous and methanol extracts of *A. digitata and B. pilosa*. Acetone extract of *B. pilosa* exhibited a promising and encouraging MIC value against *S. aureus* as compared to aqueous extract of *B. pilosa*. Methanol and acetone extracts of *R. communis* had lower MIC values against *S. aureus* compared to methanol and acetone extracts of *A. digitata*. Methanol and acetone extracts of *G. incanum* inhibited *S. aureus* with lower MIC's compared to methanol and acetone extracts of *R. communis, A. digitata* and *B. pilosa.*

The acetone extract of *M. indica* had the lowest MIC value of 0.39 mg/ml against *P. aeruginosa*. The MIC values for acetone extracts of *G. incanum* and *A. digitata* against *P. aeruginosa* were 0.781 mg/ml and 6.25 mg/ml respectively. Methanol extract of *G. incanum* had an MIC value of 1.562 mg/ml against *P. aeruginosa*. The highest MIC values (25 mg/ml) against *P. aeruginosa* were observed from methanol extracts of *M. indica*, *R. communis* and aqueous extract of *A. digitata*. The MIC values for methanol extract of *A. digitata* and aqueous extract of *B. pilosa* against *P. aeruginosa* were 12.5 mg/ml and 3.125 mg/ml respectively. The aqueous extracts of *G. incanum, M. indica* and *R. communis* demonstrated an MIC value of 1.562 mg/ml against *P. aeruginosa*.

In the present study methanol extract of *M. indica* and aqueous extract of *R. communis* had the lowest MIC values of 0.781 mg/ml against *S. typhimurium*. Aqueous extract of *B.*

pilosa had the highest MIC value of 25 mg/ml against *S. typhimurium*. Aqueous extracts of *G. incanum* and *M. indica* had MIC values of 1.562 mg/ml against *S. typhimurium*. Aqueous and methanol extracts of *A. digitata* inhibited *S. typhimurium* at an MIC value of 12.5 mg/ml. In addition, acetone extract of *A. digitata* had an MIC value of 3.125 mg/ml against *S. typhimurium*.

A lowest and highest MIC against *E. coli* was 1.562 mg/ml from aqueous extract of *M. indica* and 50 mg/ml from aqueous extract of *B. pilosa*. The MIC results of aqueous and methanol extracts of *A. digitata* against *E. coli* was 6.25 mg/ml. Nevertheless, the acetone extract of *A. digitata* had an MIC value of 3.125 mg/ml against *E. coli.* Aqueous extract of *R. communis* inhibited *E. coli* at an MIC value of 3.125 mg/ml.

The lowest and highest MIC values against *C. albicans* were 0.781 mg/ml and 12.5 mg/ml displayed by acetone extracts of *B. pilosa* and *G. incanum*. Aqueous extract of *B. pilosa* inhibited *C. albicans* at an MIC value of 6.25 mg/ml. The MIC value for aqueous and methanol extracts of *G. incanum* against *C. albicans* were 3.125 mg/ml and 1.562 mg/ml.

Acetone and methanol extracts of *B. pilosa* had the lowest MIC values of 0.39 mg/ml against *C. neoformans*. Aqueous extract of *G. incanum* displayed the highest MIC value of 6.25 mg/ml on *C. neoformans*. In the MIC assay of aqueous extract of *B. pilosa, C. neoformans* was inhibited at an MIC value of 0.781 mg/ml. The MIC results for methanol and acetone extracts of *G. incanum* against *C. neoformans* were 3.125 mg/ml and 1.562 mg/ml

Table 4.2: Minimum inhibitory concentration (MIC) for individual plant extracts

PLANT	EXTRACT	*S. aureus* ATCC 43300	*P. aeruginosa* ATCC 27853	*S. typhimurium* ATCC 49416	*E. coli* ATCC 35218	*C. albicans* ATCC 10231	*C. neoformans* clinical strain
G. incanum	**Aqueous**	1.562 mg/ml	1.562 mg/ml	1.562 mg/ml	3.125 g/ml	3.125 mg/ml	6.25 mg/ml
	Methanol	0.781 mg/ml	1.562 mg/ml	-	-	1.562 mg/ml	3.125 mg/ml
	Acetone	0.781 mg/ml	0.781 mg/ml	-	-	12.5 mg/ml	1.562 mg/ml
M. indica	**Aqueous**	0.39 mg/ml	1.562 mg/ml	1.562 mg/ml	1.562 mg/ml	-	-
	Methanol	0.39 mg/ml	25 mg/ml	0.781 mg/ml	-	-	-
	Acetone	0.39 mg/ml	0.39 mg/ml	-	-	-	-
R. communis	**Aqueous**	0.781 mg/ml	1.562 mg/ml	0.781 mg/ml	3.125 mg/ml	-	-
	Methanol	3.125 mg/ml	25 mg/ml	-	-	-	-
	Acetone	3.125mg/ml	-	-	-	-	-
A. digitata	**Aqueous**	12.5 mg/ml	25 mg/ml	12.5 mg/ml	6.25 mg/ml	-	-
	Methanol	12.5 mg/ml	12.5 mg/ml	12.5 mg/ml	6.25 mg/ml	-	-
	Acetone	6.25 mg/ml	6.25 mg/ml	3.125 mg/ml	3.125 mg/ml	-	-
B. pilosa	**Aqueous**	50 mg/ml	3.125 mg/ml	25 mg/ml	50 mg/ml	6.25 mg/ml	0.781 mg/ml
	Methanol	25 mg/ml	-	-	-	-	0.39 mg/ml
	Acetone	1.562 mg/ml	-	-	-	0.781 mg/ml	0.39 mg/ml
C. papaya	**Aqueous**	-	-	-	-	-	-
	Methanol	-	-	-	-	-	-
	Acetone	-	-	-	-	-	-
Controls	**Ciprofloxacin**	0.562 µg/ml	0.281 µg/ml	0.070 µg/ml	0.281 µg/ml	NA	NA
	Amphotericin B	NA	NA	NA	NA	1.25 µg/ml	1.25 µg/ml

MIC is given as the average of three experiments replicated five times

Key: - Resistant NA Not applicable

4.6.2 Results for minimum inhibitory concentration for combined plant extracts

In the MIC studies of combined plant extracts, the lowest MIC value of 1.562 mg/ml against *S. aureus* was obtained in five combined acetone extracts, one combined aqueous extract and one combined methanol extract. The five combined acetone extracts that displayed a lowest MIC value of 1.562 mg/ml against *S. aureus* were *A. digitata*: *M. indica, B. pilosa*: *M. indica, G. incanum*: *M. indica, R. communis*: *M. indica*, *C. papaya*: *M. indica.* Similarly, the lowest MIC value of 1.562 mg/ml against *S. aureus* was obtained from a combined methanol extract of *B. pilosa*: *M. indica*. The highest MIC result of 50 mg/ml against *S. aureus* were obtained from aqueous extracts of combinations of *A. digitata*: *R. communis, A. digitata*: *B. pilosa, C. papaya*: *B. pilosa*.

The MIC results of combined plant extracts indicated the lowest MIC value of 3.125 mg/ml against *P. aeruginosa* in seven combined acetone extracts and one methanol combined plant extracts. The seven combined acetone plant extracts that showed the lowest MIC value of 3.125 mg/ml against *P. aeruginosa* were *A. digitata*: *M. indica*, *A. digitata*: *G. incanum*, *R. communis*: *G. incanum*, *B. pilosa: M. indica, G. incanum: M. indica, R. communis: M. indica, C. papaya: M. indica.* Furthermore, methanol extract of a combination of *C. papaya*: *M. indica* demonstrated the lowest MIC value of 3.125 mg/ml against *P. aeruginosa*. Five aqueous plant combinations that displayed the highest MIC value of 50 mg/ml against *P. aeruginosa* were *A. digitata*: *R. communis*, *A. digitata: C. papaya, A. digitata*: *B. pilosa, R. communis*: *M. indica* and *C. papaya: M. indica*.

The MIC assays of combined plant extracts demonstrated the lowest MIC value of 0.781 mg/ml in two combined aqueous extracts against *S. typhimurium.* The two aqueous combined extracts that showed the lowest MIC value (0.781 mg/ml) against *S. typhimurium* were *R. communis*: *M. indica* and *G. incanum*: *M. indica*. The aqueous extract of a combination of all six medicinal plants under study displayed the highest MIC value of 12.5 mg/ml against *S. typhimurium.*

The lowest MIC value of 3.125 mg/ml against *E. coli* was obtained in three combined aqueous extracts of *B. pilosa*: *G. incanum, G. incanum*: *M. indica, C. papaya*: *R. communis*. The aqueous extract of a combination of all six medicinal plants demonstrated the highest MIC value of 12.5 mg/ml against *E. coli*. Aqueous extract of the combination

of *A. digitata: R. communis* demonstrated the highest MIC value of 50 mg/ml against *E. coli.*

The acetone extract of a combination of *A. digitata*: *B. pilosa* indicated the lowest MIC value of 0.39 mg/ml against *C. albicans*. In addition acetone extracts of the combinations of *B. pilosa: M. indica* and *B. pilosa: G. incanum* displayed an MIC value of 0.781 mg/ml against *C. albicans*. Furthermore, methanol extract of *C. papaya: G. incanum* demonstrated an MIC value of 0.781 mg/ml. The highest MIC value of 12.5 mg/ml against *C. albicans* were displayed by methanol extract of *A. digitata: G. incanum* and aqueous extract of a combination of all six medicinal plants under study.

In the present study, some combined acetone and aqueous plant extracts displayed a lowest MIC value of 0.39 mg/ml against *C. neoformans*. Three combinations of acetone extracts that demonstrated a low MIC value (0.39 mg/ml) against *C. neoformans* were *A. digitata*: *B. pilosa, B. pilosa*: *G. incanum* and *C. papaya*: *B. pilosa*. In addition, a combination of aqueous extract of *G. incanum*: *M. indica* indicated the lowest MIC value (0.39 mg/ml) against *C. neoformans*. The methanol extract of a combination of *G. incanum*: *M. indica* inhibited *C. neoformans* at a higher MIC value of 12.5 mg/ml. The MIC results for the combined plant extracts are summarised in table 4.3.

4.7 The sum of the fractional inhibitory concentration index (∑FIC)

The sum of the fractional inhibitory concentration index (∑FIC) was calculated to determine the type of antimicrobial interaction in the medicinal plant combinations. The medicinal plants were blended in 1:1 combination ratio. The ∑FIC value was calculated for each medicinal plant extract independently using the following formula (Van Vuuren & Viljoen, 2011);

$$FIC^{(i)} = \frac{\text{MIC (a) in combination with (b)}}{\text{MIC (a) independently}}$$

$$FIC^{(ii)} = \frac{\text{MIC (b) in combination with (a)}}{\text{MIC (b) independently}}$$

The letters (a) or (b) represented any of the six medicinal plants under study depending on which the two plants were combined. The ∑FIC was calculated as;

$$\sum FIC = FIC^{(i)} + FIC^{(ii)}$$

A range was used for classification of the antimicrobial interactions of the medicinal plant combinations into synergy (≤0.5), additive (>0.5-1.0), indifference (>1.0-≤4), antagonism (>4.0) (Van Vuuren & Viljoen, 2011). Van Vuuren and Viljoen (2011) highlighted that antimicrobial interactions of agents are considered to be:

a. Synergistic (≤0.5) if their combination effect is stronger than the sum of effects of the individual agents.
b. Additive (>0.5-1.0) if their combination effect improves or increases efficacy
c. Indifferent or non-interactive(>1.0-≤4) when the combination effect shows neither an additive nor antagonistic.
d. Antagonism (>4.0) if the overall combination effect is less than the sum of their individual effects

In the present study, the antimicrobial interactions ranging from synergistic to antagonistic were observed The results for the antimicrobial interactions are summarised in table 4.3.

Table 4.3: Minimum inhibitory concentration (MIC) for the combined plant extracts

PLANT COMBINATION	*S. aureus* ATCC 43300	*P. aeruginosa* ATCC 27853	*S. typhimurium* ATCC 49416	*E. coli* ATCC 35218	*C. albicans* ATCC 10231	*C. neoformans* clinical strain
A. digitata: R. communis						
Aqueous Extract	50 mg/ml (68.00)	50 mg/ml (34.00)	-	50 mg/ml (24.00)	-	-
Methanol Extract	25 mg/ml (10.00)	-	-	-	-	-
Acetone Extract	12.5 mg/ml (6.00)	-	-	-	-	-
A. digitata: M. indica						
Aqueous Extract	12.50 mg/ml (33.00)	25 mg/ml (17.00)	-	-	-	-
Methanol Extract	6.25 mg/ml (16.50)	6.25 mg/ml (0.75)	-	-	-	-
Acetone Extract	1.562 mg/ml (4.25)	3.125 mg/ml (8.50)	-	-	-	-
A. digitata: C. papaya						
Aqueous Extract	25 mg/ml (2.00)	50 mg/ml (2.00)	-	-	-	-
Methanol Extract	25 mg/ml (2.00)	-	-	-	-	-
Acetone Extract	-	-	-	-	-	-
A. digitata: G. incanum						
Aqueous Extract	6.25 mg/ml (4.50)	12.5 mg/ml (8.50)	6.25 mg/ml (4.50)	-	-	-
Methanol Extract	12.5 mg/ml (17.00)	6.25 mg/ml (4.50)	-	-	12.5 mg/ml (8.00)	12.5 mg/ml (4.00)
Acetone Extract	3.125 mg/ml (4.50)	3.125 mg/ml (4.50)	-	-	6.25 mg/ml **(0.50)**	6.25 mg/ml (4.00)

The ΣFIC values are given in brackets. Values in bold indicate combinations with synergistic activities

MIC is given as the average of three experiments replicated five times

Key

- Resistant

Table 4.3 : Minimum inhibitory concentration (MIC) for the combined plant extracts (continued)

PLANT COMBINATION	*S. aureus* ATCC 43300	*P. aeruginosa* ATCC 27853	*S. typhimurium* ATCC 49416	*E. coli* ATCC 35218	*C. albicans* ATCC 10231	*C. neoformans* clinical strain
A. digitata: B. pilosa						
Aqueous Extract	50 mg/ml (5.00)	50 mg/ml (18.00)	-	-	3.125mg/ml (**0.50)**	1.562 mg/ml **(0.25)**
Methanol Extract	25 mg/ml (3.00)	-	-	-	1.562 mg/ml	3.125 mg/ml (8.00)
Acetone Extract	25 mg/ml (20.00)	-	-	-	0.39 mg/ml **(0.50)**	0.39 mg/ml (1.00)
R. communis: G. incanum						
Aqueous Extract	6.25 mg/ml (12.00)	6.25 mg/ml (8.00)	1.562 mg/ml (3.00)	6.25 mg/ml (4.00)	1.562 mg/ml (**0.50)**	1.562 mg/ml **(0.25)**
Methanol Extract	12.50 mg/ml (20.00)	12.5 mg/ml (8.50)	-	-	3.125 mg/ml (2.00)	3.125 mg/ml (1.00)
Acetone Extract	3.125 mg/ml (5.00)	3.125 mg/ml (4.00)	-	-	3.125 mg/ml **(0.25**)	1.562 mg/ml (1.00)
B. pilosa: G. incanum						
Aqueous Extract	6.25 mg/ml (4.125)	6.25 mg/ml (6.00)	1.562 mg/ml (1.06)	3.125 mg/ml (1.06)	6.25 mg/ml (3.00)	1.562 mg/ml (2.25)
Methanol Extract	6.25 mg/ml (8.25)	6.25 mg/ml (4.00)	-	-	1.562 mg/ml (1.00)	0.781 mg/ml (2.25)
Acetone Extract	6.25 mg/ml (12.00)	6.25 mg/ml (8.00)	-	-	0.781 mg/ml (1.06)	0.39 mg/ml (1.25)
R. communis: B. pilosa						
Aqueous Extract	6.25 mg/ml (8.125)	25 mg/ml (24.00)	-	-	-	-
Methanol Extract	25 mg/ml (9.00)	-	-	-	1.562 mg/ml	1.562 mg/ml (4.00)
Acetone Extract	3.125 mg/ml (3.00)	-	-	-	-	0.781 mg/ml (2.00)

The ∑FIC values are given in brackets. Values in bold indicate combinations with synergistic activites

MIC is given as the average of three experiments replicated five times

Key:

- Resistant

Table 4.3: Minimum inhibitory concentration (MIC) for the combined plant extracts (continued)

PLANT COMBINATION	*S. aureus* ATCC 43300	*P. aeruginosa* ATCC 27853	*S. typhimurium* ATCC 49416	*E. coli* ATCC 35218	*C. albicans* ATCC 10231	*C. neoformans* clinical strain
R. communis: M. indica						
Aqueous Extract	6.25 mg/ml (24.00)	50 mg/ml (64.00)	0.781 mg/ml (1.50)	12.5 mg/ml (12.0)	-	-
Methanol Extract	3.125 mg/ml (9.00)	6.25 mg/ml **(0.50)**	-	-	-	-
Acetone Extract	1.562 mg/ml (4.50)	3.125 mg/ml (8.00**)**	-	-	-	6.25 mg/ml
B. pilosa: M. indica						
Aqueous Extract	6.25 mg/ml (16.125)	25 mg/ml (24.00)	3.125mg/ml (2.12)	12.5 mg/ml (8.25)	-	-
Methanol Extract	1.562 mg/ml (4.06)	6.25 mg/ml (**0.25)**	-	-	1.562 mg/ml	0.781 mg/ml (2.00)
Acetone Extract	1.562 mg/ml (5.00)	3.125 mg/ml (8.00)	-	-	0.781 mg/ml (1.00)	0.781 mg/ml (2.00)
G. incanum: M. indica						
Aqueous Extract	6.25 mg/ml (5.00)	25 mg/ml (32.00)	0.781 mg/ml (1.00)	3.125 mg/ml (3.00)	3.125 mg/ml (1.00)	0.39 mg/ml **(0.06)**
Methanol Extract	6.25 mg/ml (24.00)	12.5 mg/ml (8.50)	-	-	6.25 mg/ml (4.00)	12.5 mg/ml (4.00)
Acetone Extract	1.562 mg/ml (6.00)	3.125 mg/ml (12.00)	-	-	3.125 mg/ml **(0.25)**	6.25 mg/ml (4.00)
C. papaya: M. indica						
Aqueous Extract	12.5 mg/ml (32.00)	50 mg/ml (32.00)	6.25 mg/ml (4.00)	6.25 mg/ml (4.00)	-	-
Methanol Extract	6.25 mg/ml (16.00)	3.125 mg/ml **(0.125)**	-	-	-	-
Acetone Extract	1.562 mg/ml (4.00)	3.125 mg/ml (8.00)	-	-	-	1.562 mg/ml

The ΣFIC values are given in brackets. Values in bold indicate combinations with synergistic activities

MIC is given as the average of three experiments replicated five times

Key

- Resistant

Table 4.3: Minimum inhibitory concentration (MIC) for the combined plant extracts (continued)

PLANT COMBINATION	*S. aureus* ATCC 43300	*P. aeruginosa* ATCC 27853	*S. typhimurium* ATCC 49416	*E. coli* ATCC 35218	*C. albicans* ATCC 10231	*C. neoformans* clinical strain
C. papaya: R. communis						
Aqueous Extract	6.25 mg/ml (8.00)	25 mg/ml (16.00)	3.125 mg/ml (4.00)	3.125 mg/ml (1.00)	-	-
Methanol Extract	12.5 mg/ml (4.00)	-	-	-	-	-
Acetone Extract	12.5 mg/ml (4.00)	-	-	-	-	-
C. papaya: G. incanum						
Aqueous Extract	3.125 mg/ml (2.00)	25 mg/ml (16.00)	1.562 mg/ml (1.00)	6.25 mg/ml (2.00)	6.25 mg/ml (2.00)	1.562 mg/ml (1.00)
Methanol Extract	6.25 mg/ml (8.00)	6.25 mg/ml (4.00)	-	-	0.781 mg/ml (**0.50)**	3.125 mg/ml (1.00)
Acetone Extract	3.125 mg/ml (4.00)	12.5 mg/ml (16.00)	-	-	3.125 mg/ml **(0.25)**	1.562 mg/ml (1.00)
C. papaya: B. pilosa						
Aqueous Extract	50 mg/ml (1.00)	-	12.5 mg/ml **(0.50)**	25 mg/ml **(0.50)**	-	-
Methanol Extract	25 mg/ml (1.00)	-	-	-	6.25 mg/ml	1.562 mg/ml (8.00)
Acetone Extract	25 mg/ml (16.00)	-	-	-	1.562 mg/ml (2.00)	0.39 mg/ml (1.00)
All Six Plants Combined						
Aqueous Extract	25 mg/ml (114.50)	25 mg/ml (57.00)	12.5 mg/ml (33.5)	12.5 mg/ml (18.25)	12.5 mg/ml (6.00)	-
Methanol Extract	6.25 mg/ml (26.75)	6.25 mg/ml (5.00)	-	-	3.125 mg/ml (2.00)	-
Acetone Extract	3.125 mg/ml (15.50)	12.5 mg/ml (48.50)	-	-	3.125 mg/ml (4.25)	1.562 mg/ml (5.00)
Controls						
Ciprofloxacin	0.562 µg/ml	0.281 µg/ml	0.070 µg/ml	0.281 µg/ml	NA	NA
Amphotericin B	NA	NA	NA	NA	1.25 µg/ml	1.25 µg/ml

The ΣFIC values are given in brackets. Values in bold indicate combinations with synergistic activities

MIC is given as the average of three experiments replicated five times

Key: - Resistant NA Not applicable

The ∑FIC for most of the pathogens indicated antagonistic effects. However, all Gram negative bacteria and the fungal isolates under study had antimicrobial interactions worthy to be highlighted.

Methanol extracts of combinations of *R. communis*: *M. indica* (∑FIC 0.50), *B. pilosa*: *M. indica* (∑FIC 0.25), *C. papaya*: *M. indica* (∑FIC 0.125) exhibited synergistic effect with studies on *P. aeruginosa*. An additive interaction in *P. aeruginosa* was observed in methanol extract of a combination of *A. digitata*: *M. indica* (∑FIC 0.75). The greatest antagonistic effect (∑FIC 64.00) in *P. aeruginosa* was displayed in aqueous extracts of *R. communis*: *M. indica* combination.

The combination of aqueous extract of *C. papaya: B. pilosa* (∑FIC 0.50) had a synergistic antimicrobial interaction on *S. typhimurium*. Nevertheless, aqueous extracts of combinations of *G. incanum*: *M. indica* (∑FIC 1.00), *C. papaya*: *G. incanum* (∑FIC 1.00) demonstrated additive effects. The most notable antagonistic effect (∑FIC 33.5) in *S. typhimurium* was the aqueous extract of the combination of all six medicinal plants under study.

The ∑FIC of 0.50 depicting synergy was observed in aqueous extract of a combination of *C. papaya*: *B. pilosa* in *E. coli* studies. However, additive effects in *E. coli* were noted in aqueous extracts of a combination of *C. papaya*: *R*. *communis* (∑FIC 1.00*).* The largest antagonistic interaction (∑FIC 24.00) in *E. coli* was displayed on aqueous extract of a combination of *A. digitata: R. communis*.

The most interesting and promising synergistic interactions in *C. albicans* were observed in acetone extracts of combinations of *A. digitata*: *G. incanum* (∑FIC 0.50), *A. digitata*: *B. pilosa* (FIC 0.50), *R. communis*: *G. incanum* (∑FIC 0.25), *G. incanum*: *M. indica* (∑FIC 0.25), *C. papaya*: *G. incanum* (∑FIC 0.25). In addition, synergism was demonstrated in aqueous extracts of combinations of *A. digitata*: *B. pilosa* (∑FIC 0.50), *R. communis*: *G. incanum* (∑FIC 0.50). Additive interactions in *C. albicans* were demonstrated in aqueous extract of *G. incanum: M. indica* (∑FIC 1.00), methanol extract of a combination of *B. pilosa*: *G. incanum* (∑FIC 1.00), acetone extract of a combination of *B. pilosa*: *M. indica* (∑FIC 1.00). The prominent antagonistic interaction (∑FIC 8.00) in *C. abicans* was displayed in methanol extracts of *A. digitata: G. incanum*.

C. neoformans recorded the most notable synergistic effects in aqueous extracts of a combination of *G. incanum*: *M. indica* (∑FIC 0.06), *A. digitata*: *B. pilosa* (∑FIC 0.25) and *R. communis*: *G. incanum* (∑FIC 0.25). Additive interactions in *C. neoformans* were noted in all extracts of combination of *C. papaya*: *G. incanum* (∑FIC 1.00). Methanol and acetone extracts of *R. communis: G. incanum* (∑FIC 1.00) exerted additive effects in *C. neoformans* studies. Furthermore acetone extracts of combinations of *A. digitata: B. pilosa* (∑FIC 1.00) and *C. papaya: B. pilosa* (∑FIC 1.00) depicted additive effects on *C. neoformans* studies. Enormous antagonistic effects (both ∑FIC 8.00) on *C. neoformans* were observed in methanol extracts of combinations of *A. digitata*: *B. pilosa* and *C. papaya*: *B. pilosa*.

According to the results of FIC indices, most of the extracts of medicinal plant combinations displayed antagonistic effects against *S. aureus*. However, aqueous and methanol extracts of a combination of *C. papaya*: *B. pilosa* (∑FIC 1.00) exhibited additive effect in *S. aureus* studies.

In the present study some medicinal plants under study did not show antifungal activities when screened individually. The individual extracts which did not exhibit antimicrobial properties against the fungal isolates were *M. indica*, *R. communis*, *A. digitata* and *C. papaya*. No antifungal activity of the *B. pilosa* methanol extract was demonstrated against *C. albicans*. Surprisingly, when these medicinal plants were included in some combinations, favourable and enhanced activities were displayed on fungal isolates under study. Methanol extract of a combination of *R. communis*: *B. pilosa* (MIC 1.562 mg/ml), *B. pilosa*: *M. indica* (MIC 1.562 mg/ml), *C. papaya*: *B. pilosa* (MIC 6.25 mg/ml) demonstrated antifungal activity on *C. albicans*. Acetone extracts of combinations of *R. communis*: *M. indica* (MIC 6.25 mg/ml), *C. papaya*: *M. indica* (MIC 1.562 mg/ml) displayed antifungal activity on *C. neoformans*. Interestingly all combinations that included *G. incanum*, *M. indica* and *B. pilosa* were highly active compared to other combinations.

CHAPTER 5: DISCUSSION

5.1 Introduction

This chapter focuses on general discussion on the supportive facts in relation to aims and objectives of the study, literature review, practical aspects and findings of the present study.

5.2 Discussion on the research results

From time immemorial man has utilised different parts of plants for therapy and prevention of various diseases and health disorders (Ayyanar & Ignacimunthu, 2009). De la Paz, Larionova, Maceira, Barego and Echevarria (2006) highlighted the use of environmentally accepted natural products and biodegradables such as medicinal plant extracts as advantageous. Various formulations of medicinal plants have been administered by traditional healers in individual and combined preparations. Such formulations include concoctions, decoctions, infusions (Focho, Ndam & Fonge, 2009), lotions, lozenges, juices, tinctures, herbal teas, capsules, poultices and steam inhalations (Fetrow & Avila, 2000, p. 8-11).

Meningitis continues to be a feared infection due to its high morbidity, mortality and long term disability (Luksic *et al*., 2013). Globally, traditional healers have utilised medicinal plants since ancient times to treat signs and symptoms of meningitis. In the present study the antimicrobial properties of individual and combined extracts of *G. incanum, M. indica, R. communis, B. pilosa, C. papaya* and the fruit pulp of *A. digitata* were assessed by agar diffusion assay as described by Ncube *et al.* (2008) using selected bacterial and fungal strains representing Gram-positive, Gram-negative and yeast. Minimum inhibitory concentrations for all efficacious individual and combined extracts were assessed using INT microtitre plate assay described by Eloff (1998b). Sum of fractional inhibitory concentration indices (∑FIC) for all efficacious combinations were calculated and used to assess the type of antimicrobial interaction (Van Vuuren & Viljoen, 2011). Selection of the leaf parts and fruit pulp of *A. digitata* was based on the literature for their use in traditional treatment of predisposing factors of meningitis such as ear infections, managing signs and symptoms of meningitis such as headache, fever and inflammation. The bacterial and fungal strains were selected because they have been implicated in meningitis.

5.3 Preparation and extraction of the medicinal plant samples

The medicinal plants were collected from two different geographical localisations as stated in section 3.3. After drying the medicinal plant leaves, a remarkable weight loss of more than 50% was observed in leaves of *M. indica, C. papaya, R. communis,* and *B. pilosa*. A weight loss of 21.09% was noted in *G. incanum*.

In the extraction of individual medicinal plant samples, water yielded the highest mean weight of extracts in *R. communis*, *C. papaya* and *B. pilosa* compared to methanol and acetone. However, methanol extracted the highest mean weight of extract in *G. incanum* and *M. indica*. It was interesting to note that water yielded a mean weight of 4.89g from 10g of *A. digitata* fruit powder compared to methanol and acetone which extracted 7.86g and 1.45g from 50g of *A. digitata*.

In comparison to methanol and acetone, water extracted the highest mean weight of extracts in all combinations that included *A. digitata* and *G. incanum*. Among the three extractants used, methanol yielded the highest mean extracts in mixtures of *C. papaya*: *B. pilosa*, *C. papaya*: *R. communis*, *C. papaya*: *M. indica, B. pilosa*: *M. indica, R. communis*: *M. indica.*

The variations in amount of extracts obtained from the medicinal plants using the three extractants may be attributed to several factors. Anokwukuru, Anyasor, Ajibaye, Fakoya and Okebugwu (2011) highlighted that effeciency of extracting active compounds in medicinal plants depends on the plant and the extractant used. The nature of solvent, solvent concentration and polarity infuence the composition and quantity of yield extracted (Cowan, 1999). The quantity of yield extracted increases with the increasing polarity of the extractant. The highest yields demonstrated in aqueous and methanol extracts in individual and combined studies may be ascribed to increased solubility of compounds such as carbohydrates and proteins that are more soluble in water and methanol than acetone (Do, Angkawijawa, Tran-Nguyen, Huynhm, Soetaredjo, Ismadji & Ju, 2014), lectins and water soluble flavonoids such as anthocyanins (Cowan, 1999).

As stated ealier on, the medicinal plants were collected from two different geographical localisation. Geographical localisation and climatic conditions might have contributed to the highest yield of extracts in some individual and combined extracts. Medicinal plants

growing in soil rich in metallic substances may possess metallic ions or metallic compounds in their roots, bark, stem, leaves, flowers or roots (Etsuyankpa, Ndamitso, Suleman, Tijani, Idris, Shaba & Mohammed, 2013).

Etsuyankpa *et al.* (2013) reported that water soluble, heavy metallic substances were noted in aqueous extracts of *Piliostigma thonningii.* According to studies conducted by Decaluwe *et al.* (2010) *A. digitata* fruit powder contains calcium (Ca), copper (Cu), iron (Fe), potassium (K), magnesium (Mg), manganese (Mn), sodium (Na), phosphorus (P) and zinc (Zn). *C. papaya* leaves were reported to contain Ca, K, Mg, Zn, Mn and Fe (Aravind *et al*., 2013). All these water soluble metallic substances are suggested to have contributed to the highest mean weights observed in some individual and combined aqueous extracts.

5.4 Individual plant studies

In antimicrobial screening of *G. incanum* leaf extract, aqueous extracts demonstrated activity against all bacterial and fungal isolates under study. Methanol and acetone extracts of *G. incanum* exhibited activities on *S. aureus, P. aeruginosa, C. albicans* and *C. neoformans*. In the current study, screening results for aqueous, methanol and acetone extracts of *G. incanum* against *S. aureus* and *C. albicans* correlate with findings conducted by Freidberg (2009, pp. 138-140) but with contradictions on aqueous extracts against *P. aeruginosa* and *E. coli.* Agar dilution assay was employed by Freidberg (2009, p. 108) in the antimicrobial screening assays. A possible explanation on contradiction of results on aqueous extracts of *G. incanum* against *P. aeuruginosa* and *E. coli* may be ascribed to differences in methods of antimicrobial screening.

In the present study, the antimicrobial efficacy of the individual plant was assessed following a criteria which state that any medicinal plant exhibiting an MIC value below 8 mg/ml possess antimicrobial activity (Van Vuuren, 2008) and MIC values below 1.00 mg/ml are regarded as noteworthy activities (Van Vuuren, 2008; Van Vuuren, Nkwanyana & de Wet, 2015). Methanol and acetone extracts of *G. incanum* demonstrated noteworthy MIC values (both MIC value of 0.781 mg/ml) against *S. aureus. G. incanum* had the highest MIC value of 12.5 mg/ml against *P. aeruginosa*. The lowest MIC value of *G. incanum* against *C. albicans* and *C. neoformans* were displayed by the methanol and

acetone extracts (both 1.562 mg/ml). No antimicrobial properties of Methanol and acetone extracts of *G. incanum* were displayed on *S. typhimurium* and *E. coli*.

G. incanum has been reported in several studies to posses saponins and tannins (Amabeoku, 2009). Saponins contain amphiphilic compounds which can complex in biomembranes with their lipophilic terpenoid moiety and bind to surface glycoproteins and glycolipids with their sugar chain. This can result into severe tension and leakage in the biomembrane and eventually cell death. Antifungal activity was also observed in *C. albicans* and *C. neoformans* isolates under study. In addition, studies have revealed that saponins can complex with ergosterol in fungal membranes and cholesterol in animal membranes (Wink, 2015; Wink, 2008; Cowan, 1999). This leads to lysis and death of fungal cell. In view of these scientific facts, the antimicrobial activities of *G. incanum* demonstrated on the bacterial and fungal isolates under study may be ascribed to the presence of saponins and tannins.

In this study aqueous extract of *G. incanum* displayed promising antimicrobial activities in all bacterial and fungal isolates under study suggesting that this medicinal plant has a broad spectrum activities. This demonstrates that *G. incanum* can be used as a lead compound in the drug development in the pharmaceutical and drug manufacturing industries. This supports the traditional use of this plant as an anti-pyretic agent in the treatment and management of signs and symptoms of meningitis.

In antimicrobial screening studies of *M. indica* leaf, aqueous extract exhibited biological activities in all bacterial strains under study. However, lack of biological activities of methanolic extracts of *M. indica* were displayed on *E. coli, C. albicans* and *C. neoformans*. *S. typhimurium, E. coli, C. albicans* and *C. neoformans* demonstrated resistance against the acetone extracts of *M. indica* leaves. Demonstration of antimicrobial activities of acetone extract of *M. indica* leaves against *S. aureus* and *P. aeruginosa* in this study is in agreement with previous studies conducted by Bharti (2013) with contradiction on *E. coli, .* However, findings in the present study contradicts with results by Doughari and Manzara (2008) who reported lack of activity in aqueous extract of *M. indica* against *S.aureus, P. aeruginosa, and E. coli.* Nevertheless, lack of antifungal activities displayed by methanolic extract of *M. indica* on *C. albicans* in this study correlates with studies conducted by Parekh and Chanda (2008) with contradiction on *C. neoformans.*

Contradiction of results observed in the present study with previous findings from authors of other publications may be suggestive of differences in microbial strains used, methods of antimicrobial screening (Ncube *et al.,* 2008) and differences in geographical localisation from where the samples were collected (Dhami & Mishra, 2015).

All extracts of *M. indica* had a noteworthy MIC value of 0.39 mg/ml against *S. aureus*. However, *P. aeruginosa, S. typhimurium* and *E. coli* were inhibited by aqueous extract of *M. indica* at an MIC value of 1.562 mg/ml. Acetone extract of *M. indica* exhibited a noteworthy MIC value against *P. aeruginosa* (MIC value 0.39 mg/ml). Considering Van Vuuren *et al*. (2015) statement that MIC values of less than 1.00 mg/ml have clinical antimicrobial significance, it can be concluded that extract of *M. indica* leaf was significant to 50% of the pathogens under study. However, methanol extract of *M. indica* displayed the highest MIC value of 25 mg/ml against *P. aeruginosa*.

In the present study, aqueous extract of *M. indica* inhibited the growth of *S. typhimurium* and *E. coli* at an MIC value of 1.562 mg/ml. According to studies conducted by De and Pal (2014), *M. indica* inhibited *E. coli* at an MIC value of 15 mg/ml using disc diffusion assay. A study on MIC assessment conducted by Eloff (1998b) demonstrated that *Combretum molle* extracts inhibited *S. aureus* at an MIC value of 0.56 mg/ml using disk diffusion technique. An MIC value which gave 8 fold increase in activity at 0.07 mg/ml was observed when a 96 microwell plate was employed. According to Afolayan and Meyer (1997) galangin an antimicrobial compound from *Helichrysum aureonitens* displayed an MIC value between 0.5 mg/ml and 0.1 mg/ml against *S. aureus* using disk diffusion assay. However, *H. aureonitens* displayed an MIC value between 0.25 mg/ml and 0.125 mg/ml against *S. aureus* using a 96 microwell plate. Barouri, Sadiki and Ibnsouda (2016) observed that disk-diffusion assay is not appropriate for the assessment of MIC as it is impossible to quantify the amount of the antimicrobial agent diffused into the agar. The contradiction of results with findings from studies conducted by De and Pal (2014) may be suggestive of the differences in susceptibility test methods and microbial strain used.

M. indica leaves contain alkaloids, flavonoids, coumarins, cardiac glycosides, tannins, steroids (Bbosa, Kyegombe, Ogwal-Okeng, Bukenya-Ziraba, Odyek & Waako, 2007). Alkaloids have been reported to intercalate DNA (Upadhyay *et al*., 2014; Wink, 2008)

leading to impaired cell division (Upadhyay *et al.,* 2014), mutations or even cancer (Wink, 2008). Flavonoids complexes with extracellular and soluble proteins of bacterial cell wall leading to increased membrane permeability and disruption and consequently cell death (Upadhyay *et al.,* 2014; Cowan, 1999). Venugopala, Rashmi and Odhav (2013) highlighted that coumarins inhibit DNA gyrase activity. Coumarins bind to the B subunit of the DNA gyrase and inhibit DNA supercoiling by blocking the adenotriphosphatase (ATPase) activity (Maxwell, 1993). These scientific evidences justify the biological activities of *M. indica* leaves displayed on *S. aureus*, *P. aeruginosa* and *S. typhimurium*.

The low MIC value displayed by all extracts of *M. indica* against *S. aureus* (MIC value 0.39 mg/ml) and acetone extract of *M. indica* against *P. aeruginosa* (MIC value 0.39 mg/ml) may be of great significance in some treatment applications such as inhalations or topical applications (Mulyaningsih, Sporer, Zimmermann, Reichling & Wink, 2010) with regard to the traditional treatment of predisposing factors of meningitis and management of signs and symptoms of meningitis such as fever, inflammation, headache. The promising antibacterial properties displayed by *M. indica* leaf confirm its application in the traditional treatment and management of predisposing factors of meningitis such as ear infections, management and treatment of meningitis related signs and symptoms such as headache, fever. The biological properties of *M. indica* may be used to curb antimicrobial resistance against multidrug resistant organisms. Antimicrobial resistance is a global concern and it has been described as a catastrophe by medical pundits (Davies & Davies, 2010).

In the present study the antimicrobial screening of *R. communis* leaf, aqueous extract demonstrated activities on all bacterial isolates under study namely, *S. aureus*, *P. aeruginosa, S. typhimurium* and *E. coli. C. albicans*. *C. neoformans* demonstrated resistance against aqueous extract of *R. communis.* Demonstration of antibacterial properties by aqueous leaf extract of *R. communis* on *S. aureus*, *P. aeruginosa* and *E. coli* is in agreement with studies conducted by Obumselu *et al*. (2011). A possible explanation for the correlation may be that water extracted a large quantity of polar antimicrobial compounds which contributed to the inhibition of growth. However, demonstration of antimicrobial properties of aqueous leaf extract of *R. communis* in this study on *E. coli* is in contradiction with studies conducted by Mansoor *et al*. (2013). In

the present study aqueous extraction of *R. communis* leaves was performed by soaking the grounded leaves in water with constant shaking in a shaking incubator at room temperature. Mansoor *et al*. (2013) placed a mixture of the powdered *R. communis* leaves to soak at room temperature for two weeks. The contradiction of results with the present findings may be suggestive of differences in the method of extraction. According to Biavatti (2009) and Nasir, Fatima, Ahmad and Haq (2015) bioactive compounds of plants may be lost along the extraction process. Since Mansoor *et al*. (2013) performed the extraction process for two weeks it may be suggested that the bioactive constituents of medicinal plants were lost or distorted during the extraction process.

In the bacterial strains methanol extract of *R. communis* showed activity on *S. aureus* and *P. aeruginosa* but resistance was noted on *S. typhimurium* and *E. coli.* Furthermore no antifungal activities were demonstrated by methanol extract of *R. communis* against *C. albicans* and *C. neoformans.* Lack of activities displayed by methanolic extract of *R. communis* against *E. coli* contradicts with studies conducted by Malook *et al*. (2013) and Lekganyane, Matsebatlela, Howard and Masoko (2102). A study conducted by Malook *et al.* (2013) on biological activities of *R. communis* leaves collected from different locations of Pakistan, methanol extracts displayed various degrees of biological activities against *S. aureus* and *E. coli .* Contradiction of results may be attributed to differences in geographical locations from where the samples were collected. Nevertheless, further investigations of antimicrobial activities of *R. communis* leaf need to be undertaken.

On the bacterial strains under study acetone extract of *R. communis* inhibited the growth of *S. aureus* but it was interesting to note that *P. aeruginosa, S. typhimurium, E. coli,* displayed resistance against acetone extract of *R. communis* leaf. *C. albicans* and *C. neoformans* resisted against acetone extract of *R. communis*. According to studies conducted by Zarai *et al*. (2012), essential oils from fresh leaves of *R. communis* obtained by distillation and gas chromatography coupled to mass spectrometry (GC-MS) inhibited the growth of *S. aureus, P. aeruginosa,* and *E. coli*. Janssen, Scheffer and Svendsen (1987) reported that quality of the medicinal plant sample regarding freshness or being dry affect the composition of essential oil in a plant extract. Van Vuuren (2009) highlights that antimicrobial activity of volatiles obtained by distillation differ from antimicrobial activities of non-volatiles obtained by extraction. The contradiction of results with the

present study may be ascribed to differences in quality of sample used regarding freshness or being dry and differences in methods of extraction.

According to study performed by Khan and Yadav (2011) *C. albicans* demonstrated resistance against acetone extract of *R. communis* which is in agreement with this study but aqueous and methanol extracts demonstrated growth inhibition contradicting with the findings of this study. In the present study powdered *R. communis* leaves were soaked in water and left to soak at room temperature for overnight and SDA was the media of choice for screening of antifungal activities. Khan and Yadav (2011) left a mixture of *R. communis* and water to soak for three to four days and the media of choice was potato dextrose agar (PDA). Antimicrobial activity may be influenced by length of extraction time (Ncube *et al.,* 2008) agar type (Eloff,1998b) and agar composition (Noaman, Fattah, Khaleafa & Zaky, 2004). The disagreements of results may be attributed to period of extraction, agar type and agar composition. Further studies are required to establish antifungal properties of *R. communis*.

A noteworthy MIC of aqueous extract of *R. communis* was displayed on *S. aureus* and *S. typhimurium* (both MIC 0.781 mg/ml). Methanol extract of *R. communis* exhibited biological activities on *S. aureus* (MIC value 3.125 mg/ml) and *P. aeruginosa* (MIC value 25 mg/ml). Acetone extract of *R. communis* inhibited *S. aureus* at a mean concentration of 3.125 mg/ml. Zarai *et al*. (2012) reported low MIC values of essential oils of fresh leaves of *R. communis* against *S. aureus* (MIC value 150 µg/ml), *P. aeruginosa* (MIC value 270 µg/ml), *E. coli* (MIC value 240 µg/ml) and *Salmonella* (250 µg/ml). As elucidated ealier on, the higher MIC values in the present study may be attributed to differences in quality of sample used and extraction methods. Whole plant extracts containing major compounds have been found to be less active than essential oils (Van Vuuren & Viljoen, 2011). Essential oils from plants have been reported to act synergistically to produce stronger effect against microbes (Van Vuuren & Viljoen, 2011). Essential oils act as membrane permeabilisers by partitioning the bacterial cell membrane lipids. In Gram-negative bacteria, they potentiate the entry of other plant secondary metabolites to have access to their important targets such as DNA/RNA, ribosomes, proteins, receptors, ion channels (Olajuyigbe & Ashafa, 2014). The variation in the MIC values between the two studies may be attributed to differences in methods of extraction.

R. communis leaves possess, gallic acid, terpenoids of the subclasses monoterpenoids and sesquiterpenoids (Jena & Gupta, 2012), tannins (Vandita, Amin, Khyati & Monisha, 2013). Gallic acid contributes to the loss of Ca^{2+} ions from the cell leading to cell death. Chelation of metal ions may lead to loss of function of a cell (Sarjit, Wang & Dykes, 2015). Two ways have been postitulated on how gallic acid may infuence loss of Ca^{2+} ions from the cell. Lipophilic compounds may disrupt the biomembrane of a cell leading to efflux of ions from the intracellular fluid leaving behind macromolecules such as protein and nucleic acids. Examples of such ions are H^+, K^+, Na^+ and Ca^{2+}. Gallic acid may chelates with the Ca^{2+} ions leading to the loss of Ca^{2+} ions (Sarjit *et al.,* 2015). Another possibility could be the chelation of Ca^{2+} from the calcium-binding proteins from the cell surfae. Loss of Ca^{2+} from the cell can lead to cell injury or microbial death in several ways such as early degradation of DNA, modification of gene expression, interruption of cell signalling, metabolism disturbance, interference with cellular ion homoeostasis and depletion of adenosinetriphosphate (ATP) (Sarjit *et al*., 2015).

Monoterpenoids such as 1,8-Cineole, α-pinene, camphor have bee reported in *R. communis.* As elucidated ealier on terpenoids disrupts membrane permeability resulting to leakage of cell contents and eventually cell death. Tannins have been reported to complex with proteins through hydrogen bonding and hydrophobic effects as well as convalent bonding (Cowan, 1999). This result to loss of normal 3 D-structure of proteins which leads to failure of proteins to recognise their substrates, ligands or protein to protein interaction rendering the protein non-functional and eventually cell death (Wink, 2015). The mechanism of action of tannins may be associated with inactivation of microbial adhesins, enzymes, cell envelope transport protein (Wink, 2015; Cowan, 1999). These scientific facts may be ascribed to some of the inhibitory activities demonstrated by aqueous and methanol extracts of *R. communis*.

The application of the leaf of *R. communis* to the head to relieve headache (Watt & Brandwijk, 1962, p. 430) correlates with the traditional use of the plant in treatment and management of signs and symptoms of meningitis such as headache. These findings support the usefulness of *R. communis* leaves in forklore remedies in the management of signs and symptoms related to meningitis such as microbial infections. The results from the present study also confirm findings from the previous studies and reviews which

indicated that *R. communis* possesses antimicrobial properties. The results may also suggest that *R. communis* could be very promising in treating and managing predisposing factors of meningitis such as otitis media.

In the present study all extracts of fruit pulp of *A. digitata* inhibited the growth of all bacterial strains under study. The results of the present study contradicts with findings by Komolafe (2014, p. 33) who reported resistance of *S. aureus* and *E. coli* against aqueous and methanol extracts of fruit pulp of *A. digitata*. In the present study *C. albicans* and *C. neoformans* displayed resistance against all extracts of *A. digitata*. According to studies conducted by Seukep *et al*. (2013) methanol extract of fruit of *A. digitata* demonstrated weak antibacterial activities on *E. coli* and *P. aeruginosa*. Seukep *et al*. (2013) reports that the *A. digitata* fruits were bought from Bafoussam local market, western region of Cameroon. It is not known exactly when they were collected from the forest. The history relating to their collection from the forest and storage conditions remains silent. In another study conducted by Elashi (2015) *C. albicans* and *E. coli* displayed resistance against ethanolic extract of *A. digitata* fruit pulp. A possible explanation for the contradiction of results with the present study could be differences in quality of samples regarding its freshness, postharvest and storage conditions.

The aqueous, methanol and acetone extracts of *A. digitata* inhibited the growth of the bacterial strains under study at various MIC values. Acetone extract of *A. digitata* displayed a lowest MIC value of 3.125 mg/ml on *S. typhimurium* and *E. coli*. The aqueous extract of *A. digitata* indicated the highest MIC value against *P. aeruginosa* (MIC value 25 mg/ml). Methanolic extract of *A. digitata* inhibited *E. coli* at an MIC value of 6.25 mg/ml. *S. aureus, P. aeruginosa*, *S. typhimurium* were inhibited by methanolic extract of fruit pulp of *A. digitata* at an MIC value of 12.5 mg/ml. However, Sharma and Rangari (2015) reported noteworthy MIC values of methanolic extract of *A. digitata* against *S. aureus* (MIC value100 µg/ml), *P. aeruginosa* (MIC value 125 µg/ml), *E*. *coli* (62.5 µg/ml) and *C. albicans* (250 µg/ml). Sharma and Rangari (2015) defatted the fruit pulp of *A. digitata* with Petroleum ether for 24 hours followed by extraction with methanol. A possible explanation for the large variation in MIC values in the present study and a study conducted by Sharma and Rangari (2015) may be due to differences in extraction methods and microbial strains used.

The fruit pulp of *A. digitata* have been reported to possess organic acids such as citric acid, tartaric acid, malic acid, succinic acid and ascorbic acid. Afolabi and Popoola (2005) observed that an acidic medium generated by *A. digitata* fruit pulp contributed to tempe fermentation and inhibition of growth of pathogenic bacteria such as *Salmonella* species. Shokri (2011) stated that the molecular targets of organic acids and their corressponding acids are cell wall and biomembrane proteins. According to Shokri (2011) citric acid lowers the pH of microbial cell by ionisation of intact acid molecules and disruption of substrate transport by modulating biomembrane proteins or by decrease of proton motive force. As elucidated earlier on, modulation of biomembrane protein structure interferes with protein in identifying their substrates, ligands and other protein interaction partners. This renders the biomembrane protein non-functional and eventually microbial cell death. These scientific factors may be attributed to antimicrobial activities exhibited by all extracts of fruit pulp of *A. digitata* against all bacterial strains under study.

The administration of a mixture of water and fruit pulp for treatment of fever is in agreement with the traditional use of fruit pulp of *A. digitata* in management and treatment of signs and symptoms of meningitis. This indicates that *A. digitata* fruit powder may be a promising natural product in treating and managing signs and symptoms of meningitis. However, further investigations are required on the assessment of antifungal properties of *A. digitata*.

In antimicrobial screening of *B. pilosa* leaves, aqueous extract demonstrated antimicrobial activities on all bacterial and fungal strains under study but with a slight inhibition on *S. aureus*. Acetone extract of *B. pilosa* displayed strongest antimicrobial activities on *S. aureus*, *C. albicans* and *C. neoformans* with no activity on *P. aeruginosa, S. typhimurium* and *E. coli.* Methanol extract of *B. pilosa* exhibited antimicrobial activities on *S. aureus* with slight inhibition on *C. neoformans*. No antimicrobial activities were demonstrated by methanol extract of *B. pilosa* against *P. aeruginosa, S. typhimurium*, *E. coli* and *C. albicans.* In antibacterial studies of *B. pilosa* conducted by Adedapo *et al.* (2011), aqueous extract displayed biological activities on *S. aureus* which is in agreement with the present study but no activity on *E. coli* and *P. aeruginosa.* However in the same study conducted by Adedapo *et al.* (2011), acetone extract inhibited *S. aureus, E. coli* with no activity on *P. aeruginosa* and methanol extract of *B. pilosa* inhibited *E. coli* with no activity

on *S. aureus* and *P. aeruginosa*. Previously Motsei *et al*. (2003) reported antifungal activities of aqueous, ethanol, ethyl acetate and hexane extracts of *B. pilosa* leaf against *C. albicans* (ATCC 10231) and clinical strains from a 5 month-old baby and an adult. This correlate with the findings of the present study. Contradiction of results may be due to differences in microbial strain used, geographical location and purity of the extract (Van Vuureen, 2007, p. 45).

Promising MIC values of *B. pilosa* worthy to be highlighted were noted in all extracts of *B. pilosa* against *C. neoformans*. Aqueous extract of *B. pilosa* inhibited *C. neoformans* at an MIC value of 0.781 mg/ml. Growth of *C. neoformans* was suppressed by methanol and acetone extracts of *B. pilosa* at an MIC value of 0.39 mg/ml. Aqueous and methanol extracts of *B. pilosa* inhibited *C. albicans* at MIC values of 6.25 mg/ml and 0.781 mg/ml.

According to studies conducted previously*, B. pilosa* possesses polyacetylenes and numerous essential oils such as α-pinene, α-cubebene, β-linalool, β-pinene, limonene and camphene. Olajuyigbe and Ashafa (2014) highlighted that essential oils partition lipids in the microbial biomembrane rendering them more permeable leading to leakage of cellular constituents and eventually cell death.

According to studies conducted by Li, Shi, Dai, Liang, Xie, Huang, Zhao and Zhang (2016) and Tyski, Bocian, Mikucka and Grzybowska (2013) essential oils inhibit the synthesis of DNA, RNA, protein, polysaccharides and enzymatic activity. In RNA synthesis, RNA splicing is very essential for gene expression. Gene transcripts go through RNA splicing inorder to come functional mRNA with required protein coding information (Voet & Voet, 2004, p. 1257-1258). Disruption in the pathway of RNA synthesis may interfere with gene expression and other cell processes such as DNA synthesis, pyrimidine metabolism, RNA degradation, nucleotide excision repair, RNA polymerase activity, protein transport and many others (Li, *et al*., 2016). If DNA synthesis is affected, it may interfere with all microbial cell processes associated with microbial cell reproduction such as meiosis, DNA replication, cell cycle and eventually cell death (Li *et al.,* 2016). These scientific facts may have contributed to the exhibition of antimicrobial activities of *B. pilosa* on all bacterial and fungal strains under study.

The application of drops of warmed juice of the fresh plant of *B. pilosa* for earache (Watt & Brandwijk, 1962, p. 205) correlates with the traditional use of the plant in treatment and management of predisposing factors of meningitis. This validates the traditional usage of *B. pilosa* in treatment of predisposing factors of meningitis such as otitis media. These findings demonstrate that phytochemical constituents from *B. pilosa* may be utilised in synthesis of novel drugs which may be used to combat antimicrobial resistance.

In this study it was interesting to note that all bacterial and fungal isolates under study demonstrated resistance against all extracts of *C. papaya*. Aruljoth *et al*. (2014) studied and reported growth inhibitions displayed by aqueous, methanol and acetone extracts of *C. papaya* leaf against *S. aureus, P. aeruginosa* and *E. coli*. However, this contradicts with the findings of the present study. According to studies conducted by Tewari *et al.* (2014) methanol, ethylacetate and chloroform extracts of *C. papaya* leaf inhibited the growth of *E. coli, S. aureus* and *C. albicans*. Lack of activities of *C. papaya* leaf in the present study may be attributed to differences in geographical localisations and microbial strain used.

Naidoo, Van Vuuren, Van Zyl and de Wet (2013) highlighted that medicinal plants may present their mode of action in two different ways, that is, producing cidal effect to the organism or relieve signs and symptoms related to the pathology of the disease such as inflammation, fever headache and many others. According to the present study it may be suggested that *C. papaya* leaves possess phytochemicals responsible for relieving signs and symptoms related to meningitis. However, the present study was focused on biological activities and synergistic interactions of medicinal plants and it was not extended to anti-inflammatory activities of medicinal plants.

However, it cannot be ruled out in this study that *C. papaya* leaves possess phytochemicals with antimicrobial properties. Numerous studies and reviews have indicated that *C. papaya* leaves contain myricetin, kaemferol (flavonoids), linalool, 4-terpinol (monoterpenoids), carpinine, carpaine (alkaloids) and many others (Aravind *et al*., 2013). According to Semwal, Semwal, Combrinck and Viljoen (2016) myricetin has been reported to suppress DnAB helicase an enzyme responsible for DNA replication and elongation that may lead to death of a microbial cell. Biavatti (2009) highlighted that biological activities of herbal extract may be lost during preparation which may lead the

researcher to believe that the extract entirely lack activities. In addition, the loss of activity in the extracts may be due to instability of some constituents such as the volatiles (Van Vuuren, 2008) or poor handling of the extracts after reconstitution. Further investigations by controlled clinical trials need to be done as there are probabilities that in-vivo experiments may yield better results.

It was observed in this study that *S. aureus* was susceptible to most of the individual extracts compared to the Gram-negative bacteria and the fungal strains. A possible explanation for this observation may be due to the differences in the outer layers of Gram-negative and Gram positive bacteria. Gram-negative bacteria possess an outer membrane with hydrophilic surface rich in lipopolysaccharide which functions as a permeability barrier for many external hydrophobic substances such as detergents, dyes and antibiotics (Hemaiswarya *et al*., 2008). Some Gram-negative bacteria possess glycosphingolipids in the outer membrane instead of lipopolysaccharide (Hemaiswarya *et al.*, 2008).

Most of the synthetic drugs have one compound which affect only one target in a microbial cell. This is equivalent to relying upon one single golden bullet in the fight against pathogens (Van Vuuren & Viljoen, 2011; Williamson, 2001). A mono-extract or multi-extract of plant derived compounds are known to possess multitarget effects against pathogens (Wagner & Ulrich-Merzenich, 2009). Lack of activities in some crude extracts against Gram-negatives bacteria and other microorganisms under study may be suggestive of absence or insufficient quantities of essential oils to partition the cell membrane so that other SM can get access to the intracellular targets. Absence or insufficient quantities of essential oils may be due to several factors such as nature of the plant, part of the plant used, extraction techniques employed (Ncube *et al.,* 2008), handling procedures (Biavatti, 2009) just to mention but a few.

In the present study most of the aqueous extract in individual and combined studies inhibited the growth of bacterial and fungal strains under study. This contradicts with previous findings from published literature which indicate that water is not the most effective solvent for extracting active compounds in medicinal plants. It may be suggested that the low quantinty of active compounds acted in a synergistic way by affecting various cellular target leading to growth arrest of the bacteria and fungal isolates under study.

Considering the high dosages of water extracts administered by traditional healers which may be as high as four cups per adult per day water can still be regarded as a suitable extracting solvent by traditional healers (Shale, Stirk & Van Staden, 1999). The same dosage for methanol and acetone extracts could be very toxic (Shale *et al*., 1999). The biological activity of water extracts displayed on the bacterial and fungal isolates in this study supports the usage of water as an extracting solvents by traditional healers.

As it may be observed in this study, the medicinal plant under study exhibited a wide range of antimicrobial activities ranging from no inhibitions to complete inhibitions. Several investigators have studied biological activities of medicinal plants with varying results. Ncube *et al*. (2008) suggested that numerous factors may influence the results of susceptibility test. Evaluation of biological activities in plants is being challenged by lack of standard criteria for assessment of biological activities. Assessment of antimicrobial activities in medicinal plants is affected by several factors such as geographical and climatic conditions under which the plant grew, choice of extraction methods, susceptibility test methods. Furthermore, it is hard to compare results from different published literatures as different strains of microorganisms are used for assessment of antimicrobial activities (Eloff, 2004).

Liu, Liu, Yin and Zhao (2015) claim that numerous factors related to climatic conditions and geographical localisation from where the medicinal plants were collected such as soil composition, enviromental factors, seasonal variations may influence assessment of biological activities in plants. Constituents of medicinal plants may be associated with soil composition such as amount of nutients and water in the soil. Night and day temperatures, rainfall pattern, drought, duration and intensity of sunshine might have an impact on the constituents in the medicinal plant leaves under study (Soni, Brar & Gauttam, 2015; Patel *et al.,* 2009; Van Vuuren, 2008; Cantonwine & Downum, 2001). Majority of plants control the types and amount of constituents within the plant according to enviromental variations (Liu *et al*., 2015). In the present study, *M. indica, R. communis, B. pilosa, A. digitata* and *C. papaya* were collected from Malawi and *G. incanum* was collected from Port Elizabeth in South Africa. These could be suggestive as some of the factors that influenced the findings of the present study and contradictions with results from publications of other authors.

Traditional healers believe that devine powers from ancestral spirits play a great role on guiding them on which plant to collect for specific ailment, the favoured locality for the medicinal plant and time of collection (Van Wyk *et al.,* 2009, p. 14). These factors have been reported to contribute to successful treatment for the medicinal plants. Consideration of these factors when conducting studies in ethnopharmacology may give credibility to the utilisation of medicinal plants (Van Wyk *et al*., 2009, p. 14).

In the present study, some medicinal plants displayed slight to marked antimicrobial properties, however, no antimicrobial activity were displayed by some medicinal plants. According to Ncube *et al.* (2008) medicinal actions of plants are unique to a particular plant species or group, consistent with the concept that the combination of SM in a particular plant is taxonomically distinct. These SM are synthesised in a plant in a tissue, organ in a developmental specific way by biosynthetic enzymes. All plant parts such as roots, shoots, leaves, flowers, fruits and seeds synthesises SM (Gutzeit & Muller, 2014 p. 3). Sites of synthesis are not necessarily sites for storage. Some SM may be stored in specific parts such as roots, shoots, leaves, flowers, fruits and seeds, or specialised structures (Gutzeit & Muller, 2014, p. 7; Wink, 1999, p.1). Hydrophilic SM are stored in vacuoles while lipophilic compounds are stored in resin ducts, laticifers, trichomes, oil cells or cuticle (Wink, 1999, p. 1). However the amount of SM vary between tissues and organs. Higher concentrations of SM have been reported in bark, heartwood, roots, branch bases and wound tissues (Ncube *et al.*, 2008). The variation of biological activity in the present study ranging from no activity to marked activity may be ascribed to varying concentration of biological active compounds in the medicinal plant leaves under study.

Age of the medicinal plant has also an effect on the antimicrobial assessment. Wink (1999, p. 2) higlighted that alkaloids are mostly found in young or metabolically active tissues and not in dying or senescing cells. Lack of activity in some of the medicinal plant extracts may be attributed to the age of the medicinal plant leaves under study.

As elucidated in section 4.2, some of the organic extracts that were prepared towards the beginning lacked activity. It was postitulated that low concentration due to low quantity of yield extracted contributed to the lack of activity. To prove this postitulation the microrganisms under study were exposed to high concentration of the medicinal plant extract and growth inhibitions were noted in some of the microrganisms. Despite the

increase of concentration it was still surprising to note that no inhibition of growths were displayed in all extracts of *C. papaya* against all the microorganisms under study. Parekh and Chanda (2007) highlighted that other constituents in the medicinal plant extracts exert antagonistic effects or negate the positive effects of the bioactive agents and these extracts could be active against other microorganisms. This may be suggestive as one of the factors that contributed to lack of activities in some of the individual and combined plant extracts.

In the present study, results for antimicrobial screening of the plant extracts using agar diffusion assay could not corralete with the MIC values obtained using INT microtitre plate. For example, aqueous extract of *M. indica* could not inhibit *S. aureus* at a concentration of less than 60 mg/ml using agar diffusion method. However, *S. aureus* was inhibited by aqueous extract of *M. indica* at a mean concentration of 0.39 mg/ml using INT microtitre plate assay. Zones of inhibition could not collerate with the MIC values. Some of the plant extracts produced large zones of inhibitions in the agar diffusion assay but displayed large MIC values in the INT microtitre plate. Janssen *et al*. (1987) and Van Vuuren (2011) highlighted that assessment of antimicrobial activities using agar diffusion technique should be used as qualitative guide only as the method is influenced by several variables. Antimicrobial activity of a plant extract may be increased or decreased depending on agar type, temperature, medium constituents, incubation time (Eloff,1998b; Janssen *et al*., 1987) pH (Janssen *et. al*., 1987), contaminants (Eloff, 1998b). Morris, Khettry and Seitz (1979) highlighted that qualitative screening of antimicrobial activites using agar diffusion assay and quantitative assessment of MIC using broth medium are not necessarily compatible. Joshua and Takudzwa (2013) emphasised that there is more contact between the microbial cell and antimicrobial agent in the broth medium as the microorganisms are fully immersed in the mixture of broth medium and antimicrobial compound. These may be the possible explanations for the contradiction between the zones of inhibitions and the MIC values.

The medicinal plants under study displayed various MIC values against the bacterial and fungal isolates. Russell (2003) highlighted that different microorganisms respond differently to antimicrobial agents. Differences in cellular structure and physiology play a great role in antimicrobial susceptibility testing. Variation in cell wall composition, target

site and efflux pumps influences the susceptibility of microbes to antimicrobial agents (Russell, 2003). These factors are suggestive to have contributed in MIC variation and lack of total activity in some of the bacterial and fungal isolates under study.

Diffusion rate of SM in an extract may vary depending on the nature of a particular plant metabolite (Van Vuuren, 2008). Essential oils are known to be lipophilic and they do not easily diffuse through the agar even with pre-diffusion allocated time leading to inconsistent results (Van Vuuren, 2008). Certain medium components have been reported to interact with phytochemicals and inactivate them (Janssen *et al*., 1987). Lack of activities displayed by some plant extracts on the agar diffusion assay may be suggested to have been influenced by the nature of the plant metabolite or inactivation of the active compounds by the components of the media. Furthermore, lack of correlation between the zones of inhibitions and the MIC results in the microtitre plate assay may be attributed to the nature of the plant metabolite in the plant extract.

The assessment of the antimicrobial activities on the medicinal plants under study against the selected bacterial and fungal isolates was performed at 37^{o}C mimicking human body temperature. Doughari and Manzara (2008) reported that increase in temperature enhanced the activities of *M. indica* leaves against *S. aureus, P. aeruginosa* and *E. coli*. A possible explanation on the lack of activity in some plant extracts may be attributed to temperature. Further investigations are required on effect of temperature on biological activity of plant metabolite on the medicinal plants under study.

It was observed that most of the medicinal plant under study did not exhibit antimicrobial activities on *C. albicans* and *C. neoformans*. Hemaiswarya *et al*. (2008) highlighted that fungi have higher number of chromosomes and complex nuclear membrane, cell organelles and cell wall composition. Van Vuuren (2008) noted that incubation time and temperature influence antimicrobial activity particularly in fungal organisms as they require long incubation time ranging from 2 to 7 days. It has been suggested that some of the natural compounds such as hydrocarbon components of essential oils are likely to be lost due to evaporation leaving only a small portion of essential oils to be assessed for biological activity (Van Vuuren, 2008). It may be postitulated that the small portion of essential oil left behind is insufficient to exert antimicrobial effects on the complex structure of fungi leading to false negative results. This probably may be ascribed to lack

of activities in most of the medicinal plant under study against *C. albicans* and *C. neoformans.*

5.5 Combination studies

According to Van Vuuren and Viljoen, (2011) the benefits of drug combination has been recognised since ancient times and many medicine systems have depended on this concept with confidence that combination therapy may increase therapeutic effect. The potential benefits of a combination therapy are that the formulation may increase potency, reduce toxicity, decrease adverse side effects, increase bioavailability, lower the dose (Van Vuuren & Viljoen, 2011; Biavatti, 2009) and reduce the development of antimicrobial resistance (Van Vuuren & Viljoen, 2011).

The new methods of analytical chemistry and molecular biology and unanticipated transition of chemotherapy from mono-therapy to multi-drug therapy influenced synergy research in phytomedicine. Multidrug therapy has been adopted in the treatment of mixed and severe infections (Olajuyigbe & Afolayan, 2013b), AIDS, cancer, hypertension, rheumatism (Wagner & Ulrich-Merzenich, 2009), tuberculosis (Van Vuuren & Viljoen, 2011). To promote the concept of synergistic interactions which is already in existence among traditional healers, assessment of biological activities in plants should be inclined towards interactive phytochemical studies (Van Vuuren & Viljoen, 2011).

Traditional healers combine different plant species inorder to enhance efficacy. On several occassions different plant species have been combined for the treatment of microbe related infections (Van Vuuren & Viljoen, 2011). One notable example from the ethnobotanical literature is the utilisation of a bark of *Croton gratissimus* and *Ocotea bullata* in a powdered combination and blown into the womb to treat uterine disorders (Van Vuuren & Viljoen, 2011). Unfortunately, a gap still exists as very little has been done on scientific validation of synergistic effects of combined therapy involving different plant species (Van Vuuren & Viljoen, 2011). This is one of the studies which was conducted inorder to narrow the existing gap.

In the combination studies, the medicinal plants were combined in the ratio of 1:1. Sum of fractional inhibitory concentration indices (∑FIC) were calculated to assess the antimicrobial interactions. Various antimicrobial interactions were noted ranging from

synergistic to antagonistic. A range was used for classification of the antimicrobial interactions of the medicinal plant combinations into synergy (≤0.5), additive (>0.5-1.0), indifference (>1.0-≥4), antagonism (>4.0) (Van Vuuren & Viljoen, 2011). The most promising antimicrobial interactions were observed for *C. albicans, C. neoformans, E. coli, S. typhimurium* and *P. aeruginosa*. However, many antagonistic effects and very few indifference effects were observed for *S. aureus*.

In this study aqueous and acetone extracts of mixtures of *A. digitata*: *B. pilosa, R. communis: G. incanum* demonstrated synergy against *C. albicans*. Furthermore, methanol and acetone extracts of a mixture of *C. papaya*: *G. incanum* and acetone extracts of mixtures of *A. digitata*: *G. incanum*, *G. incanum*: *M. indica* displayed synergistic interactions against *C. albicans.* The most synergistic interactions for *C. albicans* were observed in methanol extract of a mixture of *R. communis: G*. *incanum* (MIC value 3.125 mg/ml, ∑FIC 0.25) and acetone extract of a mixture of *G. incanum*: *M. indica* (MIC value 3.125, ∑FIC 0.25). Individual methanol extract of *G. incanum* had an MIC value of 1.562 mg/ml against *C. albicans* with no activity from *R. communis*. When *G. incanum* and *R. communis* were combined the efficacy was enhanced (0.39 mg/ml) demonstrating a four fold increase in activity. In addition, individual acetone extract of *G. incanum* had an MIC value of 12.5 mg/ml against *C. albicans* with no activity from *M. indica* but the combination of *G. incanum*: *M. indica* demonstrated an MIC value of 3.125 mg/ml demonstrating a four fold increase in activity.

Viljoen, Van Vuuren, Ernst, Klepser, Dermici, Baser and Van Wyk (2003) highlighted that when exploring synergistic interactions of medicinal plants, studies should also extend to concentration of compounds found in that plant. As explained ealier in section 5.7.2, fruits and leaves are some of the plant parts which store large amounts of phytoconstituents. A possible explanation for the four fold increase in activity may be attributed to increased amounts of active phytoconstituents extracted from the leaf parts and the fruit pulp of *A. digitata* which interacted synergistically against *C. albicans.* Formation of hyphae is one of the virulence mechanisms of *C. albicans* and this enhances penetration of host tissues and invasive growth leading to systemic infections (Raut, Shinde, Chauhan & Karuppayil, 2012). Essential oils such as 1,8 cineole and some terpenoid contents have been reported to inhibit germ tube formation at an initial stage in formation of hyphae (Raut *et*

al., 2012). This may also be ascribed to the four fold increase of activity against *C. albicans*. The results indicate that plant mixtures of *A. digitata*: *G. incanum, G. incanum*: *M. indica* may be used in the treatment of predisposing factors of meningitis and alleviating signs and symptoms of meningitis. Further studies on antimicrobial interactions of combinations of *A. digitata*: *G. incanum, G. incanum*: *M. indica* at various ratios are required to determine the ratios with optimum activity.

The most efficacious antimicrobial combination worth highlighting is the aqueous extract of a mixture of *G. incanum*:*M. indica* against *C. neoformans* (MIC value 0.39 mg/ml, ∑FIC 0.06). Individual aqueous extract of *G. incanum* inhibited the growth of *C. neoformans* at an MIC value of 6.25 mg/ml with aqueous extract of *M. indica* displaying no activity. In combination the efficacy was enhanced drammatically (MIC value 0.39 mg/ml) demonstrating a sixteen fold increase in activity. No activity was demonstrated by aqueous extract of *R. communis* against *C. neoformans*, however, a four fold increase in activity against *C. neoformans* was observed when *G. incanum* was combined with *R. communis* (MIC value 1.562 mg/ml, ∑FIC 0.25). Van Vuuren and Viljoen (2011) indicate that antimicrobial interactions may vary depending on the ratio in which the antimicrobial agents are combined. A possible explanation for the sixteen fold increase in activity may be that the medicinal plants were combined at a maximum ratio. The results demonstrate that the combinations of *G. incanum*:*M. indica, R. communis*:*G. incanum* may serve as promising natural antimicrobial agents for treatment of predisposing factors of meningitis. However, further studies on mode of action on these efficacious antimicrobial combinations need to be explored.

Among the fungal organisms*, C. albicans* and *C. neoformans* have been the main cause of meningitis in immunocompromised patients (Kanafani & Perfect, 2008; Coelho & Casadevall, 2016; Sanchez-Portocarrero, Perez-Cecelia, Corral, Romero-Vivas & Picazo, 2000). Resistance of fungi to antifungal agents such as azoles and polyenes have been reported (Kanafani & Perfect, 2008). *C. neoformans* has also been implicated in the chronic infection of the lung (Van Vuureen, 2007, p.26). The synergistic interactions displayed by the plant combinations against the fungal strains under study will help to mitigate the burden of antifungal resistance.

The superiority of the herbal mixture demonstrated in the present study may also be attributed to the quality regarding its freshness, harvesting, processing and storage of the medicinal plant samples. The medicinal plants were collected from an area free of pesticides, microbial contamination and insect infestation. Dhami and Mishra (2015) suggest that harvesting, storage related-alterations, microbial contaminations, uncontrolled use of pesticides, insect infestations and systemic accumulation of toxic pollutants in cultivated plants may influence the quality of extracts in herbal medicine. To ensure the stability of the active phytochemicals the fine powder was stored into sterile zipped plastic bags. The zipped plastic bag containing the fine powder was placed in a sterile tight screwed opaque container.

S. typhimurium was inhibited by individual aqueous extract of *B. pilosa* at an MIC value of 25 mg/ml. No activity from aqueous extract of *C. papaya* was demonstrated against *S. typhimurium*. Interactive effects of a mixture of *C. papaya: B. pilosa* had a synergistic effect against *S. typhimurium* (MIC value 12.5 mg/ml, ∑FIC 0.50) demonstrating a two fold increase in activity. Additive effects against *S. typhimurium* were noted in aqueous extracts of mixtures of *G. incanum*: *M. indica* (MIC value 0.781 mg/ml, ∑FIC 1.00), *C. papaya*: *G. incanum* (MIC value 1.562 mg/ml, ∑FIC 1.00). The enhanced biological activities observed when the medicinal plants were combined in pairs demonstrate that their mode of action is enhanced by the mixture of phytocompounds from each of the two medicinal plant. This indicate that medical practitioners may use the combinations of *C. papaya*: *B. pilosa, G. incanum*: *M. indica* for treatment of predisposing factors and relief of signs and symptoms of meningitis.

Individual aqueous extract of *B. pilosa* inhibited the growth of *E. coli* (MIC value 50 mg/ml) but no activity was demonstrated by aqueous extract of *C. papaya*. The combination of *C. papaya*: *B. pilosa* depicted a synergistic interaction (MIC value 25 mg/ml, ∑FIC 0.50) displaying a two fold increase in activity. Additive effects were noted in aqueous mixture of *C. papaya*: *R. communis* (MIC value 3.125 mg/ml, ∑FIC 1.00). This demonstrates that the combinations of *C. papaya*: *B. pilosa, C. papaya: R. communis* may be used for treating predisposing factors as well as managing signs and symptoms of meningitis. There is a probability that combinations of antimicrobial agents that display synergistic effects in-vitro may result in successful treatment result (Aiyegoro & Oko, 2009).

Nevertheless, this contradicts with Cheesbrough (2006, p. 132) who expressed that antimicrobial assessment performed under *in-vitro* conditions do not always guarantee that the antimicrobial agent will inhibit the pathogen under *in-vivo* conditions. To validate this result the combinations would be good to be assessed in clinical trials.

Even though individual methanol extract of *C. papaya* did not exhibit antimicrobial activities against *P. aeruginosa* when screened independently, synergistic interactions worth highlighting were observed when *C. papaya* was combined with *M. indica* (MIC value 3.125 mg/ml, ∑FIC 0.125) demonstrating an eight fold increase in activity. Individual methanol extract of *B. pilosa* did not exert any biological activity against *P. aeruginosa.* Methanol extract of *M. indica* inhibited *P. aeruginosa* at an MIC value of 25 mg/ml. Furthermore, a favourable interaction was noted when methanol extract of a mixture of *B. pilosa*: *M. indica* was tested on *P. aeruginosa* (MIC value 6.25 mg/ml, ∑FIC 0.25). One interesting combination that exerted synergistic effects against *P. aeruginosa* was that of methanol extract of *R. communis*: *M. indica.* Individual methanol extract of *R. communis* inhibited *P. aeruginosa* at an MIC value of 25 mg/ml while individual methanol extract of *M. indica* had an MIC value of 1.562 mg/ml. However, synergistic and enhanced activities were displayed by a combination of *R. communis:M. indica* (MIC value 6.25 mg/ml, ∑FIC 0.50). The formulations prepared in this study demonstrated synergistic effects against *P. aeruginosa* indicating that these combinations may be effective in treating predisposing factors and managing signs and symptoms of meningitis.

It was surprising in this study to note that all individual extracts of *C. papaya* lacked activity against the bacterial and fungal isolates under study but displayed synergistic effects when combined with *B. pilosa* in a ratio of 1:1. Wagner and Ulrich-Merzenich (2009) and Van Vuuren and Viljoen (2009) expressed that some phytoconstituents in a medicinal plant combination do not exert antimicrobial effects but improve solubility of other constituents within the extract. It may be suggested that *C. papaya* possess some phytochemicals which improves the solubility of other phytochemicals within the combined extract. This resulted to increased availability of antimicrobial agents within the extract combination and enhanced uptake by the microbial cell. The high concentration of one or more active substances in the plant extract combination may affect several targets and act in a synergistic way and contribute to effective inhibition of *S. typhimurium*

and *E. coli* growth. A review conducted by Biavatti (2009) higlighted that phytoconstituents in an extract may be classified into active substances, co-effectors and matrix formers and the interaction between co-effectors and matrix formers may protect the active substances from degradation. It may be suggested that some of the phytoconstituents in *C. papaya* acted as co-factors and matrix formers that prevented degration of other phytoconstituents resulting to enhanced activities.

It is interesting to observe that some combinations exerted synergistic effects against Gram-negative bacteria under study such as *P. aeruginosa* and *E. coli.* These combinations are suggestive to enhance potency and contribute to the fight against drug resistance microbes. This validates the traditional cultural healing systems that utilise plant combinations from different species in combating infectious diseases such as meningitis. The indiscrimate use of antibiotics has contributed to the development of resistance in microorganisms and loss of potency of antibiotics that were once regarded as arsenals and effective first line antibiotics in the fight against pathogens (Olajuyigbe & Afoloyan, 2013a). This has resulted in a change to secondline or third line antibiotics that are more expensive and with many side effects (Olajuyigbe & Afolayan, 2013a). *P. aeruginosa* has been implicated in meningitis and it has often been reported as the cause of nosocomial infections in hospitals (Juhi, Bibhabati, Archana, Poonam & Vinita, 2009). *E. coli* is a commensal of the small intestines but it has been reported to cause neonatal meningitis. Furthermore, *E. coli* has been involved in intestinal and urinary tract infections (Van Vuureen, 2007, p.203; Cheesbrough, 2006, pp. 98, 113).

In this study no synergistic and enhanced activities were noted against *S. aureus.* Several antagonistic interactions predenominated with a few additive and non-interactive effects. The strongest and broadest antagonistic effect against *S. aureus* was observed on aqueous extract of a mixture of *A. digitata*: *R. communis* (MIC value 50 mg/ml, ∑FIC 68.00). A mixture of *C. papaya: B. pilosa* depicted additive effects (∑FIC 1.00). However, non-interactive effects against *S. aureus* were observed in aqueous and methanol extracts of a mixture of *A. digitata: C. papaya* (MIC value 25 mg/ml, ∑FIC 2.00) and aqueous extract of a combination of *C. papaya* and *G. incanum* (MIC value 3.125 mg/ml, ∑FIC 2.00). Most of the individual medicinal plant extracts under study inhibited the growth of *S. aureus* with lower MIC values compared to the combined extracts.

In the present study, the medicinal plants under study were combined in a 1:1 ratio. Van Vuuren and Viljoen (2011) highlighted that antimicrobial interaction may be influenced by the ratio at which the two antimicrobial agents are combined. Combinations at different ratios give more credible result of antimicrobial interactions as dose response varies among microbes (Van Vuuren & Viljoen, 2011). Therefore, all combinations in the present study need to be explored in depth at different ratios considering that combinations prepared by traditional healers in rural areas are not precisely measured.

Van Vuuren and Viljoen (2011) stressed that focus should not only be given to major constituents when embarking on studies on biological activities of medicinal plants but also minor compounds. A study conducted by Christoph, Stahl-Biskup and Kaulfer (2001) on "Death kinetics of *S. aureus* exposed to commercial tea tree oils s.l" indicated that *S. aureus* resisted against major constiuents kanuka oil, manuka oil and β-triketone oils. Van Vuuren and Viljoen (2011) indicates that antimicrobial interactions are influenced by enantiomeric configurations. Molecules of different enantiomeric configuration may affect the biological activity of medicinal plant extracts when combined at different ratios. The decrease of activity noted by most of the combined extracts of medicinal plants under study may be attributed to differences in enantiomeric configuration and a ratio at which the plant samples were combined. More investigations on antimicrobial interactions on the medicinal plants under study should be done by incorporating the inhibitors at selected ratios.

Dorman and Deans (2000) highlighted that functional groups and probable synergistic interactions influence antimicrobial activity. Some functional groups in a phytoconstituent may enhance or inactivate antimicrobial activities against microorganisms. For example, aldehydes have been reported to exert strong biological activities against microbes (Dorman & Deans, 2000). Lack of activities by some combined extracts against some bacterial and fungal isolates under study may be ascribed to factors associated with functional groups and unfavourable synergistic effects.

It was interesting to observe that all mixtures that included *G. incanum, M. indica* and *B. pilosa* were very active indicating that these species have a contributory effect in increasing potency in a combination. Plants with antioxidant potential exert synegistic effects with antibiotics due to increased amount of polyphenolic compounds (Biavatti,

2009). *M. indica* have been reported to possess antioxidant activities. Biavatti (2009) indicated that a combination of *M. indica* and tetracycline exerted synergistic interactions against *E. coli*.

The synergistic effects displayed in this study by some combined extracts correlate with the high mean weight values and mean percentage yield of extracts obtained during extraction procedure. This indicates that favourable antimicrobial effects against pathogens may depend on considerable quantities of biological active compounds in a combination. Most publications have highlighted resistance of Gram-negative bacteria against numerous plant extracts. However, demonstration of noteworthy MIC's and additive effects by certain combined extracts against Gram-negatives, *P. aeruginosa*, *S. typhimurium* and *E. coli* bring positive hope in the fight against meningitis caused by these organisms.

In the present study, Ciprofloxacin and Amphotericin B were employed as reference drugs for the bacterial and fungal strains under study. These reference drugs inhibited the growth of microorganisms under study at a very low MIC's compared to the individual and combined plant extract. However plants are able to survive and fight against bacterial, fungal and viral infections successfully due to synergistic action. This has been evidenced in the present study on assessment of individual and combined plant studies.

Surprisingly low MIC values were mostly observed in combined acetone extracts followed by methanol extracts against *S. aures, C. albicans* and *C. neoformans*. Generally, most of the aqueous extracts demonstrated high MIC's and ∑FIC's values against the bacterial and fungal pathogens under study. Eloff (1998a) assessed a variety of extractants for their potential to dissolve biological active compounds from plants. The results indicated that acetone extracted the largest number of components and inhibitors from the plants. Another investigation conducted by Masoko and Eloff (2006) where they assessed antifungal activities of twenty-four *Combretum* species from Southern Africa, the extracting solvents included were dichloromethane, acetone, methanol and hexane. The results indicated that acetone and methanol extracted more compounds compared to the other extractants. The possible explanation for the low MIC's and ∑FIC values could be that acetone and methanol used in the present study extracted a larger variety of components with a lot of inhibitors. Therefore, the low MIC and ∑FIC values may be

ascribed to large variety of inhibitors extracted by acetone and methanol that exerted increased activity synergistically.

As explained earlier some plant combination demonstrated non-interactive as well as antagonistic effects against *S. aureus* and other microorganisms under study leading to high MIC and ∑FIC values. Rabe and Van Staden (1997) believes that high MIC values are observed in antimicrobial assessment of medicinal plants because the extracts are used in impure form or the active compounds in the extract might be in low quanties. Wagner and Ulrich-Merzenich (2009) observed that proving synergy in phytomedicine might be more difficult since the plant extracts constitutes complex mixtures of major compounds, minor concomitant agents and fibres which can all participate in synergy effects.The high values of MIC's and ∑FIC's observed against *S. aureus* may be suggestive of lack of efficacy by the plant extract due to its impure form or insufficient quantities of active constiuents in the extracts. If an extract displays high MIC values against microorganisms it is an indication that the microorganisms have a potential to develop resistance against the plant extract (Joshua & Takudzwa, 2013).

Although independently the medicinal plants have exhibited antimicrobial effects and synergistic effects in combination, the methodology followed in plant extraction by numerous authors is often abandoned. The techniques employed in the antimicrobial assessment vary from author to author and important factors that affect the findings are usually neglected. Most of the traditional healers utilise water as an extractant therefore many researchers have followed wrong methods (Van Vuuren & Viljoen, 2011; Eloff, 2004). However further investigations are needed through clinical trials and follow-ups to validate these findings.

Phytocompounds are synthesised by plants for defence purposes against herbivores, bacteria, fungi, viruses and harsh conditions. In the present study, it has been observed that some plants which displayed antagonistic effects in certain combinations exerted synergistic interactions when combined with other plants. This is an indication that there is no plant that do not exert biological activities. Therefore, it should not be guaranteed that the plants in some combinations that demonstrated antagonistic effects do not possess biological activites (Mathobela, 2016). For this reason all antagonistic effects in the present study should not be ignored but warrant further investigations.

CHAPTER 6: CONCLUSION AND RECOMMENDATIONS

The discovery of antibiotics improved human health through reduced mortality and increased life span in man and reduced affordability and availability of medicine in developing countries (Eloff, 2000). However, the development of antibiotic resistance has led to exploration of natural compounds from plants. Plants have been used by man as therapeutic agents for infectious diseases since ancient times. Plants have been used by man independently and in various combinations for treatment of numerous diseases and health disorders (Van Vuuren & Viljoen, 2011).

Traditional healers administer treatment based on identified cause of the disease. Furthermore, traditional healers use specific medicinal plants to alleviate the signs and symptoms of a disease. Traditional healers use *G. incanum, M. indica, B. pilosa, R. communis*, *C. papaya* and fruit pulp of *A. digitata* in treating predisposing factors and managing signs and symptoms of meningitis such as fever, inflammation and headache. Meningitis is one of the infectious diseases causing increased morbidity and mortality (Luksic *et al*., 2013; Brouwer *et al*., 2010).

The aim of the study was to determine the antimicrobial activity of aqueous, methanol and acetone extracts of leaves of *G. incanum, M. indica, B. pilosa, R*. *communis, C. papaya* and fruit pulp of *A. digitata* and activity of their combined preparation against selected bacterial and fungal pathogens that cause meningitis. Antimicrobial screening and MIC studies of the medicinal plants were performed independently and in combination using agar well diffusion method and INT microtitre well technique.

Generally, the medicinal plants under study displayed antimicrobial activities on the bacterial and fungal isolates. Positive or negative antimicrobial effects in the individual medicinal plant studies and the type of antimicrobial interaction in the combined studies depended on the test organism and solvent used for extracting the plants. Most of the medicinal plant extracts inhibited the bacterial isolates under study independently. Synergistic and additive effects against bacterial and fungal pathogens under study were displayed by some plant combinations. Some plants which did not exert activity independently enhanced potency of other plants in combination. This validates the traditional healing practices which utilise combinations of different species of plants for

treatment of predisposing factors of meningitis, managing signs and symptoms of meningitis such as earache, headache, fever inflammation. In this study, most of the aqueous extracts displayed activity independently. Synergistic and additive effects were also displayed by some aqueous extract combinations. This supports the traditional use of water as extracting solvent by traditional healers.

In the present study, it was noted that the individual and combined medicinal plants under study exerted antimicrobial effects on the selected bacterial and fungal pathogens. Although medicinal plants have been used by man independently and in various combinations for centuries, their exact mode of action remains unclear. Nevertheless, possible pharmacological actions of medicinal plants against microbes have been postulated. These include membrane disruption and impaired cellular metabolism, inhibition of activity or production of enzymes that destroy or modify antibiotics, inhibition of efflux pumping systems (Van Vuuren & Viljoen, 2011; Hemaiswarya *et al.,* 2008). However, there are no scientific evidences that support these mode of actions due to scarcity of studies on validation of pharmacological actions of individual and combined medicinal plants (Van Vuuren & Viljoen, 2011). Further studies on mode of action of the medicinal plants under study should be conducted independently and in combination. Studies on mechanism of action of medicinal plants will pave way in development of novel therapeutic drugs.

Variation in quantity of specific phytoconstituent may influence antimicrobial activity of medicinal plant extract. Antimicrobial activity of plant extract depends on their chemical composition and the amount of individual constituent in an extract. High concentration of certain phytoconstituents have been reported to affect antimicrobial activity of medicinal plant extract (Nazzaro, Fratianni, De Martino, Coppola & De Feo, 2013). Further studies in chemical composition of medicinal plants under study need to be undertaken. This will help to correlate the quantity of specific constituent and the antimicrobial activity displayed by the extract. Such studies remain highly relevant as they will enable researchers to understand better the mechanism of action of plant SM.

Many medicinal plants possess multitude of compounds with great complexity. A mono-extract or multi-extract combination may bring desired effects or complicated outcomes. The desired effects lead to successful therapeutic effect. However, cases of self-

poisoning due to consumption of medicinal plants have been reported (Joshua & Takudzwa, 2013; Olajuyigbe & Afoloyan, 2013b). According to Van Wyk, Van Heerden and Van Oudtshoorn (2002, p. 8), 98 cases representing 4.7% of total acute poisoning reported at a university hospital in South Africa from 1996 to 2000 were related to consumption of medicinal plants. Therefore, safety and toxicity of the medicinal plants in the present study need to be investigated.

In the present study, the medicinal plants under study were combined in the ratio of 1:1. Antimicrobial interactions ranging from synergistic to antagonistic were noted. Van Vuuren and Viljoen (2011) highlighted that two antimicrobial agents may not exert antimicrobial effects at similar dosages. Antimicrobial interactions may vary according to the ratio in which the two inhibitors are combined. In future, the plant combinations that demonstrated antagonistic and indifferent antimicrobial interactions warrant further investigations in various combination ratios.

It was observed that all organisms under study displayed resistance against all extracts of *C. papaya*. As elucidated earlier on antimicrobial compounds in medicinal plants vary with season and geographical location (Van Vuuren, Viljoen, Ozek & Baser, 2007; Dhami & Mishra, 2015). Further studies on effect of season and geographical variation on *C. papaya* and other medicinal plants under study need to be done in depth. This will help researchers to take into consideration the right locality and the correct season for collection of medicinal plants for investigative purposes.

It has been noted that some of the results in the present study contradict with findings from other authors. Lack of standard criteria make it difficult to compare results of antimicrobial assessment between authors (Wachtel-Galor & Benzie, 2011, p. 6; Ncube *et al*., 2008). As stated earlier on, many authors use different microbial strains and various methods in assessment of antimicrobial activities in medicinal plant (Van Vuuren, 2011; Eloff, 2004). Furthermore, many authors have followed an incorrect lead as non-polar solvents such as hexane are employed in extraction of active compounds from plants (Eloff, 2004). Traditional healers utilise water as extracting solvent There is a need for standardisation of laboratory methods in order to have reproducibility of results.

The in-vitro antimicrobial assessment of the medicinal plants under study displayed inhibition of growth independently and in combination. However, antimicrobial activity of medicinal plant extracts demonstrated in-vitro does not affirm in-vivo activity (Cheesbrough, 2006, p. 132). There is a need to conduct regulated clinical trials to determine a therapeutic promise in all efficacious antimicrobial combinations.

Many studies involving combination of plant extracts and synthetic drugs have been highlighted in various ethnomedicinal journals. Many anecdotal reports have highlighted possible synergism between phyto-combination of different plant species (Van Vuuren, 2011). Inspite of the fact that traditional healers administer medicinal plants in combination, it is fascinating to observe that documentation of studies on this long standing cultural healing practice is lagging behind (Van Vuuren, 2011). Studies and documentation of antimicrobial interactions in plant combinations against pathogens that cause meningitis remain highly pertinent especially in this era of increasing multiple drug resistance which is a global concern to public health.

Various formulations are administered for treatment of numerous diseases and health disorders. Such formulations include decoctions, concoctions, teas, tablets, ointments, lozenges and many others. Drug formulations influence solubility, dissolution rate, permeability across the gastrointestinal tract membrane and bioavailability in the systemic circulation (Van Vuuren, 2008). Further studies on suitable formulations in the medicinal plant under study need to be conducted independently and in combined preparations to support the results of the in-vitro antimicrobial screening studies.

Furthermore, the discovery and development of treatment candidates of CNS disorders has faced more challenges compared to those intended for other treatment applications. Some CNS treatment candidates fail to display potency in clinical trials due to inability to penetrate across the blood brain barrier (Basavaraj & Betageri, 2014). The medicinal plants under study warrant further investigations in relation to permeability across the blood brain barrier.

In the present study the bacterial and fungal pathogens were selected because they are known to cause meningitis. It was observed that aqueous extracts of *G. incanum* independently inhibited the growth of all selected bacterial and fungal isolates under study

indicating a broad spectrum activity. However, other microorganisms such as *Neisseria meningitidis*, *Haemophilus influenzae*, Group B *Streptococci*, *Klebsiella pneumoniae* and many others have also been reported to cause meningitis (Nadel & Canlas, 2016). The assessment of antimicrobial activities of the medicinal plants under study should be extended to all organisms implicated in meningitis independently and in combination. Such studies remain highly relevant as they will determine the activity of the medicinal plants under study against pathogen responsible for meningitis and broad spectrum activity of *G. incanum*.

On the contrary it is difficult to assess antimicrobial activities of medicinal plants under study against fastidious organisms such as *N. meningitidis*, *H. influenzae* and Group B *Streptococci* using the traditional culture methods. However, standardisation has been made to assess antimicrobial susceptibitility testing using specific culture media, various incubation conditions and interpretative criteria for zones of inhibition (Barouiri *et al.*, 2016).

Seeking therapeutic effects from medicinal plants has been known since ancient times. Plants continue to play a great role in treating infectious diseases caused by bacteria, fungi and non-infectious diseases such as diabetes, cancer. On the other hand, some phytoconstituents in medicinal plants has been reported to alter or destroy beneficial bacteria as well as pathogenic bacteria (Cueva, Bartolome, Moreno-Arribas, Bustos, Requena, Gonzalez-Manzano, Santos-Buelga, Turrientes & Del-campo, 2015). The efficacious individual and combined medicinal plant extracts in the present study need to be explored in depth to determine if they exert antimicrobial effects on microbiota.

APPENDIX A: Media and iodonitrotetrazolium chloride preparation

MEDUIM	MANUFACTURER	QUANTITY in (g)	DISTILLED WATER in (ml)	FINAL CONCENTRATION	COMMENTS
Mueller Hinton Agar	Biolab (MERK)	38	1000	38g/1000ml	_
Mueller Hinton Broth	SGIMA-ALDRICH	23	1000	23g/1000ml	_
Nutrient Agar	Biolab (MERK)	31	1000	31g/1000ml	_
Saboraud Dextrose 4 % Agar	Biolab (MERK)	60	1000	60g/1000ml	500mg cycloheximide and 40mg chloramphenicol may be added to each litre of medium before autoclaving to inhibit non-pathogenic fungi or bacteria

Agar medium

- Boil whilst stirring until completely dissolved
- Autoclave at 121ºC for 15 minutes
- Cool rapidly to 45-50ºC
- Mix well and pour plates

Broth medium

- Sterilise by autoclaving at 121ºC for 15 minutes

Iodonitrotetrazolium chloride (INT) preparation

MANUFACTURER	QUANTITY in (mg)	DISTILLED WATER in (ml)	FINAL CONCENTRATION
SIGMA-ALDRICH	4	1	4mg/ml (0.004g/ml)

APPENDIX B: Mean extraction efficiency for individual extracts

Water extracts

MEDICINAL PLANT	WEIGHT OF POWDER	MEAN WEIGHT OF EXTRACT	MEAN % YIELD OF EXTRACTS	MEAN CONCENTRATION
G. incanum	10g	2.82g	28.2	208 mg/ml
M. indica	10g	2.13g	21.3	349 mg/ml
R. communis	10g	3.33g	33.3	303 mg/ml
C. papaya	10g	4.90g	49.0	271 mg/ml
B. pilosa	10g	3.40g	34.0	307 mg/ml
A. digitata	10g	4.89g	48.9	283 mg/ml

Appendix B: Mean extraction efficiency for individual extracts (continued)

Methanol extracts

MEDICINAL PLANT	WEIGHT OF POWDER	MEAN WEIGHT OF EXTRACT	MEAN % YIELD OF EXTRACT	MEAN CONCENTRATION
G. incanum	10g	3.52g	35.2	315 mg/ml
M. indica	10g	2.30g	23.0	288 mg/ml
R. communis	10g	1.73g	17.3	348 mg/ml
C. papaya	10g	1.53g	15.3	179 mg/ml
B. pilosa	10g	1.73g	17.3	225 mg/ml
A. digitata	50g	7.86g	15.7	371 mg/ml

Appendix B: Mean extraction efficiency for individual extracts (continued)

Acetone extracts

MEDICINAL PLANT	WEIGHT OF POWDER	MEAN WEIGHT OF EXTRACT	MEAN % YIELD OF EXTRACT	MEAN CONCENTRATION
G. incanum	10g	1.48g	14.8	301 mg/ml
M. indica	10g	1.42g	14.2	282 mg/ml
R. communis	10g	0.76g	7.6	244 mg/ml
C. papaya	10g	0.67g	6.7	221 mg/ml
B. pilosa	10g	0.45g	4.5	277 mg/ml
A. digitata	50g	1.45g	2.9	290 mg/ml

APPENDIX C: Antimicrobial screening results for individual medicinal plant

PLANT	EXTRACT	*S. aureus* ATCC43300	*P. aeruginosa* ATCC 27853	*S. typhimurium* ATCC 49416	*E. coli* ATCC 35218	*C. albicans* ATCC 10231	*C. neoformans* clinical strain
G. incanum	**Aqueous**	+ + +	+ +	+ +	+ +	+ +	+ +
	Methanol	+ + +	+ + +	-	-	+ +	+ +
	Acetone	+ + +	+ + +	-	-	+ +	+ +
M. indica	**Aqueous**	+ + +	+ +	+ +	+ +	-	-
	Methanol	+ + +	+ + +	+ +	-	-	-
	Acetone	+ + +	+ + +	-	-	-	-
A. digitata	**Aqueous**	+ +	+ +	+ +	+ +	-	-
	methanol	+ +	+ +	+ +	++	-	-
	Acetone	+ + +	+ + +	+ +	+ +	-	-
B. pilosa	**Aqueous**	+	+ +	+ +	+ +	+ +	+ +
	Methanol	+ +	-	-	-	-	+
	Acetone	+ + +	-	-	-	+ + +	+ + +
R. communis	**Aqueous**	+ +	+ +	+ +	+ +	-	-
	Methanol	+ +	+	-	-	-	-
	Acetone	+ +	-	-	-	-	-
C. papaya	**Aqueous**	-	-	-	-	-	-
	Methanol	-	-	-	-	-	-
	Acetone	-	-	-	-	-	-
Controls	**Ciprofloxacin**	+++	+++	+++	+++	NA	NA
	Amphotericin B	NA	NA	NA	NA	+++	+++

The results are given as an average of three experiments replicated five times

Key - Negative + slight positive + + positive + + + strong positive NA – Not applicable

APPENDIX D: Antimicrobial screening results for combined medicinal plants

PLANT COMBINATION	*S. aureus* ATCC 43300	*P. aeruginosa* ATCC 27853	*S. typhimurium* ATCC 49416	*E. coli* ATCC 35218	*C. albicans* ATCC 10231	*C. neoformans* clinical strain
A. digitata : R. communis						
Aqueous Extract	+ +	+ +	-	+	-	-
Methanol Extract	+ + +	_	-	-	-	-
Acetone Extract	+ + +	_	-	-	-	-
A. digitata: M. indica						
Aqueous Extract	+ +	+	-	-	-	-
Methanol Extract	+ +	+	-	-	-	-
Acetone Extract	+ + +	+ + +	-	-	-	-
A. digitata: C. papaya						
Aqueous Extract	+	+ +	-	-	-	-
Methanol Extract	+	-	-	-	-	-
Acetone Extract	-	-	-	-	-	-
A. digitata: G. incanum						
Aqueous Extract	+ +	+	+ +	-	-	-
Methanol Extract	+ +	+ +	-	-	+	+ +
Acetone Extract	+ + +	+ + +	-	-	+ +	+

The results are given as an average of three experiments replicated five times

Key: - negative + slight positive + + positive + + + strong positive

APPENDIX D: Antimicrobial screening results for combined medicinal plants (continued)

PLANT COMBINATION	*S. aureus* ATCC 43300	*P. aeruginosa* ATCC 27853	*S. typhimurium* ATCC 49416	*E. coli* ATCC 35218	*C. albicans* ATCC 10231	*C. neoformans* clinical strain
A. digitata: B. pilosa						
Aqueous Extract	+	+ +	-	-	+++	+ + +
Methanol Extract	+ +	-	-	-	+ +	+ +
Acetone Extract	+ + +	-	-	-	+ +	+ +
R. communis: G. incanum						
Aqueous Extract	+ + +	+ +	+ +	+ +	+ + +	+ +
Methanol Extract	+ + +	+	-	-	+ +	+ +
Acetone Extract	+ + +	+ +	-	-	+	+ +
B. pilosa: G. incanum						
Aqueous Extract	+ + +	+ +	+ +	+ +	+ + +	+ + +
Methanol Extract	+ + +	+	-	-	+ + +	+ + +
Acetone Extract	+ + +	+ +	-	-	+ +	+ +
R. communis: B. pilosa						
Aqueous Extract	+	+	-	-	-	-
Methanol Extract	+ +	-	-	-	+ +	+ +
Acetone Extract	+ +	-	-	-	-	+

The results are given as an average of three experiments replicated five times

Key: - negative + slight positive + + positive + + + strong positive

APPENDIX D: Antimicrobial screening results for combined medicinal plants (continued)

PLANT COMBINATION	*S. aureus* ATCC 43300	*P. aeruginosa* ATCC 27853	*S. typhimurium* ATCC 49416	*E. coli* ATCC 35218	*C. albicans* ATCC 10231	*C. neoformans* clinical strain
R. communis: M. indica						
Aqueous Extract	+ + +	+	+ +	+ +	-	-
Methanol Extract	+ + +	+ +	-	-	-	-
Acetone Extract	+ + +	+ +	-	-	-	+
B. pilosa: M. indica						
Aqueous Extract	+ + +	+ +	+ +	+ +	-	-
Methanol Extract	+ + +	+ +	-	-	+ +	+
Acetone Extract	+ + +	+ +	-	-	+ +	+ +
G. incanum: M. indica						
Aqueous Extract	+ + +	+ +	+ +	+ +	+ +	+ +
Methanol Extract	+ + +	+ +	-	-	+ +	+ +
Acetone Extract	+ + +	+ +	-	-	+	+
C. papaya: M. indica						
Aqueous Extract	+ + +	+	+ +	+ +	-	-
Methanol Extract	+ + +	+ +	-	-	-	-
Acetone Extract	+ + +	+ +	-	-	-	+ +

The results are given as an average of three experiments replicated five times

Key: - negative + slight positive + + positive + + + strong positive

APPENDIX D: Antimicrobial screening results for combined medicinal plants (continued)

PLANT COMBINATION	*S. aureus* ATCC 43300	*P. aeruginosa* ATCC 27853	*S. typhimurium* ATCC 49416	*E. coli* ATCC 35218	*C. albicans* ATCC 10231	*C. neoformans* clinical strain
C. papaya: R. cmmunis						
Aqueous Extract	+ +	+	+ +	+ +	-	-
Methanol Extract	+	-	-	-	-	-
Acetone Extract	+	-	-	-	-	-
C. papaya: G. incanum						
Aqueous Extract	+ + +	+	+	+ +	+ +	+ +
Methanol Extract	+ + +	+	-	-	+ + +	+ +
Acetone Extract	+ + +	+ +	-	-	+ +	+ +
C. papaya: B. pilosa						
Aqueous Extract	+ +	-	+ +	+ +	-	-
Methanol Extract	+	-	-	-	+ +	+ +
Acetone Extract	+ +	-	-	-	+ +	+ +
All Six Plants Combined						
Aqueous Extract	+ +	+ +	+ +	+ +	+ +	-
Methanol Extract	+ + +	+ +	-	-	+ +	-
Acetone Extract	+ + +	+ +	-	-	+ +	+ +
Controls						
Ciprofloxacin	+ + +	+ + +	+ + +	+ + +	NA	NA
Amphotericin B	NA	NA	NA	NA	+ + +	+ + +

The results are given as an average of three experiments replicated five times

Key: - negative + slight positive + + positive + + + strong positive NA Not applicable

APPENDIX E: Culture Plates

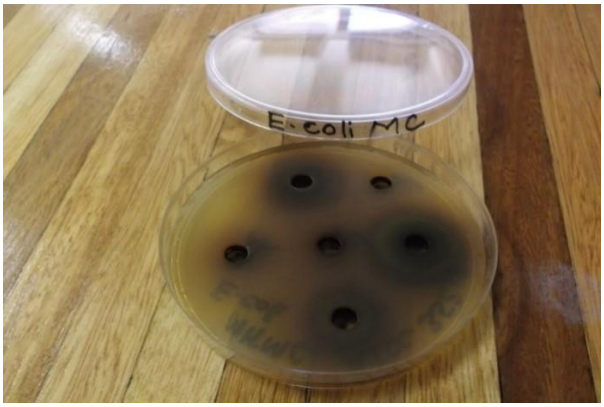


Figure E 1: *E. coli* exhibiting resistance and dark zones of colouration against combined methanol extracts

Figure E 2: *C. neoformans* inhibited by combined aqueous extracts

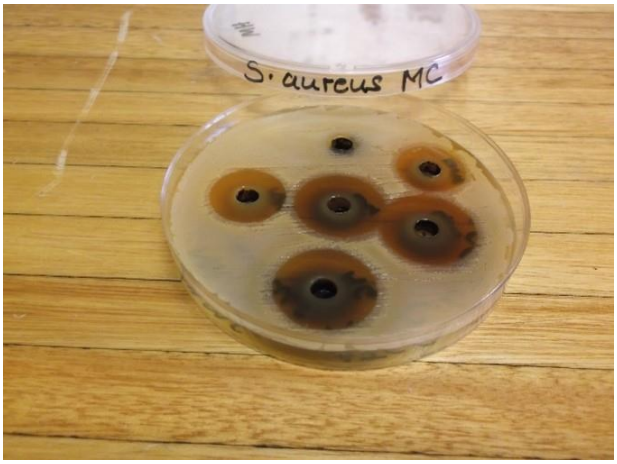


Figure E 3: *S. aureus* inhibited by combined methanol extracts

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