ANTIMICROBIAL ACTIVITY

OF
SELECTED EASTERN CAPE MEDICINAL PLANTS

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CONTENTS

ABSTRACT

Bacterial resistance to antibiotics has been a great problem for many years. The degree of resistance and the speed with which resistance develops varies with different organisms and different drugs.

Enzymes called β-lactamases are produced by bacteria and are one mechanism in which bacteria develop antimicrobial resistance. Gram-negative bacteria producing enzymes called ESBLs because of their wide substrate range are of a particular concern in nosocomial infections.

In many countries people still use traditional medicine derived from plants as an alternative to the Western medicine due to increased cost of Western medicine and microbial resistance of antibiotic treatments. Biologically active compounds isolated from plants species are used in herbal medicine.

Because of the high prevalence of the ESBLs and their increasing resistance to the antibiotics, this research study was done to test the antimicrobial activities of selected medicinal plants of the Eastern Cape; *G. incanum, D. angustifolia* and *E. autumnalis* which were traditionally used to treat various infections.

The *in vitro* antimicrobial activity of three different extracts (acetone, methanol & distilled water) and the traditional preparations of the three plants were tested against the selected strains of ESBL-producing bacteria, non β-lactamase producers and the different fungal species.

The extracts were screened against 26 Gram-positive bacterial strains, 53 Gram-negative bacterial strains and 15 fungal strains. The Gram-positive bacteria included strains from *S. aureus, B. cereus* and *E. faecalis*. The Gram-negative bacteria included strains from *E.*

coli, *E. cloacae*, *K. pneumoniae*, *P. aeruginosa* and *Acinetobacter spp*. The fungal strains included 9 strains of *Candida albicans* and a single strain of each of the following opportunistic fungi, *Mucor sp, Geotrichium sp, Penicillium sp, Fusarium sp* and *Rhizopus sp*.

The agar dilution assay was used for the antimicrobial screening of the plants extracts and for the determination of the MICs. The Ames test was performed for the determination of probable carcinogenicity of the extracts of *G. incanum* and *D. angustifolia*.

The distilled water extracts followed by acetone extracts of the plants revealed the highest antimicrobial activity against the different microbial strains. The extracts of *G. incanum* followed by the extracts of *D. angustifolia* inhibited the highest number of microbial strains. The extracts of *E. autumnalis* did not show any antimicrobial activity against all the pathogens in this study.

More of the Gram-positive bacteria were inhibited by the plant extracts. The lowest MIC was obtained with Gram-positive bacteria. The bacterial strains of *E. faecalis* and *P. aeruginosa* were not inhibited by any of the plants extracts in the agar dilution assay yet *Acinetobacter species* which are MDR were inhibited by the distilled water and methanol extracts of *G. incanum*. A single strain of *Mucor sp* was the only spore forming fungi that was inhibited by the distilled water extracts of *G. incanum*.

None of the plants extracts showed any mutagenic effects on the TA100 *S. typhimurium* strains incorporated on the Ames test.

Apart from revealing of new antimicrobial agents that may be used against resistant organisms, the proper use of antimicrobial agents should be recommended. The study has highlighted a need for further investigations on the properties of the medicinal plants used in this study.

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LIST OF ABBREVIATIONS

A

AIDS	Acquired immuno deficiency syndrome
&	And
ATCC	American type culture collection

B

β-lactamases	Beta-lactamases

C

CDC	Centre for disease control and prevention
cm	Centimeter
CNS	Central nervous system
CTX-M	Cefotaxime

D

DMSO	Dimethyl sulphoxide
^{0}C	Degrees celcius

E

ESBLs	Extended spectrum Beta-lactamases
EUCAST	European committee for antimicrobial susceptibility testing

F

Fig	Figure
4^{th}	Fourth

G

g	Grams
H	
HAART	Highly active antiretroviral therapy
HIV	Human immuno virus
his	Hisitidne
hrs	Hours
HSV	Herpese simplex virus
I	
ICUs	Intensive care units
M	
mg/ml	milligrams per milliliters
MDR	Multi-drug resistant
MIC	Minimum inhibitory concentration
µl	Microliter
MRSA	Methicillin resistant *Staphylococcus aureus*
N	
NHLS	National Health Laboratory Services
NICUs	Neonatal intensive care units
NMMU	Nelson Mandela Metropolitan University
O	
OTC	Over the counter
P	
PBPs	Penicillin-binding proteins

%	Percent
R	
rpm	Revolutions per minute
S	
SHV	Sulphydryl variable
SANBI	South African National Biodiversity Institute
Spp	Species
T	
TEM	Temoniera
3rd	Third
U	
UTIs	Urinary tract infections
V	
VISA	Vancomycin-intermediate *Staphylococcus aureus*
VRSA	Vancomycin resistant *Staphylococcus aureus*
W	
WHO	World Health Organisation
Z	
Zn2+	Zinc

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CHAPTER 1

INTRODUCTION

1.1 General review

Bacterial resistance to antibiotics has been a great problem for many years. The threats that bacterial resistance present today are greater than they were in the past. The first bacteria that were detected to be resistant to several antibiotics were reported in Japan during the 1950s. They reported resistance in *Shigella* species that were isolated from patients who had been on antibiotic treatment (Schlegel & Schmidt, 1985). The degree and the speed with which resistance now develops vary with different organisms and different drugs (Thomas, 1973; Laxminarayan &Weitzman, 2002).

According to the World Health Organisation (WHO), antibiotic resistance was first documented nearly six decades ago and became an important issue in the 1960s when resistance plasmids and plasmids transmissibility were detected (WHO, 2004a). By the beginning of the 1990s, more resistance developed in certain pathogens and most of the antibiotics were found to be ineffective against these pathogens. Antibiotic-resistant pathogens became an important and growing threat to public health which was addressed by national agencies and international bodies such as the European Commission and particularly the WHO (WHO, 2004a). Resistance development is still continuing for a number of bacterial pathogens which, apart from *Mycobacterium tuberculosis* and *Streptococcus pneumoniae*, are particularly associated with nosocomial infections such as Methicillin Resistant *Staphylococcus aureus* (MRSA) and Extended Spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae* (WHO, 2004a).

The β-lactam antibiotics are the most widely used family of antibiotics. This exposes more of the cephalosporins to microorganisms which eventually lead to antimicrobial resistance (Payne & Thomson, 1998). It is generally accepted that infections caused by ESBL-producing organisms are associated with increased risk of treatment failure with 3^{rd} or 4^{th} generation cephalosporins (WHO, 2004a).

The prevalence of ESBLs among clinical isolates varies from country to country and from institution to institution (WHO, 2004a). Eastern Europe is affected seriously by the emergence and spread of different ESBLs among hospital-acquired and some community-acquired pathogens. According to the data from international multi-centre surveys, the prevalence of ESBL producers among *Enterobacteriaceae* isolated in Intensive Care Units (ICUs) in Poland and Russia is more than 40%. In ICUs, in some Moscow hospitals, more than 90% of *Klebsiella* species isolated harbours ESBLs (WHO, 2004a). Molecular studies reveal that Sulphydryl variable (SHV)-like enzymes are predominant among ESBL producers in Poland and Russia (in 60% and 70% of isolates, respectively), Temoniera (TEM)-like enzymes in 20% and 40%, and cefotaximase (CTX-M)-like enzymes in 20% and 40%. In Russia, 45% of isolates harbours 2 or 3 different enzymes simultaneously (WHO, 2004a). Hospital outbreaks of ESBL-producing bacteria have been reported in the United States and other countries. The gastrointestinal tracts of the patients are regarded as the probable reservoirs for these organisms (Seema, 1999). Alternate reservoirs are the oropharynx, colonized wounds and urine (Joumana, Samaha, & George 2003).

Klebsiella pneumoniae is the species in which the ESBLs have been most commonly reported around the world. Mostly the TEM or SHV derived *Klebsiella pneumoniae* species are from the hospitalized patients and have caused nosocomial infections (Sirot, 1995). However, plasmid mediated ESBLs which are not TEM or SHV derived have been reported and these bacteria are resistant to all cephalosporins including cephamycins. Recent studies have investigated the risk factors in non-hospitalized patients. The predominant infection found in these patients was of the urinary tract due to community-acquired ESBL-producing enterobacteria (Moor, Roberts, Simmons Briggs, Morris, Smith & Heffernan, 2008).

According to the WHO, resistance in micro-organisms has out-spaced the development of newer antimicrobial agents. Concerns have already been expressed in many quarters that

the world may soon be heading towards a post-antibiotic era where none of the available antimicrobial agents will be effective against commonly encountered microbes. This will lead to terrible mortality and morbidity, taking the nations back to the pre-antibiotic era (WHO, 2004b).

Due to the high prevalence of the ESBLs and their increasing resistance to the antibiotics, this research study will screen selected plants for antimicrobial activity. These plants will be screened against selected strains of ESBLs and other bacterial strains that are not β-lactamase-producing as well as selected fungal strains. All the plants included in this study are medicinal and indigenous to the Eastern Cape, and have been used to treat various infections.

Plants still serve as the main source of curing agents in Africa (Schneider, 2002). Many of the South Africans still believe in the natural medicinal plants and still contact the traditional healers. About 80% of South Africans depend on traditional healers as their main contact for advice on health issues. This might be due to the limited access to Western medical care in the rural areas (Felhaber, 1997). South Africa contains about 10% of the world plant diversity but many medicinal plants have not been scientifically researched and investigated (George, Laing & Drewes, 2001). China is comparatively one of exceptional regions where there is much documentation about medicinal plants (Yavin & Bachrach, 2005). Plants included in this study are *Geranium incanum*, *Dodonaea angustifolia* and *Eucomis autumnalis*.

In this study, the following Gram-negative bacteria were included: *Escherichia coli*, *Enterobacter cloacae, Klebsiella pneumonia, Pseudomonas aeruginosa* and *Acinetobacter species* which are a Gram-negative coccal-bacillus. *Staphylococcus aureus* and *Enterococcus faecalis* represented the Gram-positive cocci and *Bacillus cereus* was the only Gram-positive bacillus. *Candida albicans* was the only yeast and the other fungi included were *Mucor, Geotrichium, Penicillium, Rhizopus* and *Fusarium.* The fungi were included due to their potential capability of causing the opportunistic infections and to represent the eukaryotes.

No matter how sophisticated Western medicine becomes, common ailments like fungal infections can exceed the best of the world's antifungal agents. In people with compromised immune systems (like premature babies, AIDS victims or those undergoing chemotherapy for cancer) the risk is very high: contracting a fungal infection can be fatal. Fungal infections are very difficult to treat and they are considered to be among the most resistant microbes (Tau Researchers, 2009).

1.2 Aim

This study is aimed at revealing plants that can be used to overcome microbial resistance which is a major problem and which at present moment promotes the mortality rate especially of the patients with immunosuppressive infections like HIV/AIDS.

1.3 Objectives

- Screening the different plants extracts for *in vitro* antimicrobial activity against ESBLs, non β-lactamase producing bacteria and fungi.
- To determine the minimum inhibitory concentration of the plants.
- To test the plants extracts for any mutagenic effects.

CHAPTER 2

LITERATURE REVIEW

2.1 TRADITIONAL MEDICINE

2.1.1 Introduction

To survive on earth, there has to be a suitable relationship between people and the environment. As the human population grows and people strive for improved living standards, this relationship is prone to many stresses and strains (Bromilow, 2001). Since the beginning of civilization, survival of the human race was dependent on plants not only as a source of food and oxygen, but also as a source of natural remedies. Books, magazines and many sources of information give a genuine tidal wave of information, declaring the merits and medicinal effectiveness of plants (Schneider, 2002).

Common sense and experience have shown that plants freshly picked, which involve a minimum amount of processing are better able to retain their active principles and therapeutic value (Schneider, 2002). Antimicrobials of plant origin have an extremely large therapeutical potential. They are effective in the treatment of infectious diseases while simultaneously alleviating many of the side effects that are often connected with synthetic antimicrobials (Iwu, Duncan & Okunji, 1999).

Southern Africa is exceptionally rich in plant diversity with some 30 000 species of flowering plants, accounting for almost 10% of the higher plants in the world. Southern Africa also has great cultural diversity, with many people still using a wide variety of plants in their daily lives for food, shelter, fuel, medicine and the other necessities of life (Van Wyk & Gericke, 2000). In the early 1930s, there was a marked increase in the output of research, especially from South African laboratories, on the chemistry, pharmacology and toxicology of plants. It is believed that anything which leads to a

greater utilization of natural products of South Africa should be encouraged (Watt & Breyer-brandwijk, 1932). The more recent development is the increasing commercial exploitation of the medicinal plants. Many of the plants used are cultivated and are often quite harmless, but in a small percentage of cases they are toxic and sometimes deadly. Some plants can cause contact dermatitis or allergic responses in susceptible people and such conditions can sometimes be very intense (Munday, 1988).

Use of medicinal plants as a source of relief and cure from variety of illnesses is as old as humankind itself. Even today, medicinal plants provide a cheap source of drugs for the greater number of the world's population. Plants have provided and will continue to provide not only directly usable drugs, but also various chemical compounds that can be used as starting points for the synthesis of new drugs with improved pharmacological properties (Mukherjee, 2002). Many modern medicines have their origins in plants (Mukherjee, 2002).

Although South Africa contains about 10% of the world's plant diversity, there is still much work that needs to be done on the medicinal plants from this region (George, Laing & Drewes, 2001). Recently ethnopharmacology is progressing in South Africa (Louw, 2002) but in general, there is limited research and investigation regarding the therapeutic potential of medicinal plants (Lin, Opoku, Geheeb-Keller Hutchings, Terblanche, Jager & van Staden, 1999).

2.1.2 Traditional medicine knowledge

According to Grenier, (1998), traditional medicine is the diagnosis and treatment of psychological and medical illness based on local knowledge and socio-cultural and religious beliefs, developed over time by local people within their belief systems and specific environmental conditions of a particular area. Traditional medicine is said to be a well-established system of medicine, parallel to the western or orthodox medicinal system and still in active use by rural communities in developing countries (Iwu & Laird, 1998 : Tabuti, Lye & Dhillion, 2003).

An essential part of traditional medicine knowledge refers to the properties of natural materials used in their wild form, as part of a preparation or mixture. Such medicines include plant based or “herbal medicines” as well as animal parts and minerals (Correa, 2002). Knowledge and the identities of traditional medicines are usually kept as personal secrets or within certain expert circles of traditional medicine practitioners. Bio-prospectors have extracted knowledge by different means and contacts with or without benefits to the owners of the knowledge (ten Kate & Laird, 1999; Svarstad & Dhillion, 2000).

Traditional medicinal knowledge is produced by individuals without any interface with the community or outsiders and therefore will be held by individuals (Correa, 2002). On the other hand, traditional medicine knowledge is in the possession of some but not all members of the group. In some groups, traditional medicine system healers compare notes and share remedies across quite wide geographic areas (Bonabeau, & Theraulaz, 1994). At the end of the day, knowledge may be available to all members of a group, including where knowledge of herbal home remedies is held by multitudes of people, often concentrated among women and the elderly. This kind of knowledge may even spread across national boundaries (Bonabeau, & Theraulaz, 1994).

According to Correa (2002), there is no clear demarcation between what belongs to the general community, specific community, or individual within the communities. Certainly for herbalists, herbal knowledge is treated as personal property. However some of the knowledge they possess is relatively available in the same form in the general community due to older tradition of sharing knowledge (Correa, 2002). On the other hand Sharma (2000) says the herbalists have continuously innovated what is available in the general community and hence they possess special rights to their innovations. Contrarily, he says it is hard to determine how the benefits should be shared if there is no clarity in the ownership (Sharma, 2000).

Internationally traditional medicine has received much attention the last decades. In 1977, the World Health Assembly urged member states to utilize their traditional

systems of medicine. In 1978, the international conference on Primary Health Care, held in Alma-Ata, recommended that governments give high priority to utilization of traditional medicine practitioners and traditional birth attendants, and incorporate proven traditional remedies into national drug policies and regulation (Akerele, 1987).

In Africa, during occupied periods, colonial powers connected traditional medicine practitioners to the use of supernatural forces or witchcraft. Therefore traditional medicine was subjected to discredit and legal prohibitions. When colonization ended, independence made some nations more tolerant towards traditional medicine, regaining African identity and developing national and cultural values. Two nations that fully incorporate traditional medicine in their systems are Ghana and Mali (Diallo & Paulsen, 2000; Romero-Daza, 2002). Due to the lack of proper conventional health care systems, traditional medicine is often the first choice for countries like Tanzania. In such countries, the accessibility to conventional medical doctors is limited as compared to that of traditional medicine practitioners (Marshall, 1998).

The intervention of recognizing traditional medicine has been further heightened by the HIV/ AIDS pandemic (Romero-Daza, 2002) as many HIV positive individuals use herbal remedies to boost the immune system and to fight opportunistic infections (Irin, 2006).

During colonization, traditional medicine was not officially accepted in Africa. Under the British rule, provision for traditional medicine practice was recognized. Medical practitioners and dentist advanced, but co-operation between traditional medical practitioners and conventional doctors was forbidden. The witchcraft ordinance of 1928 was operational, forbidding witchcraft. However, during the period 1970-1988, approximately 3690 people reportedly died in witchcraft related incident (Marshall, 1998).

In South Africa and Zimbabwe, the responsible people in authority allow substantial recognition to healers through national efforts designed to integration of traditional and

Western medical systems. In other countries, healers are not recognized therefore their status exists purely within the custom of local communities (Correa, 2002).

In the year 2008, there was a transformation by the South African Government for formal recognition of traditional medicine. South African Government is committed to institutionalizing African traditional medicine in the health care system. The main objective of the plan of action is to recognize, accept, develop and integrate the African traditional medicine by all member states into public health care system in the region by the year 2010 (Ministry of Health, 2008).

2.1.3 Importance of traditional medicine

In many parts of the world, there is a rich tradition in the use of herbal medicine for the treatment of many infectious diseases (Schneider, 2002).

Plants are used in many different ways by the traditional herbalists; the simplest and most effective ways have survived the passage of time. Some of the areas in which they can be readily found are:

- In cooking in the form of seasoning salads, and eaten as fruits.
- In beauty and personal-hygiene products. e.g., bubble baths, tooth paste and soaps. The best perfumes are made from essential oils derived from distilled flowers.
- In farming, as a compost activator and a pesticide.
- In medicine, about 40% of synthetic medications are derived from plants. Cardio tonic is made from foxglove, a remedy for cancer is derived from yew, and that for leukemia is taken from periwinkle (Schneider, 2002).

The WHO has estimated that about 80% of the population living in the developing countries relies on traditional medicine for their health care needs (WHO, 2002) and there is an estimation that about 80% of all South Africans use traditional medicine derived from plant species indigenous to the region. Traditional medicine is beneficial

even in developed countries and has also influenced pharmaceutical products. Extracts of plants and algae have been incorporated in the products and plants in particular are an indispensable source of pharmaceuticals (Lambert, Srivastava, & Vieyer, 1997). Recently, there has been a dramatic increase in the demand for "herbal medicines" (WHO, 1996). According to one estimate, the world market for "herbal medicine" has reached 60 billion US dollars, with annual growth rates of between 5% and 15% (WHO, 2000).

The importance of traditional medicine is being recognized in most parts of the world. The public policies which are based on traditional medicine vary from country to country. Different policies exist, in particular, in relation to the integration of traditional medicine in national health care systems (Correa, 2002). Countries such as China, the Republic of Korea and Vietnam have adopted measures to promote this integration. These countries are aiming to exploit the complementarities of traditional medicine in their health care systems. Measures have included procedures for the registration of traditional healers or herbalists. They have established specialized hospitals and higher institutions like colleges and universities and they are involved in research programmes encouraging the development of traditional medicine (Xu & Yang, 2009). Validation and certification of traditional medicine products and good manufacturing practices are encouraged. They have incorporated medical doctors who have graduated from traditional medical universities into the hospitals to promote the use of traditional medicine in combination with the practice of Western medicine. In order to formalize the position of traditional medicine within the health system of the state, a necessary first step is the establishment of the standards of quality, safety and efficacy (Scott, Springfield & Coldrey, 2004).

Traditional medicine according to Carlos Correa (2002) includes knowledge and practices either codified in writing or transmitted orally (Correa, 2002; Zhang, 1998). Traditional medicine serves the vital needs for multitudes of people in developing countries since the economic and cultural factors limit people from accessing 'modern' health care services. In such countries, traditional medicine is used by most of the people because it is available and accessible even to the poorest population and to those in

remote areas (Correa, 2002). There is an anticipated increase of the usage and relevance of traditional medicine as high prices are generally charged for patented medicines (Kettler, 2002).

2.1.4 The role of traditional healers in the community

In ancient times, traditional healers often served as advisors to chiefs especially in rural areas. They were also decision makers and were fulfilling the role of arbiter within their communities. Healers were consulted in cases of natural disasters such as drought, and were observers of social order and harmony amongst families and individuals. Traditional healers were of vital importance to their society because they do not only look after the individual's well-being, but even after the health of the community as a whole. Today traditional healers still hold positions of respect within their communities, and provide guidance on health matters and other issues important to the people. Traditional healers are taken as guardians of their cultural norms and rules (Mayeng, 1997).

In many countries, traditional healers are established health care workers within their communities. Rough estimations show that about 80% of South African's population consults traditional healers for advice and health treatment (WHO, 2002; Lambert, Srivastava; Vieyer, 1997).

Traditional medicine is readily available and affordable and that enable traditional healers to treat people of all age groups, presenting with any problem. Their treatment is said to be holistic, they treat the whole person not only the symptoms of the disease (surroundings inclusive). In countries like Chile, Mexico, Peru, Philippines South Africa and Zimbabwe, traditional medicine is being actively promoted with the aim of making it part of the national health care system (Ministry of Health, 2008). According to Isaac Mayeng (1997), the role of traditional healers in primary health care can be formalized if the government of such countries as mentioned above could fully recognize the services that the traditional healers provide.

2.1.5 Traditional medicine and Westernized biomedicine

2.1.5.1 Concept of disease

Traditional belief is that disease occurs when body functioning deviates from normal or when there is something foreign present in the body. It is believed that the abnormal functioning of the body may be organic, physiological, emotional or psychological in nature, and may manifest itself in the form of one being sick or ill. Traditionally, the cause of disease may or may not be obvious, and can also be either contagious or non-contagious. Traditional belief is that disease may be caused by several factors. These causes or factors can be divided into natural causes and unnatural causes (Mayeng, 1997 & Brown, 1997). Natural causes of disease include age-related diseases and accidents, while unnatural causes of disease include: sorcery, and spirits. Unnatural causes of diseases have not been scientifically followed up or proven (Mayeng, 1997).

Western concept of disease indicates that illness usually results from a combination of causes. According to Western medical science, it may be easy to understand the cause of the disease if divided into infectious disease and non-infectious diseases (Jensen, Wright & Robison, 1997). Infectious diseases are the diseases that are caused by micro-organisms. There are many types of micro-organisms that cause diseases. Bacteria cause tuberculosis, cholera, typhoid, septic wounds and some types of pneumonia. Viruses cause colds, influenza, coughs, diarrhoea and HIV/AIDS. Fungi, helminthes and protozoa living inside the body are the other causative organisms of infectious diseases (Jensen, Wright & Robison, 1997). Micro-organisms may enter the body through the nose or mouth, from the gastrointestinal tract or the urogenital tract, and through breaks in the lining of the mouth, vagina or anus and the skin. Most of the non-infectious diseases are not spread from one person to another. Examples of non-infectious disease include diseases that are caused by: something that wears out in the body (arthritis) or goes wrong within the body (cancer) and something from outside the body that is harmful or irritating to the body (allergies, poisons, asthma). A lack of something that the body needs to function properly (Vitamins, minerals) can also be the principal cause of non

infectious disease. Some people are born with the problems (called congenital problems) and some problems start in the mind (anxiety, depression) (Mayeng, 1997).

2.1.5.2 Diagnosis

Traditional diagnosis is a system that is both an art and a method of seeking to discover the origins of disease and what it is. The diagnostic process seeks answers to the questions of how the disease originated (in relation to natural causes), and why the disease is manifesting itself (the deeper meaning or motivation behind the causes). The diagnosis is made after combining information gathered from observing the patient, patient self-diagnosis and divination (Mayeng, 1997).

In Western medicine, the diagnosis of illness in a patient is based upon the patient's history, a physical examination of the patient, and any special investigations or tests that thought to be necessary. A patient's diagnosis is based on differential diagnosis which is a list of possible illness that fit the patient's symptoms (Lombardo & Buckeridge, 2007).

2.1.5.3 Treatment

Traditional treatment is holistic in nature because of its comprehensive approach. It is holistic because it cannot be reduced to a single dimension of treatment or remedy. The purpose of traditional treatment is aimed not only at curing the disease, but also at healing the patient. The relationship between various organs or the importance attached to specific organs is considered. The states of the patient's social and spiritual relationships, as well as certain internal psychic states such as guilt and anxiety, are all taken into consideration when diagnosing and determining treatment (Mayeng, 1997).

Traditional treatment has elements of being curative, protective and preventive, and can be either natural or ritual, or both ritual and natural. The treatment of the disease depends on the diagnosis as to what the cause of the disease is. Administration of the treatment is specific to the illness but is flexible and adapted to the daily changes in the patient's

health. It involves proper follow-up, with alertness to any changes in symptoms or in the patient's psychic conditions (Mayeng, 1997).

Traditional treatment comprises a variety of procedures and methods that are used in various combinations to treat disease. Traditional treatment can be performed in a form of traditional medicine formulas (Mayeng, 1997), bathing (Samson, 1998), behavior; this method refers to the role of counseling and encouragement in obtaining the desired drug compliance from the patient (Mayeng, 1997). Blood cleansing, charm (Taafaki, Flower & Thaman, 2006), cuts, dancing (Tansey, 2003; Ganapathy, 2006), diet (Dragon, 2009), emetics (Ndubani & Hojer, 1999; Taafaki, Flower & Thaman, 2006), enemas (Edler & Mukherji, 1997), intelezi, reassurance, rest and ncinda (Mayeng, 1997) are the other traditional ways of treatment. Traditional healers also use prayers to ancestors (Ganapathy, 2006), sacrifices to ancestors, drumming (Mayeng, 1997; Ganapathy, 2006), smoke ihalation, steaming (Bhootra & Kitinya, 2009), snuff (Balisunset, 2009), and piercing (Tansey, 2003) for the treatment of some diseases.

On the other hand, the Western approach to treatment is diagnostic-based. This means the treatment chosen is specific for the illness diagnosed. Treatment vary with any number of factors, some of which include: the type of sickness, how severe the illness is, what other illnesses the individual may be suffering from, what other medications the person might be taking, and any allergies. There are almost as many types of treatments as there are diseases. Treatments are being continually evaluated and improved, and it is up to the health professional to keep up to date with current trends in treatment. The body also has a natural way of fighting the disease by using the immune system. Individual can help the body to fight illness by keeping clean (bathing everyday), getting plenty of rest, drinking plenty of liquid (especially water) and eating well. Even when there is a more serious illness that requires medicine, it is still the body that must overcome the disease; the medicine only helps the body to do it (Lombardo & Buckeridge, 2007).

When medicines are needed to treat an illness, some guidelines for their use must be remembered. Any medicine is said to be potentially dangerous if not used correctly. The

guidelines for proper use of medicine are: medicine should only be used when necessary, knowledge to how to use the medicine correctly and the precautions for its use are recommended, right dosage is required, most importantly, using of medicine should be stopped right away if it does not help or causes more problems, and lastly, if in doubt, a patient must seek a professional help of a health worker (Moberg & Cohn, 1990).

Another means of treatment used in Western medicine is surgery. The best treatment for most illness is prevention (Lombardo & Buckeridge, 2007).

Western medicines are the drugs used to treat illnesses. There are thousands of drugs available. The World Health Organisation has drawn up a model list of essential drugs. South Africa has its own essential drugs list which is available from the department of Health. The objective of an essential drugs list is to ensure that the drugs on it are those that can be used to treat the majority of the people. Medicines are available over the counters (OTC) or by prescription from a doctor. In South Africa, the regulatory body called the Medicine Control Council decides how a drug should be classified, and what controls should limit its availability. These decisions are based upon research and trials testing using the drugs prior to use (Mayeng, 1997).

Antimicrobial drugs have also proven remarkably effective for the control of bacterial infections. Different drugs may include antibiotics which are used to fight certain infections and diseases caused by bacteria. The different types of antibiotics work in different ways against specific infections; treatments must be chosen with care. Antibiotics should not be used unless the prescriber knows which group of antibiotics it belongs to, what disease the group is used to treat, and the precautions that must be taken when the antibiotics are used. Otherwise they will not work, may be unsafe to use, and the organism causing the illness may develop resistance to treatment (Moberg & Cohn, 1990).

When antibiotics are used to treat an infection, they must be taken for the whole length of time for which they have been prescribed, even if the person feels better few days after

taking them. This is to make sure that all bacteria have been treated properly. Stopping the drug before the prescribed time can cause bacteria that are resistant to the drug to grow out of control, resulting in difficulty in treating the illness the second time (Terrie, 2004).

Antibiotics should be limited to when they are absolutely needed. Like any medicine, they can cause side-effects or allergic reactions. They can also upset the natural balance in the body by killing body's normal flora together with the infectious bacteria. This is one of the reasons why some people develop diarrhoea while taking antibiotics; the medicine kills the bacteria in the gut that are necessary for digestion (Moberg & Cohn, 1990).

The most important reason why the use of antibiotics should be limited is that there is too much use of antibiotics already. The treatments of minor infections that can get better on their own do not need antibiotics. All over the world, microbes are becoming resistant to antibiotics because they have been overused or used incorrectly (Mayeng, 1997; Terrie, 2004).

Pain medicine, decongestants and medicines used to treat gastrointestinal illnesses are the other alternative medicine in the treatment of illnesses by the westernized medicine. Combination usage of pain medicine should be avoided; even those put together in a single tablet, as they are no better for treating pain and may even cause new problems (Tansey, 2003).

Decongestants may be taken as tablets or may be used as spray in the nose and should not be used for more than 3 to 5 days at the time, because after that they may cause a worse runny or stuffy nose than before they were used. Decongestants are not recommended for people with high blood pressure because they can exacerbate the situation. Medicines used to treat gastrointestinal illnesses must only be used under the control of pharmacist or doctor (Mayeng, 1997)

2.2 MEDICINAL PLANTS

Many plants are cultivated and used as food, medicine and compounds activators in farming. Most of them are often quite harmless, but in a small percentage of cases they are toxic and to some extent lethal (Munday, 1988). Most of the traditional medicine systems use a large number of medicinal plants (Shankar, Hafeel, & Suma, 1999). Therefore, most of the cases traditional medicine is mostly linked to plant-based medicines. Animal medicines have also played a vital role in healing practices, magic rituals, and religions of many societies. It is estimated that of 252 essential medicines selected by the WHO, 11.1% are derived from plants and 8.7% from animals (Correa, 2002). Plants are the reliable sources of antimicrobial compounds and hence are used as antibiotics.

2.2.1 Plants as alternative source of treatment

There is a continuous and urgent need to develop new antibiotics and immune modulating compounds, with widely varied chemical structures and different mechanisms of action, because there is an alarming increase in the frequency of new and re-emerging infectious diseases (Rojas, Bustmate, Bauer, Fernandez & Alban, 2003). Due to the side effects and the resistance that pathogenic micro-organisms build against the antibiotics (Essawi & Srour, 2000), there is increased attention on extracts and biologically active compounds isolated from plants species and used in herbal medicine.

The WHO, (2002) has also emphasized development and utilization of herbal drugs and traditional medicines for the benefit of the world population, in terms of the cost effectiveness and side effects of the drugs. In traditional systems of medicine, a single drug or combinations in raw forms are preferred over compound formulation (Mukherjee, 2002; Mukherjee & Wahile, 2006). An example of such medicines as mentioned before is cardio tonic made from foxglove (Schneider, 2002).

2.2.2 Trade in medicinal plants of South Africa

The trade in traditional medicines forms part of a multi-million rand 'hidden economy' in Southern Africa (Cunningham, 1997) stimulated by high population growth, rapid urbanization, unemployment, and the high cultural value of traditional medicine. Demand generates a species-specific trade network that can extend across national boundaries (Cunningham, 1997). Consequently the trade in traditional medicines is now greater than at any time in the past and is certainly the most complex resource management issue facing conservation agencies, health care professionals and resource users in South Africa today (Cunningham, 1997). It was estimated that there are 27 million indigenous medicine consumers in South Africa with a large supporting industry (Mander, 1998). The use and the trade of plants for medicine is therefore no longer confined to traditional healers but has entered both the informal and formal entrepreneurial sectors of the South Africa economy (Cunningham, 1989; Danskardt, 1990; Cocks & Dold, 2000), resulting in an increase in the number of herbal gathers and traders.

2.2.3 Medicinal plants under investigation

All the plants that are included in this study are indigenous to the Eastern Cape and have been traditionally used as medicine to treat ailments especially those that are caused by infections. The following plants were selected for the study: *Geranium incanum* VanWyk, Oudtshoorn & Gericke, (1997) (Geraniaceae), *Dodonaea angustifolia*, VanWyk *et al*., (1997) (Sapindaceae) and *Eucomis autumnalis* VanWyk *et al*., (1997) (Hyacinthaceae).

2.2.3.1 *Geranium incanum***:**

Geranium incanum Taken from (VanWyk, Oudtshoorn & Gericke, 1997).

Common names: (Afrikaans) Bergtee, amarabossie (Sotho) Ngope setsoha, tlako (VanWyk *et al*., 1997).

This is an attractive sprawling perennial shrub with finely divided, silvery leaves. The flowers are white, pale pink violet or magenta in colour and borne on long slender stalks. The elongated fruit resembles a stork's bill.

The hair on the exterior of the flowers and flower stalks lie flat and are not spreading as in related species (VanWyk *et al*., 1997). An infusion of the leaves of the plant and rarely the roots are traditionally used to relief diarrhoea and colic, to treat venereal disease and most importantly to relief bladder infections in women and for gynaecological problems (VanWyk *et al*., 1997) & (Hillard & Burt, 1985).

The results of an investigation of cytotoxicity and antiviral activity of 16 South African plant species showed that aqueous extracts of *Geranium incanum* showed cytotoxicity. The aqueous extracts in a cell culture antiviral assay were found to be effective on both coxsakie B2 virus and herpes simplex virus-1(HSV-1). The cytotoxicity of aqueous extracts may possibly be ascribed to presence of polyphenols (tannins) and account for *Geranium incanum* as an abortifacient (Treurnicht, 1997). Some studies reported no *in vitro* antimicrobial activity of aqueous extracts of *Geranium incanum* when tested against *Pseudomonas aeruginosa* and *Candida albicans* and weak activity were noted against *Staphylococcus aureus* (Treurnicht, 1997).

2.2.3.2 *Dodonaea angustifolia*

Dodonaea angustifolia Taken from (VanWyk *et al*., 1997).

Common names: (English) Sand olive (VanWyk *et al*., 1997).

The sand olive is a small shrub or small tree of about five metres in height, occurring in a wide range of habitats, from deserts to forest margins. The long, narrow leaves are pale green and shiny as a result of resinous exudates on the surface. The inconspicuous flowers are yellowish-green and bear small, winged papery fruits roots (VanWyk *et al*., 1997). Leaves of this plant are traditionally used to relief fever, sore throats, chest complaints, influenza, stomach disorder and cancer (VanWyk *et al*., 1997). *Dodonaea angustifolia* has been found to have antifungal properties (Heymann, Hussein, Meyer, & Lall, 2009). The plant is also traditionally used to treat oral Candidiasis and is reported as being more effective than commercially available mouth rinses.

Dodonaea angustifolia is said to have antibacterial activity against MRSA (Heymann *et al*., 2009). However, in other preliminary *in vitro* assays there were no antimicrobial

activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans* (Asres, Bucar, Edelsbrunner, Kartnig, Hoger & Thiel, 2001).

2.2.3.3 *Eucomis autumnalis*

Eucomis autumnalis Taken from (VanWyk *et al*., 1997).

Common names: (English) Pineapple flower
(Afrikaans) Wilde pynappel (VanWyk *et al*., 1997).
This bulbous plant has long, broad, soft-textured leaves with weavy margins. Numerous small, yellowish-green flowers are borne on a thick central stalk. A rosette of green leaves can be found above the flowers, a characteristic feature that gives the flower a cluster that resembles a pineapple roots (VanWyk *et al*., 1997). Decoctions of the plant are used for urinary disease, stomach aches, fevers, colic, and syphilis and also in facilitating child birth. It was also used as a wound therapy (VanWyk *et al*., 1997).

Eucomis autumnalis is reported to have antifungal activities when tested against pathogenic plant fungi (Pretorius, Craven, & Van der watt, 2002). Ethnopharmacological

investigations have revealed antibacterial properties of the leaves and the underground parts (Zsche, Rabe, Taylor, Jager, & Van Staden, 2000; Louw, 2002).

2.3 ANTIMICROBIAL RESISTANCE

Pathogenic bacteria develop resistance to antimicrobial drugs by at least one of the following mechanisms: The bacteria may delay or inhibit the entrance of the drugs into cells. The bacteria may alter the receptor site of the drug so that a drug is not able to bind or has less affinity to the bacterial cell. Bacteria may alter its metabolic pathways so as to trigger the production of enzymes that may effectively reduce the power of the drug. The other way in which bacteria may be resistant to drugs is by pumping the drug out of the cells before the drug is active. Bacteria may also produce enzymes that destroy or deactivate the drug. This is a common mode of resistance in β-lactamase producing bacteria (Bauman, 2007).

β-lactamases are the enzymes produced by some bacteria and are responsible for bacterial resistance to β-lactam antibiotics such as penicillins, cephalosporins, cephamycins and carbapenem (Bradford, 2001). There are over 200 different lactamases which have been identified (Bauman, 2007).

β-lactam antibiotics are a broad class of antibiotics which are used to treat various infections. At first, β-lactam antibiotics were mainly active only against Gram-positive bacteria. The recent development of resistant Gram-negative bacteria called ESBLs increased their usefulness. β-lactam antibiotics have a common element in their molecular structure which is a four-atom ring known as β-lactam. The β-lactamases produced by the bacteria break that ring open, and deactivate the antibacterial properties of the β-lactam antibiotics (Bradford, 2001).

β-lactam antibiotics are bactericidal and act by inhibiting the synthesis of the peptidoglycan layer of the bacterial cell wall. The peptidoglycan layer is important for structural integrity of the cell wall especially in Gram-positive bacteria. The final transpeptidation step in the synthesis of the peptidoglycan is facilitated by the transpeptidases known as penicillin-binding proteins (PBPs) (Bush, Jacoby & Amedeiros, 1995). The effectiveness of these antibiotics relies on their ability to reach their target (PBPs) intact, their ability to bind the PBPs (Emery, & Weymouth, 1997) and their resistance of inactivation by β-lactamases (Prober, 1998). The common ways in which the β-lactamase producing bacteria become resistant to drugs are discussed in more detail below.

2.3.1 Classification of resistance mechanisms in β-lactamase producing bacteria

Resistance mechanisms can be broadly classified into three main types:

- The bacterial target site may be altered

The ultimate target of β-lactam antibiotics is a group of proteins called penicillin-binding proteins (PBPs) found in the bacterial cell wall. The proteins are penicillin-sensitive enzymes involved in cell wall biosynthesis. Resistance at β-lactamase producing bacteria target sites for the β-lactam antibiotics may be secondary to alteration in PBP structure of β-lactamase producing bacteria or to a decrease in the β-lactam antibiotics affinity for PBPs (Washington, 1986). The target may be altered so that it has a lowered affinity for the antibacterial agent, but still functions adequately for normal metabolism to proceed. Alternatively an additional target may also be synthesized (Mims, Dockrell, Goering, Roitt, Wakelin & Zuckerman, 2004). Methicillin resistant staphylococci synthesize an additional PBP, which has a much lower affinity for β–lactam antibiotics than the normal PBPs and is therefore able to continue cell wall synthesis when the other PBPs are inhibited (Mims *et al*., 2004). Methicillin resistant *Staphylococcus aureus* (MRSA) is resistant to most of β-lactam antibiotics including methicillins and other related penicillins (Centre for Disease Control (CDC), 2009). Methicillin resistance in *Staphylococcus aureus* is caused by the acquisition of the exogenous (*mec A*) gene. This gene encodes for an additional penicillin-binding protein, referred to as PBP 2a (or PBP

2), with a low binding affinity for β-lactam antibiotics and consequently these strains are resistant to all β-lactam antibiotics (Grisold & Kessler, 2006).

- Access to target site of bacteria may be altered

This mechanism is found in Gram-negative cells where β-lactam antibiotics gain access to their target PBPs by diffusion through protein channels (porins) in the outer membrane. This mechanism involves decreasing the amount of drug that reaches the PBPs by either altering the porins or pumping the drug out of the cell (Mims *et al*., 2004). Mutations in porins genes result in a decrease in permeability of the outer membrane and hence resistance. Strains resistant by this mechanism may exhibit cross-resistance to unrelated antibiotics that use the same porins (Mims *et al*., 2004).

- Enzymes modifying or destroying antibacterial agents may be produced

There are many examples of such enzymes, the most important being β-lactamases, aminoglycoside-modifying enzymes and chloramphenicol acetyl tranferase (Mims *et al*., 2004). The β-lactamases of Gram-positive bacteria are released into extra-cellular environment and resistance will only be manifested when a large population of cells is present. The β-lactamases of Gram-negative cells, however, remain within the bacterial periplasm (Mims *et al*., 2004). The amide bond in β-lactam ring of cephalosporins and penicillins is a target for β-lactamases that hydrolise the amide bond resulting in acidic derivatives which have no antibacterial activity (Washington, 1986). This will be discussed further in 2.3.2 below. β-lactamase producing bacteria are said to be the major determinants of the resistance of β-lactam antibiotics of most bacterial pathogens (Medeiros, 1984; Prober, 1998).

2.3.2 β-lactamases and antibiotics

β-lactamases are the enzymes that catalyze hydrolysis of the β-lactam ring of penicillin antibiotics to yield microbiologically inactive products. These enzymes are mainly produced by Gram-positive bacteria. Genes encoding these enzymes are widespread in the bacterial kingdom and are found on the chromosomes and on plasmids (Mims *et. al*.,

2004). Gram-negative bacteria also started producing the β-lactamases called Extended Spectrum β-lactamases (ESBLs) because of their greatly extended substrate range (Sirot, 1995). Extended spectrum β-lactamases were first recognized in Europe, in Germany and in France, and have been reported world wide (Sirot, 1995). ESBLs are capable of efficiently hydrolyzing penicillins, narrow spectrum cephalosporins, many extended-spectrum cephalosporins, monobactams and the oxiamino group containing cephalosporins such as cefotaxime and cefazidime (Livermore, 1995). Strategies for overcoming microbial resistance to β-lactam antibiotics have not only included the development of new β-lactam antibiotics with increased stability to β-lactamases but also the use of β-lactamase inhibitors such as clavulanic acid to enhance β-lactam activity (Washington, 1986).

2.3.3 Mechanism of bacterial resistance to cephalosporins

Cephalosporins are the most frequently prescribed class of antibiotics. They are structurally and pharmacologically related to the penicillins. Like the penicillins, cephalosporins have a β-lactam ring structure that interferes with synthesis of the bacterial cell wall and hence they are bactericidal (Dancer, 2001).

The antibacterial activity of cephalosporins depends on their ability to penetrate the bacterial cell wall, resist inactivation by β-lactamases and bind to and activate penicillin-binding proteins (PBPs). Bacterial resistance may develop at each of these steps. The outer wall of Gram-negative bacteria, composed of lipids, proteins and polysaccharides presents a powerful barrier. Cephalosporins penetrate this complex cell wall through the channels formed by the proteins called porins. The number and size of porins vary among different Gram-negative organisms. Decreased cell wall penetration is an important mechanism of cephalosporins resistance of *Pseudomonas aeruginosa* and *Enterobacter cloacae* (Prober, 1998). *Staphylococcus aureus* is another example of very resistant organisms to cephalosporins.

2.3.4 Classification of β-lactamases

During the 1980s, β-lactamases were classified according to their plasmids, which are transferred from cell to cell by phages (transduction). First strains that were found to produce β-lactamase were the strains of *Streptococcus umbers* and two strains of *Enterococcus faecalis*. One of these strains has been shown to be plasmid-determined. At that time, the enzymes were not yet extensively characterized (Medeiros, 1984). The wide variety of β-lactamases, produced by Gram-negative bacteria, has led to a number of classification schemes. The earliest were based primarily on a substrate profile that is the rate of hydrolysis of penicillins versus cephalosporins. β-lactamases, with additional distinction provided by parameters such as enzyme inhibition and reaction to antisera were also found (Medeiros, 1984).

On the other hand, β-lactamases were classified on the basis of amino acid and nucleotide sequencing data and the results of the enzymatic analysis. There were three distinct classes of β-lactamases that had been defined (Washington, 1986). Class A had a molecular weight of approximately 29,000 daltons that particularly hydrolyze penicillins, and include TEM-1 broad spectrum β-lactamases from Gram-negative bacteria and Gram-positive bacteria and the "penicillinases" of staphylococci and of *Bacillus licheniformis* (Washington, 1986). Class B enzymes had a molecular weight of 23,000 daltons and break down cephalosporins and can only be produced by *Bacillus cereus* (Washington, 1986). Class C enzymes that included the chromosomal cephalosporinases of Gram-negative bacteria had molecular weights of 39,000 daltons (Washington, 1986).

During the 1990s, a classification scheme for β-lactamases based on functional characteristics was made. Three major enzymes were defined by their substrate and inhibitor profile. Group 1 enzymes are the cephalosporinases. Group 2 enzymes are penicillinases, cephalosporinases, and broad spectrum β-lactamases that are generally inhibited by active site-directed β-lactamase inhibitors. Group 3 are the metallo-β-lactamases that hydrolise penicillins, cephalosporins and carbapenems that are poorly inhibited by most of β-lactam-containing molecules (Bush, Jacoby & Amedeiros, 1995).

To date, hundreds of different β-lactamase enzymes have been described. All have the same function but with different amino acid sequence that influence their affinity for different β-lactam substrates. Some enzymes specifically target penicillins or cephalosporins while others are especially troublesome in broadly attacking most β-lactam compounds (Mims *et al*., 2004).

According to the currently accepted classification, β-lactamases fall into four distinct classes, which are A, B, C and D, respectively. Of these the members of classes A, C and D utilizes an active-site serine residue as a nucleophile in their catalytic mechanism involving an acyl-enzyme intermediate. The class B β-lactamases, in contrast, are metalloproteins with Zn^{2+} present in their active sites and are often referred to as metallo-β-lactamases (Viswanata, Laura, Goodyellow & Gary, 2007).

2.3.5 Plasmid mediated β-lactamases

The synthesis of large numbers of antibiotics over the past three decades has caused complacency about the threat of bacterial resistance. Bacteria have become resistance to antimicrobial agents as a result of chromosomal changes or the exchange of genetic material via plasmids and transposons (Michael & Simor, 2009).

The first *Klebsiella* isolate with plasmid mediated resistance to broad-spectrum cephalosporins was reported in the Federal Republic of Germany in 1983. Since then this has been a growing problem all over the world. In the United States, the frequency of resistance to ceftazadime has increased from 1.5% (1987 to 1990) to 3.6% (1990 to1991) as reported by the National Nosocomial Infections Surveillance system. A surveillance trial involving 102 medical centers in the United States detected 10.3% and 23.8% ceftazadime resistant *E. coli* and *K. pneumoniae* respectively (WHO, 2004b).

Due to plasmid-mediated resistance, members of *Enterobacteriaceae* and *Pseudomonas* families are resistant to virtually all of the existing antibiotics. Perhaps of greater concern is the development of resistance because of the acquisition of new genetic material.

Genes mediating antimicrobial resistance may be found on transferable segments of DNA such as plasmids, transposons or integrons. Plasmids are extrachromosomal molecules of DNA that replicate independently from the bacterial chromosome. They may carry genes that convey resistance to antibiotics, as well as genes that may enhance bacterial fitness or virulence. Transposons carry antibiotic resistance genes along with genes that allow them to replicate and transpose, to other regions of the chromosome or to plasmids. An integron is a DNA structure capable of capturing genes. Although integrons are not themselves mobile, they may be carried by plasmids or transposons to other bacteria. These mobile genetic elements may be transferred from organism to organism, and even from one bacterial species to another. Multiple antibiotic resistance genes may be transferred at the same time. There are numerous examples of this type of resistance, including plasmid-mediated production of β-lactamase enzymes, which are capable of inactivating penicillins or cephalosporins found in *Staphylococcus aureus*, *Escherichia coli* or *Enterobacter species* (Michael & Simor, 2009).

2.3.6 Extended spectrum β-lactamases

The Gram-positive bacteria were the only β-lactamase producing bacteria mainly by the strains of *Staphylococcus aureus* called MRSA. Later there was an emergence of Gram-negative β-lactamase producing bacteria with a wide substrate range called ESBLs. The ESBLs pose resistance to most of the drugs with β-lactam ring.

The number and variety of ESBLs and mechanisms that regulate their spread and production have been rapidly increasing (Sirot, 1995). According to the project of WHO involving the monitoring of antimicrobial resistance 2004, India and Sri Lanka show the occurance of 50-90% of resistance to antimicrobial agents which are commonly used to treat infections (WHO, 2004b). Multiple studies in a wide range of settings have shown that failure or delay in adequate therapy results in an adverse mortality outcome which is also the case with infections caused by ESBL-producing bacteria (Pitout & Laupland 2008). The outcomes of the previously undertaken studies show that there is a great problem concerning the treatment of resistant strains of ESBL-producing bacteria. It is suggested that some special care measures should be followed in the selection of β-

lactam antibiotics for the treatment of infections caused by ESBL-producing strains because presence of ESBL enzyme does not prevent other mechanisms of resistance from emerging (for example decreasing permeability to cephamycins) (Sirot, 1995).

ESBLs grant variable levels of resistance to monobactams such as aztreonam, cefotaxime and cetazidime (Sirot, 1995). In the past 20 years, increasing attention was directed towards plasmid-mediated and quite recently chromosome-mediated β-lactamases, especially in Gram-negative bacilli. In countless studies, investigators have looked for antibiotics capable of inhibiting Gram-negative β-lactamases (Washington, 1986).

2.4 SELECTED PATHOGENS

In this study, ESBL-producing bacteria were obtained from the National Health Laboratory Service (NHLS), in Port Elizabeth. The antimicrobial screening of ESBL producers as well as non-ESBL producers were performed on both Gram-positive and Gram-negative bacteria. Antimicrobial studies have shown that Gram-negative bacteria show a higher resistance to plant extracts than Gram-positive bacteria (Palambo & Semple, 2001). Gram-negative bacteria have an outer membrane that is composed of high density lipopolysaccharides that serve as a barrier to many environmental substances including antibiotics (Palambo & Semple, 2001).

Certain Gram-positive bacteria are more susceptible to antibiotics than Gram-negative bacteria. Other Gram-positive bacteria like MRSA which is now referred to as a ‘super bug’ causes a range of ailments like pneumonia, mastitis, meningitis, urinary tract infections and post operational infections (Heymann *et al*., 2009). Strains of *Escherichia coli, Enterobacter cloacae, Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococus aureus, Candida albicans, Bacillus cereus, Enterococcus faecalis,* and *Acinetobacter species* were tested. The following ATCC strains were used: *Escherichia coli* 35218, *Pseudomonas aeruginosa* 27853 and *Staphylococcus aureus* 43300. Certain opportunistic fungi were included in this study.

2.4.1 *Escherichia coli*

Escherichia coli (*E. coli*) belongs to the family of *Enterobacteriaceae* (Bauman, 2007). The bacteria in this family are facultative anaerobic Gram-negative rods. Most of the bacteria are commernsals or normal flora of the human and animal gastrointestinal tract and may occasionally be associated with disease in humans (Centers for disease control and precention (CDC), 2007). Others are opportunists that can become pathogenic in other body sites. *E. coli* are among the most medically important bacteria and causes 12-50% of nosocomial infections. Virulent strains of *E.coli* have genes that code a variety of exotoxins, which enables these strains to colonize human tissue and cause disease. *E.coli* is responsible for causing numerous diseases which include septicemia, UTI's, neonatal meningitis and gastroenteritis. Gastroenteritis which is mediated by exotoxins called enterotoxins is regarded as the most common disease associated with *E.coli*. Enterotoxins trigger the production of watery diarrhoea. Some *E.coli* antigens like 0157:H7 can result in diseases which ranges from bloody diarrhea to severe or fatal hemorrhagic colitis, to a severe kidney disorder called hemolytic uremic syndrome which results in renal function failure. *E.coli* 0157:H7 also produces shiga-like toxin, which inhibits protein synthesis in host cells. The use of antimicrobial drugs induces *E.coli* 0157:H7 to increase its production of shiga-like toxin, which then results into disease exacerbation (Bauman, 2007).

2.4.2 *Enterobacter cloacae*

Enterobacter cloacae are the Gram-negative bacteria that reside in the digestive system of animals and humans as well as in soil, water, decaying vegetation and sewage. Enterobacter is involved in nosocomial infections, e.g. of the blood, wounds, surgical incisions, and urinary tract of immuno compromised patients (Bauman, 2007). Enterobacter can be a contaminant of diary products. Certain strains of *Enterobacter cloacae* are resistant to expanded spectrum cephalosporins and have been implicated in outbreaks of serious infections in neonatal intensive care units (NICUs) (Finnstrom, Isacsson, Haeggman, & Burman, 1998; Hervans, Ballesteros, Alorma, Gil, Benedi & Alberti, 2001). In this species stable depression of the chromosomal Ampler class C β-lactamase is the major cause of resistance to most of the drugs (Pfaller, Jones, Marshall, Coffiman, Hollis, Edmon & Wenzel, 1997). On the other hand, production of class A

extended spectrum β-lactamases (ESBLs) has also been detected in Enterobacters, though their prevalence is generally very low (Coudron, Moland & Sanders, 1997; Hibbert-Rogers, Heritage, Gascoyne-Binzi, Hawkey, Todd, Lewis & Bailey, 1995). Recently a novel integron-associated class A β-lactamase with extended-spectrum properties, designated IBG-1, has been described for *Enterobacter cloacae* clinical strains in Greece (Giakkoupi, Tzouvelekis, Tsakris, Lakova, Sofianou & Tzelepi, 2000).

2.4.3 *Klebsiella pneumonia*

Klebsiella pneumonia is a member of the family *Enterobacteriaceae*. They are non-motile, rod-shaped, Gram-negative bacteria. Klebsiella are ubiquitous in nature. In humans, they may colonize the skin, pharynx and gastrointestinal tract. They may also colonize sterile wounds and urine. They are the opportunistic pathogens found in the environment and in mammalian mucosa surfaces. The principal pathogenic reservoirs of infections are the gastrointestinal tract of patients and the hands of hospital personnel. In recent years, Klebsiellae have become important pathogens in hospital acquired infections where *Klebsiella pneumonia* is the most medically important species of Klebsiella group. *Klebsiella species* are said to account for about 8% of all hospital-acquired infections which may be due to presence of an indwelling catheter, feeding tube, poor health status, and treatment in an intensive care unit or nursing home. Nosocomial infections such as UTIs, lower respiratory tract, billiary tract and surgical wound site infections are also common (Umeh, 2006). *Klebsiella pneumonia* and *Klebsiella oxytoca* are competitively the principal causes of human infections. They have a prominent polysaccharide capsule which makes them resistant to many host defense mechanisms. Extensive use of broad-spectrum antibiotics in hospitalized patients has led to both increase carriage of *Klebsiella species* and subsequently, the development of multi-drug-resistant strains that produce ESBLs. These strains are highly virulent, and have extraordinary ability of spreading. Bacteremia and significant increased mortality have resulted from infection with these species (Umeh, 2006).

2.4.4 *Pseudomonas aeruginosa*

Pseudomonas is a Gram-negative rod that belongs to the family *Pseudomonadaceae* which has a distinctive sweet smell (Qarah, 2009). This organism is mostly found in moist places like bodies of water and moist soil (Bauman, 2007). These bacteria form part of normal flora of the human skin. *Pseudomonas aeruginosa* has become an important cause of infections, especially in patients with compromised host defense mechanism. Bacteremia occurs in association with chemotherapy, AIDS, burn wounds sepsis and diabetes. Pseudomonas infections can involve any parts of the body (Qarah, 2009). The predisposing factors include placement of intravenous wires, severe burns, urinary tract catheterization, surgery, trauma and premature birth. *Pseudomonas aeruginosa* accounts for approximately 10.1% of all hospital-acquired infections such as pneumonia, UTI's and bacteremia (Bauman, 2007; Qarah, 2009). *Pseudomonas aeruginosa* is regarded as a very resistant bacterium which is very difficult to treat. It can thrive in solutions of antibacterial drugs and disinfectants (Bauman, 2007). Pseudomonas is said to be 'notoriously' resistant to various antibacterial agents including, antimicrobial drugs, soaps, antibacterial dyes, and quaternary ammonium disinfectants. The ability of Pseudomonas to metabolize many drugs in the presence of non specific proton or drug antiports that pump some types of drugs out of the bacterium, and to the ability of Pseudomonas to form biofilms, which resist the penetration of antibacterial drugs and detergents, account for its resistance (Qarah, 2009). ESBL enzyme encoding genes SHV-29 and TEM-42 have been found in *P. aeruginosa*, suggesting that ESBL reservoir is not restricted to *Enterobacteriaceae* family (Naas, Phiippon, Poirel, Ronco & Nordmann, 1999; Mugnier, Dubrous, Casin, Alert & Collatz, 1996).

2.4.5 *Staphylococcus aureus*

Staphylococcus aureus are Gram-positive cocci (Peddie, Donald & Burger, 2004) and part of normal flora of humans. They are normally found on the skin and in the noses of healthy people (Peddie, Donald & Burger, 2004). Staphylococcus causes variety of medical problems, depending on the site of infections, the immune state of the host, and toxins and enzymes a particular species or strain secretes (Bauman, 2007). *Staphylococcus aureus* can be isolated from pus, blood or other fluids of the patients. *Staphylococcus aureus* are one of the most common causes of skin infections such as

pimples and boils. On the other hand, *Staphylococcus aureus* causes serious and sometimes fatal infections such as sepsis and pneumonia (Peddie, Donald & Burger, 2004). During the 20th century, genes for β-lactamase which convey resistance to natural penicillins were recognized in *Staphylococcus aureus* (Elseivier, 2006). In 1945, 90% of staphylococci were susceptible to penicillin of which 5% are comparatively susceptible today. As an alternative, a semi-synthetic form of penicillin/methicillin, which is not inactivated by β-lactamase, was used for Staphylococcal infections. However, MRSA emerged, and posed resistance to various antibiotics, including penicillin, macrolides, amino-glycosides and cephalosporins (Centre for Disease Control (CDC), 2009). Vancomycin was then used as a drug of choice in the treatment of MRSA infections (Bauman, 2007). Recently, more resistant strains of *Staphylococcus aureus* have been found. They are called vancomycin-resistant *Staphylococcus aureus* (VRSA) and vancomycin-intermediate *Staphylococcus aureus* (VISA). These are said to be resistant to vancomycin and most of the antibiotics to which they are exposed to (Peddie, Donald & Burger, 2004; CDC, 2009). Traditionally, MRSA infections have been associated with hospitalization, but in recent years there is an increase in number of people with MRSA infections who have acquired them in the community without a history of hospital admissions (Peddie, Donald & Burger, 2004). *Staphylococcus aureus* is distinguished from *Staphylococcus epididimis* which is the normal inhabitant of a human by a biochemistry test called coagulase test, where *S. aureus* is coagulase positive and *S. epidermidis* is coagulase negative (Bauman, 2007).

2.4.6 *Bacillus cereus*

Members of the genus Bacillus are aerobic spore-forming rods which are ubiquitous in nature. This group of organisms is normal flora of the human skin which rarely causes infections. *Bacillus cereus* however, can cause food poisoning and post-traumatic endophthlamitis. *Bacillus cereus* causes opportunistic infections especially of immunocompromised patients (Will, Van Der Zwet, Parlevliet, Savelkoul, Stoof, Kaizer, Van Furth & Vandenbroucke-Grauls, 2000). Although *Bacillus cereus* is not a β-lactamase producing bacteria, it was included in the study to represent other Gram-positive bacilli.

2.4.7 *Enterococcus faecalis*

Enterococcus faecalis is Gram-positive, facultative anaerobic, coccus which occurs singly, in pairs or short chains. It is a normal inhabitant of the intestinal tract and female genital tract. *Enterococcus faecalis* is said to be a leading cause of bacterial infections among hospital patients. A concern about the dangers of *Enterococcus faecalis* antibiotic resistance and its implications for the return of infectious diseases that cannot be effectively treated has long been there. Researchers have identified a group of genes that contributed to the bacterium's transformation from being harmless in the gut, to a menacing invader. A virulence region never seen before was scientifically identified in the genome of *Enterococcus faecalis*. However, until the entire genome was sequenced, scientists did not realize a quarter of *Enterococcus* genomes (William, 2003).

2.4.8 *Acinetobacter species*

Acinetobacter is a short plump aerobic bacillus that grows in soil, water sewage and forms part of a normal flora of a human skin (Seifert, Dijkshoorn, Gerner-smidt, Pelzer, Tjernberg & Vaneechoutte, 1997; Bauman, 2007). Acinetobacter species are rarely responsible for community-acquired infections. It is an opportunistic pathogen that causes infections of the respiratory, urinary and central nervous systems (Kepler, Kimberly, McAllister & Paula, 2005). These distributions seem to be very similar to that of other nosocomial Gram-negative bacteria (Bergogue-Berezin & Towner, 1996). Acinetobacter is also reported to be a causative organism of endocarditis, septicemia (Bauman, 2007), wound infections and osteomyelitis. Such infections are difficult to treat due to extensive antimicrobial drug resistance (Kepler, Kimberly, McAllister & Paula, 2005). *Acinetobacter baumannii* has emerged as important organisms in ICU settings and this may result from adaptation by the organisms to ICU invasive diagnostic and therapeutical procedures (Bergogue-Berezin & Towner, 1996) as *Acinetobacter species* appear to be multi-drug resistant (MDR) bacteria (Kepler *et al*., 2005; Bauman, 2007). Nosocomial infections caused by *Acinetobacter species* are of increasing concern in critically ill patients, and the risk factors for these infections are not well established (Prashanth & Badrinath, 2006).

2.4.9 *Candida albicans*

Candida species are ubiquitous single cellular yeast cells that are a member of normal flora of skin, mouth, vagina, stool and other mucus membranes. *Candida albicans* are the principal causes of opportunistic infections. *C. albicans* represent the most common fungal pathogen that affects humans and results into the disease called candidiasis. On daily basis, virtually all physicians are confronted with a positive Candida isolate obtained from one or more various anatomical sites. High-risk areas for Candida infections include neonatal, pediatric, and adult ICUs, both medical and surgical (Pappas, Rex & Lee, 2003). Patients who are critically ill and in medical and surgical ICUs have been the prime targets for opportunistic nosocomial fungal infections, primarily due to *Candida species*. The increased prevalence of local and systemic disease caused by *Candida species* has resulted in numerous new clinical syndromes, which mostly affects immuno-compromised people. More than 90% of persons infected with HIV who are not receiving highly active antiretroviral therapy (HAART) eventually develop one episode of oesophangeal candidiasis (De Repentigny, Lewandowski & Jolicoeur, 2004). *Candida albicans* was included in this study as it is a eukaryotic organism that is very difficult to treat and most of the drugs used on patients with *Candida albicans* infections have toxic side effects (Neal, 2009).

Fungi normally found in the environment can cause opportunistic infections. The screening of the plants for antimicrobial activity against other fungi such as *Mucor*, *Geotrichium*, *Penicillium*, *Fusarium* and *Rhizopus* was also performed. Most of the fungi are the opportunistic fungi that are normally found in the living environment. *Mucor* and *Rhizopus* are classified under the division Zycomycota because they cause opportunistic infection called Zycomycoses (Bauman, 2007). Zycomycoses are commonly seen in patients with uncontrolled diabetes, in people who inject illegal drugs, in cancer patients and those that are receiving antimicrobial agents. *Penicillium species* is an important ascomyte that is involved in the production of penicillin. On the other hand, *Penicillium marneffei* is a dimorphic, invasive fungus that causes pulmonary disease upon inhalation. In Southern Asia, pulmonary diseases are commonly caused by *Mycobacterium*

tuberculosis, *Cryptococcus neoformans* and other opportunistic fungi. *Fusarium* cause respiratory distress, disseminated infections and fungemia (fungi in the blood stream). They also produce toxins that accumulate to dangerous levels when excreted in food. *Fusarium* is resistant to all antifungal agents including amphoteric B (De Repentigny *et al*., 2004). According to Bauman (2007), it is very difficult to treat the eukaryotic infections with available antibiotics because of their adverse side effects on human.

CHAPTER 3

METHODOLOGICAL VALIDATION

The laboratory results obtained when testing the sensitivity patterns of bacteria using antibiotic discs or screening plants for their antimicrobial activity can be influenced by many factors (Joumana *et al*., 2003, McGowan, 2006; Lautenbach; Polk, 2007). The media, the density of the bacteria, (McGowan, 2006; Lautenbach & Polk, 2007), the collection of plants and extraction methods are some of the factors (Nostro, Germano, D'angelo, Marino & Cannatelli, 2000). The materials and methods used in this study were selected with the most important factors taken into consideration.

3.1 PLANTS

Roots, bark, leaves and various plants materials are involved in ethno-medicine (Onyeagba, Ugbegu, Okeke & Oroakasi, 2004). Medicinal plants, *Geranium incanum*, *Dodonaea angustifolia* and *Eucomis autumnalis* were selected in this study on the basis of their ethno-medical application in the treatment of diseases (Van Wyk et al., 2002 & Van Wyk: Gericke, 2000). The selected medicinal plants are indigenous to the Eastern Cape region and are easily accessible. Mature plants were collected from various sites in the Summerstrand area in Port Elizabeth region, Eastern Cape Province, South Africa.

Leaves of these plants are traditionally used as decoctions or as infusions in the healing of different microbial and non-microbial ailments (Van Wyk *et al*., 2002).

Table 3.1 summarizes the essential medicinal parts and the traditional medicinal uses of the plants under investigation.

Table 3.1 Plants under investigation, their essential medicinal parts and traditional medicinal uses

Family and Scientific names	**Used parts**	**Medicinal uses**
1. *Geraniaceae* (***Geranium incanum***)	Leaves (rarely the roots or the fruits)	Leaves used as a tea substitute. Traditionally used in the treatment of bladder infections, venereal diseases and menstrual related ailments. It has also been used in the treatment of colic, diarrhoea, fever and bronchitis.
2. *Sapindaceae* (***Eucomis autumnalis***)	The leaves (Taylor & Van Staden, 2001), and the bulb	Used for lower backache, to assist in post operative recovery and to aid in the healing of fractures, urinary disease, stomach ache, fevers, colic flatulence, hangovers. Treatment of syphilis and facilitating child birth.
3. *Hyacinthaceae* (***Dodonaea angustifolia***)	The leaves and twigs	Decoctions of the leaves for fever, for colds, influenza, stomach troubles and even measles. Treatment of arthritis, and used as a gargle for sore throat and oral thrush. An important remedy for treating pneumonia, tuberculosis and externally as an anti-pruritic in skin rashes.

(Compiled from: Van Wyk *et al*., 2002; Taylor & Van Staden, 2001)

After the picking of the herb from its original destination, the next task is to prolong its potency. The most efficient way to accomplish this is consumption on the spot, or soon thereafter. If the usage of the plant material is intended to be postponed until later dates,

dehydrating (drying) plants is the alternative method of preserving and storing the harvest. Obtaining herbs that closely resemble the living plant in colour, aroma, taste and texture, dehydrating must be done correctly. There are two reliable ways of drying plants for preservation. Plants can either be dried at room temperature, by avoiding direct sunlight, or plants can on the other hand be oven dried using low temperatures (Green, 2000). The temperature used can have different effects on the plants. Herbs that are dried too quickly by using too much heat roast and loose their potency. When plants are dried too slowly, they can become infected with mold. Alternatively, there can be self-destruction by enzymatic actions, which also seriously impoverishes their quality. In this study, freshly picked plants were processed as soon as possible. Prior to extraction of the compounds in the plants, leaves dehydration was considered to be necessary. Plants leaves were oven dried at 37^0C as to avoid both the delay in drying as well as quick drying. According to Green (2000) after the proper plant drying, plant must be crushed as to allow for proper plant extraction. The leaves of these particular plants were crushed in to smaller macroscopic particles as to allow a maximum surface area for the penetration of extracting solvent.

3.2 MEDICINAL PLANT EXTRACTION

A number of different plant extraction methods all involve centrifugation but with different solvents. Centrifugation is a cheap, readily available and an efficient method of extraction to use (Karakousis & Langridge, 2003). Centrifugation is a good method of extraction because laboratory centrifuges are used for separating particles from solutions according to their size, shape, density, viscosity of the medium and rotor speed. The theoretical basis of this technique is the effect of gravity on particles (including macromolecules) in suspension. Centrifugal force is used to increase the settling rate of the particles depending on the rotation to induce centrifugal force, which accelerates the separation of the liquid from the solids. Centrifugal force causes the solid phase to move though the liquid phase in a straight line and away from the centre of rotation (New Zealand Qualification Authority, 2006).

All solvents used were of analytical grade and varying polarities. There are many chemicals or compounds used as extraction solvents in research. According to the literature, the most effective solvent is acetone, methanol and distilled water respectively (Akowuah, Ismail, Norhayati & Sadikun, 2004). Acetone is mostly used in pharmaceuticals as a solvent and methanol is used in pharmaceuticals as the organic solvent. Roughly 95% of the available acetone is incorporated with other chemicals and 12% is used as a solvent. Since water is the most abundant molecule on earth's surface in liquid, solid and gaseous state, various substances dissolve in water and it is commonly referred to as the universal solvent. According to Putman (1985), a commonly used extracting solvent is water in which dried or living plants are soaked (Putman, 1985).

Rusak, Komes Likic, Horzik & Kovac, 2008 mention that the extraction efficiency of the extracting solvents depends strongly on the time of extraction and the solvents used Rusak *et al*. (2008)

The extracting solvents in this study were selected bearing in mind that there is no perfect solvent; each one has its drawbacks (Hughes, 2002). According to Hughes (2002), when considering the suitability of a solvent it should meet the following criteria:

- It should display low toxicity to a higher life forms
- It should not cause the extract to complex or dissociate
- It should be a preservative in action
- It should promote rapid physiologic absorption of the extract
- It should be easily evaporated at low heat

In this study, acetone, methanol and distilled water were used and were selected to be suitable solvents for plant extraction.

Infusions are prepared by adding plant material in boiling or cold water. It is left to stand for some time until the solid matter is strained off and the liquid is drunk as herbal tea. Infusions are said to be vulnerable to fungal contamination so they need to be used within 12 hours following their preparation (Van wyk *et al*., 2002). Decoction as opposed to

infusions is the method which is normally used with a plant material which is woody and very hard to extract, e.g. plant roots and bark (Chiej, 1984). In this study infusions were used and were suitable because extraction was performed on the leaves and freshly prepared infusions were used to avoid fungal contamination.

3.3 ANTIMICROBIAL SCREENING

According to Basson, Nicolaas, Grobler & Sias, 2008 the antimicrobial activity can be effective against a broad spectrum of microbes, especially of medical importance. It is important to test for the antimicrobial activity of plants extracts on selected microbial strains (Basson *et al*., 2008). In this study, the antimicrobial activity of three selected plants was performed against specific microbial strains. The standard ATCC strains and other microbial strains, specifically β-lactamase producing bacteria were used in the screening of antimicrobial activity of *Geranium incanum*, *Dodonaea angustifolia* and *Eucomis autumnalis* extracts.

3.4 MINIMUM INHIBITORY CONCENTRATION (MIC)

Minimum Inhibitory Concentration (MIC) is defined as the highest dilution or least concentration of the extracts that inhibit growth of organisms. Determination of the MIC is important in diagnostic laboratories because it helps in confirming resistance of micro-organism to an antimicrobial agent and it monitors the activity of new antimicrobial agents (EUCAST, 2000). According to European committee for Antimicrobial susceptibility Testing (EUCAST), MIC for drugs is established using either the agar dilution method or the microtitre-plate broth dilution method. In agar dilution tests (used in this study), microorganisms are tested for their ability to produce visible growth on a series of dilutions incorporated in agar plates (agar dilution). On the other hand, microtitre-plate wells (broth micro-dilution) containing dilutions of the antimicrobial agent can be used (EUCAST, 2000). The agar dilution method has an advantage over the microtitre-plate method when interpreting the results. The agar dilution method uses the

all or nothing phenomenon of reading the results while microtitre-plate uses the changes in colour of tetrazolium salts which are visually determined and can sometimes be confused with the colour of the extract itself (EUCAST, 2000). MIC was achieved by using agar dilution assay as a subsequent test on the extracts of *Geranium incanum* and *Dodonaea angustifolia* that showed the antimicrobial activity in the antimicrobial screening tests.

3.5 AMES TEST

The Ames test is a biological assay to assess the mutagenic potential of chemical compounds. Testing chemical compounds for potential mutagenic and carcinogenic effects is very difficult and expensive. Ames test is a very important test. The testing of probable carcinogenicity by using Ames test is quick yet it indicates the carcinogenic potential of many chemicals. Ames test is advantageous as it uses bacteria, which is cheap to culture and grow rapidly. The Salmonella-*his* reversion test (Ames test), a standard reverse mutation assay, was implemented to test plants extracts for mutagenicity (Chan, Pelczar, and Krieg, 1993). Ames test was performed on the extracts of the three plants including the plants that did not display any antimicrobial activity. Sodium azide and 4-nitroquinoline are the well known toxic compounds that are used in the preparation of the positive control in Ames test. Ames test can be performed using the *Salmonella typhimurium* TA 98 or TA100. According to Mortelmans and Zeiger (2000), TA 98 works well with sodium azide while TA 100 works properly with 4-nitroquinoline in the preparation of positive controls in the Ames test. In this study, TA 100 was used with either sodium azide or nitroquinoline in the preparation of the positive controls of the Ames test.

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Leaves ready for being processed.

Weighing balance

-Weighing powdered plants and media

Centrifuge

- Plants extraction by spinning

Water bath

-Evaporation of extracts

-Maintains media at specific temperatures

Vortex mixer

-General mixing of plants material with liquids

Mast multi-point inoculator

-Inoculating microbes on Mueller-Hinton Agar

Incubator
-Incubation of microbes
-Drying of plants leaves

Fume cabinet
-Plants extraction

Fig 3.1 Summary of the procedures and the equipment used in the study

CHAPTER 4

MATERIAL AND METHODS

A Pilot study was done on the methods selected for this research project to ensure that conditions in the laboratory were suitable for conducting this research project.

4.1 Collection and identification of micro-organisms

Geranium incanum, Dodonaea angustifolia and *Eucomis autumnalis* were screened for antimicrobial activity against various bacterial and fungal strains. A total of 94 micro-organisms were screened. This included 13 strains of *Escherichia coli*, 4 strains of *Enterobacter cloacae*, 12 strains of *Klebsiella pneumoniae*, 11 strains of *Pseudomonas aeruginosa*, 11 strains of *Staphylococus aureus*, 4 strains of *Bacillus cereus*, 11 strains of *Enterococcus faecalis*, 13 strains of *Acinetobacter spp*, 9 strains of *Candida albicans* and a single strain of *Mucor, Geotrichium, Penicillium, Fusarium* and *Rhizopus*. The selected strains included the β-lactamase- and non β-lactamase producing bacteria. *Bacillus cereus*

was included to represent Gram-positive bacilli while Candida and the filamentous fungi represent the eukaryotes.

All the micro-organisms included in this study, except the ATCC strains, were clinical strains obtained from the National Health Laboratory Services (NHLS), Port Elizabeth. The fungal cultures and the following ATCC strains *E. coli* 35218, *P. aeruginosa* 27853 and *S. aureus* 43300, were obtained from the department of Biomedical Technology and Radiography at the NMMU.

The micro-organisms were stored on the beads called Microbank vials purchased from Davies Diagnostics. The microbes were sub-cultured onto different agar medium. The following media were used: Blood agar (NHLS) for the initial subculturing of Gram-positive bacteria, MacConkey agar (Biolab) for the Gram-negative bacteria and Sabouraud Dextrose agar (Biolab) for fungi. Nutrient agar was used after the initial subculturing for either Gram-positive or Gram-negative bacteria. The microorganisms were then incubated at 37^0C for 24 hours or 48 hours for *Candida albicans*. All the tests performed in this study were done in triplicate as to guarantee the reliability of the obtained results.

4.2 Media preparation

Nutrient agar (Biolab), Davies Minimum salt agar (Fluka), Sabouraud Dextrose agar (Biolab) and Mueller-Hinton agar were prepared according to the manufacture's instructions as illustrated in appendix A. MacConkey agar (Biolab) and blood agar (NHLS) were already prepared from the NHLS.

Davies Minimum salt agar (Fluka) was used in the Ames test. In order to perform the plant antimicrobial assays, 25ml of Mueller-Hinton agar (Biolab) or Sabouraud Dextrose agar (Biolab) were dispensed into McCartney bottles, sterilized and allowed to cool to about 45^0C before the addition of plant extracts. The plant extracts were added at an initial concentration of 20mg/ml. The bottles were shaken gently and poured into sterile Petri dishes. The agar was allowed to set at room temperature. The medium was stored in

the refrigerator at 4^0C. The surface of the agar was dried for 10 minutes in an incubator at 37^0C prior to inoculation.

4.3 Plants extraction procedure:

Day-1

After drying of the plant leaves in the incubator at 37^0C, the dried leaves were crushed into powder using a pestle and mortar. Leaves were then extracted using methanol, acetone and distilled water. Two grams of powdered leaves were weighed and then transferred to a screw cap plastic tube. The extracting solvents were then added to the powdered leaves in the plastic tubes (20ml of a single solvent per tube). The plastic tubes were tightly closed and shaken vigorously for 5 minutes to allow plant-solvent interaction. The tubes were centrifuged at 4000 rpm for 5 minutes to separate a solid phase and the liquid phase of a mixture. The Supernatant of plant extracts was transferred into the sterile pre-weighed beakers using a pasture pipette to avoid the re-mixing of the solid phase and the supernatant. The procedure was repeated twice using the same amount of a solvent in each tube. The beakers with all the supernatants were then placed into 45^0C water-bath overnight or up to two days to allow the evaporation of the extractant.

Day-2

After evaporation, the beakers containing the solvent free residues were weighed. The residues were re-dissolved in DMSO. DMSO is a universal solvent (Gad, 2005). The amount of DMSO added to a dry extract was dependent on an individual extract. Extracts were further diluted to obtain an initial concentration of 20mg/ml and was incorporated into the Mueller-Hinton agar plates.

4.4 Traditional method of preparation

Traditional method of preparation was included to represent the ancient way in which the traditional healers were preparing and using the plants. Traditionally, plants extracts were

prepared with water as infusions or decoctions. In this study, the infusions of the leaves of the plants *Geranium incanum, Dodonaea angustifolia* and *Eucomis autumnalis* were prepared according to traditional method described by Scott and Springfield (2004). The infusions were prepared by adding 5g of powdered leaves of a single plant to 1000ml boiling water and left to infuse until cool. Mueller-Hinton agar was mixed with the infusions to obtain a final concentration of 20mg/ml.

4.5 Multipoint method

Antimicrobial assay was performed using the Mast multipoint inoculator. The antimicrobial screening was done on Mueller-Hinton agar. This agar is normally used for sensitivity testing (Carlo, Dorothy & Fred, 1972). A standardized inoculum was prepared by suspending colonies in 5ml normal sterile saline to obtain a turbidity of 0.5 McFarland standard.

The bacterial suspensions were tranfered to the sterile multipoint inoculator buckets that contains 36 wells. 30µl of a specific bacterial suspension was transferred to a single well using aseptic technique as to prevent cross-contamination of the wells. From the wells, 36 sterile inoculating pins which are embedded on the head of a Mast multipoint inoculator were used to transfer the inoculum to the media. The multipoint inoculator can transfer 3µl of each of the 36 samples simultaneously. After inoculation, the plates were incubated at 37^0C for 24 hours for bacteria and for 48 hrs for *Candida albicans*.

Control plates were also prepared where the highest and the lowest DMSO volumes that were used in the plant extraction were mixed with Mueller-Hinton agar. The other control plate was a pure Mueller-Hinton agar plate. Purity plates were prepared from the same suspensions of microorganisms as used for the inoculation and incubated. Purity plates were prepared to obtain pure cultures and to make sure that there is growth of every microorganism tested.

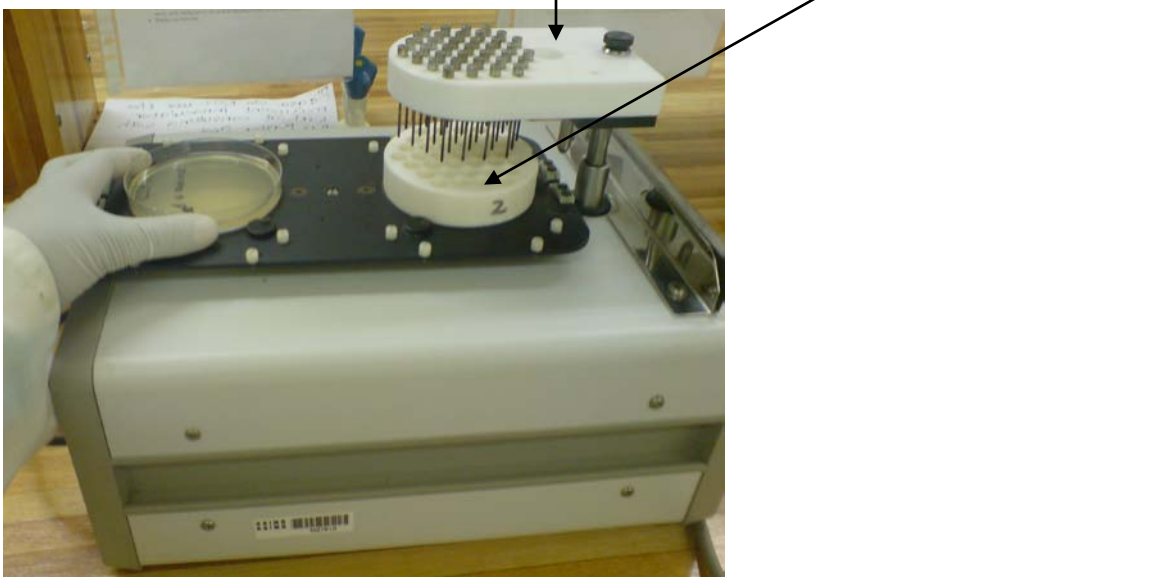


Figure 4.1 Mast multipoint inoculator

All the plates were read after the incubation time (24hrs for bacteria and 48 hours for *C. albicans*). It was first ensured that all the organisms had purely grown in all the purity plates. If there was no visible growth on purity plates for a certain organism, or contamination of a certain organism was seen, such a test was repeated with further sub-culturing on purity plates. The organism was considered to be either inhibited or not inhibited. The organism was inhibited if there was no growth observed on the plate containing the extract and there is growth on the control plates. On the other hand, the organism was considered not inhibited when there was a visible growth on both the control plates and the extract containing plate. The main limitation of using the multi-point inoculator is its all or nothing results, which masks finer degree of susceptibility.

4.5.1 Fungi

The fungal cultures were obtained from NHLS or stock cultures in the Biomedical Laboratory and Radiography at NMMU. Verification of the fungi was achieved by staining with lactophenol cotton blue and by colonial morphology. Antimicrobial testing of the fungi was done using the agar dilution method. A sterile knife was used for transfering the fungi from their original plates to the test plates. The agar from the original plates was cut into the same dimensions (1cmx1cm) and plated on to the centre of the test plates. All of the work was performed in the safety cabinet because of all the spore forming fungi except for the *C. albicans*. Incubation was done at 25^0C for 48 hours or more depending on the time required by each fungal strain to grow. Fungi was considered to be susceptible if there was an up-right growth that originated from the (1cmx1cm) agar cut from the original plate, as opposed to the spread of the fungi on the control plate (De Repentigny *et al*., 2004).

4.6 MIC

MIC was performed on two of the three plants and the inhibited microorganisms. *Geranium incanum* and *Dodonaea angustifolia* showed the inhibition properties against micro-organisms therefore their MIC was determined. MIC was achieved by making

serial dilutions of extracts such that the concentration is halved in each agar plate containing 0.5, 0.25, 0.125 and 0.0625 dilutions, which corresponded with the concentrations of the extracts 10mg/ml, 5mg/ml, 2.5mg/ml and 1.25mg/ml respectively (Table 4.1). MIC was expressed in milligrams per ml. Inocula were applied on a Mueller-Hinton agar with a Mast multipoint inoculators resulting in a final inoculum of 0.3µl per single inoculum spot. MIC end points were read as visible growth or no growth. Every concentration was done in triplicate. The MIC was obtained from the lowest concentration of the extract that inhibited the growth of the organism.

4.7 AMES TEST

The lowest minimum inhibitory concentration of every plant extract was used to determine whether a plant had mutagenicity effects on *Salmonella typhimurium* TA100 used. The adapted whole plate method instead of the top overlay (Krug, 2007) was used. The 4 McFarland standard suspension of *Salmonella typhimurium* TA100 was added to 100ml Davis Minimum salt agar (Fluka) that contain traces of histidine (0.008g) at 45-48^{0}C. The two positive controls used were the 4-nitroquinoline at the concentration of 20µg/ml and sodium azide at the concentration of 0.0001g/ml. 20ml of the mixture was then added to the plates and allowed to set. The negative control plates contained 20ml Davis Minimum salt agar (Fluka), with traces of histidine and bacterial suspensions. The plates were then incubated for 48 hours and the histidine positive revertants were counted using a colony counter. API test was used to confirm the identity of *Salmonella typhimurium* revertant colonies.

CHAPTER 5

RESULTS

5.1 Antimicrobial screening assay results

The antimicrobial activity of three medicinal plants (*Geranium incanum*, *Dodonaea angustifolia*, and *Eucomis autumnalis*) was determined by using different solvents extracts as well as an infusion of different plants. The extracts were screened against 26 Gram-positive bacterial strains, 53 Gram-negative bacterial strains and 15 fungal strains. The Gram-positive bacteria included strains from *S. aureus, B. cereus* and *E. faecalis*. The Gram-negative bacteria included strains from *E. coli*, *E. cloacae*, *K. pneumoniae*, *P. aeruginosa* and *Acinetobacter species*. The fungal strains included *Candida albicans* and various other opportunistic fungi.

The freshly picked plants leaves were dried and processed. Powdered leaves were extracted thrice with each extraction solvent (acetone, distilled water & methanol) respectively. However, the infusions of the plants leaves were also used to represent traditional way of preparation. The antimicrobial screening assays were performed in

triplicate on a total of 94 microbial strains. The antimicrobial screening was performed at the concentration of 20mg/ml. Extracts that inhibited some of the bacteria were then diluted to determine their MIC's. The Ames test was performed on these specific extracts to determine the mutagenicity which indicate possible toxicity of the plants. *Geranium incanum* and *Dodonaea angustifolia* showed antimicrobial activity against some organisms while *Eucomis autumnalis* did not show any antimicrobial activity. The results of antimicrobial screening assay which were based on visible growth and no growth of the micro-organisms are reported in Table 5.1 and Table 5.6.

A total of 79 bacterial strains of different bacteria (*S. aureus, B. cereus, E. faecalis, E. coli, E. cloacae, K. pneumoniae, P. aeruginosa* & *Acinetobacter species*) were screened for antibacterial activity using the agar dilution assay (Table 5.1).

Table 5.1 Antibacterial activity of plants extracts tested against bacterial strains using agar dilution assay at initial concentration of 20mg/ml

		G. incanum			*D. angustifolia*			*E. autumnalis*		
Gram + ve		**Acetone**	**D. water**	**MeOH**	**Acetone**	**D. water**	**MeOH**	**Acetone**	**D. water**	**MeOH**
S. aureus	1.	-	-	-	+	+	+	+	+	+
	2.	-	-	-	+	+	+	+	+	+
	3.	-	-	-	+	+	+	+	+	+
	4.	-	-	-	+	+	+	+	+	+
	5.	-	-	-	+	+	+	+	+	+
	6.	-	-	-	+	+	+	+	+	+
	7.	-	-	-	+	+	+	+	+	+
	8.	-	-	-	+	+	+	+	+	+
	9.	-	-	-	+	+	+	+	+	+
	10.	-	-	-	+	+	+	+	+	+
	11.	-	-	-	+	+	+	+	+	+
								+	+	+
B. cereus	1.	-	-	-	-	-	-	+	+	+

	2.	-	-	-	-	-	-	+	+	+
	3.	-	-	-	-	-	-	+	+	+
	4.	-	-	-	-	-	-	+	+	+
E. faecalis	1.	+	+	+	+	+	+	+	+	+
	2.	+	+	+	+	+	+	+	+	+
	3.	+	+	+	+	+	+	+	+	+
	4.	+	+	+	+	+	+	+	+	+
	5.	+	+	+	+	+	+	+	+	+
	6.	+	+	+	+	+	+	+	+	+
	7.	+	+	+	+	+	+	+	+	+
	8.	+	+	+	+	+	+	+	+	+
	9.	+	+	+	+	+	+	+	+	+
	10.	+	+	+	+	+	+	+	+	+
	11.	+	+	+	+	+	+	+	+	+
Gram – ve										
E. coli	1.	+	-	+	+	+	+	+	+	+
	2.	+	-	+	+	+	+	+	+	+
	3.	+	-	+	+	+	+	+	+	+
	4.	+	-	+	+	+	+	+	+	+
	5.	+	-	+	+	+	+	+	+	+
	6.	+	-	+	+	+	+	+	+	+
	7.	+	-	+	+	+	+	+	+	+
	8.	+	-	+	+	+	+	+	+	+
	9.	+	-	+	+	+	+	+	+	+
	10.	+	-	+	+	+	+	+	+	+
	11.	+	-	+	+	+	+	+	+	+
	12.	+	-	+	+	+	+	+	+	+
	13.	+	-	+	+	+	+	+	+	+
									+	
E. cloacae	1.	+	-	+	+	+	+	+	+	+
	2.	+	-	+	+	+	+	+	+	+
	3.	+	-	+	+	+	+	+	+	+
	4.	+	-	+	+	+	+	+	+	+
K. pneumoniae	1.	+	-	+	+	+	+	+	+	+
	2.	+	-	+	+	+	+	+	+	+
	3.	+	-	+	+	+	+	+	+	+
	4.	+	-	+	+	+	+	+	+	+

	5.	+	-	+	+	+	+	+	+	+
	6.	+	+	+	+	+	+	+	+	+
	7.	+	-	+	+	+	+	+	+	+
	8.	+	-	+	+	+	+	+	+	+
	9.	+	-	+	+	+	+	+	+	+
	10.	+	+	+	+	+	+	+	+	+
	11.	+	-	+	+	+	+	+	+	+
	12.	+	-	+	+	+	+	+	+	+
P. aeruginosa	1.	+	+	+	+	+	+	+	+	+
	2.	+	+	+	+	+	+	+	+	+
	3.	+	+	+	+	+	+	+	+	+
	4.	+	+	+	+	+	+	+	+	+
	5.	+	+	+	+	+	+	+	+	+
	6.	+	+	+	+	+	+	+	+	+
	7.	+	+	+	+	+	+	+	+	+
	7.	+	+	+	+	+	+	+	+	+
	8.	+	+	+	+	+	+	+	+	+
	9.	+	+	+	+	+	+	+	+	+
	10.	+	+	+	+	+	+	+	+	+
	11.	+	+	+	+	+	+	+	+	+
Acinetobacter species	1.	+	-	-	+	+	+	+	+	+
	2.	+	-	-	+	+	+	+	+	+
	3.	+	-	-	+	+	+	+	+	+
	4.	+	-	-	+	+	+	+	+	+
	5.	+	-	-	+	+	+	+	+	+
	6.	+	-	-	+	+	+	+	+	+
	7.	+	-	-	+	+	+	+	+	+
	8.	+	-	-	+	+	+	+	+	+
	9.	+	-	-	+	+	+	+	+	+
	10.	+	-	-	+	+	+	+	+	+
	11.	+	-	-	+	+	+	+	+	+
	12.	+	-	-	+	+	+	+	+	+
	13.	+	-	-	+	+	+	+	+	+

Key: MeOH = Methanol D. water = Distilled water (+) = Not inhibited (-) = inhibited

The different extracts of *Geranium incanum* showed the antibacterial activity against fifty-five of seventy-nine bacterial strains. The extracts inhibited 13 strains of *E.coli*, 4 strains of *E. cloacae*, 10 strains of *K. pneumoniae*, 11 strains of *S. aureus*, 4 strains of *B. cereus*, and 13 strains of *Acinetobacter species*. The strains of *E. faecalis* and *P. aeruginosa* were not inhibited by any of the extracts of *Geranium incanum* and hence resulted in total of 24 bacterial strains that were not inhibited by any of the extracts of *Geranium incanum* (Table 5.2 & fig 5.1).

The acetone, distilled water and methanol extracts of *Geranium incanum* inhibited the eleven strains of *S. aureus* and four strains of *B. cereus* (Table 5.1).

All of the strains of *E.coli* and the four strains of *E. cloacae* were only inhibited by the distilled water extract of *Geranium incanum*. The distilled water extracts of *Geranium incanum* inhibited ten of the twelve strains of *K. pneumoniae*. Strains numbers 6 and 10 were not inhibited. However, the distilled water and methanol extracts of *Geranium incanum* inhibited all the strains of the *Acinetobacter species* (Table 5.1).

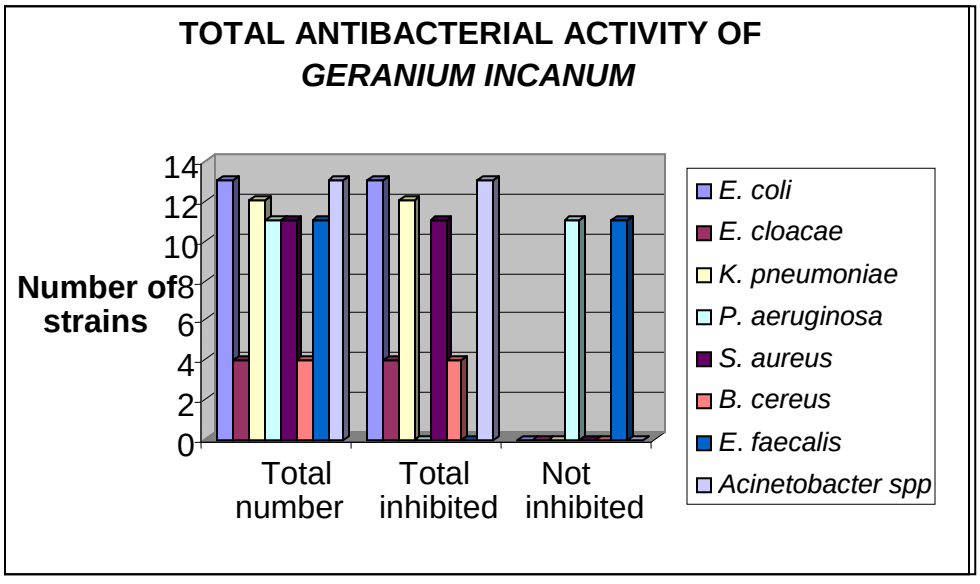


Fig 5.1 Number of each strain inhibited by the plant extracts of *Geranium incanum*

Table 5.2 Summary of the total number of bacteria inhibited by the extracts of *Geranium incanum* and *Dodonaea angustifolia* at a concentration of 20mg/ml

		Geranium incanum		*Dodonaea angustifolia*	
Bacteria	Total number	Total inhibited	not inhibited	Total inhibited	not inhibited
E. coli	13	13	0	0	13
E. cloacae	4	4	0	0	4
K. pneumoniae	12	10	2	0	12
P. aeruginosa	11	0	11	0	11
S. aureus	11	11	0	0	11
B. cereus	4	4	0	4	0
E. faecalis	11	0	11	0	11
Acinetobacter species	13	13	0	0	13
TOTAL	**79**	**55**	**24**	**4**	**75**

Total number of 79 bacterial strains was tested against three different extracts of *Dodonaea angustifolia*. The acetone, methanol and distilled water extracts of *Dodonaea angustifolia* only inhibited all the four strains of *B. cereus*. None of the strains of 13 *E. coli*, 4 *E. cloacae*, 12 *K. pneumoniae*, 11 *S. aureus*, 13 *Acinetobacter species*, 11 *E. faecalis* and 11 *P. aeruginosa* were inhibited by any of the extracts of *Dodonaea angustifolia* (Table 5.2 & fig 5.2).

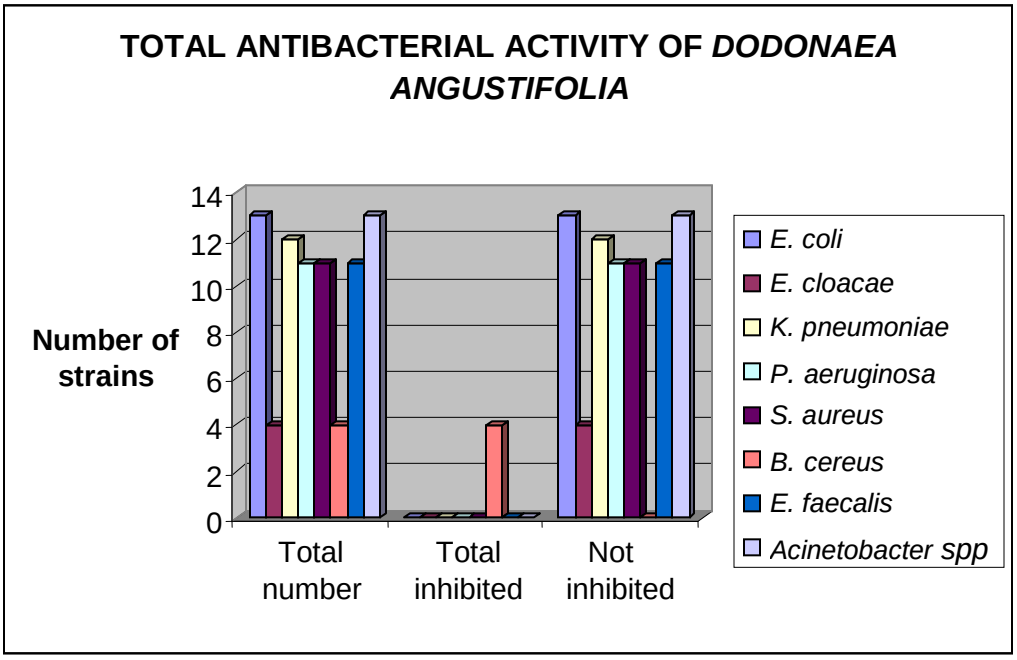


Fig 5.2 Number of each strain inhibited by the plant extracts of *Dodonaea angustifolia*

The extracts of *D. angustifolia* only inhibited the strains of *B. cereus* (fig 5.2) and therefore activity of the plant extracts of this plant is very limited in the range of inhibiting bacteria in this study.

The extracts of *Eucomis autumnalis* were the only extracts that did not inhibit any of the bacterial strains tested in this study as illustrated in table 5.3 and in fig 5.3.

Table 5.3 Summary of total number of each of the organisms inhibited by the extract of *Eucomis autumnalis* at a concentration of 20mg/ml

Bacteria	Total number	Total inhibited	not inhibited
E. coli	13	0	13
E. cloacae	4	0	4
K. pneumoniae	12	0	12
P. aeruginosa	11	0	11
S. aureus	11	0	11
B. cereus	4	0	4
E. faecalis	11	0	11
Acinetobacter species	13	0	13
	79	**0**	**79**

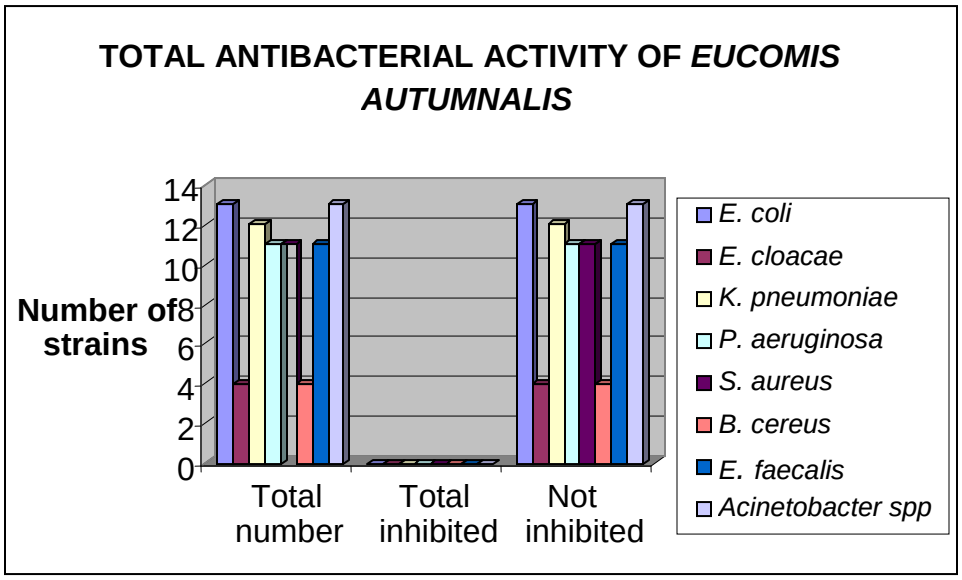


Fig 5.3 Number of each strain inhibited by the plant extracts of *Eucomis autumnalis*

All the plant extracts were tested against the following fungal strains; *C. albicans, Mucor, Geortrichium, Penicillium, Fusarium* and *Rhizopus*.

As illustrated on the table 5.4 *Geranium incanum* inhibited all the strains of *C. albicans* and the strain of *Mucor species*. On the other hand, none of the strains of *Geotrichium, Penicillium, Fusarium* and *Rhizopus* were inhibited by the extracts of *Geranium incanum* (Table 5.4).

The extracts of both *Dodonaea angustifolia* and *Eucomis autumnalis* showed no inhibition properties against any of the selected fungi.

Table 5.4 Total number of the fungus that were inhibited by the extracts of *Geranium incanum*, *Dodonaea angustifolia* and *Eucomis autumnalis* respectively

Plants	Fungi	Total number	Total inhibited	Total not inhibited
G. incanum				
	C. albicans	9	0	9
	Mucor	1	1	0
	Geotrichium	1	0	1
	Penicillum	1	0	1

	Fusarium	1	0	1
	Rhizopus	1	0	1
D. angustifolia				
	C. albicans	1	0	1
	Mucor	1	0	1
	Geotrichium	1	0	1
	Penicillum	1	0	1
	Fusarium	1	0	1
	Rhizopus	1	0	1
E. autumnalis				
	C. albicans	1	0	1
	Mucor	1	0	1
	Geotrichium	1	0	1
	Penicillum	1	0	1
	Fusarium	1	0	1
	Rhizopus	1	0	1
		1	0	1

5.2 Traditional way of preparation

The infusions of the leaves of the plants were included to represent the traditional way of preparing medicinal plants. The infusions of all the plants (*Geranium incanum*, *Dodonaea angustifolia* & *Eucomis autumnalis*) were tested for antimicrobial activity. None of the microorganisms were inhibited by any of the plants infusions against which they were tested (Table 5.5).

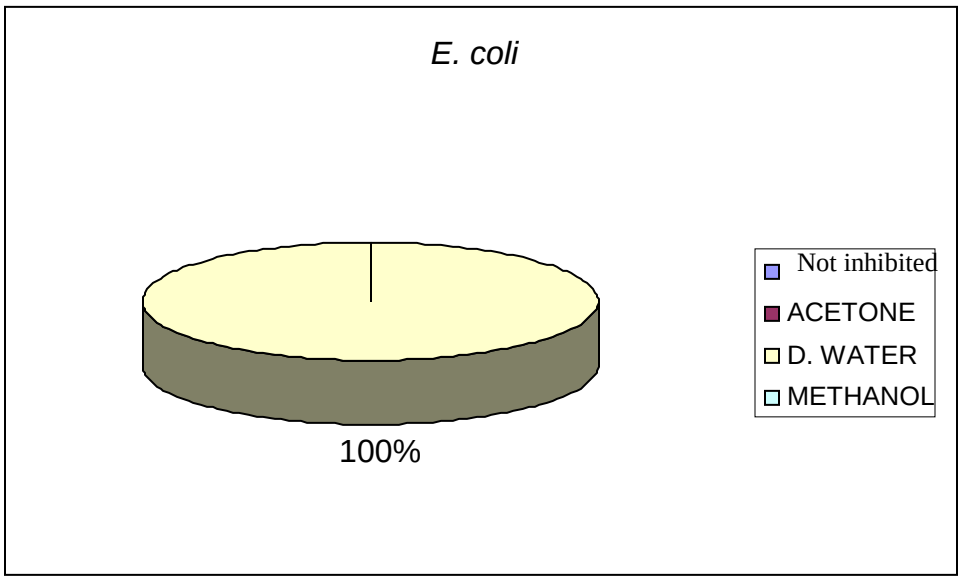
Table 5.5 Summary of the results of the antimicrobial activity of infusions of *G. incanum*, *D. angustifolia* and *E. autumnalis*

Gram +ve	***G. incanum***	***D. angustifolia***	***E. autumnalis***
S. aureus	+	+	+
B. cereus	+	+	+
E. faecalis	+	+	+
Gram -ve	+	+	+
E.coli	+	+	+
E. cloacae	+	+	+

K. pneumoniae	+	+	+
P. aeruginosa	+	+	+
Acinetobacter spp	+	+	+
Fungi	+	+	+
C. albicans	+	+	+
Mucor	+	+	+
Geotrichium	+	+	+
Penicillium	+	+	+
Fusarium	+	+	+
Rhizopus	+	+	+

(+) Not inhibited

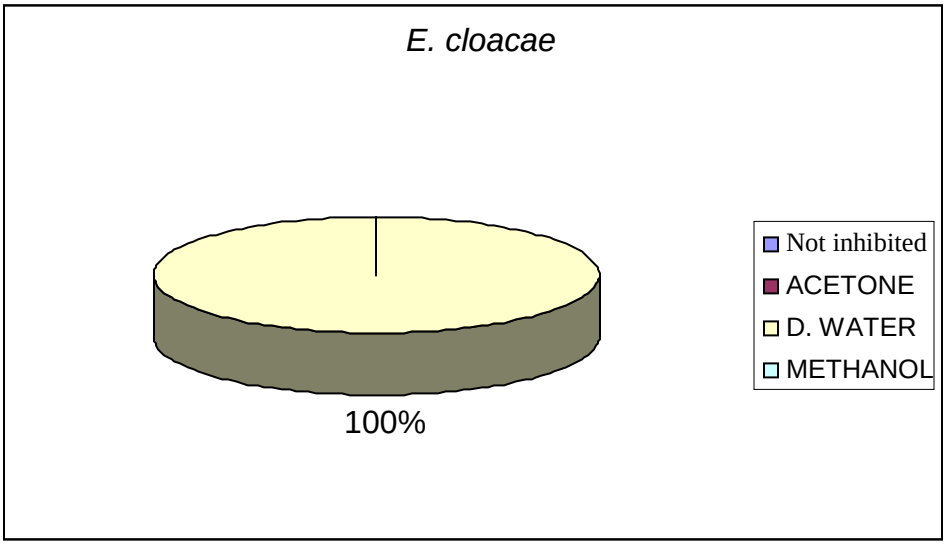
The percentage of bacteria that were inhibited by the plant extracts (Acetone, Distilled water and Methanol) of the three plants tested at the initial concentration of 20mg/ml as well as the bacteria that were not inhibited are illustrated by means of the pie charts.



Key: Acetone and methanol showed 0% inhibition of *E. coli* strains

Fig 5.4 Pie chart indicating percentage of the strains of *E. coli* inhibited by different extracts of *Geranium incanum*

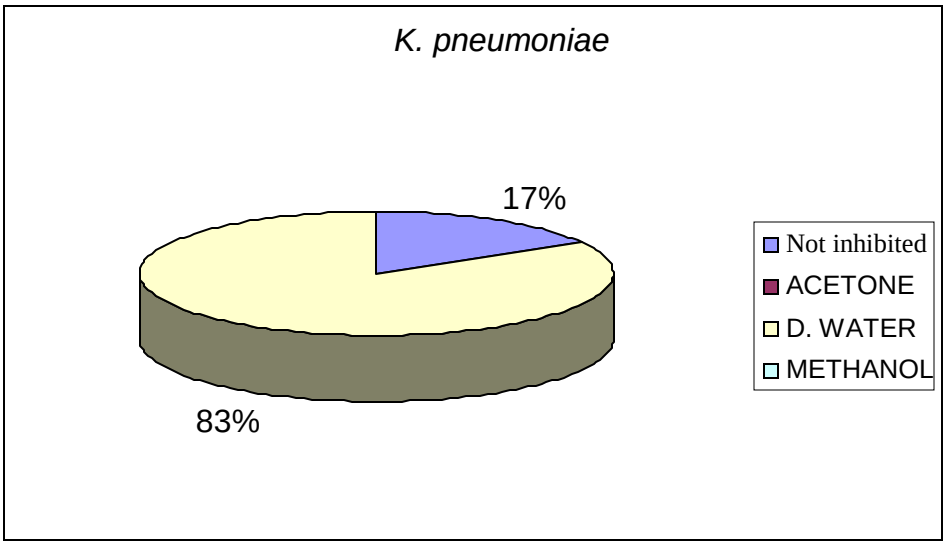
Hundred percent of the *E. coli* strains were inhibited by the distilled water extracts of *Geranium incanum* while 0% of *E. coli* strains were inhibited by the acetone and methanol extracts of *Geranium incanum* (Fig 5.4)



Key: Acetone and methanol showed 0% inhibition of *E. cloacae* strains

Fig 5.5 Pie chart indicating percentage of the strains of *E. cloacae* inhibited by different extracts of *Geranium incanum*

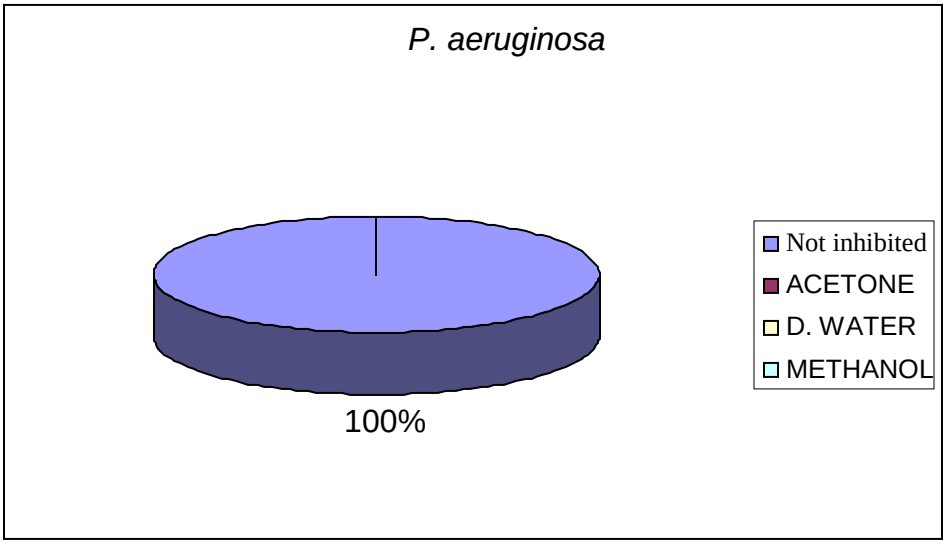
All of the strains of *E. cloacae* were inhibited by the distilled water extracts of *Geranium incanum* which resulted in the 100% inhibition indicated in figure 5.2, however, none of the strains of *E. cloacae* were inhibited by both acetone and methanol extracts of *G. incanum*.



Key: Acetone and methanol showed 0% inhibition of *K.pneumoniae* strains

Fig 5.6 Pie chart indicating percentage of the strains of *K. pneumoniae* inhibited by different extracts of *Geranium incanum*

Eighty-three percent of the strains of *K. pneumoniae* strains were inhibited by the distilled water extracts of *Geranium incanum*. No inhibition was detected by acetone and methanol extracts of these plant. 17% of the strains of *K. pneumoniae* species were not inhibited by the distilled water extracts of *Geranium incanum* (Fig 5.6).



Key: Acetone and methanol and distilled water inhibited none of the strains of *P. aeruginosa*

Fig 5.7 Pie chart indicating percentage of the strains of *P. aeruginosa* inhibited by different extracts of *Geranium incanum*

None of the strains of *P. aeruginosa* were inhibited by any of the extracts of *Geranium incanum*. (Fig 5.7)

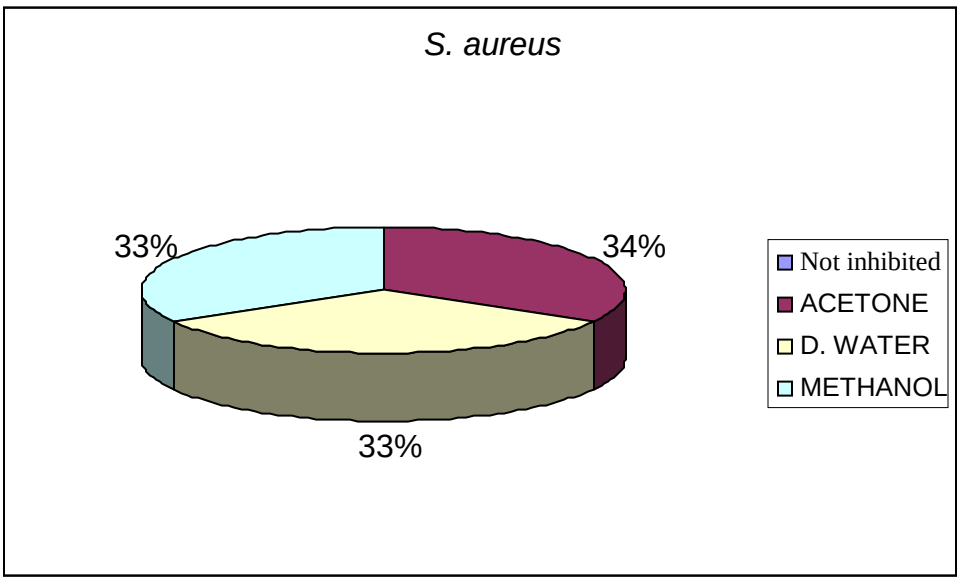


Fig 5.8 Pie chart indicating percentage of *S. aureus* inhibited by different extracts of *Geranium incanum*

Thirty-four percent of the strains of *S. aureus* were inhibited by the acetone extracts of *Geranium incanum* while both distilled water and methanol extracts of *Geranium incanum* inhibited 33% of the strains of *S. aureus* respectively (Fig 5.8).

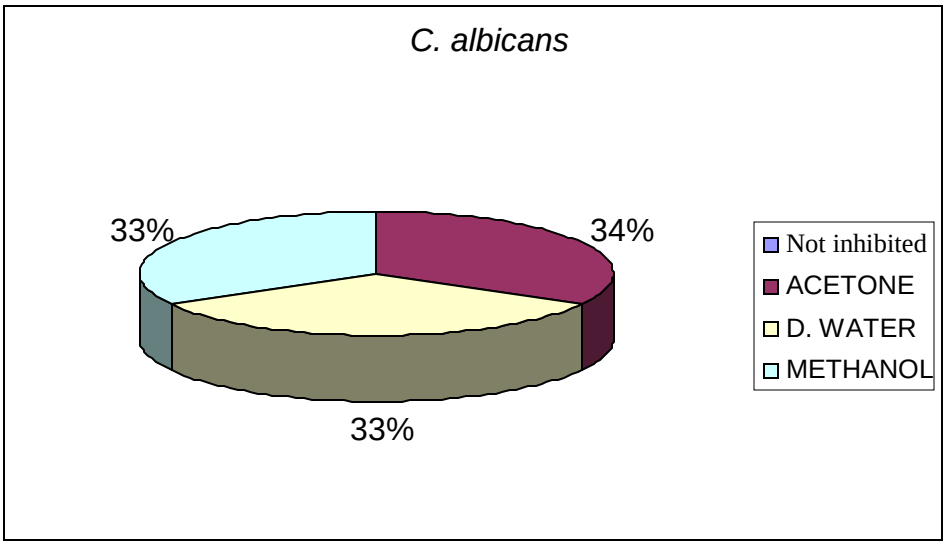
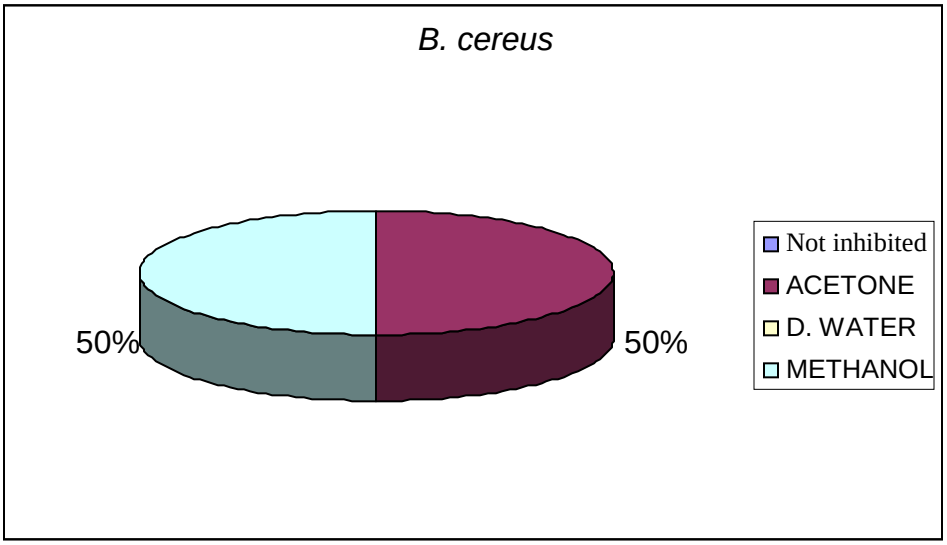


Fig 5.9 Pie chart indicating percentage of the strains of *C. albicans* inhibited by different extracts of *Geranium incanum*

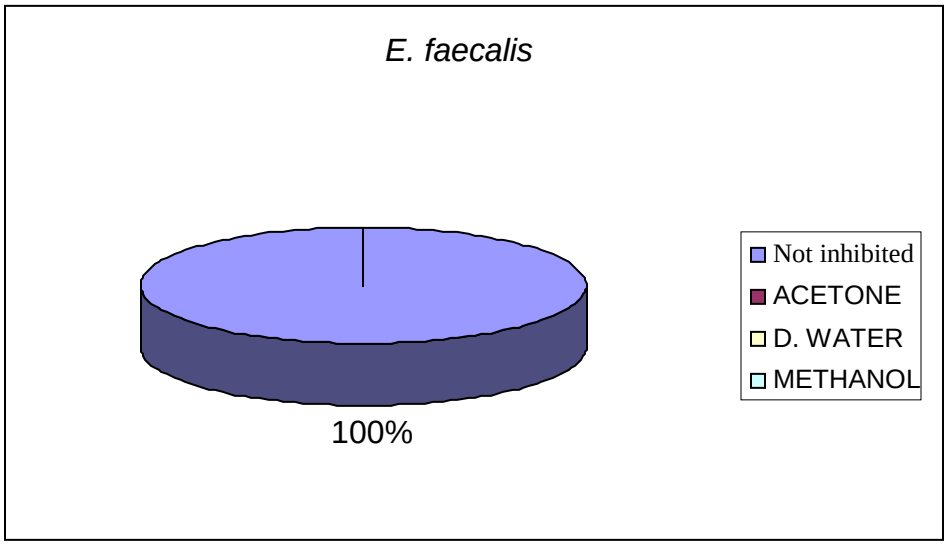
Thirty-four percent of the strains of *C. albicans* were inhibited by the acetone extracts of *Geranium incanum* while both distilled water and methanol extracts of *Geranium incanum* inhibited 33% of the strains of *C. albicans* respectively (Fig 5.9).



Key: Distilled water showed 0% inhibition of *E. cloacae* strains

Fig 5.10 Pie chart indicating percentage of the strains of *B. cereus* inhibited by different extracts of *Geranium incanum*

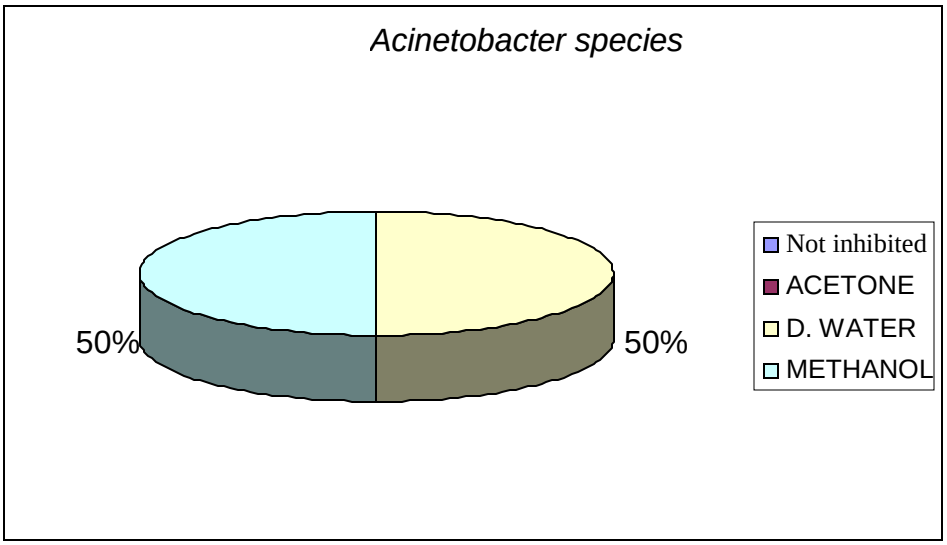
Methanol and distilled water extracts of *Geranium incanum* inhibited 50% of the strains of *B. cereus* respectively. Acetone extracts of *Geranium incanum* did not display any antimicrobial activity against the strains of *B. cereus* (Fig 5.10).



Key: Acetone, methanol and distilled water showed 0% inhibition of *E. faecalis* strains

Fig 5.11 Pie chart indicating percentage of the strais of *E. faecalis* inhibited by different extracts of *Geranium incanum*

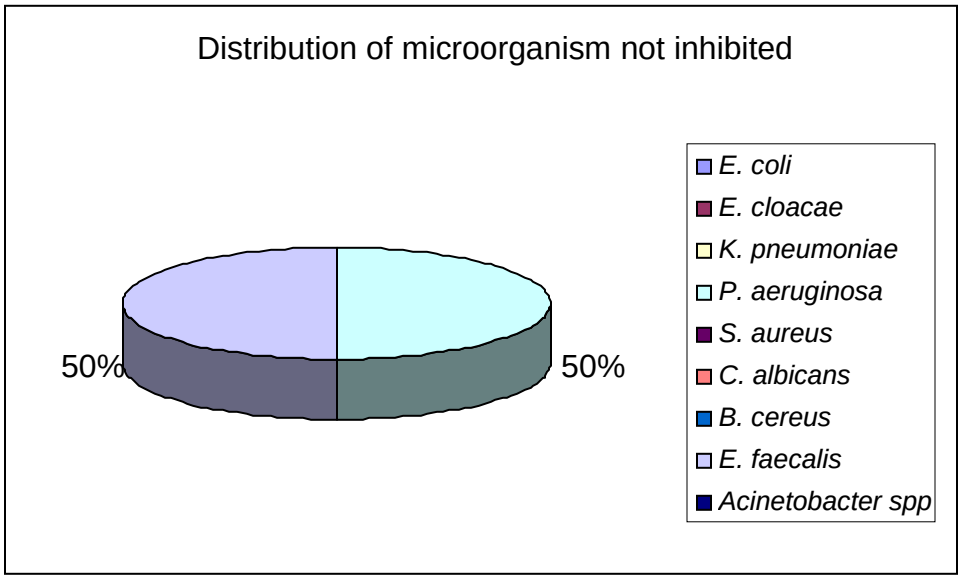
None of the strains of *E. faecalis* (100%) were inhibited by any of the extracts (acetone, methanol & distilled water) of *Geranium incanum* respectively (Fig 5.11).



Key: Acetone extracts showed 0% inhibition of Acinetobacter spp

Fig 5.12 Pie chart indicating percentage of the strains of *Acinetobacter species* inhibited by different extracts of *Geranium incanum*

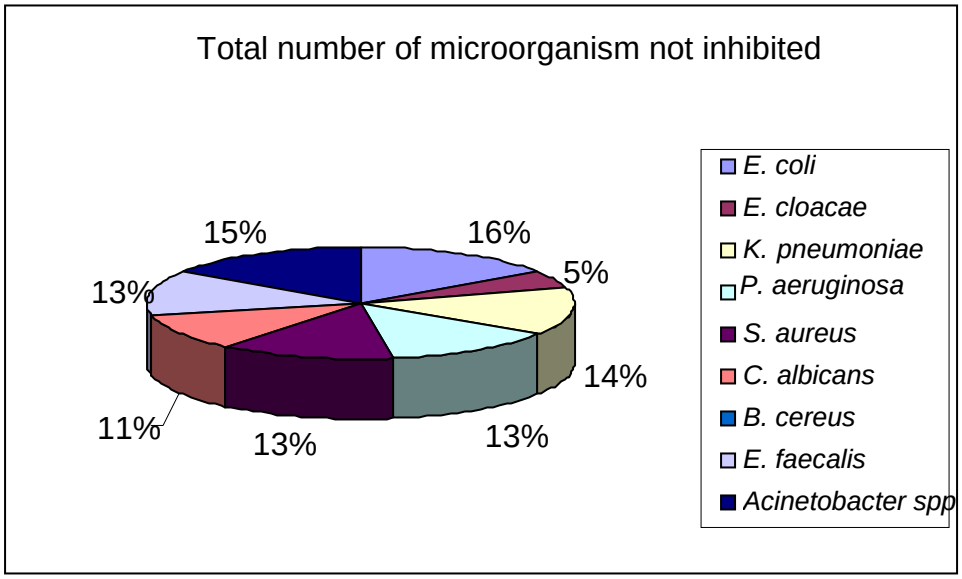
Fifty percent of the strains of *Acinetobacter species* were inhibited by the distilled water and methanol extracts of *Geranium incanum* respectively. None of the strains of *Acinetobacter species* were inhibited by acetone extracts of *Geranium incanum* (Fig 5.12).



Key: *P. aeruginosa* and *E. faecalis* were the only microbial strains which were not inhibited

Fig 5.13 Pie chart indicating percentage distribution of the organisms that were completely not inhibited by the extracts of *Geranium incanum*

The only strains of micro-organisms that were not inhibited by the extracts solvents of *G. incanum* were 11 strains of *E. faecalis* and 11 strains of *P. aeruginosa*. All the other bacterial strains and the strains of *C. albicans* were inhibited by the extracts of *G. incanum* (Fig 5.13).



Key: All the stains of *B. cereus* were inhibited by the extracts of *Dodonaea angustifolia*

Fig 5.14 Pie chart indicating percentage of the organisms that were not inhibited by the extracts of *Dodonaea angustifolia*

None of the 88 microbial strains were inhibited by the extracts of *Dodonaea angustifolia* and their results are displayed in figure 5.14 in percentage form. Of the 84 microorganisms that were not inhibited, 16% were *E. coli* strains, 5% were the *E. cloacae* strains, 14% were the strains of *K. pneumoniae*, 13% were the strains of *P. aeruginosa*, 13% were the strains of *S. aureus*, 11% were the strains of *C. albicans*, 13% were the strains of *E. cloacae* and 15% were the strains of *Acinetobacter species*.

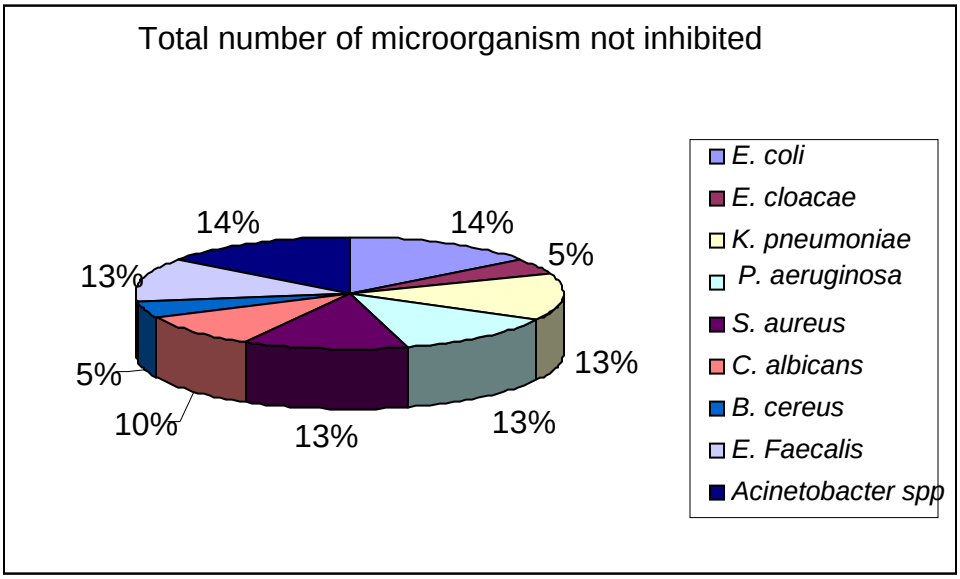


Fig 5.15 Pie chart indicating percentage of the organisms that were not inhibited by the extracts of *Eucomis autumnalis*

None of the 88 microbial strains were inhibited by the extracts of *Eucomis autumnalis*. Figure 5.15 shows 88 microorganisms that were not inhibited by the extracts of *Eucomis autumnalis* of which 14% were strains of *E. coli*, 5% were strains of *E. cloacae*, 13% were strains of *K. pneumoniae*, 13% were strains of *P. aeruginosa*, 10% were strains of *C. albicans*, 5% were strains of *B. cereus*, 13% were strains of *E. faecalis* and 14% were strains of *Acinetobacter species*.

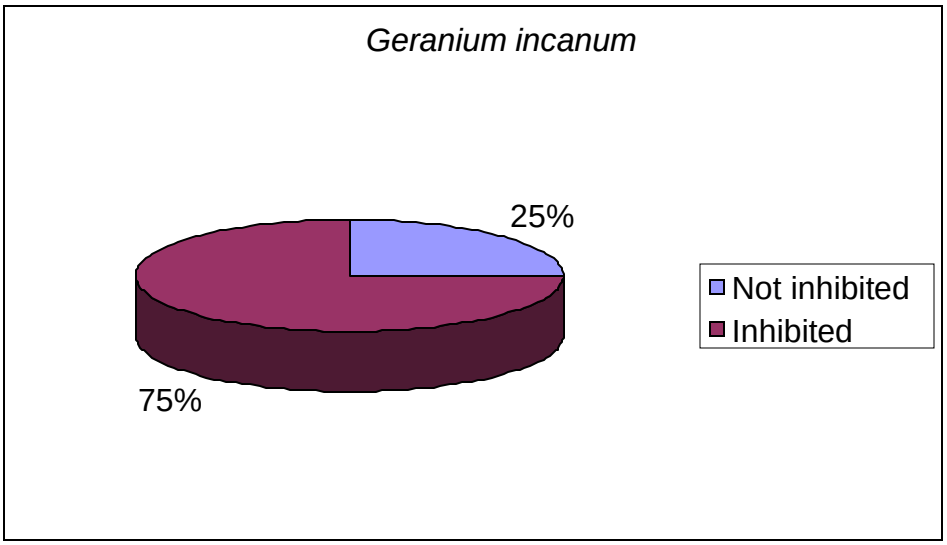


Fig 5.16 Pie chart indicating percentage of the strains that were inhibited and those that were not inhibited by the extracts of *Geranium incanum*

The total number of the microorganisms (All the bacteria and *C. albicans*) in percentage inhibited by the plant extracts of *G. incanum* was 75% and the total number of microorganisms that were not inhibited by the plant extracts of *G. incanum* was 25% (Fig 5.16).

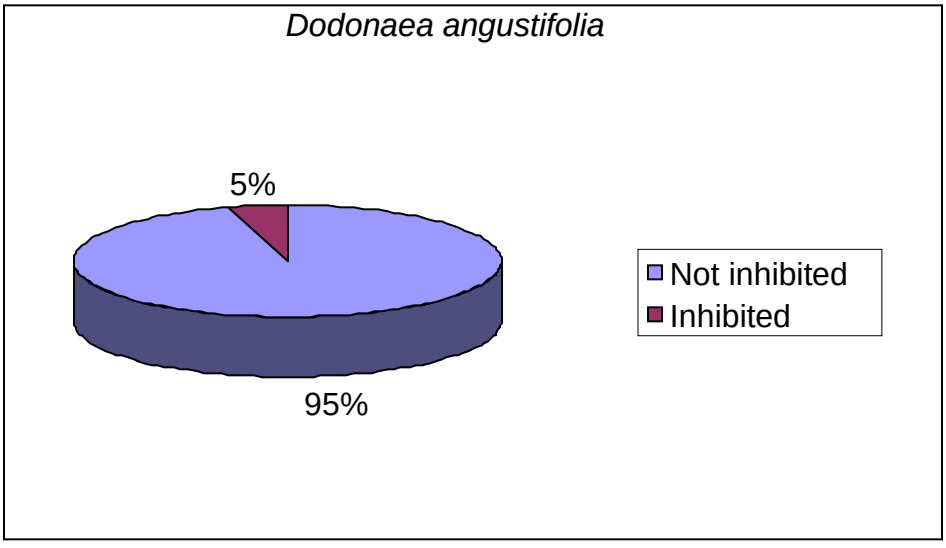


Fig 5.17 Pie chart indicating percentage of the strains that were inhibited and those that were not inhibited by the extracts of *Dodonaea angustifolia*

Ninety-five percent of the microorganisms were not inhibited by the extracts of *D. angustifolia* and only 5% were inhibited by the extracts of *D. angustifolia* (Fig 5.17).

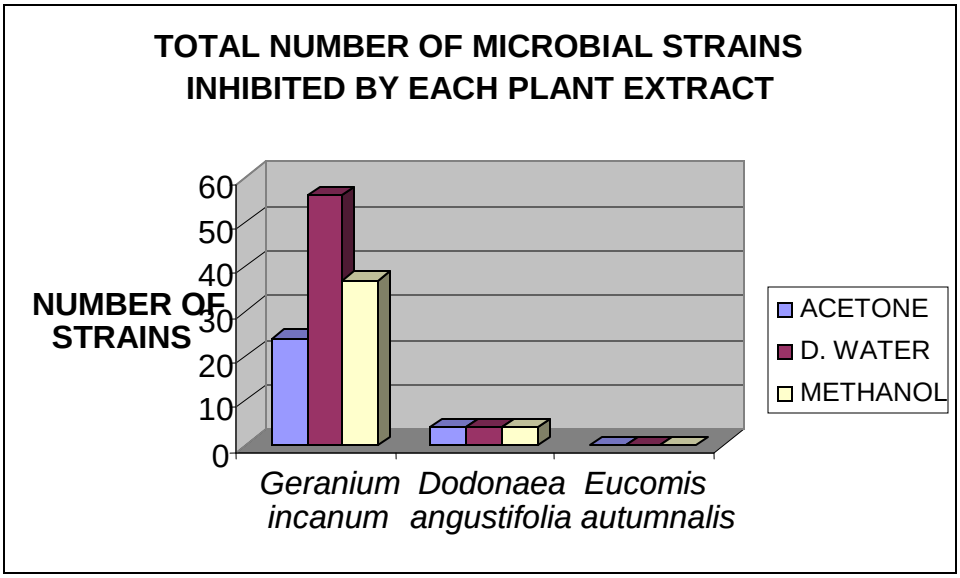


Fig 5.18 Total number of bacterial strains inhibited by the different plant extracts

Summary of the organisms in number that were inhibited by plant extracts are illustrated in figure 5.18. *G. incanum* extracts inhibited 51 bacterial strains and 9 strains of *C. albicans* which add up to a total of 60 microorganisms. Distilled water extracts of *G. incanum inhibited* all of the 60 microorganisms, methanol extracts inhibited 37 microorganisms and acetone extracts of *G. incanum* inhibited 24 microorganisms (Fig 5.18).

Each of the *D. angustifolia* extracts (distilled water, acetone & methanol) inhibited only 4 of all the microorganisms tested (Fig 5.18).

5.3 Antifungal activity of the plant extracts

Agar diffusion assay was used to screen the extracts of the three plants (*Geranium incanum, Dodonaea anjustifolia* and *Eucomis automnalis*) for any antifungal activity. The following fungi were included in the study. Nine strains of *C. albicans* and a single strain of each of the following, *Mucor species*, *Geotrichium species*, *Penicillium species*, *Fusarium species* and *Rhizopus species*. The strains of the yeast, *C. albicans*, were inoculated with the Mast multipoint inoculator and the other fungi were inoculated by using a sterile knife. The extracts of *Dodonaea agustifolia* and *Eucomis autumnalis* did not inhibit any of the fungal strains (Table5.6). However, the extracts of *Geranium incanum* inhibited some of the fungal strains. The methanol, acetone and distilled water extracts of *G. incanum* inhibited all the strains of *C. albicans*. The strain of *Mucor species* was inhibited only by the distilled water extracts and not by any of the other extracts of *G. incanum* (Table 5.6).

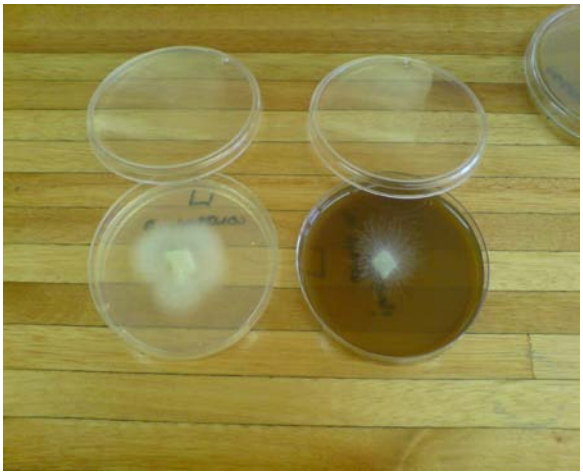
Table 5.6 Antifungal activity of plant extracts using agar dilution assay at the initial concentration of 20mg/ml

		G. incanum			*D. angustifolia*			*E. autumnalis*		
Fungi	**Strains**	**Acetone**	**D. water**	**MeOH**	**Acetone**	**D. water**	**MeOH**	**Acetone**	**D. water**	**MeOH**
C. albicans	1.	-	-	-	+	+	+	+	+	+
	2.	-	-	-	+	+	+	+	+	+
	3.	-	-	-	+	+	+	+	+	+
	4.	-	-	-	+	+	+	+	+	+
	5.	-	-	-	+	+	+	+	+	+
	6.	-	-	-	+	+	+	+	+	+
	7.	-	-	-	+	+	+	+	+	+
	8.	-	-	-	+	+	+	+	+	+
	9.	-	-	-	+	+	+	+	+	+
Mucor	1.	+	-	+	+	+	+	+	+	+
Geotrichium	1.	+	+	+	+	+	+	+	+	+
Penicillium	1.	+	+	+	+	+	+	+	+	+
Fusarium	1.	+	+	+	+	+	+	+	+	+
Rhizopus	1.	+	+	+	+	+	+	+	+	+

Key: (+) = Not inhibited (-) = Inhibited D. water = Distilled water MeOH = Methanol

The antifungal activity of the plant extracts was determined by the growth pattern of the fungi. Under normal circumstances the fungi colonies will spread across the culture plate. This can be seen in fig 5.20. When an inhibiting substance is included in the agar plate,

the fungi will not spread over the agar, but will grow in an upward direction on the piece of agar which was inoculated onto the plate from the original culture plate. See fig 5.19 for the comparison of the growth.



Key: control plate on the left and distilled water extract plate on the right

Fig 5.19 Inhibition of the strain of *Mucor species* by the distilled water extract of *Geranium incanum*

Figure 5.19 shows the inhibition of the strain of *Mucor species* by distilled water extracts of *Geranium incanum* at the concentration of 20mg/ml. Figure 5.20 indicate the non inhibition of the strain of *Rhizopus species* with the methanol extracts of *Dodonaea angustifolia* at the concentration of 20mg/ml.

Key: control plate on the left and methanol extract plate on the right

Fig 5.20 No inhibition of the strain of *Rhizopus species* with the methanol extracts of *Dodonaea angustifolia*

5.4 Standard antimicrobial sensitivity patterns of Gram-negative and Gram-positive β-lactamase and non β-lactamase producing bacteria

Antibiotic sensitivity patterns of all the β-lactamase producing bacteria were received from the National Health Laboratory Services (NHLS) Port Elizabeth. Table 5.8 display the antibiotic sensitivity patterns of the selected Gram-negative ESBLs and non-β-lactamase producing bacteria while Table 5.9 displays the antibiotic pattern of the selected Gram-positive β-lactamase producing bacteria together with Non-β-lactamase producing bacteria.

Listed are twenty-seven antibiotics which were used for sensitivity testing of β-lactamase producing Gram-negative and Gram-positive bacteria respectively.
Twenty of the twenty-five antibiotics were tested against the Gram-negative bacteria namely:

- Amikacin (AMIK)
- Ampicillin/Amoxycillin (AMP/AM)
- Cefazolin/Cephalexin (CEFAFZ/CEPH)
- Cefazolin/Cephalexin (CEFAZ)
- Cefepime (CEFEPIM)

- Cefotaxime/Ceftriaxone (CEFO/CEF)
- Ceftazidime (CEFTA)
- Cefuroxime (CEFURO)
- Ciprofloxacin (CIPRO)
- Co-Amoxiclav (CO-AMOX)
- Colistin (COLIST)
- Cotrimoxazole (COTRIM)
- Gentamicin (GENTA)
- Ertaponem (ERTAP)
- Imipenem (IMIPEN)
- Meropenem (MEROPE)
- Nalidixic acid (NALIDIX)
- Nitrofurantoin (NITROF)
- Piperacillin-Tazobactam (PIP-TAZ)
- Tobramycin (TOBRA)

All the strains of *E.coli* displayed resistance to Ampicillin/Amoxicillin except for strains no. 3 and no. 8 which were sensitive. The strains no. 2 and strain no. 4 were sensitive and resistant to Ampicillin/Amoxicillin respectively (Table 5.7).

All the strains of *E.coli* displayed sensitivity to cefuroxime, piperacillin-tazobactam, cefotaxime/ceftriaxone, ciprofloxacin, ceftazidime, gentamicin, amikacin, tobramycin, nitrofurantoin co-amoxicillin, cotrimoxazole, and cefazolin/cephalexin except for strains no.2 which was resistant to co-amoxiclav and intermediate to cefazolin/cephalexin, no. 6 and no. 9 which were resistant to cefuroxime, cotrimoxazole, and cefazolin/cephalexin, no.10 resistant to cotrimoxazole and intermediate to co-amoxiclav while strain no. 4 displayed resistance to cotrimoxazole (Table 5.7).

All the stains of *E. cloacae* are sensitive to ciprofloxacin, imipenem, meropenem, ertaponem, nalidixic acid, colistin and piperacillin/ampicillin except for strain no. 4 which showed intermediate results to piperacillin/ampicillin. On the other hand, *E.*

cloacae strains displayed resistance to cefazolin/cephalexin, cotrimoxazole, gentamicin, cefepime, co-amoxiclav, ceftazidime, cefotaxime/ceftriaxone, cefuroxime, ampicillin/amoxicillin and tobramycin except for strain no. 2 which displayed intermediate results to tobramycin. Furthermore, *E.cloacae* strains displayed intermediate results to amikacin except for the strain no. 2 which was sensitive (Table 5.7).

Strains of *K. pneumoniae* were sensitive to ampicillin/amoxicillin, cefuroxime, ceftazidime, cefepime, cefotaxime/ceftriaxone, gentamicin, cotrimoxazole, nalidixic acid and cefazolin/cepalexin and strains no.1 displayed sensitivity to cefotaxime/ceftriaxone, strain no. 9 displayed sensitivity to gentamycin, strains no. 7 and no. 8 showed sensitivity to cotrimoxazole while strain no. 2 displayed sensitivity to nalidixic acid (Table 5.7).

Strains of *K. pneumoniae* were sensitive to piperacillin-tazobactam, ciproflaxin, co-amoxicillin, amikacin, meropenem, ertapenem, tobramycin and colistin. Exceptions were strains no. 1 which was resistant to piperacillin-tazobactam, while strain no. 11 was intermediate to piperacillin-tazobactam. Strains no.1 and no.11 were resistant to ciproflaxin while strain no. 9 was intermediate to ciproflaxin. Strains no. 1, no. 3, no. 7 and no. 12 were intermediate to co-amoxiclav while strains no.9 and no.11 were resistant to co-amoxicillin. Strain no. 1 was resistant to amikacin, ertaponem and tobramycin, and was intermediate to meropenem. Strain no. 9 displayed resistance to both amikacin and tobramycin while strain no. 11 was intermediate to amikacin and resistant to tobramycin (Table 5.7).

Strain no. 2 of *P. aeruginosa* which was the only strain tested against imipenem displayed resistance to imipenem. On the other hand, *P. aeruginosa* showed sensitivity to the following antibiotics with the exceptions: piperacillin-tazobctam, ciprofloxacin, ceftazidime, cefepime gentamicin, amikacin, meropenem and tobramycin. Strain no. 2 was intermediate to piperacillin-tazobactam and resistant to ciprofloxacin, gentamycin, amikacin, meropenem and tobramycin. Strain no. 4 was intermediate to piperacillin-tazobactam, and ceftazidime and resistant to cefepime. Strain no. 10 displayed intermediate results to both piperacillin-tazobzctam and ceftazidime (Table 5.7).

Strains of *Acinetobacter spp* were resistant to piperacillin-tazobactam, ciproflaxin, ceftazidime, cefepime imipenem, gentamycin and meropenem except for strains no. 3 and no. 4 which are sensitive to piperacillin-tazobactam, ciproflaxin, ceftazidime, cefepime and meropenem. Strain no.10 displayed intermediate results to imipenem and meropenem. Strain no. 12 showed intermediate results to imipenem, meropenem. Strains no. 3, no.1 and no. 7 were sensitive to gentamycin. On the other hand strains of the *Acinetobacter spp* were sensitive to amikacin, tobramycin and colistin except for strain no.6 which was intermediate to amikacin, strain no. 10 which was resistant to amikacin, tobramycin and colistin while strain no. 12 was resistant to amikacin while intermediate to tobramycin. Strain no. 4 was resistant to tobramycin while strain no. 11 was resistant to tobramycin (Table 5.7).

Table 5.7 Standard antimicrobial sensitivity patterns of ESBLs Gram-negative and non β-lactamase producing Gram-negative bacteria

Gram –ve β-lactamase producing bacteria	**strains**	**AMP/AM**	**CEFURO**	**PIP-TAZ**	**CEFO/CE**	**CIPRO**	**CEFTAZ**	**CO-AMOX**	**CEFEP**	**IMIPEN**	**GENTA**	**COTRIM**	**AMIK**	**MEROP**	**ERTAP**	**TOBRA**	**NALIDIX**	**COLIST**	**CEFAZ**	**FLAMAZ**	**NITROF**	**FLAMAT**	**BETAD**
E.coli	1.	R	S	S		S	S	S			S	R							S				
	2.	R/S	S					R /S			S	R /S							I/ S				
	3.	S	S		S		S				S	S							S				
	4.	R/S	S	S				S			S	R /S							S				
	5.		S		R		S	S				S							S		S		
	6.	R	R	S	S	S	S	S			S	R	S			S			R				
	7.	R	S	S	S		S	S			S	S	S						S				
	8.	S	S			S	S	S			S	S							S				
	9.	R	R			S		S			S	R							R				
	10.	R	S					I			S	R							S				
	11.	R	S					S			S	S							S				
E. cloacae	1.	R	R	S	R	S	R	R	R	S	R	R	I	S	S	R	S	S					
	2.	R	R	S	R	S	R	R	R	S	R	R	S	S	S	I		S					
	3.	R	R	S	R	S	R	R		S	R	R	I						R				

	4.	R	R	I	R	S	R	R		S	R	R	I	S					R				
K. pneumoniae	1.	R	R	R	S	R	R	I	R	R	R		R	I	R	R	R	S					
	2.	R	R	S	R	S	R	S	R	S	R	R	S	S	S	S	S	S					
	3.	R	R	S	R	S	R	I	R	S	R	R	S	S	S	I		S					
	4.	R	R	S	R	S	R	S	R	S	R	R	S	S	S	I							
	5.	R	R	S	R	S	R	S	R	S	R	R	S	S	S	S		S					
	6.	R	R	S	R	S	R	S	R	S	S	R	S	S	S	S		S					
	7.	R	R	S	R	S	R	I	R	S	R	S	S	S		S							
	8.	R	R	S	R	S	R	S		S	R	S	I	S					R				
	9.	R	R	S	R	I	R	R		S	I	R	R	S		R			R				
	10.	R	R	S	R	S	R	S		S	R	R	S	S					R				
	11.	R	R	I	R	R	R	R	R	S	R	R	I	S	S	R			R				
	12.	R	R	S	R	S	R	I		S	R	R	S	S					R				
P.aeruginosa	1.			S		S	S		S		S		S	S									
	2.			I		R	S		S	R	R		R	R		R							
	3.			S		S	S		S		S		S	S									
	4.			I		S	R		R		S		S	S		S							
	5.			S		S	S				S		S	S									
	6.			S		S	S				S												
	7.			S		S	S		S		S		S	S									
	8.			S		S	S		S		S												
	9.			S		S	S		S		S		S	S									
	10.			I		S	I				S		S			S							
Acinetobacter spp	1.			R		R	R		R	R	S		S	R		S							
	2.			R		R	R		R	R	R		S	R	R	S		S					
	3.			S		S	S		S	S	S		S	S		S							
	4.			S		S	S		S	S	R		S	S		I							
	5.			R		R	R		R	R	R		S	R		S							
	6.			R		R	R		R	R	R		I	R		S							
	7.			R		R	R		R	R	S		S	R		S							
	8.			R		R	R		R	R	R		S	R		S							
	9.			R		R	R		R	R	R		S	R		S							
	10.			R		R	R		R	I	R		R	I		R				R			
	11.			R		R	R		R	R	R		S	R		T		S					
	12.			R		R	R		R	I	R		R	I		I				S			
	13.			R		R	R		R	R	R		S	R		S							

Key: (R) = Resistant (S) = Sensitive (I) = Intermediate (I/S) = Two isolates (R/S) = Two isolates (R/I) = Two isolates

Listed below are the six of twenty-seven antibiotics tested only against Gram-positive β-lactamase producing MRSA and non β-lactamase producing bacteria:

- Betadine (BETA)
- Clindamycin (CLINDA)
- Cloxacillin (CLOXA)
- Cotrimoxazole (COTRIM)
- Erythromycin (ERYTHRO)
- Flamazine (FLAMAZ)
- Penicillin/Ampicillin (PEN/AMP)
- Vancomycin (VANCO)

There were 26 Gram-positive bacterial strains included in this study. There were 11 strains of *S. aureus*, 4 strains of *B.cereus* and 11 strains of *E. faecalis*. *S. aureus* which was the only Gram-positive β-lactamase producing bacteria included in this study was tested against the eight antibiotics and displayed sensitivity to most of the antibiotics including, cloxacillin, erythromycin, clindamycin, cotrimoxazole, flamazine and betadine except strain no. 6 which was resistant to cloxacillin and erythromycin while strain no. 11 showed resistance to erythromycin. *S. aureus* strains were resistant to penicillin/ampicillin except for strain no. 4 which was sensitive (Table 5.8).

Table 5.8 Standard antimicrobial sensitivity patterns of Gram-positive MRSA and non β-lactamase producing Gram-positive *Staphylococcus aureus*

Gram +ve β-lactamase producing bacteria	**strains**	**CLOXA**	**PEN/AMP**	**ERYTH**	**CLINDA**	**COTRIM**	**VANCO**	**FLAMAZ**	**BETA**
S.aureus	1.								
	2.	S	R	S	S	S			
	3.	S	R	S	S	S			
	4.	S	S	S	S	S			
	5.	S	R	S	S	S			
	6.	R	R	R	S	S		S	S
	7.	S	R	S	S	S			
	8.	S	R	S	S	S			
	9.	S	R	S	S	S			
	10.	S	R	S	S	S			

	11.	S	R	R	S	S			

Key: (R) = Resistant (S) = Sensitive

5.5 Minimum Inhibitory Concentration of plant extracts

The following section displays the results of the Minimum Inhibitory Concentration (MIC) test which was performed on two plants (*G. incanum* & *D. angustifolia*). All the tests were done in triplicate. The MIC was performed as a subsequent test after performing the antimicrobial assays of the three plants. The lowest concentration of the plants extracts that showed the inhibition of the microorganisms is represented in (mg/ml). All the 79 bacterial strains were initially screened for antibacterial activity with the respective antibacterial assays before the determination of the MIC's of the plant extracts. MIC was only performed on the plants that displayed antimicrobial properties as well as the inhibited microorganisms. The two fold serial dilution of the plant extracts were performed to determine the lowest concentration of the plant extracts that inhibited the specific microbial strain. The highest concentration of the plant extract used was 20mg/ml which was the initial antimicrobial screening concentration.

Table 5.9 indicates the number of microbial strains included to determine the MIC of the plant extracts using agar dilution assay.

Table 5.9 Summary of all the plants and the microorganisms included in the MIC assay

		G. incanum		*D. angustifolia*		*E. autumnalis*	
Gram + ve:	Strains	Screened	MIC Determin	Screened	MIC Determin	Screened	MIC Determin
S. aureus	1.	S	T	S	NT	S	NT
	2.	S	T	S	NT	S	NT
	3.	S	T	S	NT	S	NT
	4.	S	T	S	NT	S	NT
	5.	S	T	S	NT	S	NT
	6.	S	T	S	NT	S	NT
	7.	S	T	S	NT	S	NT
	8.	S	T	S	NT	S	NT
	9.	S	T	S	NT	S	NT
	10.	S	T	S	NT	S	NT
	11.	S	T	S	NT	S	NT
B. cereus	1.	S	T	S	T	S	NT
	2.	S	T	S	T	S	NT
	3.	S	T	S	T	S	NT
	4.	S	T	S	T	S	NT
E. faecalis	1.	S	NT	S	NT	S	NT
	2.	S	NT	S	NT	S	NT
	3.	S	NT	S	NT	S	NT
	4.	S	NT	S	NT	S	NT
	5.	S	NT	S	NT	S	NT
	6.	S	NT	S	NT	S	NT

	7.	S	NT	S	NT	S	NT
	8.	S	NT	S	NT	S	NT
	9.	S	NT	S	NT	S	NT
	10.	S	NT	S	NT	S	NT
	11.	S	NT	S	NT	S	NT
Gram – ve:							
E. coli	1.	S	T	S	NT	S	NT
	2.	S	T	S	NT	S	NT
	3.	S	T	S	NT	S	NT
	4.	S	T	S	NT	S	NT
	5.	S	T	S	NT	S	NT
	6.	S	T	S	NT	S	NT
	7.	S	T	S	NT	S	NT
	8.	S	T	S	NT	S	NT
	9.	S	T	S	NT	S	NT
	10.	S	T	S	NT	S	NT
	11.	S	T	S	NT	S	NT
	12.	S	T	S	NT	S	NT
	13.	S	T	S	NT	S	NT
Ent. cloaca	1.	S	T	S	NT	S	NT
	2.	S	T	S	NT	S	NT
	3.	S	T	S	NT	S	NT
	4.	S	T	S	NT	S	NT
K. pneumo	1.	S	T	S	NT	S	NT
	2.	S	T	S	NT	S	NT
	3.	S	T	S	NT	S	NT
	4.	S	T	S	NT	S	NT
	5.	S	T	S	NT	S	NT
	6.	S	T	S	NT	S	NT
	7.	S	T	S	NT	S	NT
	8.	S	T	S	NT	S	NT
	9.	S	T	S	NT	S	NT
	10.	S	T	S	NT	S	NT
	11.	S	T	S	NT	S	NT
	12.	S	T	S	NT	S	NT
P. pyogens	1.	S	NT	S	NT	S	NT
	2.	S	TN	S	NT	S	NT
	3.	S	NT	S	NT	S	NT
	4.	S	NT	S	NT	S	NT
	5.	S	NT	S	NT	S	NT
	6.	S	NT	S	NT	S	NT

	7.	S	NT	S	NT	S	NT
	7.	S	NT	S	NT	S	NT
	8.	S	NT	S	NT	S	NT
	9.	S	NT	S	NT	S	NT
	10.	S	NT	S	NT	S	NT
	11.	S	NT	S	NT	S	NT
Acinetobacter species	1.	S	T	S	NT	S	NT
	2.	S	T	S	NT	S	NT
	3.	S	T	S	NT	S	NT
	4.	S	T	S	NT	S	NT
	5.	S	T	S	NT	S	NT
	6.	S	T	S	NT	S	NT
	7.	S	T	S	NT	S	NT
	8.	S	T	S	NT	S	NT
	9.	S	T	S	NT	S	NT
	10.	S	T	S	NT	S	NT
	11.	S	T	S	NT	S	NT
	12.	S	T	S	NT	S	NT
	13.	S	T	S	NT	S	NT
Fungi							
C. albicans	1.	S	NT	S	NT	S	NT
	2.	S	NT	S	NT	S	NT
	3.	S	NT	S	NT	S	NT
	4.	S	NT	S	NT	S	NT
	5.	S	NT	S	NT	S	NT
	6.	S	NT	S	NT	S	NT
	7.	S	NT	S	NT	S	NT
	8.	S	NT	S	NT	S	NT
	9.	S	NT	S	NT	S	NT
Mucor	1.	S	NT	S	NT	S	NT
Geotrichium	1.	S	NT	S	NT	S	NT
Penicillium	1.	S	NT	S	NT	S	NT
Fusarium	1.	S	NT	S	NT	S	NT
Rhizopus	1.	S	NT	S	NT	S	NT

Key: (NT) = Not tested (S) = Screened (T) = Tested

G. incanum and *D. angustifolia* are the only plants that were tested for the MIC. Only the bacterial strains and the strains of *C. albicans* that were inhibited by the highest concentration of the plants extracts used were included in the MIC assay.

Table 5.10 indicates the various concentrations of plants extracts to which the micro-organisms were subjected to.

Table 5.10 Concentrations of the plants extracts that were used on every single microbial strain

		G. incanum						***D. angustifolia***					
Gram + ve:	Strains	20mg/ml	10mg/ml	5mg/ml	2.5mg/m	1.25mg/	0.625mg	20mg/ml	10mg/ml	5mg/ml	2.5mg/m	1.25mg/	0.625mg
S. aureus	1.	I	I	I	I	I	NI	NI	-	-	-	-	-
	2.	I	I	I	I	I	NI	NI	-	-	-	-	-
	3.	I	I	I	I	I	NI	NI	-	-	-	-	-
	4.	I	I	I	I	I	NI	NI	-	-	-	-	-
	5.	I	I	I	I	I	NI	NI	-	-	-	-	-
	6.	I	I	I	I	I	NI	NI	-	-	-	-	-
	7.	I	I	I	I	I	NI	NI	-	-	-	-	-
	8.	I	I	I	I	I	NI	NI	-	-	-	-	-
	9.	I	I	I	I	I	NI	NI	-	-	-	-	-
	10.	I	I	I	I	I	NI	NI	-	-	-	-	-
	11.	I	I	I	I	I	NI	NI	-	-	-	-	-
B. cereus	1.	I	I	I	I	NI	-	I	I	I	I	NI	-
	2.	I	I	I	I	NI	-	I	I	I	I	NI	-
	3.	I	I	I	I	NI	-	I	I	I	I	NI	-
	4.	I	I	I	I	NI	-	I	I	I	I	NI	-
E. faecalis	1.	NI	-	-	-	-	-	NI	-	-	-	-	-
	2.	NI	-	-	-	-	-	NI	-	-	-	-	-
	3.	NI	-	-	-	-	-	NI	-	-	-	-	-
	4.	NI	-	-	-	-	-	NI	-	-	-	-	-

	5.	NI	-	-	-	-	-	NI	-	-	-	-	-
	6.	NI	-	-	-	-	-	NI	-	-	-	-	-
	7.	NI	-	-	-	-	-	NI	-	-	-	-	-
	8.	NI	-	-	-	-	-	NI	-	-	-	-	-
	9.	NI	-	-	-	-	-	NI	-	-	-	-	-
	10.	NI	-	-	-	-	-	NI	-	-	-	-	-
	11.	NI	-	-	-	-	-	NI	-	-	-	-	-
Gram – ve:													
E. coli	1.	I	NI	-	-	-	-	NI	-	-	-	-	-
	2.	I	NI	-	-	-	-	NI	-	-	-	-	-
	3.	I	NI	-	-	-	-	NI	-	-	-	-	-
	4.	I	NI	-	-	-	-	NI	-	-	-	-	-
	5.	I	NI	-	-	-	-	NI	-	-	-	-	-
	6.	I	NI	-	-	-	-	NI	-	-	-	-	-
	7.	I	NI	-	-	-	-	NI	-	-	-	-	-
	8.	I	NI	-	-	-	-	NI	-	-	-	-	-
	9.	I	NI	-	-	-	-	NI	-	-	-	-	-
	10.	I	NI	-	-	-	-	NI	-	-	-	-	-
	11.	I	NI	-	-	-	-	NI	-	-	-	-	-
	12.	I	NI	-	-	-	-	NI	-	-	-	-	-
	13.	I	NI	-	-	-	-	NI	-	-	-	-	-
E. cloacae	1.	I	NI	-	-	-	-	NI	-	-	-	-	-
	2.	I	NI	-	-	-	-	NI	-	-	-	-	-
	3.	I	NI	-	-	-	-	NI	-	-	-	-	-
	4.	I	NI	-	-	-	-	NI	-	-	-	-	-
K. pneumoniae	1.	I	NI	-	-	-	-	NI	-	-	-	-	-
	2.	I	NI	-	-	-	-	NI	-	-	-	-	-
	3.	I	NI	-	-	-	-	NI	-	-	-	-	-
	4.	I	NI	-	-	-	-	NI	-	-	-	-	-
	5.	I	NI	-	-	-	-	NI	-	-	-	-	-
	6.	I	NI	-	-	-	-	NI	-	-	-	-	-
	7.	I	NI	-	-	-	-	NI	-	-	-	-	-
	8.	I	NI	-	-	-	-	NI	-	-	-	-	-
	9.	I	NI	-	-	-	-	NI	-	-	-	-	-
	10.	I	NI	-	-	-	-	NI	-	-	-	-	-
	11.	I	NI	-	-	-	-	NI	-	-	-	-	-
	12.	I	NI	-	-	-	-	NI	-	-	-	-	-

P. aeruginosa	1.	NI	-	-	-	-	-	NI	-	-	-	-	-
	2.	NI	-	-	-	-	-	NI	-	-	-	-	-
	3.	NI	-	-	-	-	-	NI	-	-	-	-	-
	4.	NI	-	-	-	-	-	NI	-	-	-	-	-
	5.	NI	-	-	-	-	-	NI	-	-	-	-	-
	6.	NI	-	-	-	-	-	NI	-	-	-	-	-
	7.	NI	-	-	-	-	-	NI	-	-	-	-	-
	7.	NI	-	-	-	-	-	NI	-	-	-	-	-
	8.	NI	-	-	-	-	-	NI	-	-	-	-	-
	9.	NI	-	-	-	-	-	NI	-	-	-	-	-
	10.	NI	-	-	-	-	-	NI	-	-	-	-	-
	11.	NI	-	-	-	-	-	NI	-	-	-	-	-
Acinetobacter species	1.	I	I	NI	-	-	-	NI	-	-	-	-	-
	2.	I	I	NI	-	-	-	NI	-	-	-	-	-
	3.	I	I	NI	-	-	-	NI	-	-	-	-	-
	4.	I	I	NI	-	-	-	NI	-	-	-	-	-
	5.	I	I	NI	-	-	-	NI	-	-	-	-	-
	6.	I	I	NI	-	-	-	NI	-	-	-	-	-
	7.	I	I	NI	-	-	-	NI	-	-	-	-	-
	8.	I	I	NI	-	-	-	NI	-	-	-	-	-
	9.	I	I	NI	-	-	-	NI	-	-	-	-	-
	10.	I	I	NI	-	-	-	NI	-	-	-	-	-
	11.	I	I	NI	-	-	-	NI	-	-	-	-	-
	12.	I	I	NI	-	-	-	NI	-	-	-	-	-
	13.	I	I	NI	-	-	-	NI	-	-	-	-	-
Fungi													
C. albicans	1.	I	I	I	I	I	NI	NI	-	-	-	-	-
	2.	I	I	I	I	I	NI	NI	-	-	-	-	-
	3.	I	I	I	I	I	NI	NI	-	-	-	-	-
	4.	I	I	I	I	I	NI	NI	-	-	-	-	-
	5.	I	I	I	I	I	NI	NI	-	-	-	-	-
	6.	I	I	I	I	I	NI	NI	-	-	-	-	-
	7.	I	I	I	I	I	NI	NI	-	-	-	-	-
	8.	I	I	I	I	I	NI	NI	-	-	-	-	-
	9.	I	I	I	I	I	NI	NI	-	-	-	-	-

Key: (I) = Inhibited (NI) = Not inhibited (-) = Not tested

Table 5.11 The lowest MIC obtained from plant extracts with a single microbial species

	Geranium incanum			***Dodonaea angustifolia***		
ORGANISMS	ACETONE	D.WATER	METHANOL	ACETONE	D.WATER	METHANOL
E. coli	-	20mg/ml	-	-	-	-
Ent. cloacae	-	20mg/ml	-	-	-	-
K. pneumoniae	-	20mg/ml	-	-	-	-
S. aureus	2.5mg/ml	0.125mg/ml	0.125mg/ml	-	-	-
C. albicans	0.125mg/ml	2.5mg/ml	2.5mg/ml	-	-	-
B. cereus	2.5mg/ml	5mg/ml	5mg/ml	2.5mg/ml	2.5mg/ml	2.5mg/ml
Acinetobacter species	-	10mg/ml	10mg/ml	-	-	-

Key: (-) = Not done

The highest MIC's of the plant *Geranium incanum* was seen with the Gram-negative microorganisms using the distilled water extracts and the lowest MIC of the same plant was seen in Gram-positive bacteria with both methanol and distilled water extracts (Table

5.12). The highest MIC of the plant *Geranium incanum* was the same as the initial extract concentration (20mg/ml) hence some Gram negative bacterial strains were only inhibited in the initial plates of the agar dilution assay containing the highest plant extracts concentrations (Table 5.12).

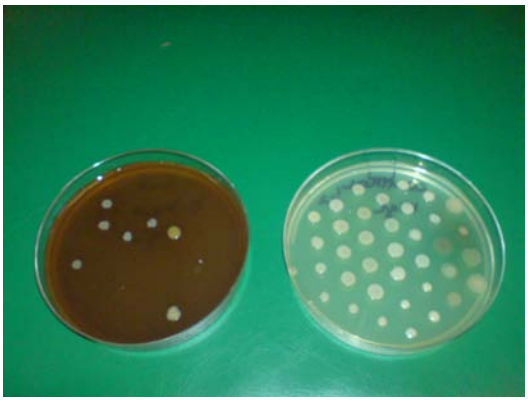
Dodonaea angustifolia displayed an inhibition only against Gram-positive bacteria. The highest MIC of the plant *Dodonaea angustifolia* were obtained with distilled water extracts while the lowest were obtained with both acetone and methanol extracts (Table 5.12).

Table 5.12 The highest and the lowest MIC's of the extracts of *G. incanum* and *D .angustifolia*

			Gram +ve bacteria		Gram –ve bacteria	
Medicinal Plants	**Extracts Solvents**	**Initial Extract mg/ml**	**MIC Low mg/ml**	**MIC High mg/ml**	**MIC Low mg/ml**	**MIC High**
G. incanum	Acetone	20	2.5	2.5	0.125	0.125
	Methanol	20	0.125	5	2.5	10
	D. water	20	0.125	5	2.5	20
D. angustifolia	Acetone	20	2.5	2.5	-	-
	Methanol	20	2.5	2.5	-	-
	D. water	20	10	10	-	-

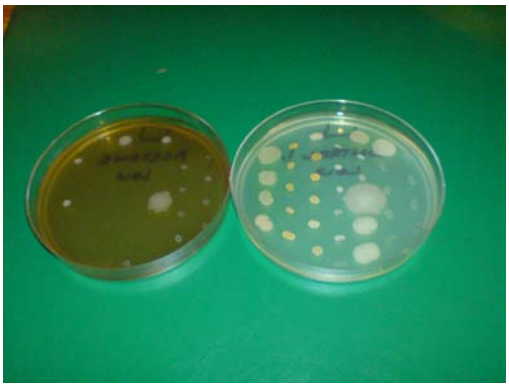
Key: (Low) = Lowest (High) = Highest (-) = Not tested

The MIC was performed using the agar dilution assay. Figures 5.21, 5.22 & 5.23 illustrate the results of the agar dilution assay of different extracts of *G. incanum*



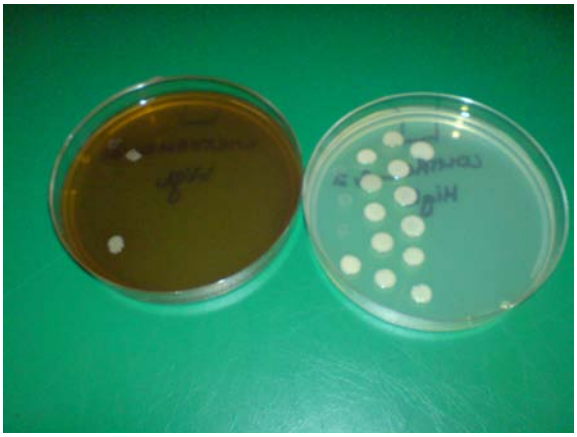
Key: Positive control plate on the right and the distilled water extract plate on the left

Fig 5.21 Inhibition of most *Staphylococcus aureus* and *B. cereus* strains by the distilled water extracts of *G. incanum* at 2.5mg/ml



Key: Positive control plate on the right and the acetone extract plate on the left

Fig 5.22 Inhibition of most Gram-positive bacteria by the acetone extracts of *G. incanum* at 20mg/ml



Key: Positive control plate on the right and methanol extract plate on the left

Fig 5.23 Inhibition of all the *Acinetobacter species* by the methanol extracts of *G. incanum* at 10mg/ml

5.6 Ames test results

Extracts of *Geranium incanum* and *Dodonaea angustifolia* were tested for the possible carcinogenicity by using the Ames test which indicate the mutagenic properties. The Ames test results were read based on the guidelines published by Mortelmans and Zeiger (2000) and Bulmer, Reid, Coombes, Blanchfield, Toth and Wagner (2007). An extract was classified as mutagenic if the number of the revertant colonies was equal or greater than two times of the negative control (Moltenmans & Zinger, 2000; Bulmer *et al*., 2007). The test was done in triplicate and the average of the revertant colonies from the negative control plates, positive control plates and each plant extract were counted respectively using a colony counter (Fig 5.25). The confirmation that the revertant colonies were of the *S. typhimurium* was done by performing the API test (Fig 5.24). There were some revertant colonies displayed by the extracts of the two plants, by the negative control as well as the positive control. The number of the revetant colonies displayed on the positive control plates was higher than that on the negative control plates and the plate containing plant extracts. Furthermore, the number of the revertants on the negative control was higher than that obtained from the plates containing plant extracts (Table 5.13) hence all the plants extracts were not mutagenic in this study.

Fig 5.24 Verification of the *S. typhimurium* colonies using the API test

Fig 5.25 Colony counter used to count the revertant colonies on the Ames test

Table 5.13 Number of revertant colonies of *S. typhimurim* indicated on Ames test

G. incanum	**Number of colonies/per plate**
Acetone	28
Methanol	27
Distilled water	24
D.angustofolia	
Acetone	39
Methanol	40
Distilled water	64
Positive control	361
Negative control	70

CHAPTER 6

DISCUSSION AND CONCLUSION

Medicinal plants were once the primary source of medicine in the world. Currently natural products and their derivatives still play an integral role in the pharmaceutical industry. The increased cost of Western medicine and resistance of bacteria to antibiotics have caused renewed interest in alternative therapies. Traditionally, the medicinal plants in this study have been used to treat infections and other non-microbial disorders ranging from bladder infections to stomach disorders. The infusions of the plants *G. incanum*, *D. angustifolia* and *E. autumnalis* were traditionally used in the healing of different ailments.

Antibiotics that once readily cured a wide range of infectious diseases are becoming less useful, mainly due to improper use of antibiotics and the development of antibiotic resistance (Nostro *et al*., 2000). The spread and growing threat by a wide range of common pathogens has lead to increased investigations into traditional medicinal plants as an alternative.

An investigation into the antimicrobial properties of the Eastern Cape medicinal plants traditionally used in the treatment of different microbial and non-microbial disorders could provide a starting point for the recognition of these remedies as essential tools to combat and control ESBL-producing bacteria. Antimicrobial activity of the selected plants of the Eastern Cape, South Africa against ESBLs has not been reported by other researches. This shows the importance of this study and the impact towards the improvement in medical science.

This study has revealed plants that can be used to treat some infections caused by resistant and opportunistic pathogens.

In this study, the antimicrobial activities of three selected plants were tested against specific microbial strains. The standard ATCC strains and clinical isolates of microbial

strains, mainly β-lactamase producing bacteria, were used to screen the antimicrobial activity of the extracts of *Geranium incanum*, *Dodonaea angustifolia* and *Eucomis autumnalis*. A total of 79 strains of different bacteria (*S. aureus, B. cereus, E. faecalis, E. coli, E. cloacae, K. pneumoniae, P. aeruginosa* & *Acinetobacter species*) were used to screen the extracts of the three plants for antibacterial activity using the agar dilution assay (Table 5.1). Most of the bacteria selected in this study form part of the normal human flora and are the primary cause of opportunistic infections. They mostly cause nosocomial infections and are either Gram-negative or Gram-positive bacteria.

In this study, more Gram-positive bacteria than Gram-negative bacteria were inhibited by the plant extracts. The lowest MIC was obtained with Gram-positive bacteria (Table 5.9 & Table 5.10). Most of β-lactamse producing bacteria in this study are Gram-negative bacteria. This correlates with the literature in that the Gram-positive bacteria are more susceptible to antimicrobial agents than Gram-negative bacteria (Heymann *et al*., 2009).

The antimicrobial compounds from plants may inhibit bacteria by a different mechanism than the presently used antibiotics and may therefore have clinical value in the treatment of resistant microbial strains (Eloff, 1999). This was observed in this study where the antibiotics sensitivity patterns when tested against the same bacterial strains, differed from the antibacterial activity patterns of the plant extracts.

Production of shiga-like toxins by *E. coli* when exposed to antimicrobial agents may account for the absence of antibacterial activity during the testing of the extracts of *D. angustifolia* and *E. autumnalis* against the strains of *E.coli* in this study. The strains of *E. coli* on the other hand displayed resistance to ampicillin/amoxicillin except for strains no. 3 and strain no. 8 which were sensitive in this study. There was inhibition of the strains of *E.coli* by all three extracts of *G. incanum*. *E. coli* is a common cause of urinary tract infections and ampicillin/amoxicillin is commonly used in the treatment of UTIs hence there is a probability that *E. coli* developed resistance to ampicillin/amoxicillin due to frequent treatment with ampicillin/amoxicillin (Reddy, 2008). The results obtained from the study undertaken by Smith (2005), were similar to the results obtained in this study

where the distilled water extracts of *G. incanum* revealed antimicrobial properties against strains of *E. coli*.

In this study, the strains of *Enterobacter cloacae* were not inhibited by the extracts of both *D. angustifolia* and *E. autumnalis* but were inhibited by the extract of *G. incanum*. On the other hand, most of the strains of *E. cloacae* displayed resistance to most of the antibiotics including cefazolin/cephalexin, cotrimoxazole, gentamicin, cefepime, co-amoxiclav, ceftazidime, cefotaxime/ceftriaxone, cefuroxime, ampicillin/amoxicillin and tobramycin (Table 5.7). Similar results were seen in some studies where some strains of *Enterobacter cloacae* were resistant to expanded spectrum cephalosporins and were implicated in outbreaks of serious infections in neonatal intensive care units (NICUs) (Finnstrom, Isacsson, Haeggman, & Burman, 1998; Hervans, Ballesteros, Alorma, Gil, Benedi & Alberti, 2001). *E. cloacae* in the study undertaken by Wang, Shangwei, Xue, Ping & Yunde, (2009) were resistant to piperacillin, ciprofloxacin, amikacin, cefazidime and ampicillin.

According to the results obtained in this study, strains of *Klebsiella pneumonia* were not inhibited by the extracts of all the three selected plants except for the distilled water extracts of *G. incanum*. The extensive use of broad-spectrum antibiotics in hospitalized patients accounts for the development of multi-drug resistant strains of *K. pneumonia* that produce ESBLs (Umeh, 2006). In the study conducted by Pillay, Adhikari, & Sturm (1998), *Klebsiella pneumoniae* were resistant to piperacillin/tazobactam and cefotaxime. Results in table 5.7 indicate that the strains of *Klebsiella pneumoniae* were sensitive to most of the antibiotics except for strain no. 1 which was resistant to piperacillin-tazobactam, while strains no.1 and no.11 were resistant to ciproflaxin. Strains no.9 and no.11 were resistant to co-amoxicillin while strain no. 1 was resistant to amikacin, ertaponem and tobramycin as indicated in table 5.7. On the other hand strain no. 9 displayed resistance to both amikacin and tobramycin (Table 5.7). The acetone and the methanol extracts in this study did not show any antimicrobial activity against any strain of *K. pneumonia* (Table 5.1). The distilled water extracts prepared showed antibacterial activity against all the strains of *K. pneumonia* except strains no. 6 and no.10.

P. aeruginosa is not a member of the family of *Enterobactriaceae* but forms part of ESBL producing bacteria (Naas, Phiippon, Poirel, Ronco & Nordmann, 1999; Mugnier, Dubrous, Casin, Alert & Collatz, 1996). In the study of Carmeli, Troillet, Eliopoulos & Samore (1999) *P. aeruginosa* was resistant to penicillins, cephalosporins carbapenems, fluroquinnolones and ciprofloxacin. In contrast, in this study, *Pseudomonas aeruginosa* was sensitive to piperacillin-tazobactam, ciprofloxacin, ceftazidime, cefepime gentamicin, amikacin, meropenem and tobramycin except for strain no.2 which was resistant to ciprofloxacin, gentamycin, amikacin, meropenem and tobramycin and strain no.4 which was resistant to cefepime (Table 5.7). These findings were not similar to those of the study done by Carmeli *et al*. (1999) as no inhibition of the strains of *P. aeruginosa* was observed with different extracts of all selected plants. As indicated in Table 5.1, the strains of *P. aeruginosa* were not inhibited by any of the plant extracts tested.

The development of antibiotic resistance in *S. aureus* has led to the use of development of many antibiotics to treat infections caused by different strains of *S. aureus* (Peddie *et al*., 2004; CDC, 2009; Bauman, 2007). The results obtained by Russell & Suller (2000) indicate that methicillin resistant *Staphylococcus aureus* showed resistance to penicillin, erythromycin, methicillin streptomycin and tetracycline. The antimicrobial pattern of the *Staphylococcus aureus* (Table 5.8) investigated in this study similar to from the above mentioned study because strain no. 6 was resistant to cloxacillin and erythromycin while strain no. 11 showed resistance to erythromycin. All the strains of *S. aureus* in this study were resistant to penicillin and ampicillin except for strain no. 4 which was sensitive (Table 5.8). Russell & Suller (2000) tested MRSA against triclosan, which is an antimicrobial agent used in hygiene products and for the treatment of MRSA infections, and found that low-level resistance to triclosan by *Staphylococcus aureus* have emerged. The current study revealed no inhibition of *S. aureus* strains by the extracts of *D. angustifolia* and *E. autumnalis*. These plants are traditionally used in the treatment of infections. Despite the resistance of all the strains of *S. aureus* to penicillin and ampicillin (Table 5.8), acetone, methanol and distilled water extracts of *G. incanum* inhibited all the

strains of *S. aureus* (Table 5.1). However, in this study strains of *S. aureus* was not inhibited by the infusions of *G. incanum* as found by Freidberg, (2009). The use of dried leaves in this study might account for the difference of the results obtained from the study conducted by Freidberg (2009) where fresh leaves were used.

Although *Bacillus cereus* is not a β-lactamase producing bacteria, it was included in the study to represent other Gram-positive bacilli. *B. cereus* strains were inhibited by all the extracts of *G. incanum* and *D. angustifolia* and were not inhibited by the extracts of *E. autumnalis*. Gram-negative bacteria are generally more resistant to plants extracts than Gram-positive bacteria (Palombo *et al*., 2001), that might be the reason why *B. cereus* strains were inhibited by all of the extracts of *G. incanum* and *D. angustifolia*. Similarly, *B. cereus* was inhibited by the methanol, acetone and distilled water extracts of *G. incanum* in the study of Freidberg (2009) using the same agar diffusion method.

E. faecalis also is not β-lactamase producing bacteria, but has developed resistance to antibiotics (William, 2003). This was indicated by the absence of inhibition of the strains of *E. faecalis* by the extracts of *G. incanum*, *D. angustifolia* and *E. autumnalis* observed in this study (Table 5.1). These results are similar to the results obtained by Smith (2005) where the acetone, methanol and distilled water extracts of *G. incanum* did not show any inhibition of the strains of *E. faecalis* at the same concentration of 20mg/ml.

Acinetobacter species were reported as multi-drug resistant (MDR) bacteria (Kepler *et al*., 2005; Bauman, 2007) which cause infections that are difficult to treat (Kepler *et al*., 2005). Go and Cunha (1999) found that antibiotics to which *Acinetobacter* species were usually sensitive include: meropenem, colistin, polymyxinB, amikacin, rifampicin, minocycline and tetracycline. Burk (2009) indicate that first, second and third-generation cephalosporins, macrolides and penicillins have little or no anti-bacterial activity against *Acinetobacter* species and their use may predispose to *Acinetobacter* colonization. In this study, most of the strains of the *Acinetobacter* species were resistant to piperacillin-tazobactam, ciproflaxin, ceftazidime, cefepime imipenem, gentamycin and meropenem (Table 5.7). The resistance of *Acinetobacter* species to plant extracts has been recorded in

literature. Results obtained by Kudi, Umoh, Eduvie & Gefu, (1998) show that crude extracts from eight Nigerian medicinal plants did not inhibit any of the *Acinetobacter* species included in that study Kudi *et al.* (1998). In the present study the strains of *Acinetobacter* species were not inhibited by the extracts of *D. angustifolia, E. autumnalis* and the acetone extracts of *G. incanum.* However in this study, the distilled water and methanol extracts of *G. incanum* inhibited all the strains of *Acinetobacter* species selected (Table5.10).

Candida albicans was included in this study as it is a eukaryotic organism that is very difficult to treat and most of the drugs used to treat patients with *Candida albicans* infections were found to have toxic side effects (Neal, 2009). *C. albicans* is the principal cause of many opportunistic infections (Pappas, Rex & Lee, 2003). The strains of *C. albicans* were not inhibited by the three extracts of *E. autumnalis* and *D. angustifolia* as indicated in table 5.6 yet the extracts of *G. incanum* displayed antimicrobial activity against all the strains of *C. albicans*. In a study done by Daman, Gideon & Patel (2005) *D. angustifolia* inhibited all the strains of *C. albicans* isolated from HIV positive and HIV negative patients. The difference in the results obtained in the two studies may be due to the different methods, agar diffusion compared to agar dilution. Although there was no inhibition of the strains of *C. albicans* by the extracts of *G. incanum* observed in the study by Smith (2005), extracts of *G. incanum* in the present study displayed antimicrobial activity against all the strains of *C. albicans* indicated in table 5.6. Again the reason may be that different methods were used. In this study, agar dilution assay was used while in the Smith (2005) study agar-well diffusion assay was incorporated. The difference in the strains of *C. albicans* may also account for the difference in the results obtained. Fungi are the primary cause of opportunistic infections. Therapeutically active plant extracts of *G. incanum* showed inhibition properties against fungal strains of *C. albicans* and *Mucor* in this study. Following further *in vivo* investigations, *G. incanum* might prove to be a useful antifungal agent.

Mucor, Geotrichium, Penicillium, Fusarium and *Rhizopus* were also included in this study to represent the spore forming opportunistic fungi that may affect man. According

to Portillo, Vila, Freixa, Adzet and Canigueral, (2001) there is a distinct need for the discovery of new safer and more effective antifungal agents due to low potency, poor solubility, the emergence of resistant strains and drug toxicity that limit the use of the available antimycotic drugs. *Geotrichium, Penicillium, Fusarium* and *Rhizopus* were not inhibited by any of the extracts of the plants included in this study. In this study, the distilled water extracts of *G. incanum* inhibited *Mucor* strain tested, indicating the potential of this plant in combating emerging diseases.

According to Paterson (2005) the use of solvents allows quite precise manipulation of herbal material and without their use herbal therapeutics would not have advanced far beyond a primitive art.

There are many chemicals or compounds used as extraction solvents in research. Acetone is mostly used in pharmaceuticals as a solvent and methanol is used in pharmaceuticals as an organic solvent (Putman, 1985). Since water is the most abundant molecule on the earth's surface, various substances dissolve in water and it is commonly referred to as the universal solvent. According to Putman (1985), a commonly used extracting solvent is water in which dried or living plants are soaked. According to Akowuah *et al.* (2004) the most effective solvent in the antimicrobial screening of plant extracts is acetone, followed by methanol and then distilled water. Demiray, Pintado and Castro (2009) reported acetone to be the best extract solvent while they reported the distilled water as the least active solvent.

In contrast to a general antimicrobial pattern of the extracts obtained from using the solvents indicated above, distilled water extracts inhibited a higher percentage of pathogens in current study, followed by the acetone extracts (Fig. 5.18). Methanol extracts inhibited the smallest percentage of the pathogens as indicated in figure 5.18 and as mentioned above in other studies.

It became clear through this study that *Geranium incanum* and *Dodonaea angustifolia* have antimicrobial activity to some extent against selected pathogens.

Although Treurnicht (1997) reported no *in vitro* antimicrobial activity of aqueous extracts of *Geranium incanum* against *Pseudomonas aeruginosa* the distilled water extracts of *G. incanum* in this study showed antimicrobial activity against most of the Gram-positive bacteria and some of the Gram-negative bacteria except for *P. aeruginosa* like in the findings reported by Treurnicht (1997). In the present study, the strains of *E. faecalis* were also not inhibited by the extracts of *G. incanum*.

Freidberg (2009) reported similar results to the results obtained in this study where the strains of *C. albicans*, *E. coli*, *S. aureus* and *B. cereus* were inhibited by the extracts of *G. incanum* using the same agar dilution method with different concentrations incorporated. Freidberg (2009) was using a higher concentration of 100mg/ml in contrast to the concentration of 20mg/ml used in this study.

In the *in vitro* assays performed by Asres *et al*. (2001), *Dodonaea angustifolia* showed no antimicrobial activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans*. Results obtained in this study were similar since no antimicrobial activity of the extracts of *D. angustifolia* was detected against the above mentioned pathogens except for the strains of *B. cereus* (Table 5.1). *Dodonaea angustifolia* is traditionally used to treat oral candidiasis and is said to have antifungal properties. In contrast to the findings of Asres *et al*. (2001), in this study; *D. angustifolia* did not show any inhibition to any of the selected fungi (Table 5.6). The reason to account for this difference maybe different antibacterial screening techniques that were used in both studies and maybe the plant parts used in both studies could differ and hence different constituents may account for the absence of inhibition encountered in this study. Since *Dodonaea angustifolia* is said to have antibacterial activity against MRSA (Heymann *et al*., 2009), it was selected as plant of choice to inhibit most β-lactamase producing bacteria selected. However, *Dodonaea angustifolia* did not show any antibacterial activity against β-lactamse producing bacteria in this study.

Ethnopharmacological investigations have revealed antibacterial properties of the leaves and the underground parts of *E. autumnalis* (Zsche *et al.*, 2000; Louw, 2002). In this study, *E. autumnalis* revealed no antimicrobial activity to any of the selected bacteria and fungi though it was reported to have antifungal activities when tested against pathogenic plant fungi (Pretorius, *et a.l*, 2002). The reason for different results maybe that in this study pathogenic human fungi were used but in the study of Pretorius *et al*. (2002) pathogenic plant fungi were used.

Although testing of probable carcinogenicity by using Ames test is quick and indicates the carcinogenic potential of many chemicals (Chan, *et al.*, 1993), it is not possible to make a direct correlation between the observed activity of the extracts *in vitro* and the actual effects when used *in vivo*. Results *in vitro* may be encouraging while different biochemical pathways may be activated or utilized *in vivo*. The extracts of *G. incanum* and *D. angustifolia* did not show any probable carcinogenicity on the *S. typhumirium* TA 100 incorporated as indicated in table 5.13. It is important that the probable carcinogenicity of the plants species that showed antimicrobial activity in this study be further investigated. Preclinical *in vivo* and clinical trials should be performed using the selected medicinal plants. Furthermore, patients that are considering the use of traditional remedies should be well informed of the side effects that may occur. The Ames test in this study did not incorporate liver extracts (s9) and hence might not reflect the *in vivo* activity of the plant extracts. In spite of no mutagenic effects of the extracts of *G. incanum* detected in this study, the aqueous extracts of *G. incanum* were found to be cytotoxic in the study performed by Treurnicht (1997). The cytotoxicity of aqueous extracts was said to be possibly ascribed to presence of polyphenols (tannins) and hence account for *G. incanum* as an abortifacient (Treurnicht, 1997). Determination of plants constituents by performing further tests may be helpful in the determination of the selected plants compounds that might be harmful or even toxic to human. The medicinal plants used in this study may have additional properties that might be displayed by their single constituents or at least synergistic effects of two.

The antimicrobial activity results were read based on visible growth or no growth of selected pathogens. The finer degree of susceptibility might be masked by the all or nothing phenomenon that is used in the reading of the results obtained from the agar dilution assay. Further antimicrobial screening of the plant extracts may be performed using other methods like the micro-titer plate method which might indicate further antimicrobial activity of the selected plant extracts.

Although the extracts of *E. autumnalis* did not show any antimicrobial properties against selected pathogens in this study, ethnopharmacological investigations have revealed antibacterial properties of the leaves and the underground parts (Zsche *et al*., 2000; Louw, 2002). The crude extracts of *E. autumnalis* displayed antibacterial activity in the study perfomed by Zschocke *et al*., 2000). *E. autumnalis* was also found to have antifungal activities against *C. michiganense* (Zschocke *et al*., 2000). Further studies, using different methodology and different plant parts may be undertaken regarding the potential antimicrobial properties that the extracts of *E. autumnalis* might have as reported in the literature.

Apart from revealing of new antimicrobial agents that may be used against resistant organisms, the proper use of antimicrobial agents should be recommended as suggested by the WHO (2004b).

Although the relationship between antibiotic use and antibiotic resistance is complex and dynamic, there is no doubt that more intense antibiotic use will eventually lead to higher microbial resistance that may be associated with treatment failure.

There is increasing evidence showing that antibiotic use in human medicine does not only vary according to patient characteristics (medical determinants) but also according to:

- Geographic location associated with different health systems
- Reimbursement systems
- Medical education
- Primary care structural components

- Cultural expectations and market characteristics (socioeconomic and cultural determinants)

Indiscriminate and irrational use of antimicrobial agents is primarily responsible for emergence and spread of antimicrobial resistance in almost all the micro-organisms (WHO, 2004b).

This study was aimed at revealing plants that can be used to overcome microbial resistance which is a major problem and that promotes the mortality rate of patients with immunosuppressive infections like HIV/AIDS.

In this investigation the extracts of *G. incanum* and *D. angustifolia* were promising potential microbial inhibitors. The high antimicrobial activity against a wide range of pathogens by the extracts of *G. incanum* especially the inhibition of all the strains of *Acinetobacter species* which are multi-drug resistant (Kepler *et al*., 2005; Bauman, 2007; Kudi, *et al*., 1998; Burk, 2009) and the strains of *C. albicans* and *Mucor* makes *G. incanum* a promising plant for further investigations to cure diseases caused by ESBL producing organisms and maybe even MDR *Mycobacterium tuberculosis*.

APPENDIX A

Media preparation

Medium	**Quantity (Powdered)**	**Distilled water in ml**	**Final concentration**	**Autoclave temperature in ^{0}C**
Nutrient agar	31g	1000ml	31g/1000ml	121
Mueller-Hinton agar	38g	1000ml	19g/500ml	121
DMA	26g	1000ml	13g/500ml	121
SabouraudDextrose agar	60g	1000ml	3g/50ml	121

- Powdered media was dissolved in distilled sterile water
- Sterilized in an autoclave at 121^{0}C
- Cooled to about 45^{0}C in the water bath
- Poured the plates

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