ANTICANCER, ANTIOXIDANT AND ANTIMICROBIAL SCREENING OF
EXTRACTS FROM *ZIZIPHUS MUCRONATA*, *HELIOTROPIUM CILIATUM* AND
GNIDIA POLYCEPHALA FROM THE OSHIKOTO REGION OF NAMIBIA

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Secilia K. Ilonga

DEDICATION

I dedicate this work to my family for always believing in me and encouraging me even in instances when I felt like giving up.

To my brothers and sisters, you can go as far as your imagination goes. Do not give up on your dreams.

ABSTRACT

Plants have long been used to treat ailments such as headaches, stomach-ache, diarrhoea, tumours, wounds and sexually transmitted diseases, just to mention a few. *Ziziphus mucronata*, *Heliotropium ciliatum* and *Gnidia polycephala* are traditionally used to treat tumours and wound-related illnesses. Tumours and persistent wounds can be a sign of cancer. Microbial wound infections can also bring fatal consequences if unattended. This study evaluates the antioxidant, anticancer and antimicrobial potential of extracts of these three plants. The leaves (*Z. mucronata*) and aerial parts (*G. polycephala* and *H. ciliatum*) were collected from Oshikoto region, grinded and sequentially extracted with hexane, dichloromethane, ethanol and methanol. Water extracts were also prepared. The antioxidant potential was evaluated by 1,1-diphenyl-picrylhydrazyl (DPPH) assay whereas the Brine shrimp lethality test (BST), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromine (MTT) assay and APOP*ercentage*^{TM} flow cytometry assay were used to evaluate the anticancer potential of the extracts. The antimicrobial potential of the plant extracts against eight wound pathogens: *Candida albicans*, *Clostridium tetani*, *Escherichia coli*, Methillicin-resistant *Staphylococcus aureus*, *Mycobacterium terrae*, *Pseudomonas aeruginosa*, *Staphylococcus epidermidis* and *Streptococcus A*, was evaluated using the broth micro-dilution method. Methanol extracts of *Z. mucronata* and *G. polycephala* showed good antioxidant activity, comparable to that of a control, butylated hydroxyl toluene. Dichloromethane and hexane extracts of *Z. mucronata* and *H. ciliatum* as well as ethanol extracts of *H. ciliatum* showed high cytotoxicity, with LC_{50} values < 250 µg/ mL. Water extracts showed the least cytotoxic activity. In addition, dichloromethane extract of *Z. mucronata* also demonstrated a broad spectrum antimicrobial activity, obtaining MIC values ≤ 1 mg/ mL against six of the eight tested pathogens. The low toxicity of water extracts of the three plants and the antimicrobial activity recorded validates the use of these extracts in traditional medicine. Some extracts also appears to be good sources of potential antioxidant and anticancer agents. However, more studies are required.

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LIST OF ABBREVIATIONS

µg – Microgram

µL – Microliter

µM – Micro molar

AIDS – Acquired Immune Deficiency Syndrome

APOP*ercentage*^{TM} – Apoptosis Percentage

AST – Antimicrobial Susceptibility Test

BC – Before Christ

BHT – Butylated Hydroxyl Toluene

BSAC – British Society for the Antimicrobial Chemotherapy

BST – Brine Shrimp lethality Test

CA – *Candida albicans*

Caco-2 – Colon Adenocarcinoma

CARNEIGE-RISE – The Carnegie Corporation of New York as a Regional Initiative in Science and Education

CFU – Colony Forming Unit

CHO – Chinese Hamster Ovary

CO_2 –Carbon dioxide

CT – *Clostridium tetani*

DMEM – Advanced Dulbecco's Modified Eagle's Medium

DMSO – Dimethyl sulfoxide

DNA – Deoxyribonucleic acid

DPPH – 1,1-diphenyl-2-picrylhydrazyl

DRC – Democratic Republic of Congo

EC – *Escherichia coli*

EDTA – Ethylenediamine tetraacetic acid

EUCAST – European Committee for Antimicrobial Susceptibility Testing

FBS – Foetal Bovine Serum

FSC – Forward Scatter

g – Gram

GD – *Gnidia polycephala* dichloromethane extract

GE – *Gnidia polycephala* ethanol extract

GH – *Gnidia polycephala* hexane extract

GM – *Gnidia polycephala* methanol extract

H157 – Lung carcinoma

H_2O_2 – Hydrogen peroxide

H4 – Small intestinal foetal tissue

H_a – Alternative hypothesis

HCl – Hydrochloric acid

HD – *Heliotropium ciliatum* dichloromethane extract

HE – *Heliotropium ciliatum* ethanol extract

HeLa – Human cervical cancer

HEPES – 4-(2- hydroxyl ethyl) piperazine-1-ethanesulfonic acid

HepG-2 – Human heptoma cells

HH – *Heliotropium ciliatum* hexane extract

HIV – Human Immunodeficiency Virus

HM – *Heliotropium ciliatum* methanol extract

H_o – Null hypothesis

HT-29 –Human colon adenocarcinoma

IC_{50} – 50 % Inhibitory concentration

KMST-6 – Non-tumorigenic immortalised human diploid fibroblasts

LC – Lethal concentration

LC_{50} – 50 % Lethal concentration

LCL – Lower confidence limit

mg – Milligram

MIC – Minimum inhibition concentration

mL – Millilitre

MRSA – Methicillin-resistant *Staphylococcus aureus*

MSCRAMMs –Microbial surface components recognizing adhesive matrix molecules

MT – *Mycobacterium terrae*

MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

$NADP^+$ - Nicotinamide adenine dinucleotide phosphate

NADPH – Nicotinamide adenosine dinucleotide phosphate

NBCS – New-born calf serum

NCCLS – National Committee for Clinical Laboratory Science

NCI – National Cancer Institute

NCI-H640 – Human lung cancer cells

NETHA – National Eagle Traditional Healers Association

nm – Nanometre

NO – Nitrogen monoxide

NR – Nitrate reductase

^{o}C – Degree Celsius

OD – Optical Density

PA – *Pseudomonas aeruginosa*

PBS – Phosphate buffered saline

POL-SABINA – Policy and Support Actions for Southern African Natural Product Partnership

RNA – Ribonucleic acid

ROS – Reactive Oxygen Species

RPMI 1640 – Roswell Park Memorial Institute 1640 medium

SA – *Streptococcus* A

SABINA – Southern African Biochemistry and Informatics for Natural Products Network

SARs – Structure Activity Relationships

SE – *Staphylococcus epidermidis*

SSC – Side Scatter

TMB – 3,3',5,5'-Tetramethylbenzidine

UCL – Upper Confidence Limit

WHO – World Health Organization

YEPG – Yeast Extract Peptone Glucose

ZD – *Ziziphus mucronata* dichloromethane extract

ZE – *Ziziphus mucronata* ethanol extract

ZH – *Ziziphus mucronata* hexane extract

ZM – *Ziziphus mucronata* methanol extract

CHAPTER 1

1. INTRODUCTION

1.1 Orientation of the study

Plants have been an important source of medicine since ancient times. Early written reports on the use of plants as medicine appeared about 2600 BC when plants were used as medicine by Sumerians and Akkaidians (Shoeb, 2006). Since then, plants have been used to treat ailments such as headaches, toothaches, stomach aches, diarrhoea, wounds, tumours and sexually transmitted diseases (Van Wyk and Gericke, 2000, Wuyang, 2008; Khaleeliah, 2001; Von Koenen, 2001), just to mention a few. However, the potential of several plants as medicinal agents has not been fully characterized and established. This is due to the fact that most scientific studies carried out on plants focused on specific diseases, thereby revealing only a narrow spectrum of active compounds. This is often attributed to the limited resources, labour and time allocated for the study. An example of this is the screening of over 35, 000 plants extracts by the National Cancer Institute (NCI) of the United States in the 1960s which only targeted anticancer compounds. Compounds with other medicinal abilities were left uninvestigated (Hostettmann, Wolfender, Rodriguez and Marston, 1996). Screening of plants for medicinal purposes is important as plants are an important source of lead compounds- backbone compounds used in the synthesis of new drugs in pharmaceutical industries (Potier, Gueritte-Voegelein and Guenard, 1996). A review by Funnell, Lindsey, McGraw, Sparg, Stafford, Elgorashi, *et al*., (2004), states that about 122 drugs were estimated to have been discovered through ethnobotanical leads of 94 plant species. The screening of plant extracts by the NCI during the early 1960s alone led to the discovery of important anticancer compounds such as Camptothecin, Taxol and Vinblastine, which are used clinically in the treatment of cancer. Camptothecin, Taxol and Vinblastine were isolated from extracts of *Camptotheca acuminate*, *Taxus brevifolia* and *Catharanthus roseus*,

respectively (Sarkar, Sharma and Talukder, 1996; Hostettmann *et al*., 1996). In Namibia, plants were and are still being used among different indigenous groups to treat different diseases and ailments. Despite this, the compounds responsible for the healing actions in most of the Namibian medicinal plants are not yet investigated due to lack of scientific studies and exposure.

Cancer is a disease characterized by uncontrolled cell growth (Boyd, 1995). In most cases, these cells divide uncontrollably forming lumps or tumours, and consequently interfering with the normal functioning of the cells. In leukaemia, the cancerous cells do not form lumps or tumours but rather causes cell deformation, resulting in interference with normal blood functions. Not all tumours are cancerous. Cells of cancerous tumours differ from cells of non-cancerous tumours in their ability to relocate to other parts of the body and invade healthy cells (What is cancer, 2009). Cancerous uncontrolled cell division is primarily due to oxidative cell damage caused by free radicals. Free radicals are acquired from the environment through exposure to cigarette smoke and other products of incomplete combustion, ultraviolet radiation and are also produced in the body during the normal process of food breakdown (Tsang, 2009). Anticancer compounds help prevent or slow down the development of cancer by inhibiting or regulating cell division (Wall and Wani, 1995) and by killing cancerous cells (Shoeb, 2006). Antioxidants help prevent cancer by neutralizing the free radicals and repairing the oxidatively damaged cells (NCI, 2004).

1.2 Problem statement

Namibia is a semi-arid developing country, situated between the Namib and the Kalahari deserts. This makes it one of the hottest and driest countries in the world. Under such harsh conditions, plants develop survival mechanisms to help them survive under these

conditions. One such survival mechanism is the production of toxic compounds, which not only help the plant adapt to these harsh conditions but may also offer it protection from being fed on by herbivores (Balandrin, Klocke, Wurtele and Bollinger, 1985). This makes some Namibian plants medicinally promising. In fact, being a poor developing country with many cultural diversities and traditional practices, different plants were and are still being used among the different Namibian traditions to treat different ailments and diseases. There is also a wide usage and dependence on medicinal plants for primary health care worldwide, which motivated the World Health Organization (WHO) to encouraged the use of medicinal plants in primary health care (Shoeb, 2006).

The effects of cancer is quite devastating, as cancer is one of the leading causes of death worldwide causing over 6.7 million cancer deaths annually worldwide. Namibia has an average prevalence rate of over 1000 new cancer cases being reported every year (Carrara, Stein, Sitas and Ferlay, 2009). Although cancer is found among people of all age groups, it is common in people above 15 years of age, prevalence increasing with age. This constitutes the majority of the working age group. Thus cancer has a negative impact on the economy of the country. There is also a lack of proper medication for cancer as most treatment agents used today have numerous side effects, creating an urgent need for the search of safer medication. Despites this, plants which have the potential to treat a lot of ailments including cancer are still under investigated. In addition, traditionally used medicinal plants are constantly being destroyed by human activities and may become extinct. *Ziziphus mucronata*, *Heliotropium ciliatum* and *Gnidia polycephala* are among some traditional medicinal plants used in the treatment of wounds and or tumour (Neuwinger, 1996). The complexity of cancer makes it difficult for a traditional healer to distinguish between ordinary wounds and wounds resulting from cancer. In addition, wound microbial infections are common in Africa. Different forms of cancer and wound pathogens are constantly gaining resistance to current drugs, creating a need for the discovery of new drugs. This study was aimed at evaluating the

antioxidant, anticancer and antimicrobial potential (against wound pathogens) of extracts of the three traditional medicinal plants.

1.3 Objectives of the study

1. To prepare crude extracts from the leaves of *Z. mucronata* and aerial parts of *H. ciliatum* and *G. polycephala*
2. To assess anticancer and antioxidant activities of the extracts *Z. mucronata*, *H. ciliatum* and *G. polycephala*
3. To assess antimicrobial activity of extracts of *Z. mucronata*, *H. ciliatum* and *G. polycephala* against wound pathogens

1.4 The hypotheses

1. H_o: The method of extraction has no effect on the amount of crude extract extracted.

   H_a: The method of extraction has an effect on the amount of crude extracts extracted.

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2. H_o: Crude extracts of *Z. mucronata*, *H. ciliatum* and *G. polycephala* do not exhibit anticancer and antioxidant activity.

   H_a: Crude extracts of *Z. mucronata*, *H. ciliatum* and *G. polycephala* exhibit anticancer and antioxidant activity.

3. H_o: Crude extracts of *Z. mucronata*, *H. ciliatum* and *G. polycephala* do not exhibit antimicrobial activity against wound pathogens.

   H_a: Crude extracts of *Z. mucronata*, *H. ciliatum* and *G. polycephala* do not exhibit antimicrobial activity against wound pathogens.

1.5 Relevance and significance of study

Plants are seen as an important source of useful medicinal natural products; as well as therapeutic and pharmaceutical novel compounds needed in the discovery of new drugs. The chances of obtaining pharmacologically active compounds from these traditional medicinal plants are high, thus documentation of these plants is of great importance (Hostettmann *et al*., 1996). The assessment of the antioxidant, anticancer and antimicrobial potential of traditional medicinal plants serve as the baseline for the chemical identification of active molecules which may be used as anticancer, antioxidant or antimicrobial compounds; or lead compounds which could be chemically manipulated into effective anticancer, antioxidant or antimicrobial drugs. These drugs could be used to slow down cancer growth or even cure cancer and manage microbial infections; consequently contributing positively to the economy of the country and the world at large. Obtaining anticancer compounds from plants will be a great move towards reducing the side effects associated with current cancer treatment methods as natural and semi-synthetic products are considered safer than synthetic drugs (Khaleeliah, 2001).

In addition, these plant products could be included in the primary health care, as encouraged by WHO (Shoeb, 2006). Though there are anticancer products of plant origins in clinical testing at the moment, the search for new products is of outmost importance as diseases, including cancer, are constantly developing resistance to existing drugs. Screening plants for other biologically active compounds such as antifungal and antibacterial activities and not just anticancer and antioxidants will also help in the implementation of conservation measures for medicinally useful plants.

CHAPTER 2

2. LITERATURE REVIEW

2.1 Medicinal plants and indigenous knowledge systems

A plant is said to be medicinal if it produces active compounds which are therapeutically effective (Wuyang, 2008; Khaleeliah, 2001). In addition to the use of plants as medicine by the Sumerians and Akkaidians (2600 BC), other ancient literature on the use of plants as medicine include the Egyptian Ebers Papyrus, dated 1500 BC upwards, with records of over 700 drugs and the Chinese Materia Medica dating 1100 BC, recording over 600 medicinal plants. The Indian Ayurvendic system dating 1000 BC and Greek about 100 BC are also other records on the ancient use of plants as medicine (Shoeb 2006).

Traditionally, medicinal plants were used in the treatment of various diseases. Plant parts such as leaves, stems, roots, barks, twigs, tubers, bulbs, exudates, flowers and fruits were all used in the treatment of different ailments. These plant materials are used to prepare enemas, extracts, infusions, teas, snuffs and in many other forms which are administered in different ways (van Wyk and Gericke, 2000). Enemas are oily or aqueous suspensions introduced rectally. Extracts are preparations containing active principles of a crude drug, prepared by extracting the plant material with a suitable solvent such as water or alcohol. Infusions on the other hand are prepared by soaking of the plant material. Teas are prepared by soaking the plant material in hot water for a few minutes. Snuff constitutes finely powdered medicinal plant material which can be inhaled through the nostrils (van Wyk, van Oudtshoorn and Gericke, 1997).

Epilepsy, malaria, dysentery, pneumonia, inflammations, ulcers, wounds, cancer and sexually transmitted diseases among others, are some of the conditions and diseases reported to have been treated traditionally with plants (von Koenen, 2001; Khaleeliah, 2001; van Wyk and Gericke, 2000). The use of traditional medicine in primary health

care is commonplace, especially in developing countries (Shoeb, 2006; Chinsembu and Hedimbi, 2010). Mdlolo (2009) estimated that up to 80% of the population in most developing countries may be using traditional medicine in primary health care. Developed countries have also developed interest in the use of plants as medicine due to their reduced toxicity, availability and affordability compared to manufactured drugs (Khaleeliah, 2001).

2.2 The use of traditional medicines in Namibia

Namibia is among countries which up to date has a strong dependency on traditional medicine, especially in rural areas where modern clinics are situated several kilometres away from the villages. This is true for most regions in northern and north-eastern parts of Namibia such as the Oshikoto, Kavango and Caprivi region, though the use of traditional medicine is also common in central regions. In these regions, traditional medicine is used in the treatment of ailments such as headaches, pneumonia, inflammation, mental illnesses, sexually transmitted diseases and management of HIV/AIDS related illnesses (Cheikhyoussef, Shapi, Matengu and Ashekele, 2011; Chinsembu and Hedimbi, 2010). Marshall (1998 cited by Cheikhyoussef *et al*., 2011) reported that about 53 plant species are in demand in Namibia's capital city Windhoek for medicinal trade. Most of these plants are indigenous to Namibia and comes from the northern and north-eastern part of the country, with some others being imported from neighbouring countries. The use of these medicinal plants is often associated with consultations with traditional healers, though in case of minor ailments such as coughs and fever, an ordinal member of the community may help with the identification and administration of the medicinal plant products. Currently, there are about 2500 traditional medical practitioners in Namibia registered with the National Eagle Traditional Healers Association (NETHA), though the actual number of traditional medical practitioners may be higher. Some of these traditional medical practitioners

originated from other southern African countries such as Malawi, DRC, Tanzania, Zambia and Zimbabwe (Cheikhyoussef *et al*., 2011).

2.3 Natural products

Natural products can either be primary or secondary plant or animal metabolites. Primary metabolites unlike secondary metabolites are essential for biochemical pathways necessary for the normal growth and development of plants or animals (Martin, 1995). Secondary metabolites are derived from primary metabolites through biosynthetic processes, and in most cases, they are restricted to certain taxonomic groups. Secondary metabolites have no role in the normal growth of the plant but, may play important ecological roles depending on the conditions under which they were produced, though some may be just mere waste products from physiological processes. Ecological roles played by plant secondary metabolites include attracting pollinators, acting as chemical defence against disease-causing microorganisms and insects, as well as facilitating survival under environmental stresses (Balandrin *et al*., 1985). In addition, secondary metabolites are responsible for the characteristic smells, colours, flavours and medicinal properties of plants (Martin, 1995).

Generally, primary metabolites are produced in large amounts compared to secondary metabolites. Due to this, primary plant products constitute a large portion of raw materials in scientific, technological and commercial applications. An example of a scientific application of a metabolite is the study of the effects of a metabolite on the growth of organisms. Commercial applications on the other hand include the use of plant extracts as flavourants, fragrances, and pesticides as well as in pharmaceuticals (Balandrin *et al*., 1985). Because of their various applications, natural products have the

potential to alleviate most global crises such as malnutrition, poverty and disease outbreaks (Potier *et al*., 1996).

2.4 Medicinal plants natural products

As mentioned above, a plant is considered medicinal if it produces active compounds which are therapeutically effective. Plants produce a wide range of secondary metabolites. The medicinal properties of plants are attributed to the presence of secondary metabolites such as terpenoids, steroids, saponins, tannins, flavonoids, alkaloids and phenolic compounds (Mdlolo, 2009; Fawole, 2009). These metabolites are often restricted to certain taxonomic groups such as family, genus, species or subspecies. Adelaja, Ayoola, Otulana, Akinola, Olayiwola and Ejiwunmi (2008) isolated alkaloids from *Heliotropium indicum*, which when tested possessed anti-inflammatory, antiseptic as well as antimicrobial activities among others. Alkaloids, sterols, triterpenoids, saponins and tannins have been isolated from leaves and roots of *Ziziphus* species (Neuwinger, 1996; van Wyk *et al*., 1997).

During the last two decades, more than 50% of new drugs introduced to the market for the use against different ailments were of natural origin (Wuyang, 2008). These drugs are important as they are believed to have the potential to prevent and cure diseases. This has intensified the interest in the study of biological effects, isolation and structure determination of natural compounds. Though studies of this nature were initially complicated, they are made much easier by the establishment of new and comprehensive screening methods (Sarkar *et al*., 1996).

2.5 Cancer

Cancer is one of the devastating diseases in the world and in southern Africa in particular. According to Parkin *et al*. (2005) (cited in Shoeb, 2006), about 6.7 million cancer deaths were reported worldwide in 2002 and although the population-based cancer registry is still not yet fully established in most developing countries, especially in southern Africa, cancer is considered to be the leading cause of death worldwide (Carrara *et al*., 2009; Wiredu and Armah, 2006). The World Health Organisation estimates that about 10% of deaths in developing countries are caused by cancer related illnesses and about 10 million new cancer cases are reported every year (Carrara *et al*., 2009). In Ghana alone, more than 2500 cancer deaths were reported between the year 1991 and 2000 (Wiredu and Armah, 2006).

In Namibia, the annual cancer cases increased from 1 to 402 cases between the years 1969 and 1994 to over 1000 cases between 1995 and 1998 (Carrara *et al*., 2009). During the latter period, 5,144 cases were reported, showing an average of 1028.8 cases per annum. Malignant neoplasm had the highest prevalence, accounting for 4,949 cases of 4969 cancer cases reported in Namibia between the year 2000 and 2005 (Carrara *et al*., 2009).

2.6 Natural products and Cancer

The discovery of Penicillin, an antibiotic produced by fungi of the *Penicillin* species, during the World War II opened the search of natural products as potential drug candidates. Plants were among the sources of natural products targeted in the search for potential natural drug candidates. This led to the discovery that plants are not only a source of potential drugs, but rather an important tool for therapeutics, as they are the source of most novel compounds used by pharmaceutical industries today (Ginsberg,

2005). It is estimated that more than 80% of pharmaceutical and therapeutic lead compounds are derived from natural products (Potier *et al*., 1996). The screening of about 35000 plant extracts for anticancer activity by the NCI in the 1960s resulted in the isolation of approximately 2619 compounds (Sarkar *et al*., 1996). Consequently, a number of clinically active anticancer drugs were discovered. Among those drugs was taxol, camptothecin, vinblastine, vincristine and some natural products derived drugs such as taxotere and navelbine (Balandrin, Kinghorn and Farnsworth, 1993; Potier *et al*., 1996). Cyclosporine, a drug used to prevent rejection of transplanted organs, FK506 and rapamycin, the two commonly used immune-modulating drugs are also of plant origin (Potier *et al*., 1996).

2.7 Oxidants/ Free radicals

Free radicals sometimes referred to as oxidants, are chemical species containing unpaired electrons. These chemical species are abundant in nature, and they play a significant role in chemical reactions. Reactions such as combustion and food spoilage are all known to proceed through free radical reactions (Prasad, Hao, Yi, Zhang, Qui, Jiang, *et al*. 2009; Min and Boff, 2002). Radical reactions are also important in biology and medicine. Molecular oxygen (O_2), a molecule needed for all aerobic reactions in the body is diradical. Another radical that play an important role in survival is nitric oxide also known as nitrogen monoxide (NO). Although often seen as a potentially toxic gas, NO plays an important role in signalling pathways. NO acts as a chemical messenger in living system and it is involved in the regulation of blood pressure and blood clotting, neurotransmission, and the immune response against tumour cells. Superoxide radical is also another radical involved in the immune response against pathogens (Solomons and Fryhle, 2008).

Free radicals are produced in the body during food breakdown processes. It can also be acquired from the environment, such as from cigarette smoke, industries, car exhausts or other polluted environments. Two processes which produce free radicals *in vivo* have been identified, namely Fenton and Haber-Weiss reaction (see **figure 2.1**).

$$
\begin{array}{llllll}
Fe^{3+}/Cu^{2+} & + & O_2 & \rightarrow & Fe^{2+}/Cu^+ & + & O_2 & & & (1) \\
Fe^{2+}/Cu^+ & + & H_2O_2 & \rightarrow & Fe^{3+}/Cu^{2+} & + & OH & + & OH & (2) \\
O_2 & + & H_2O_2 & \rightarrow & O_2 & + & OH & + & OH & (3)
\end{array}
$$
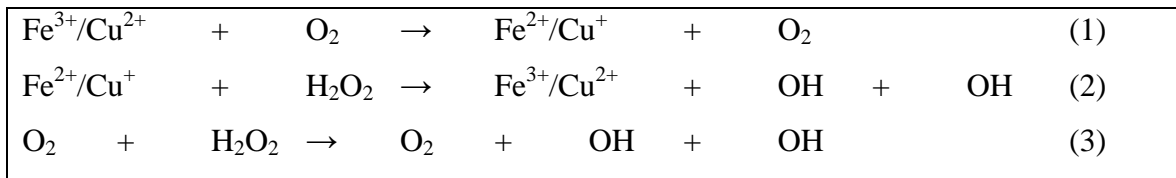
Figure 2.1: Processes which produce free radicals *in vivo*, Fenton reaction (2) and Haber-Weiss reaction (3). Reaction (1) is a normal metal reduction reaction (Naidoo, 2005).

It is estimated that up to 5% of O_2 inhaled daily may be converted to free radicals, often referred to as reactive oxygen species (ROS). ROS are generated endogenously from cellular metabolism and inflammatory responses or by exposure to exogenous agents such as ionising radiation and xenobiotics (Bellion, Digles, Will, Dietrich, Baum, Eisenbrand and Janzowski, 2010). Examples of ROS are hydroxyl, peroxyl, singlet oxygen and superoxide radicals (Prasad *et al*., 2009, Bellion *et al*., 2010). In the body, these radicals are neutralised to harmless forms through enzymatic actions. Some enzymatic systems known to play a role in the radical neutralization include glutathione S-transferase, glutathione peroxidase, superoxide dismutase and catalase system (Lee, Cha, Kim, Park, Park, and Lee, 2007). The enzyme superoxide dismutase for example, oxidises superoxide to hydrogen peroxide and oxygen. Another enzyme, a catalase, converts the potentially harmful hydrogen peroxide to water and oxygen (Solomons and Fryhle, 2008). This way, the free radicals cannot cause damage to cells.

However, if free radicals accumulate in the body to a level beyond the one that is controllable by antioxidants systems, they take part in oxidation reactions, that can be harmful to the body, hence the name oxidants. Oxidation processes are intrinsic in the energy management of all living organisms and are therefore kept under strict control by several mechanisms (Solomon and Fryhle, 2008). However, the excessive production of free radicals and the unbalanced mechanisms of antioxidant protection result in cellular oxidative stress, which causes cell damage, often termed oxidative cell damage. This damage is cumulative and may be the trigger for diseases like inflammation, diabetes, arteriosclerosis, cancer, Parkinson's diseases, multiple sclerosis and lupus (Prasad *et al*., 2009; Min and Boff, 2002; Naidoo, 2005; Lee *et al*., 2007). Though the actual mode of progression is not known, the accumulated free radicals are believed to contribute to the initiation of these progressive and chronic diseases through binding to cellular structures such as DNA, RNA, protein and cell membrane. This leads to lipid oxidation and DNA and protein damage which in the end results in the onset of these various diseases (Reynertson, Basile, and Kennelly, 2005; Goze, Alim, Tepe, Sokmen, Sevgi, and Tepe, 2009).

2.7.1 Oxidants as potential carcinogens

Oxidants are potential carcinogens. The binding of an oxidant to cellular structures (mentioned in **section 2.7**) may cause structural damage to DNA or mutate cancer-related genes. Oxidative damage is also believed to be the causative agent for cancer development by initiating chemical reactions leading to DNA mutations. Prompted by increased oxidative stress, DNA oxidation damage occurs through reaction with ROS leading to mispairing of DNA bases or breaking of DNA strands (Bellion *et al*., 2010). At the same time, oxidants activate signal transduction pathways and alter the expression of growth and differentiation-related genes. Indeed, the carcinogenic action of oxidants

results from the superposition of these genetic and epigenetic effects (Naidoo, 2005; Lee *et al*., 2007).

2.7.2 The role of antioxidants in cancer prevention

Antioxidants are substances that help protect the body from oxidative damage caused by free radicals. Antioxidants are a very important part of our diets as they help prevent oxidative cell damage by acting as free radical scavengers and thereby preventing oxidation and repairing the damage caused by free radicals (Tsang, 2009). Laboratory studies involving chemical and cell cultures, and results obtained from animal studies have shown that antioxidants have the potential to slow down or even prevent the development of cancer. However, results obtained from clinical trials with humans do not support the above finding thus further investigation is required to confirm this (National Cancer Institute, 2004).

2.7.3 Plant natural products as potential anti-carcinogens

It is important that food rich in antioxidants are taken so that the antioxidants will neutralise the free radicals in case of enzymatic mechanisms failure or inadequate efficiency (Goze *et al*., 2009). Coloured fruits, vegetables, fish, red wine and some teas are well known sources of antioxidants. It is estimated that consumption of these products reduces the risk of cancer and heart diseases by up to 25% (Tsang, 2009). Plants are a rich source of antioxidants; that help protect them from ultraviolet damage and against lipid peroxidation, which would otherwise negatively affect the fruits, especially for plants found in tropical regions growing under high temperatures and intense sunlight (Reynertson *et al*., 2005). Polyphenolic compounds such as phenolic acids, flavonoids, anthocyanidins and tannins, produced as secondary metabolites by plants possess remarkable antioxidants and anticancer activities (Prasad *et al*., 2009).

2.8 Medicinal plant families

2.8.1 *Boraginaceae* (Forget-me-not family)

Boraginaceae is a family of flowering plants, consisting of about 148 genera and more than 2700 species (Boraginaceae, 2012). It is mostly found in Europe, Asia and along the Mediterranean region. This family has a common characteristic of corolla colour change during aging, a characteristic brought about by pH change in the cell sap. Some of this family members are used as garden flowers (e.g. *Heliotropium*, *Martensia*, *Myosotis* and *Pulmonaria*), some are medicinal (e.g. *Borago officinalis*, *Symphytum officinalis* and *Lithospermum*) and the majority of this group are poisonous (Boraginaceae, 2012). Pyrrolizidine alkaloids are common in *Boraginaceae* and these alkaloids exhibit pronounced toxic effects on the liver and lungs, whereas cytotoxic, mutagenic and carcinogenic activities have also been reported (Gurib-Fakim, 2006).

The genus *Heliotropium* is part of the *Boraginaceae* family. There are about 20 species of this genus, of which eighteen are known in Namibia. These include *Heliotropium albiflorum*, which is one of the few endemic species to Namibia, and *H. curassavicum*, *H. indicum* and *H. supinum*, species which are alien to Namibia (Kyffhauser, 2011). *Heliotropium ciliatum* (**figure 2.2**) is an erect perennial herb, which is indigenous to Namibia, Botswana, Zimbabwe, Angola, South Africa and Mozambique. It can grow at an altitude of about 1050 m above sea level. This species has appeared in earlier literature with names such as *H. tuberculosum* and *Tournefortia tuberculosa*. *H. ciliatum* grows in sandy or rocky soils along riverbanks and in dry open woodland, attaining the height of up to 80 cm and leaves the length of 2-5 cm long. Its flowering season is between September and February, producing curled elongated inflorescence with flowers about 19 cm long (Hyde, Wursten and Ballings, 2012).

In Namibia, *H. ciliatum* is found in Owamboland area (von Koenen, 2001), where it is locally known as *Ohanauni* (Kwanyama) or *Etadido* (Ndonga). The extracts of boiled roots and /or aerial parts are used as a steam bath or as a decoction to treat oedema resulting from a weak heart. The decoction can also be administered rectally to treat painful legs. According to C. Nghidipo (personal communication, June 25, 2009), the powder of the dried aerial parts can be mixed with butter or any other oil and be applied to wounds of *Ondhiya*. Among the Ovambo tribe, the aqueous extract of *H. supinum* is used to treat all kind of tumours (von Koenen, 2001).

Figure 2.2: *Heliotropium ciliatum* growing in the field.

2.8.2 *Rhamnaceae* (Buckthorn family)

Ziziphus mucronata is a dense leafy small to medium-size tree, which is 2-5 m tall (see **figure 2.3**). This plant belongs to family *Rhamnaceae* and genus *Ziziphus*. *Z. mucronata* is found in tropical area and along moist riverbanks. This plant is widely distributed in southern Africa. In Namibia, *Z. mucronata* is widespread throughout Namibia, except in the Namib Desert, north-west of Etosha and a few areas in the southeast (Curtis and

Mannheimer, 2006; Von Koenen, 2001). Van Wyk and colleagues (1997) recorded *Z. mucronata* to be the most widely distributed tree in South Africa. This plant flowers between March and June producing small, round, red-brown berries with thin dry flesh which appears all year round but is mostly common between December and June (Curtis and Mannheimer, 2006; Van Wyk and Gericke, 2000). Local names for *Z. mucronata* includes buffalo thorn (English), *Omukaru* (Herero), *≠aros* (Damara/Nama), *omukekete* (Kwanyama), *mukalu* (Silozi) and *omusheshete* (Ndonga) (Curtis and Mannheimer, 2006; von Koenen, 2001).

The leaves and fruits of *Z. mucronata* are a source of food for livestock, especially goats. The fruits are also edible to humans. In Ovamboland, the dried fruits are fermented and distilled to make alcoholic liquor called *ombike* (Ndonga), which is sold by the locals, serving as a source of income. The woods of *Z. mucronata* are a fuel source in rural areas.

Buffalo thorn is widely known as a medicinal plant. Its leaves and roots have been used to treat diarrhoea, tumour, cough, chest complaints, dysentery, sores, glandular swellings, skin diseases, open and swollen wounds, ear inflammation, asthma, syphilis, gonorrhoea, lumbago, measles, as well as rheumatic pains and fever (von Koenen, 2001; van Wyk *et al*., 1997; Neuwinger, 1996). Other reports include the use of the roots by the Bambara and Malinke tribes in Tanzania for the psychiatric treatment, and the use of the leaf juice to prevent abortion (Neuwinger, 1996). Several other *Ziziphus* species are known for their medicinal properties. Strong sedative effects have been reported from *Z. vulgaris* and *Z. jujube* (van Wyk *et al*., 1997). *Z. mauritiana* and *Z. abyssinica* have a range of medicinal uses which include wounds, fever, abdominal pains, venereal diseases and diarrhoea treatment; diuretic effects has also been reported for the two plants, just to mention a few (Neuwinger, 1996). A number of active ingredients have been isolated from leaves, roots, stems and seeds of *Ziziphus* species. Among them are

alkaloids, sterols, triterpenoids, saponins and tannins (Neuwinger, 1996; van Wyk *et al.*, 1997).

Figure 2.3: *Ziziphus mucronata* tree about 3 m tall

2.8.3 *Thymelaeaceae* (Daphne family)

The genus *Gnidia* is part of the *Thymelaeaceae* family. This genus consists mainly of perennial herbs, commonly found along wet riverbanks. *Gnidia* species are well known for their poisons. *G. kraussiana* and *G. latifola* are used as hunting poisons for wild animals and fish (Neuwinger, 1996). In northern Cameroon and Nigeria, the hunting poisonous is prepared by boiling the roots of *G. kraussiana* with the seeds of *Strophanthus hispidus*. The powder of the roots or young leaves, are reportedly used as a human poison in some African countries such as Nigeria, Kenya and Tanzania (Neuwinger, 1996). *Gnidia* species are a big concern to farmers. A common example is

the cattle and sheep-poisoning outbreak in South Africa caused by consumption of *G. kraussiana* and *G. burchellii* (Coetsee and van der Westhuizen, 2007).

According to Neuwinger (1996), poisonous plants are a very important source of medicine in Africa. In agreement with this statement, a number of medicinal applications of *Gnidia* species have been reported. *G. kraussiana* is used in many African countries to treat burns, snakebites, stomach complaints, measles, cough, easing childbirth and as a pain reliever (Neuwinger, 1996; van Wyk *et al*., 1997). Chemical studies done on some *Gnidia* species indicated the presence of diterpene esters and coumarins, and since these compounds could be limited to a particular taxonomic group, family, genus or species (Balandrin *et al*., 1985); these could be some of the compounds we could expect to be isolate from this particular species, *Gnidia polycephala* (Neuwinger, 1996). *G. polycephala* is shown in the **figure 2.4** below.

Figure 2.4: *Gnidia polycephala* plant growing along a plain bed

2.9 Antioxidants and anticancer assays in natural products studies

2.9.1 Antioxidants assay

Tests for antioxidant activity are performed both *in vivo* and *in vitro*. Both methods give reliable data, and the choice of the method depends on the resources and time available for the study. Kandasamy, Yeligar, Maiti and Maity (2005) tested the antioxidant activity of some plant extracts *in vivo* using Swiss mice. Assays performed *in vivo* included lipid peroxidation, reduced glutathione, superoxide dismutase and catalase assay. Most studies however tests for the antioxidant activity through *in vitro* procedures. Some of the common *in vitro* antioxidant assays are the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay, superoxide anion radical scavenging activity, reducing power, ferric thiocyanate assay and total antioxidant activity (Goze *et al*., 2009; Saha, Hasana, Aktera, Hossaina, Alamb, Alam and Mazumderc, 2008; Prasad *et al*., 2009; Li, Wang, Yu, Fang, Xin, Yang *et al*., 2008).

Among the above mentioned *in vitro* assays, the DPPH activity assay is the widely used assay (Goze *et al*., 2009; Saha *et al*., 2008; Prasad *et al*., 2009; Li *et al*., 2008). DPPH is a radical, which in the presence of antioxidants, is reduced from a stable free radical, 1,1-diphenyl-2-picryl hydrazyl (purple) to a diphenyl picryl hydrazine (yellow). Reaction of DPPH with hydroxyl group (R-OH) and $R-NO_2$ produces 2-(4-hydroxylphenyl)-2-phenyl-1-picryl hydrazine and 2-(4-nitrophenyl)-2-phenyl-1-picryl hydrazine, respectively (Naidoo, 2005). This concept is illustrated in **figure 2.5**. The amount of DPPH reduced is equivalent to the amount of antioxidants present in the study sample, thus the results obtained gives a true reflection of the extract's ability as an antioxidant (Goze *et al*., 2009; Prasad *et al*., 2009).

1,1-diphenyl-2-picryl hydrazyl

Diphenyl picryl hydrazine

* = OH or NO$_2$
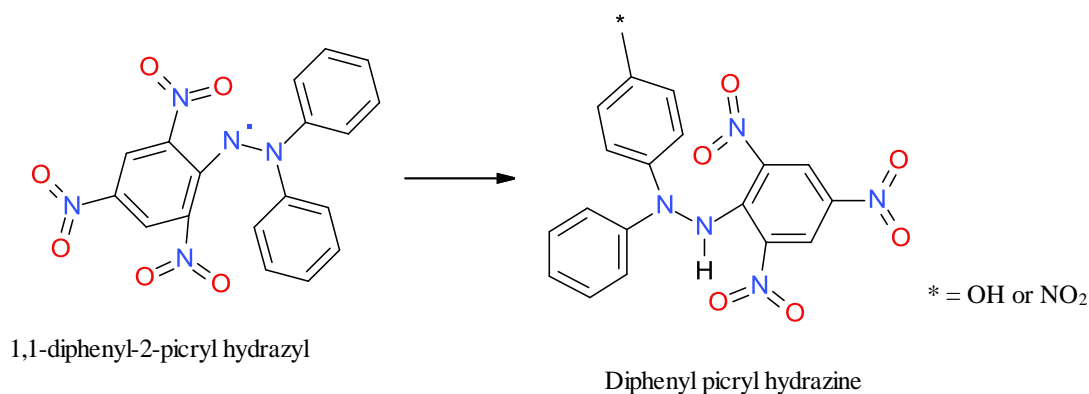
Figure 2.5: In the reaction of DPPH with antioxidants, the purple DPPH radical is converted to a yellow hydrazine (Naidoo, 2005).

2.9.2 Anticancer bioassay

A compound is considered to have anticancer activity when it shows some inhibitory activities against cancer cell growth. The mechanisms of inhibition are not fully understood, but they are speculated to involve microtubules- filaments formed during cell division (Schiff and Horwitz, 1980). Anticancer activity bioassay can be performed both *in vitro* and *in vivo*. *In vitro* anticancer activity bioassay is performed on a laboratory bench and it is therefore much quicker. The effect of the extract or a particular compound against cancer cells is studied by adding a known amount of the extract to laboratory grown cancer cells. The effectiveness of the extract is equivalent to the inhibiting effects of the extract on the growth of the cancer cells. *In vivo* anticancer activity is done in living systems. One such assay involves the use of albino mice. The albino mice are infected with cancer cells and then treated with the extract or compound under study to study its effects on the growth of the cancer cells (Kandasamy *et al*., 2005).

Often, due to limited resources and expertise, *in vitro* assays are opted for. Some of the commonly used *in vitro* anticancer assays are MTT assay, H_2O_2 and NO assay. The cells of choice are first cultured and are then used for the experiments when they are in an exponential growth stage, which is attained between 1-4 days depending on the growth rate of the cells (Lee *et al*., 2007; Li *et al*., 2008; Prasad *et al*, 2009).

2.9.2.1 Cell culture

Culturing cells in laboratory serves as a mean of multiplying cells to a reasonable number of cells to be able to conduct the experiments safely (Li *et al*., 2008; Uddin, Grice and Tiralongo, 2011). Cells need to be provided with favourable conditions for them to proliferate. Among these conditions are nutrients, CO_2 and optimum temperature. Examples of growth media used in cell culture are: Advanced Dulbecco's modified Eagle's medium (DMEM) and Roswell Park Memorial Institute 1640 medium (RPMI 1640). The media is supplemented with serum, antibiotics and amino acid. Foetal bovine serum (FBS) and new-born calf serum (NBCS) are used. Penicillin and/or streptomycin are added as antibiotic supplements depending on the growth requirements for the cells. L-glutamine is often added as amino acid supplement (Li *et al*., 2008; Noguira, Leao, Vieira, Benfica, da Cunha, and Valadares, 2008; Uddin *et al*., 2011; Prasad *et al*., 2009).

The growth medium is colourless and an indicator is added to aid monitoring cell growth and cell activity during the culturing process. The waste products of the utilisation of the nutrients by the cells alter the pH of the media, which is observed through media colour change (Haynes, Worthington, Morris, and Newell, 1997). Phenol red indicator is added to DMEM giving it a red colour which turns orange and then yellow as nutrients get depleted. The media need to be replaced as soon as it changes colour to keep cells in the

exponential growth phase. In a culture vessel, the cells form a monolayer which is attached to the vessel surface. These adherent cell lines continue to grow until they have covered the surface available for growth or they have depleted the nutrients from the surrounding medium. At this point, the cells must be cultured into new vessels to avoid intoxication which will eventually result in rapid cell death (Morris, Griffiths, Warburton, West, Al-Rubeai, Clarke, *et al*., 1997).

To harvest cells for sub-culturing or for use in experiments, the cell monolayer is disrupted to release the cells into the suspension. A protease, trypsin is used to detach the cells from the vessel. Trypsin is a pancreatic serine protease. In laboratories, purified trypsin is used for this purpose. However, crude trypsin (mixture of proteases, polysaccharides, nuclease and lipases) is also available commercially. Trypsin, like other proteolytic enzymes, also undergoes autolysis, and for this reason it is recommended that it be dissolved immediately just before use. To enhance tissue disaggregation, ethylenediamine tetraacetic acid (EDTA, 0.02% w/v) in calcium and magnesium free phosphate buffered saline can be used in conjunction with trypsin. EDTA acts as a chelating agent, which dissociates intercellular links (Haynes *et al*., 1997; Morris, 1997; Morris *et al*., 1997).

Despite the use of trypsin and the chelating agent, EDTA, problems such as slow detachment and cell aggregation may occur with some cell lines. Morris (1997) listed two contributing factors:

(i) Trypsin in solution gradually loses activity above 4 ^{o}C and repeated warming and cooling also increases the loss of enzyme activity

(ii) Cell aggregation or clumping is due to surface membrane adhesion of carbohydrate components produced during protease activity.

Cells in the exponential growth phase are used in experiments. An important procedure in ensuring that the cells are in an exponential growth phase is examining cell viability. Trypan blue is used for this procedure to test for non-viability of cells. This dye crosses membranes of dead, nonviable cells, staining them blue. In trypan blue, live healthy cells appears round, refractile and relatively small in comparison with dead cells which appear larger and non-refractile (Morris, 1997).

2.9.2.2 MTT assay

2.9.2.2.1 MTT test for anticancer screening

Anticancer assays are based on measuring the number of intact cell survival after treatment with a drug of interest. Three common methods namely the 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), trypan blue staining and radioactive method have been used in this area. Trypan blue staining is a simple way to evaluate cell membrane integrity and thus assume cell proliferation or death. The method is not very sensitive and cannot be adapted for high-throughput screening. On the other hand, measuring the uptake of a radioactive substance usually tritium-labelled thymidine is accurate but it is time consuming and involves handling of radioactive substances (Wallert and Provost Lab, 2007).

The MTT test is a widely accepted, rapid, reliable and sensitive method which is not only used to screen for anticancer compounds but has also found application in medicine such as the in selection of effective chemotherapy in patients with acute leukaemia (Prasad *et al*., 2009; Hongo, Fujii and Igarashi, 1990). The MTT method is also useful in studying cell growth in response to mutagens, antigenic stimuli, growth factors and other cell growth promoting reagents, cytotoxicity studies and in derivation of cell growth curves. This colorimetric assay measures the reduction of yellow MTT by mitochondrial

succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, dark-purple formazan. The mitochondrial dehydrogenase of viable cells cleaves the tetrazolium ring yielding purple MTT formazan crystals that are insoluble in aqueous solutions. The cells are then solubilised with an organic solvent (e.g. isopropanol) and the solubilised formazan reagent is measured spectrophotometrically at a wavelength between 500 and 600 nm. Since mitochondrial reductase enzymes are only active in metabolically active cells, the reduction of MTT is directly related to the number of viable (living) cells (Wallert and Provost lab, 2007; Al-Rubeai, 1997). The reduction of a tetrazolium salt to a formazan is illustrated in **figure 2.6**.
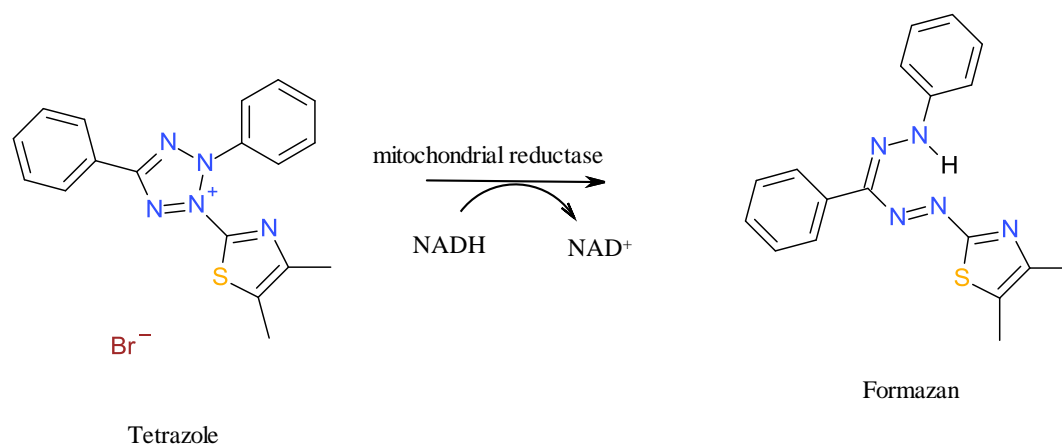
Figure 2.6: The reduction of MTT tetrazole (yellow) to a formazan (purple) by mitochondrial reductace (Wallert and Provost Lab, 2007).

2.9.2.2.2 Significance of the MTT test

The MTT assay is based on the ability of mitochondrial reductase to cleave the MTT formazan dye (yellow) to a tetrazole (purple). Since this enzyme is only active in viable cells, the MTT assay is often used as a measure of viable (living) cells. However, it is

important to keep in mind that other viability tests (such as the CASY cell counting technology) sometimes give completely different results, as many different conditions can affect the level of cell metabolic activity. Changes in metabolic activity can significantly affect the results of this assay even if the number of viable cells remains constant. When the amount of purple formazan produced by cells treated with an agent of interest is compared with the amount of formazan produced by untreated control cells, the effectiveness of the agent in causing cell death, or altering metabolism of cells can be deduced through the production of a dose-response curve. However, care must be taken when interpreting these results due to the limitation of the MTT method such as change in the physiological state of the cells or the variances in mitochondrial dehydrogenase activity in different cell types (Wallert and Provost Lab, 2007).

2.9.2.3 The release of Nitric oxide (NO)

2.9.2.3.1 Inorganic Chemistry of NO

NO is a relatively stable radical, which reacts readily with O_2, superoxide radical or H_2O_2 yielding NO_2, peroxynitrite and NO_2/NO_3 respectively. NO is soluble in water, thus it has a high partition coefficient and for this reason it tends to exist as a gas. NO has great potential as a biological messenger, because of its small size which facilitate cell entry and once within the cell it avidly binds to transition metals such as Fe, Cu, Co and Mn, which are crucial to the functioning of many cytochromes and oxidases. NO can act as an oxidant (NO to N_2O) or a reducing agent (NO^{2-} to NO), depending on the redox environment in which it exists (Archer, 1993).

NO acquired from the environment such as from area polluted with incomplete combustion of fuels, can be potentially toxic. NO is however also produced in the body. In mammalian cells such as microphages, endothelial cells, neutrophils and in the

cerebellum, NO is synthesized via the enzyme NO synthase with the basic amino acid L-arginine acting as substrate and molecular oxygen (O_2) as co-substrate. This process can be inhibited by the N-substituted L-arginine analogues such as N^6-monomethyl L-arginine (Archer, 1993).

Although endogenous NO is also a potentially harmful gas, its action in biological fluid is minimised by the rapid deactivation of this gas by oxidation to nitrite (NO_2^-) and nitrate (NO_3^-) by physically dissolved oxygen and water. Moreover, it has become apparent in recent years that NO plays a role in a number of biologically important functions. It was established that NO acts as an intercellular and intracellular messenger substance with a broad physiological action spectrum. In addition to being a potent endogenous vasodilator, NO has a role in inflammation, thrombosis, immunity and neurotransmission (Solomons and Fryhle, 2008; Molecular probes, 2003; Archer, 1993).

2.9.2.3.2 The significance of NO assay

The NO assay is based on the photometric determination of NO via its oxidation products, nitrite and nitrate (**figure 2.7**). This test uses the Griess diazotization reaction to spectrophotometrically detect nitrile formed by the spontaneous oxidation of NO under physiological conditions (Molecular probes, 2003). In the presence of the enzyme nitrate reductase (NR), the nitrate present in the sample is reduced to nitrite by reduced nicotinamide adenine dinucleotide phosphate (NADPH). The nitrite formed reacts with sulphanilamide and N-(1-naphthyl)-ethylenediamine dihydrochloride to give a red-violet diazo dye, whose absorbance is measured spectrophotometrically at 550 nm. The Griess diazotization reaction is illustrated in **figure 2.8**.

$$NO \ + \ O_2^- \ \longrightarrow \ ONO_2^- \xrightarrow{\ H^+\ } NO_3^- \ + \ H^+$$

$$2NO \ + \ O_2 \ \longrightarrow \ N_2O_4 \xrightarrow{\ H_2O\ } NO_2^- \ + \ NO_3^- + 2H^+$$

$$NO \ + \ NO_2^- \ \longrightarrow \ N_2O_3 \xrightarrow{\ H_2O\ } 2NO_2^- \ + 2H^+$$
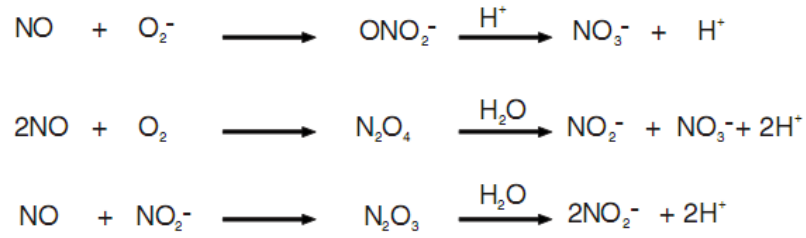
Figure 2.7: The oxidation of NO to nitrite and nitrate
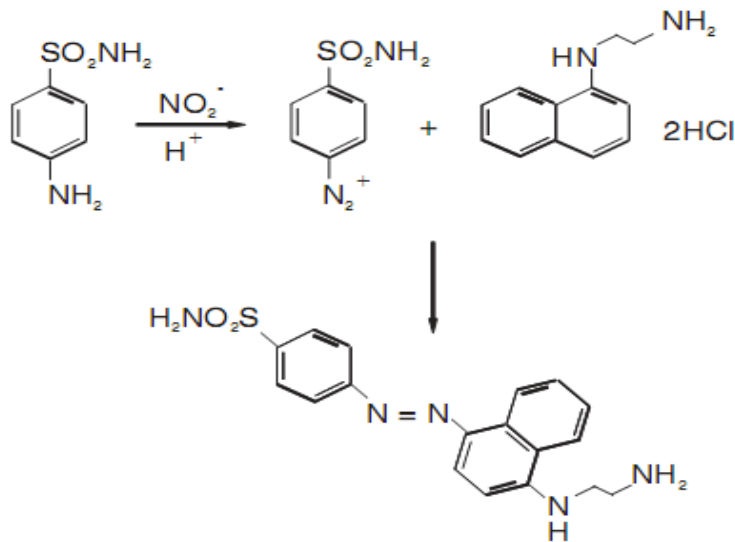
Figure 2.8: The Griess diazotization reaction (Molecular Probe, 2003)

2.9.2.4 Release of Hydrogen peroxide (H₂O₂)

The intracellular metabolic cascade, which produces large amounts of reactive oxygen intermediates in a process known as the respiratory burst, is a characteristic feature of vertebrate phagocytes (Babior, 1984). Hydrogen peroxide is a reactive oxygen metabolic

by-product that serves as a key regulator for a number of oxidative stress-related states. **Figure 2.9** below illustrates the production of H_2O_2.

$$O_2 \xrightarrow{\text{NADPH} \quad \text{NADP}^+ \quad (1)} O_2^- \xrightarrow{\text{H}^+ \quad (2)} H_2O_2 + O_2$$
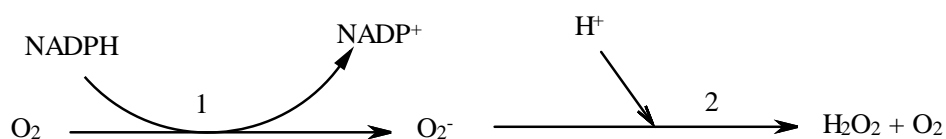
Figure 2.9: The production of H_2O_2 is facilitated by NADPH oxidase (1) and Superoxide dismutase (2) (Ortuno *et al*., 2000)

Peroxidases catalyse the decomposition of H_2O_2 coupled to the oxidation of a variety of organic and inorganic substrates. One of the most stable, highly active enzyme and used is the horseradish peroxidase. It allows activity measurements by colorimetric and fluorometric methods. Among the numerous colorimetric assays available, one of the most sensitive uses the colourless benzidine-based substrate 3,3',5,5'-tetramethylbenzidine (TMB). TMB is oxidized during the enzymatic degradation of H_2O_2 by peroxidase. A blue charge-transfer complex is formed instantaneously, followed by final oxidation to a yellow stable product. For the detection of oxidized TMB, it is necessary to determine the OD of the yellow colour at 450 nm.

2.9.2.5 APOPercentage™ assay

APOP*ercentage*™ assay is a detection and measurement system to monitor the occurrence of apoptosis in mammalian cells during *in vitro* culture. Apoptosis also known as programmed cell death, is defined as a process characterised by cell shrinkage, membrane blebbing and nuclear condensation. Apoptosis can be seen as a physiological mode of cell death that affects many aspects of natural life from embryonic development

through cellular homeostasis and disease. It allow multicellular organisms to smoothly get rid of old, superfluous, damaged or infected cells in a process which is clearly distinct from necrosis, the result of accidental cell death. The induction of apoptosis is one approach used in cancer therapy (Chinkwo, 2005; Bicolour, 2008).

Mammalian cell membrane is composed of a phospholipid bilayer, which is asymmetric in composition, structure and function. The outer monolayer is composed of choline containing phospholipids (phosphatidylcholine and sphingomyelin) which are in contact with the extracellular matrix (*in vivo*) or with cell culture medium (*in vitro*). The inner monolayer is composed of phosphatidyl ethanolamine and phosphatidylserine which is in contact with the cellular cytoplasm. The non-polar, hydrophobic fatty acid tails of the phospholipids of both layers make up the interior volume of the membrane, making the typical bilayer structure. This bilayer is partially permeable to molecules and it is thus essential in maintaining viable cells. The normal functioning of this transmembrane is regulated by flippases, which catalyse the active transport of amino-phospholipids from the outer to inner monolayer. In cells undergoing apoptosis, the activity of the flippase is overwhelmed by the action of another enzyme, floppase or scramblase. This result in the scrambling of the phospholipid distribution between the inner and outer monolayer, action referred to as the flip-flop mechanism. The exposure of phosphatidylserine to the exterior surface of the membrane has been linked to the onset of the execution phase of apoptosis. The flip-flop action results in the uptake of the APOP*ercentage* dye by the apoptotic committed cells. The dye entry does not take place until the flip-flop mechanism has occurred. The dye enters the cell following this event and dye uptake continues until blebbing occurs. This is a one way entry and the trapped dye is not released (Bicolour, 2008).

To study the apoptotic potential of a drug of interest, cells treated with this drug are analysed using a Fluorescence Activated Cell Sorter (FACS). The FACS is designed for cell counting and cell sorting. This equipment mechanically separates cells into different regions based on their cell surface and cell density and presents the data in a form of a

histogram. The histogram has a scale of four quadrants. Normal cells remains in the first quadrant (10^1) and the percentage indicates the number of normal cells in the population. The second quadrant (10^2) and third (10^3) indicates cells death and percentage indicates the number of cells that died naturally within the population. Cells scattered into the fourth quadrant indicate cell death due to mechanical injury, necrosis (Bicolour, 2008; Chinkwo, 2005).

2.9.3 Toxicity evaluation

Bioactive compounds are almost always toxic at high doses, and pharmacology is simply toxicology at lower doses or vice versa. In efforts to screen for cytotoxic compounds, *Artemia salina* eggs or their larvae (nauplii) have been used in a test commonly known as the Brine shrimp lethality test (BST) (Meyer, Ferrigni, Jacobsen, Nichols and McLaughlin, 1982). The BST assay is an efficient, rapid and inexpensive test which is widely accepted as a convenient probe for preliminary assessment of toxicity, detection of toxins, heavy metals, pesticide and its results can be extrapolated for cell toxicity and anticancer activity (Manilal, Sujith, Kiran, Selvin and Shakir, 2009; Maridas, 2008, Rahman, Arslan, Saha, Talukder, Khaleque and Ali, 2006; Mutha, Shimpi and Jadhav, 2010). This cytotoxic assay is used as an indicator for a wide range of pharmacological activities such as anticancer, antiviral, insecticidal, pesticidal activities, just to mention a few (Mutha *et al*., 2010; Rahman *et al*., 2006). BST is a proposed simple, rapid and convenient general bioassay for active plant constituents due to the following reasons:

(a) It is a selective method for cytotoxicity, various pharmacological actions and pesticidal effects,

(b) It gives the IC_{50} (concentration at which 50% of growth of the nauplii is inhibited) of the tested sample and,

(c) It is a useful technique in bioactive-guided isolation of natural products.

2.10. Recent developments in natural products and antimicrobial activity

Microbial infections are one of the challenges the medicine world is faced with today. Millions of lives are lost every year owing to infectious diseases, more so due to pathogens developing resistance to existing drugs (Yu, Zhang, Li, Zheng, Guo and Li *et al*., 2010; Nicolaou, Chen, Edmonds and Estrada, 2009; Ncube, Afolayan and Okoh, 2008). Plants and other natural products have been used by different traditions to manage infectious diseases. Because of this, ethno-pharmacologists, botanists, microbiologist and natural product chemists has turned to natural products in search for new drugs which are expected to be renewable, non-petrochemical, eco-friendly and easily obtainable (Yu *et al*., 2010; Mancini, Defant and Guella, 2007; Leeds, Schmitt and Krastel, 2006; Ncube *et al*., 2008).

The use of natural products as a source of modern medicine started with the discovery of Penicillin, an antibiotic that saved the lives of million soldiers during World War II and was later made available for use by civilians (Nicolaou *et al*., 2009). Since then, several natural antimicrobial drugs such as tetracyclines, phenylpropanoids, macrolides and glycopeptides were discovered and brought to clinics. Endophytes in particular has been the target and so far they have been a source of many antimicrobial compounds such as aliphatic compounds, alkaloids, flavonoids, peptides, phenols, quinones, steroids and terpenoids (Yu *et al*., 2010; Ncube *et al*., 2008). Marine natural products have also been a significant source of antimicrobial compounds (Mancini *et al*., 2007).

The development of antimicrobial drugs begin with the identification of bioactive properties, detailed biological assays and dose formulation, followed by clinical studies to establish safety and efficacy and pharmacokinetic profile of the new drug (Ncube *et al*., 2008). Different methods are used in the screening of natural products for biological activity and the results obtained strongly depend on the technique, the method and solvent of extraction as well as the part of the plant used (in case of plant natural

products). This makes it difficult for researchers to compare their findings. For this reason, there is a need to standardise the method to allow for intra- and inter-laboratory result comparison (Ncube *et al*., 2008; Yu *et al*., 2010).

However with natural products studies, although there are standard methods approved by various bodies such as the National Committee for Clinical Laboratory Science (NCCLS), the British Society for the Antimicrobial Chemotherapy (BSAC) and the European Committee for Antimicrobial Susceptibility Testing (EUCAST), these might not be exactly applicable to natural products and modifications have to be made (Ncube *et al*., 2008). One such standard method is the Antimicrobial Susceptibility Test (AST), a technique used in ethno-pharmacology research to determine the efficacy of the novel antimicrobials against microorganisms especially those of medical importance. AST is divided into two: diffusion and dilution methods. Diffusion methods include the agar well diffusion, agar disc diffusion and bio-autography. Dilution methods include the agar dilution and the broth micro/ macro-dilution methods (Ncube *et al*., 2008). These are the methods currently used in screening natural products for antimicrobial activity. Hidayathulla, Chandra and Chandrashekar (2011) evaluated antimicrobial activity of leaf extracts of *Pterospermum diversifolium* using the disk diffusion method. Suliman (2010) evaluated the antimicrobial activity of fifteen traditional medicinal plants using the broth micro dilution and the bio-autography method. Other commercial custom prepared methods like agar screen plate, Epsilometer test and the vitek system can be used, but are not common in use with plant extracts (Ncube *et al*., 2008).

In recent years, a lot of new antimicrobial targets, antimicrobial drugs and screening methods which are simpler, faster and more efficient have been invented (Zhou, Luo, Li, Wu, Huang, Ding *et al*., 2012). New technologies in high-throughput cultivation, genetic approaches to biodiversity and discovery of relatively untrapped sources of natural products are expanding the ability to find novel, potent and highly selective antibacterial structures (Leeds *et al*., 2006). Genomics has identified several highly conserved,

essential bacterial genes most of which have not yet been targeted as means to combat bacteria. Today novel compounds can even be searched for with a particular mechanism of action (Zhou *et al*., 2012; Nicolaou *et al*., 2009). Advances in purification, dereplication and structure elucidation combined with the ability to chemically or biologically derivatise hits, aim to make the timeline for natural product derived drug discovery shorter than that expected for small synthetic molecules (Leeds *et al*., 2006).

Natural antibiotics have been produced and modified through total and partial synthesis. The synthetic means of producing natural antibiotics overcomes the challenge of inefficiency of the fermentation method used to produce antibiotics naturally. In some cases, it offers a direct and cost effective way for the large scale production of the next generation compounds. Analogs of these natural products are also prepared in an attempt to produce compounds with improved activity and/ or reduced side effects (Nicolaou *et al*., 2009). Just from the beginning of the year 2000, a number of naturally occurring antibiotics have been discovered and tried through total and partial synthesis. These includes tetracyclines, thiopeptides, pseudomonic acids, kanamycin C, ramoplanin A2, lysobactin and abyssomicins, pestalone, squalamine and sitosterol just to mention a few (Nicolaou *et al*., 2009; Mancini *et al*., 2007). Some of these drugs have a history of extensive use in human and veterinary medicine; some are in clinical trials while others are yet to be developed into drugs. Total and partial synthesis plays a significant role in natural antibiotic discovery as most of the antibiotics available today are next-generation versions of established drugs, and many structural classes are now in their third or fourth generation of development, a phenomenon demonstrating the immense potential of the existing natural product derived leads (Nicolaou *et al*., 2009). The *de novo* synthesis of naturally occurring antibiotics and their analogs plays a critical role in understanding the mechanism of action and the Structure Activity Relationships (SARs) of many naturally occurring antibiotics.

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2.11. Natural products in wound treatment

Wounds are the physical injuries that result in an opening or the breaking of the skin. Left unattended, wounds are at risk of microbial infection. **Section 2.12** presents the virulence and pathogenicity of some microorganisms responsible for microbial wound infections. Microbial wound infections prolong the healing process and thus appropriate method for the healing of wounds is essential for the restoration of disrupted anatomical continuity and disturbed functional status of the skin. The wound healing process can be broadly divided into three stages: the inflammation phase, the proliferation phase and the remodeling phase (Ayyanar and Ignacimuthu, 2009).

Reports on the traditional use of plants by different tribes in the treatment of wounds and related injuries such as cuts, burns, bruises caused by external injury, boils, sores and wound resulting from delivery, among other exist (Ayyanar and Ignacimuthu, 2009; Neuwinger, 1996; van Wyk *et al*., 1997; van Wyk and Gericke, 2000). Ayyanar and Ignacimuthu (2009) documented 46 plants belonging to 44 genera and 26 families that are traditionally used by the people of Tirunelveli hills, India, in the management of wounds. Other plant products and natural products such as honey, aloe, cocoa and oak bark extracts are widely used in the treatment of wound and extensive scientific research has been done on investigating their roles in wound healing (Davis and Perez, 2009). Research on honey for instance has proven honey to possess antimicrobial effects against *Escherichia coli*, *Staphylococcus aureus* and MRSA; clinically proven to possess anti-inflammatory activity and; to have demonstrated both *in vitro* and *in vivo*, the ability to retain proper amount of moisture needed during wound healing. Leaf extracts of aloe have been shown to possess anti-inflammatory and antibacterial properties both *in vitro* and in animals. According to Biswas and Mukherjee (2003 cited in Ayyanar and Ignacimuthu, 2009), 70 % of wound healing Ayurvedic drugs are derived from plants.

However, the active ingredients in most traditional medicinal plants are not standardized making it difficult to determine their true efficacy. In addition, natural products are often seen as dietary supplements and thus not regulated (Davis and Perez, 2009). Research in modern biomedical sciences aim at identifying and assessing the validity of traditionally claimed therapeutic properties including wound healing of these plant products for the development of effective wound treatments (Rose, van Houten and Watts, n.d). Due to many social, economic and ethical problems faced in the use of live animals in experiments, laboratory wound models have been developed (Ayyanar and Ignacimuthu, 2009; Rose, van Houten and Watts, n.d). The use of wound models is an essential tool in natural product research as apart from its reduced cost and controlled wound environment; it also allows a high-throughput screening of natural products with wound healing properties, with the determination of mode of action at cellular level, an advancement vital in drug discovery (Rose, van Houten and Watts, n.d).

2.12 Pathogenicity and virulence of microbial pathogens used in this research study

Pathogenicity is the ability of a microorganism to infect and cause disease by overcoming the defences of the host (Tortora, Funke and Case, 2007; Spicer, 2000). Virulence refers to a property of the pathogen that enables it to multiply and cause harm to its host (Spicer, 2000). The pathogenicity of an organism depends on its ability to adapt to tissue environment and to withstand the lytic activity of the host defences and it is aided by virulence factors, either acting together or individually at different stages of infection (Wu, Wang and Jennings, 2008). Although a lot of study has been done on the virulence factors of different pathogens, the virulence and mechanism of pathogenicity of some pathogens are yet to be understood. The discovery of microbial virulence factors plays an important role in understanding microbial pathogenesis and also in the identification of targets for novel drugs (Wu *et al*., 2008). A number of approaches has been made to the discovery of microbial virulence factors and these includes comparative genomics, transcriptomics and proteomics (Yao, Sturdevant, Villaruz, Xu,

Gao and Otto, 2005; Khan, Ahmad, Aqil, Owais, Shahid and Musarrat, 2010; Wu *et al*., 2008). Generally, four crucial steps are necessary for microbial infections. These are (1) entry and adherence to the host tissue, (2) invasion of the host tissue, (3) multiplication, colonisation and dissemination in tissues and (4) evasion of the host immune system and damage to tissues (Khan *et al*., 2010). Virulence factors play different roles in these stages of infection, and can be divided into several groups on the basis of the mechanism of virulence. These include (1) membrane proteins, which plays a role in adhesion, colonization and invasion of host cells; promote adherence to host cell surfaces, are responsible for resistance to antibiotics and promote intercellular communication; (2) polysaccharide capsules that surround the bacterial cell and have anti-phagocytic properties and (3) secretory proteins, such as toxin, which can modify the host cell environment and are responsible for some host cell-bacteria interactions (Wu *et al*., 2008).

Some microorganisms are inhabitants on our skins and play a protective role by keeping pathogenic microorganisms from invading our skin. However, if the microbial balance is disrupted, these microorganisms can cause infections ranging from simple infections to serious conditions depending on the length and depth of tissue exposure as well as on the pathogenicity of the invading microorganism. Disruption of the skin barrier either by cuts or by surgery may introduce the otherwise harmless microorganisms into deeper tissues. The use of broad-spectrum antimicrobials also disturbs the microbial balance of microorganisms resulting in fungal overgrowth. Microbial infections by otherwise harmless microorganisms are also high among people with compromised immunity, people on immunosuppressive agents after organ transplant and cancer chemotherapy patients among others (Karkowska-Kuleta, Rapala-Kozik and Kozik, 2009; Spicer, 2000).

Microorganisms may also invade traumatic wounds, surgical wounds, cuts, and burns. Traumatic wound infections are best classified as those infected from skin flora,

perforated viscus, water and animal and those infected by soil pathogens. *S. aureus* and *S. pyogenes* are some of the skin flora that causes wound infections resulting in infections such as erysipelas and cellulitis. *C. tetani* can cause tetanus in soil contaminated wounds. Surgical wound infections are caused by infections with *S. aureus*, *S. epidermidis*, *Streptococcus* A, *E. coli*, *P. aeruginosa* and *C. albicans*. Burns can be infected by *S. aureus*, *S. pyogenes*, *E. coli* and *P. aeruginosa*. Microbial wound infections may cause wound sepsis, injury to the tissue and consequently interference with the normal functioning of the host and if untreated it may lead to chronic wounds (Akinjogunla, Adegoke, Udokang and Adebayo-Tayo, 2009; Spicer, 2000). The pathogenicity of these microorganisms is described below.

2. 12.1 *Escherichia coli*

Escherichia coli is a common component of aerobic bowel flora. Depending on the antigens they produce, some *E. coli* strains are pathogenic. *E. coli* strains pathogenic to humans are grouped into six pathotypes, the most important being the enteroadhesive, enteroinvasive, enterotoxogenic and enterohaemorrhagic, of which the last two are considered to be the most severe strains. *E. coli* has been reported to cause a number of infections including urinary tract, wound, lung, meningeal and septicaemic infections. Some *E. coli* stains are also identified as a cause of traveller's diarrhoea and the uraemic syndrome (Spicer, 2000; Olaniran *et al*., 2011; Mittal *et al*., 2009). The pathogenicity of a particular *E. coli* strain is primarily determined by specific virulence factors which include adhesins, invasions, haemolysins, toxins, effacement factors, cytotoxic necrotic factors, capsules and siderophores (Olaniran, Naicker. and Pillay, 2011, Mittal, Aggarwal, Sharma, Chhibber and Harjai, 2009).

2. 12.2 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is an aerobic gram-negative rod, which is widespread in soil and water. *P. aeruginosa* is an opportunistic and a serious pathogen, especially to patients with burns and genetic lung disease cystic fibrosis (Tortora *et al*., 2007; Spicer, 2000). It was also reported to cause skin diseases Pseudomonas dermatitis and otitis externa, ear infections, wound infections, acute pneumonia and urinary tract infections especially catheter-associated urinary tract infections (Tortora *et al*., 2007; Mittal *et al*., 2009). *P. aeruginosa* produce endotoxins and exotoxins, though the later accounts for much of its pathogenicity. It also has the ability to form a biofilm and for this reason it is a common cause of hospital-acquired infections due to its ability to form biofilms on medical devices (Tortora *et al*., 2007; Spicer, 2000).

Other virulence factors of *P. aeruginosa* include cell-associated factors like alginate, lipopolysaccharide, flagellum, pilus and non-pilus adhesins, as well as secretory virulence factors such as protease, elastase, phospholipase, pyocyanin, hemolysins and siderophores. Depending on the site of infection, different amount of virulence factors are produced. A quantitative study for the production of four virulence factors, elastase, phospholipase C, toxin A and exoenzyme S among strains isolated from wound infections, respiratory tract infections and urinary tract infections showed that, although all four virulence factors were produced in all strains, the amounts differed depending on the site of infection. Wound and urinary tract isolates were found to produce higher levels of elastase and phospholipase C compared to isolates of the respiratory tract infections. Wound isolates also produced significantly higher amounts of toxin A (Mittal *et al*., 2009).

2. 12.3 *Mycobacterium terrae*

Mycobacteria are non-motile, nonsporing, strictly aerobic rods. They have a special cell wall rich in lipids, which gives them resistance to environmental stresses, host defences

and antibiotics. They can colonise, contaminate and cause pulmonary and systemic infections as well as skin and soft tissue ulcers. They cause prominent infections of the immune-compromised. *Mycobacterium tuberculosi*s and *M. leprae* causes tuberculosis and leprosy respectively. *M. terrae* have been reported to cause wound infections (Spicer, 2000).

2. 12.4 Methicilin-resistant *Staphylococcus aureus*

Staphylococcus aureus is a group of gram positive, coagulase positive staphylococci bacteria, which are commonly found on the skin and mucus membrane. *S. aureus* is documented to be the cause of ear infection, sinusitis, lower respiratory tract infections such as bronchitis and influenza; scalded skin syndrome, toxic shock syndrome and secondary bacterial infections (Tortora *et al.*, 2007; Boyd, 1995; Gordon and Lowy, 2008). Pathogen can enter the body through a natural opening in the skin barrier and hair follicle passage through the epidermal layer. Methicilin-resistant *Staphylococcus aureus* (MRSA) strains are a common cause of lung infection in hospital (Suliman, 2010; Tortora *et al.*, 2007).

Virulence factors of *S. aureus* include toxins, enzymes and the actual structure of the organism. The lack of antigenicity and the consequent lack of protective antibodies also contribute to its pathogenicity (Spicer, 2000). Colonisation increases the risk of subsequent infections and although the basis of colonisation is not completely understood, it appears to involve host-pathogen contact and the ability of the pathogen to adhere to the host cells and to invade the immune response. Microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) play a significant role in establishing the infection through adhesion. MSCRAMMs appear to play a key role in initiation of endovascular infections, bone and joint infections, and prosthetic-device infections. Other virulence factors include the zwitterionic capsule, leukocidins,

chemotaxis inhibitory protein, elastases, proteases, lipases and the formation of biofilms, which enables it to evade the host's defence. Peptidoglycan, lipoteichoic acid and *α*-toxin may all play a role. In addition, *S. aureus* strains may also produce superantigens, resulting in various toxinoses, such as food poisoning and toxic shock syndrome (Gordon and Lowy, 2008).

MRSA is a strain of *S. aureus* that is resistant to methicillin and other β-lactam drugs. The methicillin resistance is conferred by the *mecA* gene which encodes a penicillin-binding protein with decreased affinity for β-lactam antibiotics. This gene is part of the staphylococcal cassette chromosome (SCC) *mec*, which was transferred into methicillin susceptible *S. aureus* to produce different clones of MRSA. MRSA is more virulent than *S. aureus* and even among the different strains of MRSA, the hospital-acquired MRSA strains are more virulent compared to community-acquired MRSA as hospital-acquired strains have been exposed to different antibiotics (Gordon and Lowy, 2008).

2. 12.5 *Staphylococcus epidermidis*

Staphylococcus epidermidis makes up 90 % of normal skin microbiota. Large populations are present in the armpits, anterior nares and perineum though it may also be found in the throat, mouth, vagina, intestinal tract and mammary glands but in smaller numbers than on the skin (Boyd, 1995, Tortora *et al*., 2007). However, if introduced into the body when skin barrier is broken or invaded by medical procedure, it can be a serious opportunistic pathogen (Tortora *et al*., 2007, Spicer, 2000). *S. epidermidis* particularly infects by attaching to foreign, synthetic materials like intravascular catheter or joint prostheses often assisted by slime production and biofilm formation (Spicer, 2000).

2. 12.6 *Streptococcus* A

Streptococci are gram positive bacteria which are part of the normal flora of skin and mucosal surface (Tortora *et al*., 2007; Boyd, 1995; Spicer, 2000). Streptococcal infections are the cause of meningitis, pneumonia, sore throats, otitis media, endocarditis, puerperal fever and dental caries. Streptococcal infections of the throat, wound and burn has been reported (Spicer, 2000). *Streptococcus* A is a group of streptococci, which produces β-hemolysins. B-Hemolytic streptococci are often associated with human diseases, and they are further differentiated into serological groups A to T according to antigenic carbohydrates in their cell walls. Group A streptococci are synonymous with the species *Streptococcus pyogenes*, the most common human pathogen which is responsible for a number of human diseases, some deadly. *S. pyogenes* is the cause of skin diseases such as erysipelas, impetigo and streptococcal toxic shock syndrome. *Streptococcus* A is an aggressive pathogen with numerous virulence factors which gives it the ability to adhere, invade and damage tissues. These include toxins, enzymes and structural components. The M protein, exotoxin A and the capsule of hydraluronic acid are some of these virulence factors. Infections are generally localised but can be highly destructive if bacteria reach deeper tissues (Tortora *et al*., 2007; Spicer, 2000).

2. 12.7 *Candida albicans*

Candida albicans is a yeast opportunistic pathogen that forms part of the normal flora of the mucus membrane in the respiratory, gastrointestinal and female genital tracts and rarely on the skin, though its growth is often suppressed by bacterial microbiota (Suliman, 2010; Tortora *et al*., 2007; Spicer, 2000). Although naturally non-pathogenic, the change in pH or use of broad spectrum antibacterial drugs may shift microbial balance leading to overgrowth of this fungus. Colonisation by this fungus can lead to medical conditions such as skin, nails, vaginal and oral candidiasis. The fungus may also

directly enter the bloodstream from the epithelium after tissue damage or by dissemination from biofilms formed on medical devices introduced into the patient for example catheters, disseminate with the blood flow and infect and cause tissue damage to inner organs such as lungs, kidney, heart, liver, spleen and brain, causing fungamia and life-threatening septicaemia (Suliman, 2010; Khan *et al*., 2010; Tortora *et al*., 2007; Karkowska-Kuleta *et al*., 2009).

A number of virulence factors play a role in the pathogenesis of this fungus. The adherence to the tissue, formation of a biofilm, secretion of hydrolytic enzymes, phenotypic switching and morphological dimorphism all contribute to the virulence of *C. albicans*. *C. albicans* is known to produce adhesions which help with adherence to host cells; to switch between yeast cells, pseudohyphae and septate hyphae to increase its virulence during adhesion and infection establishment as well as to secrete enzymes such as aspartyl proteinases, phospholipases, farnesol, catalases and superoxide dismutases which helps with nutrient uptake, tissue invasion, adherence and dissemination and prevention of oxidative damage among others (Spicer, 2000; Karkowska-Kuleta *et al*., 2009; Khan *et al*., 2010).

2.12.8 *Clostridium tetani*

Clostridium tetani is a large anaerobic gram-positive rod that is particularly found in the soil. *C. tetani* can cause tetanus if the wound is contaminated with the spores and the tissue conditions are suitable for germination. The germinated and growing organisms produce tetanospasmin, one of the two most potent poisons known, which when released binds to peripheral nerve membranes then moves by retrograde neuronal transport to anterior horn cells where it blocks the release of inhibitory neurotransmitters thus causing spasms and spastic paralysis (Spicer, 2000).

CHAPTER 3

3. MATERIALS AND METHODS

3.1 Preparation and extraction of plant material

3.1.1 Samples collection and preparation

Plant samples were collected from the Oshikoto region in northern Namibia in February 2010. The leaves of *Ziziphus mucronata* and aerial parts of *Gnidia polycephala* and *Heliotropium ciliatum* were collected. The fresh samples were chopped in small pieces and dried in the shade on laboratory benches. The samples were then ground to a powder using a Waring® commercial heavy duty blender. The crushed samples were stored in sealed plastic bag, which were kept inside the sample paper bags in the cupboards at room temperature until use. **Figure 3.1** shows a map of Namibia indicating the location of the Oshikoto region.

Figure 3.1: The map of Namibia indicating the Oshikoto region, sample collection site (Cheikhyoussef *et al*., 2011).

3.1.2 Extraction

3.1.2.1 Hot water extraction

Aqueous extracts were prepared according to the method of Li *et al*. (2008) with slight modifications. Briefly, 20 g of the powdered plant material was mixed with 200 mL of distilled water in a conical flask, which was incubated in a hot water bath at 70 ^{o}C for 2 hours. The resulting mixture was allowed to cool to room temperature before being filtered using Whatman filter paper No. 1. The extracts were concentrated by heating in a water bath. The concentrated extracts were kept in a fridge until use.

3.1.2.2 Organic extraction (Method 1)

Plant materials were extracted following the method of Goze *et al*. (2009), with slight modifications. Briefly, plant materials were extracted sequentially with hexane, dichloromethane, ethanol and methanol, in order of increasing polarities. Fifty grams (50 g) of the powdered plant samples were soaked in 200 mL of the extraction solvent for three days, after which the extracts were filtered through Whatman$^®$ filter paper. Extraction per solvent was only carried out once and the residues were air dried before soaking with the next extraction solvent.

3.1.2.3 Organic extraction (Method 2)

As above, plant materials were also sequentially extracted with hexane, dichloromethane, ethanol and methanol in order of increasing polarities. About 25-35 g of the dry powdered plant materials were extracted with 150 mL of organic solvents in a Soxhlet apparatus for 6 hours each. The residues were also air dried before extraction with the next solvent.

All organic extracts were concentrated in rotary evaporator, and later left in the fume hood for a few days to dry off excess solvent. The extracts were kept in a fridge until use.

3.2 Bioassays

3.2.1 Antioxidant bioassay

3.2.1.1 DPPH radical scavenging activity

The inhibition effects of the extract on a free radical DPPH was studied using the DPPH radical-scavenging method as described in Goze *et al*. (2009). Two millilitre (2 mL) of different concentrations (25, 50, 75, 100 and 500 µg/ mL) of the plant extracts in methanol were mixed with 2 mL of DPPH solution, shaken vigorously and allowed to stand for 30 minutes before measuring the absorbance with a UV spectrophotometer at 517 nm. A sample of the dissolving solvent (methanol) with no plant extract was used as a negative control. Butylated hydroxyl toluene (BHT) was used as a positive control. The inhibition effects of the extract on free radical DPPH were expressed as follows:

% inhibition = $[(A_{control} - A_{sample})/A_{control}] \times 100$, where, $A_{control}$ is the absorbance of the control and A_{sample} is the absorbance of the sample.

3.2.2 Anticancer bioassay

3.2.2.1 Cytotoxicity assay using BST

The brine shrimp lethality assay was performed following the reported procedure (Meyer *et al*., 1982) with some slight modifications (Silva, Camara, Barbosa, Soares, Cunha, Pinto and Vargas, 2005). The shrimp were grown in a small tank divided into

two compartments filled with artificial sea water. The shrimp eggs were added to the covered compartment. A lamp was placed over the open side of the tank to attract hatched shrimps through perforations in the partition wall. The shrimps were allowed to grow for 48 hours, after which they were matured, now being called nauplii, and are ready to be used for the test. The plant extracts were dissolved in 2 mL of DMSO. Artificial sea water prepared by dissolving 3.8 g of sea salt in 1 L of distilled water was added to complete 5 mL of total volume. Appropriate volumes of the resulting solution were added to tubes, in duplicate, with 5 mL of saline solution containing 10 nauplii each to afford the final sample concentrations of 24, 40, 80, 120 and 240 µg/ mL. The negative control contained brine shrimp, artificial sea water and 0.6% DMSO, under the same conditions but no plant extracts. The samples were incubated under light conditions for 24 hours and the number of dead and surviving brine shrimps in each tube was recorded. A graph of percentage mortality against logarithm concentration was plotted using a computer program Microsoft Excel. The lethal concentration causing 50 % mortality (LC_{50}) was determined by taking the anti-logarithm of logarithm concentration corresponding to 50% mortality. An LC_{50} value greater than 1000 µg/ mL were considered to represent an inactive extract.

3.2.2.2 Anticancer potential of the extracts against cancer cell

3.2.2.2.1Cell lines and cell culture

Colon adenocarcinoma (Caco-2), small intestinal foetal tissue (H4), non-tumorigenic immortalised human diploid fibroblasts (KMST-6), human cervical cancer (HeLa), lung carcinoma (H157) and Chinese hamster ovary (CHO) cell lines were used to assess the anticancer potential of the plant extracts. Assays using Caco-2 and H4 cell lines were performed at the Department of Microbiology, Biochemistry, Molecular Biology and Biotechnology, University of Maribor, Slovenia. The APOP*ercentag*e™ assays using CHO, H157, HeLa and KMST-6 cell lines were performed at the Department of

Biotechnology, University of the Western Cape, South Africa. The cells were grown in 100 mL conical flasks in an appropriate medium, supplemented as indicated in **table 3.1** and were incubated in a humidified 5% CO_2 incubator at 37°C. When the cells reached ~90% confluency, they were trypsinised and cells counted before plating.

Table 3.1: Growth media and supplements for the cell lines used

Cell line	Species	Media	Supplements
H4	Human	DMEM	5% Foetal Bovine Serum, 100 U/ml Penicillin, 100 μg /ml Streptomycin, 2 mM L-glutamine
Caco-2	Human	DMEM	5% Foetal Bovine Serum, 100 U/ml Penicillin, 100 μg /ml Streptomycin, 2 mM L-glutamine
CHO	Chinese Hamster	F-12Hams	10% Foetal Bovine Serum, 50 U/ml Penicillin, 50 μg /ml Streptomycin
H157	Human	DMEM	10% Foetal Bovine Serum, 50 U/ml Penicillin, 50 μg /ml Streptomycin
HeLa	Human	DMEM	10% Foetal Bovine Serum, 50 U/ml Penicillin, 50 μg /ml Streptomycin
KMST-6	Human	DMEM	10% Foetal Bovine Serum, 50 U/ml Penicillin, 50 μg /ml Streptomycin

3.2.2.2.2 Cell tryptinization and subculturing

After 2-4 days of incubation the cells had reached ~90% confluency and are ready for tryptinization. The media was discarded and the cell monolayer was washed with 2 mL of trypsine-EDTA solution. The cell monolayer was incubated with 1 mL of trypsine to allow for cells to detach from the flask walls. To the detached cells solution, 9 mL of

fresh media was added to stop tryptinization and the cell suspension was centrifuged at 800 rpm at 22 ^{o}C for 5 minutes to obtain a cell pellet. The pellet was re-suspended in 10 mL of fresh media supplemented with appropriate amount of FBS (see **table 3.1**). One fifth of the resuspended cells (2 mL) solution was transferred back into the culture flask to which 8 mL of supplemented media was added for subculturing into a 100 mL flask. The cells were incubated at 37 ^{o}C in the 5% CO_2 incubator. The rest of the cells were seeded into plates and used for the experiments.

3.2.2.2.3 Cell counting

To determine cell viability, the trypan blue staining technique was used. In an Eppendorff tube, 100 µL of the cell suspension was mixed with 900 µL of 0.1 % trypan blue. A drop of this suspension is added to a sealed hematocytometer. The number of live cells in the 25 squares was then counted under the light microscope, following the rule of counting on only two sides of the square for cells trapped between the squares. The number of cells counted had to be between 10 and 25 for accuracy in calculations. If the cell number was higher than 25 cells in the 25 squares, the cell solution was diluted with 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid - buffered saline solution (HEPES-BSS) and the cells were recounted. The cell concentration (number of cells per mL of cell suspension) was determined using the following equation:

Number of cells/ mL cell suspension = number of cells counted $\times 10^6$/ 25 squares

3.2.2.2.4 Cell plating

From the concentration obtained from the step above, the cells were diluted to the desired concentration with supplemented media. The cells were seeded in 96-well plates at a density of 111 cells/ mL for the cell growth inhibition assay and 6×10^4 cells/ mL

for the MTT assay. For the APOP*ercentage*™ apoptosis assay, cells were seeded in 12-well plates at a density of 2.5×10^4 cells/ mL. For the 96-well plates, 100 µL of the cell suspension was delivered into each well using a multichannel pipette. To ensure even cell distribution, the cell suspension was poured into a sterile petri dish which was shaken regularly before drawing out the cell suspension. For the 12-well plate, 1 mL of the cell suspension was delivered into each well. The plates were incubated for 24 hours at 37 ^{o}C in a 5% CO_2 incubator or until they reached 90% confluency before the treatment with plant extracts.

3.2.2.2.5 Testing plant extracts for anticancer activity

3.2.2.2.5.1 Cell growth inhibition assay

As described above, 100 µL of 111 cells/ mL of Caco-2 and H4 cells solution was seeded into 96-well plates. The outer wells of the plates i.e., rows A and H and columns 1 and 12, were filled with 100 µL of distilled water. The inner wells were filled with 100 µL of cell solution (**figure 3.2**). The plates were incubated for 24 hours at 37 ^{o}C at 5% CO_2. After the incubation, the media was replaced with fresh, complete media and the cells were treated with plant extracts.

The extracts were dissolved in dimethyl sulfoxide to make a stock solution of 50 mg/ mL. Row B was used as a control. Eleven microliters (11 µL) of plant extract stock solution was added into well in row C, performing a 1: 10 serial dilution through to well in row G (**figure 3.2**). The experiment was performed in five replicates. The plates were incubated at 5% CO_2, 37 ^{o}C for 8 days, where cell growth was clearly visible under a light microscope. The media was discarded and the cells were treated with crystal violet for 5 minutes. The crystal violet was washed off under running tap water and the plates were dried on a towel paper over night. The number of colonies observed in wells under the light microscope was recorded. The highest concentration in which some selective

toxicity against Caco-2 cells was observed (500 μg/ mL) was used as the highest concentration in the cytotoxicity (MTT) assay and only extracts which showed selective toxicity towards Caco-2 cell lines were used for the cytotoxicity (MTT) assay.

	1	2	3	4	5	6	7	8	9	10	11	12	
A													
B													⇐ Control
C													⇐ 1:10 dilution
D													⇐ 1:100 dilution
E													⇐ 1:1 000 dilution
F													⇐ 1:10 000 dilution
G													⇐ 1:100 000 dilution
H													

Figure 3.2: The treatment of cells in the 96-well plate for the growth inhibition assay.

3.2.2.2.5.2 Cytotoxicity assay

Due to the slow growth of Caco-2 cells, only H4 cells were used for this assay. Extracts that showed selective toxicity against Caco-2 in the previous assay were used in this assay. The cells were seeded and incubated as stated in **section 3.2.2.4**. At the end of the incubation period, media was discarded and the cell monolayer was washed twice with 200 μL of sterile PBS. One hundred and twenty microliters (120 μL) of DMEM, only supplemented with L-glutamine (no FBS, no indicator) was added.

Nine microliters (9 uL) of the extract stock solution (50 mg/ mL) was mixed with 291 µL of colourless DMEM media to prepare working solution of 1.5 mg/ mL. Row A was used as a control. To row B, 60 µL of extract solution was added (making the starting concentration of 500 µg/ mL). A 1: 4 serial dilution was performed through to wells in row H, always discarding 60 µL from the last well to afford a final volume of 120 µL per well. The experiment was performed in triplicate and the plates were incubated for 24 hours. The effects of the extract dissolving solvent (DMSO) was also tested, which was treated in a similar manner as the plant extracts.

After incubation, two sets of 40 µL of the incubation media from each well were transferred into sterile 96 well plates in a sequence corresponding to that of the original plate, and these were reserved for the H_2O_2 and NO assay. The left over media was discarded and the cells were used for the MTT assay. The three assays were performed following the provided protocol as briefly described below.

3.2.2.2.5.2.1 MTT assay

The cell monolayer was washed with PBS. To each well, 220 µL of a mixture DMEM (only supplemented with L-glutamine) and 5 mg/ mL MTT solution (in the ratio 10: 1) was added. The plates were incubated for 5 hours at 5% CO_2 and 37 ^{o}C to allow for the development of purple formazan. The media was discarded and the plates were dried on a towel paper overnight. One hundred microliters (100 µL) of 0.04% HCl in isopropanol was added to each well to dissolve the formazan. The plates were shaken on a rotating shaker for 5 minutes, before being further incubated at 5% CO_2 and 37 ^{o}C for 20 minutes. The absorbance was measured at 570 nm with background wavelength set at 630 nm.

3.2.2.2.5.2.2 NO assay

To the 40 µL of the overnight incubation media, 40 µL of Griess reagent was added. The plates were gently shaken on a shaker for 20 minutes and the absorbance was measured at 540 nm.

3.2.2.2.5.2.3 H_2O_2 assay

To the 40 µL of the overnight media, 40 µL of 0.01% peroxidise was added, followed by the addition of 100 µL of TMB-H_2O mixture (1:1). The control was prepared by mixing 50 µL of 0.001% H_2O_2, 50 µL of 0.01% peroxidase and 100 µL of mixture of 1:1 TMB and H_2O_2 in one well. The mixture was place on a shaker for 20 minutes before measuring the absorbance at 450 nm.

3.2.2.2.6 APOPercentage^{TM} assay (Flow cytometric analysis of apoptosis)

3.2.2.2.6.1 Extract preparation

Plant extracts (20 mg) was dissolved in 200 µL DMSO, and 800 µL of an appropriate media was added to make a stock solution of 20 mg/ mL. From the stock, working solutions (2.5 and 5.0 mg/ mL) were prepared by diluting the stock solution with the appropriate media. The working solution was filtered through a filter paper (pore size: 0.45 µm).

3.2.2.2.6.2 APOPercentage^{TM} assay

Chinese hamster ovary (CHO), lung carcinoma (H157), human cervical cancer (HeLa) and non-tumorigenic immortalised fibroblasts (KMST6) cell lines were used. As

mentioned above, when the cells in the culture flask were grown to 90% confluency, they were trypsinized and the pellet was resuspended in complete media to a cell density of 2.5 $\times 10^4$ cells per mL. The cells were seeded in 12- well tissue culture plates, 1 mL per well. The cells were then incubated at $37\,^{\circ}C$ in a humidified CO_2 incubator for 24 hours.

At the end of the incubation period, the media was replaced with working extract solution (2.5 and 5.0 mg/ mL). Complete media with no plant extract was used as a negative control, whereas 150 µM ceramide was used as a positive control. The cells were incubated at 37 $^{\circ}C$ and 5 % CO_2 for 24 hours. The negative control cells were used to aid in properly distinguishing normal cells from apoptotic cells. Following incubation, floating (apoptotic) cells were transferred to 15 mL centrifuge tubes and the adherent cells were trypsinized and added to tubes containing floating cells. The tube was centrifuged at 300 x g for 3 minutes to obtain cell pellet which was washed twice with PBS, centrifuging at 300 x g for 3 minutes for each wash. At the end of the last wash, the pellet was resuspended in the residual PBS and 250 µL APOP*ercentage*™ dye (a 1: 160 dilution in complete media) was added. The cells were incubated for 30 minutes at 37 $^{\circ}C$ in a humidified 5 % CO_2 incubator. After the incubation period, 500 µL of PBS was added to the tube and cell mixture was spun down for 5 minutes at 300 x g to get a cell pellet. The pellet was washed one more time with PBS. After which the pellet was resuspended in 400 µL of PBS and the cells were acquired and analysed using a FASCan™ (Becton Dickson) instrument equipped with a 488 nm Argon Laser as a light source within one hour. Acquisition was done by setting forward scatter (FSC) and side scatter (SSC) on a log scale dot plot to differentiate population of cells and cellular debris. On a linear histogram dot plot, APOP*ercentage*™ (FL-3 channel) was measured against relative cell numbers. Negative control cells were used to set the cells in the negative quadrant before all samples were acquired. A minimum of 10, 000 cells per sample was acquired and analysed using CELLQUEST Pro software by setting the non-stained (untreated) cell population in the first quadrant (10^1) of the forward side scatter histogram dot plot and

cells which appeared in the second (10^2) or third quadrant (10^3) were regarded as APOP*ercentage* [TM] positive (apoptotic/necrotic) cells.

3.2.3 Antimicrobial test

The antimicrobial effects of the extracts of the three selected traditional medicinal plants against different microbes were determined by the broth micro-dilution method as described by Chingwaru, Duodu, van Zyl, Schoeman, Majinda, Yeboah *et al*. (2011) (as established by the University of Maribor, Slovenia, with few modifications). Briefly, *M. terrae*, *E. coli, P. aeruginosa, Streptococcus A, C. tetani,* MRSA and *S. epidermidis* were grown in nutrient broth (10 mL) and *C. albicans* in yeast extract peptone glucose (YEPG) broth (10 mL) for 24 hours at 37 $^{\circ}$C. The optical density of the microbial suspension was measured at 600 nm and the microbial suspension was diluted with appropriate media to yield microbial suspensions of 1×10^3 colony forming unit (CFU)/ mL for bacteria and 1×10^3 CFU/ mL for yeast, which were then used for the experiments. Before addition of the microbe, the microbial suspension was shaken to evenly distribute the microbes.

Plant extracts were dissolved in DMSO to make a stock solution of 50 mg/ mL. The extracts were diluted with nutrient broth for bacteria (or YEPG for *C. albicans*) to yield a working solution at concentration of 20 mg/ mL for *E. coli* and *M. terrae*; and 5 mg/ mL for all the other microbes. In a 96-well plate, 40 µL of the appropriate media was delivered into each well using a multichannel pipette. To the first well, 40 µL of working extract solution was delivered and a 1:2 serial dilution was performed through to the last well, always discarding 40 µL from the last well. Twenty microliters (20 µL) of the bacterial suspension (1×10^5 CFU/ mL) or yeast (1×10^3 CFU/ mL) was added. The plates were incubated at 37 $^{\circ}$C, measuring the microbial growth at 600 nm every hour for the first 10 hours and also at the end of the experiment, i.e. at 24 hours. DMSO

and appropriate growth media were used as negative control whereas gentamycin (4 mg/ mL) and streptomycin (10 mg/ mL) were used as positive control. The experiment was performed in triplicate and the absorbances were expressed as mean ± standard deviation. The minimum inhibition concentration (MIC) was identified as the lowest concentration which inhibited the growth of the microbe.

CHAPTER 4

4. RESULTS

4.1 Natural products extraction

Some plant natural products were successfully extracted during the extraction process (**table 4.1**). In each case, method 1 is the room temperature extraction and method 2 is the Soxhlet extraction. As stated in the methodology, the amount of starting material used for these two extraction methods ranged between 25-35 g for method 2 and about 50 g for method 1. To allow for statistical comparison, all yields were expressed as yield fraction, i.e. yield fraction = amount of extract/ amount of starting material. The percentage yields were expressed as means ± standard deviation and are tabulated in the **tables 4.1 A-D** below. Although the yield of the Soxhlet extraction (method 2) were slightly higher than that obtained from cold extraction (method 1), the yield per extraction solvent were not statistically significant except for hexane extracts of *G. polycephala* (t= -4.708, p=0.009), ethanol extract of *H. ciliatum* (t= -5.533, p=0.005) and hexane and methanol extracts of *Z. mucronata* (t= -8.688, p= 0.001 and t= -3.466, p= 0.026 respectively). The accumulated yields for the two extraction methods however were statistically significant, with p values of 0.001, 0.004 and 0.005 for *Z. mucronata, G. polycephala* and *H. ciliatum* respectively. Because the yields per extraction solvent for the three plants were not statistically significant with a few exceptions, only extracts of the first method of extraction (Method 1) were used in the assays for this study. These extracts were given codes shown in **table 4.2** and were referred to by these codes throughout this document.

Table 4.1: Comparative analysis of extraction yield of different extracts of the three traditional medicinal plants using two extraction methods, cold extraction by soaking for three days (method 1) and soxhlet extraction for six hours (method 2). The yield per extraction solvent per plant are shown in **table 4.1 A** (*G. polycephala*), **table 4.1 B** (*H. ciliatum*) and **table 4.1 C** (*Z. mucronata*). The pooled yields per plant are shown in **table 4.1 D**. * Values were mean ± standard deviation of three replicates.

Table 4.1 A

	Percentage yield (%)	
Extract	**Method 1**[*]	**Method 2**[*]
Hexane	1.90±0.31	3.42±0.47
Dichloromethane	2.27±0.25	2.95±0.55
Ethanol	2.51±1.09	4.14±0.47
Methanol	5.77±0.52	12.02±1.94

Table 4.1 B

	Percentage yield (%)	
Extract	**Method 1**[*]	**Method 2***
Hexane	1.10±0.19	1.23±0.68
Dichloromethane	1.03±0.32	1.27±0.12
Ethanol	1.06±0.36	2.68±0.35
Methanol	2.70±0.12	4.26±1.16

Table 4.1 C

Extract	Percentage yield (%)	
	Method 1[*]	Method 2[*]
Hexane	1.50±0.10	3.35±0.36
Dichloromethane	1.57±0.16	1.88±0.86
Ethanol	4.98±0.09	14.2±0.16
Methanol	9.70±0.92	12.9±1.3

Table 4.1 D

Plant	Cumulative percentage yield (%)	
	Method 1[*]	Method 2[*]
Z. mucronata	17.74±1.20	32.34±2.16
H. ciliatum	5.88±0.52	9.45±1.01
G. polycephala	12.45±1.44	22.53±2.64

Table 4.2: Codes assigned to hexane, dichloromethane, ethanol, methanol and water extracts of *G. polycephala*, *Z. mucronata* and *H. ciliatum*.

Plant	Extraction solvent				
	Hexane	Dichloromethane	Ethanol	Methanol	Water
Z. mucronata	ZH	ZD	ZE	ZM	ZW
G. polycephala	GH	GD	GE	GM	GW
H. ciliatum	HH	HD	HE	HM	HW

4.2 Antioxidant activity: DPPH assay

Ethanol and methanol extracts of the three traditional medicinal plants were tested for their ability to scavenge DPPH radical. The extracts showed dose-dependent DPPH scavenging activities. Methanol extracts generally exhibited higher antioxidant activity compared to ethanol extracts (**figure 4.1 - 4.3**). The DPPH scavenging activity for the methanol extract of *G. polycephala* (GM) and *Z. mucronata* (ZM) were comparable to the activity of the synthetic antioxidant butylated hydroxyl toluene (BHT), although ZM exhibited slightly higher activity than that of GM (**figure 4.4**). For all extracts, *Z. mucronata* showed the highest DPPH scavenging activity, followed by *G. polycephala* with *H. ciliatum* showing the least activity (**figure 4.4 and 4.5**). The concentration of the extracts that was able to scavenge at least 50% of the DPPH dye (IC_{50}) was calculated. ZM and GM were the most active with low IC_{50} of 45.19 µg/ mL and 50.20 µg/ mL respectively. *Z. mucronata* possessed the most DPPH radical scavenging activity among the three plants with both extracts showing $IC_{50} < 85$ µg/ mL and *H. ciliatum* exhibited the least DPPH radical scavenging activity with IC_{50} for both extracts > 300 µg/ mL. The results are summarized in **table 4.3**.
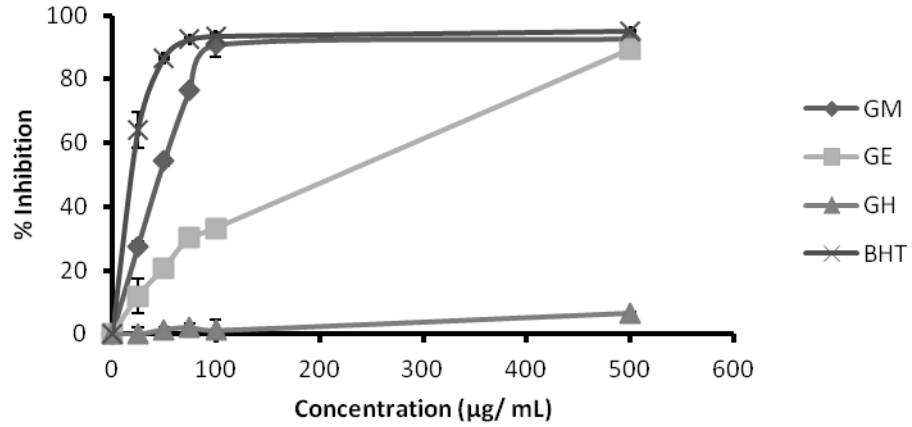
Figure 4.1: DPPH scavenging activity of GE, GM and GH extracts as compared to that of BHT.
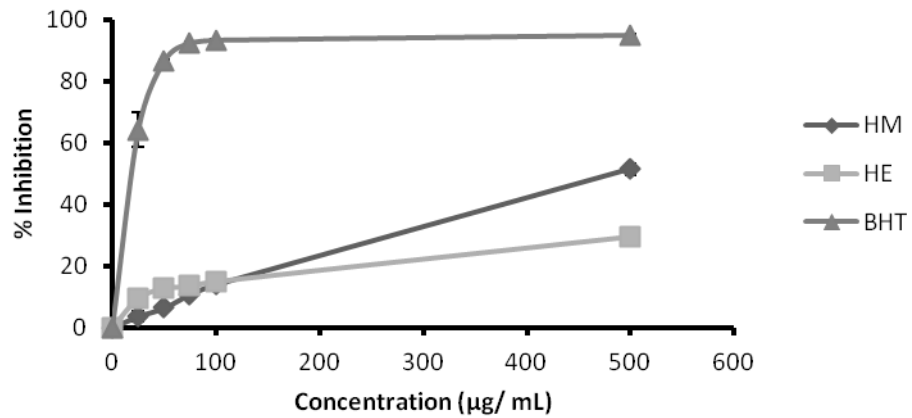

Figure 4.2: DPPH scavenging activity of HE and HM extracts as compared to that of BHT.
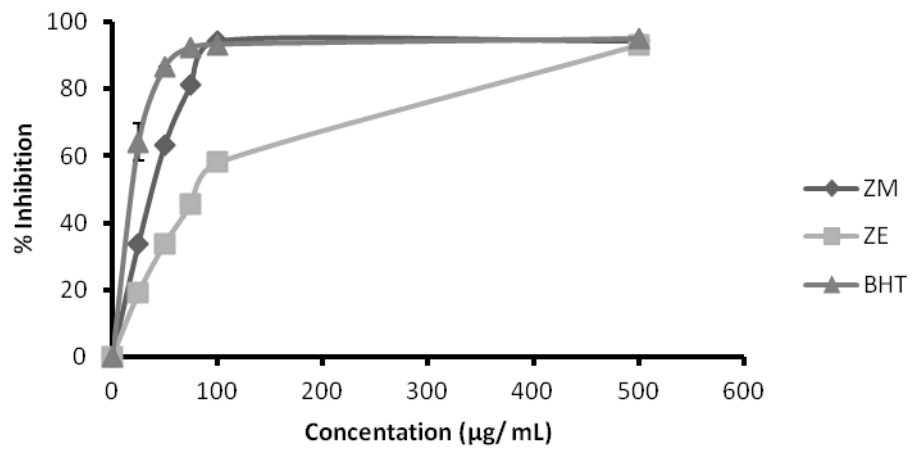
Figure 4.3: DPPH scavenging activity of ZE and ZM extracts as compared to that of BHT.
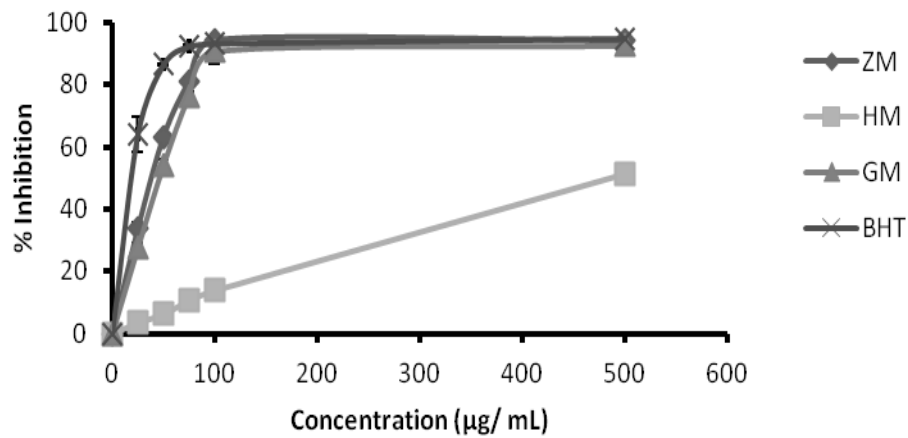
Figure 4.4: DPPH scavenging activity of methanol extracts of the three indigenous plants
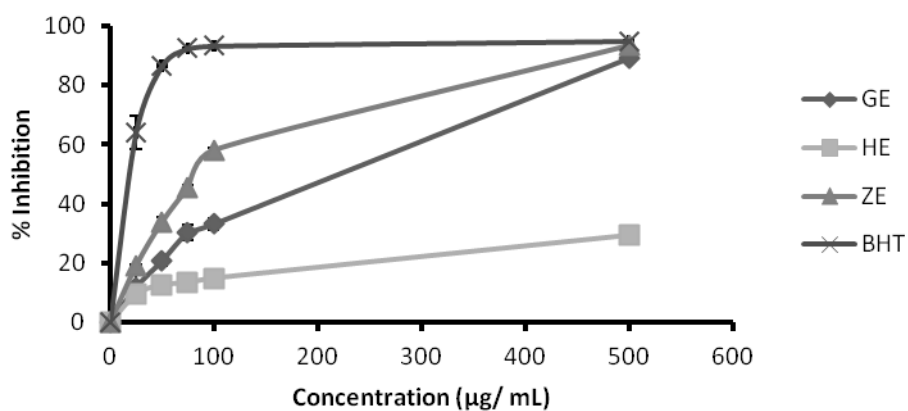
Figure 4.5: DPPH scavenging activity of ethanol extracts for the three indigenous plants

Table 4.3: IC$_{50}$ values (µg/mL) for the DPPH scavenging activity

Extract	IC$_{50}$ (µg/ mL)
BHT	29.92
GM	50.20
GE	140.68
GH	> 1000
HM	357.59
HE	343.81
ZM	45.19
ZE	82.68

4.3 Anticancer activity

4.3.1 Brine shrimp lethality test

ZM, ZW, ZE and GM extracts were relatively inactive either showing lethal concentration to at least 50 % (LC_{50}) of the *A. salina* larvae at a concentration of or more than 1000 µg/ mL. An LC_{50} value of $\geq$ 1000 µg/ mL was taken as inactive and extracts which obtained this score were considered safe to the tested organism. *H. ciliatum* showed the most toxicity with LC_{50} for the hexane, ethanol and methanol extracts < 200 µg/ mL. Other extracts that showed an LC_{50} < 200 µg/ mL are hexane and dichloromethane extracts of *Z. mucronata*. Extracts of *G. polycephala* showed moderate toxicity with LC_{50} values for ethanol, dichloromethane and hexane extracts all above 250 µg/mL. The results are tabulated in **table 4.4.**

Table 4.4: Brine shrimp activity of extracts of the three selected traditional medicinal plants. LC= Lethal concentration; LCL= Lower confidence limit; UCL= Upper confidence limit; *= No activity (mortality) up to the maximum tested concentration (240 µg/ mL); # = $LC_{50} \geq$ 1000 µg/ mL.

Extract	LC_{50} (µg/ mL)	95% Fiducial Limit (µg/ mL)
		LCL — UCL
ZW	#	#
ZM	#	#
ZE	*	*
ZD	165.64	44.50—616.43
ZH	198.54	119.04—331.12
GM	*	*
GE	264.46	48.39—1445.34
GD	590.85	181.862—442.95

GH	729.27	100.945—268.53
HM	169.71	51.52—558.99
HE	142.67	54.723—71.93
HD	230.27	40.863—76.46
HH	131.84	94.991—82.99

4.3.2 Cell growth inhibition assay

Screening for selective activity against cancerous Caco-2 cells and not healthy H4 cells identified GH, GE, GD, GM, ZD, ZE, ZM, HH, HE and HM. These were the extracts further tested in the cytotoxicity assay. The effect of plant extracts on the proliferation of the two cell types are shown **figure 4.6** below. The control has no plant extract, d1 to d5 are plant extracts dilutions where; d1 is a 1:10 dilution, d2 is 1:100 dilution, d3 is 1:1000, d4 is 1:10 000 and d5 is a 1: 100 000 dilution. The extracts that showed selected toxicity towards Caco-2 cells are given in **table 4.5.**

However, the basis of identifying which extracts are active against Caco-2 cells and not H4 cells is quite tricky as the proliferation of any two different cell lines differs. In addition, it is hard to keep a constant concentration of diluted cell solution and only a fraction of the transferred cells survive and proliferate, making it hard to compare absolute colonies obtained with different cell lines. The spectrophotometric method is also unreliable as much of the absorbance recorded is rather due to the dye attached to the walls of the plate than the dye retained by the cell colonies.
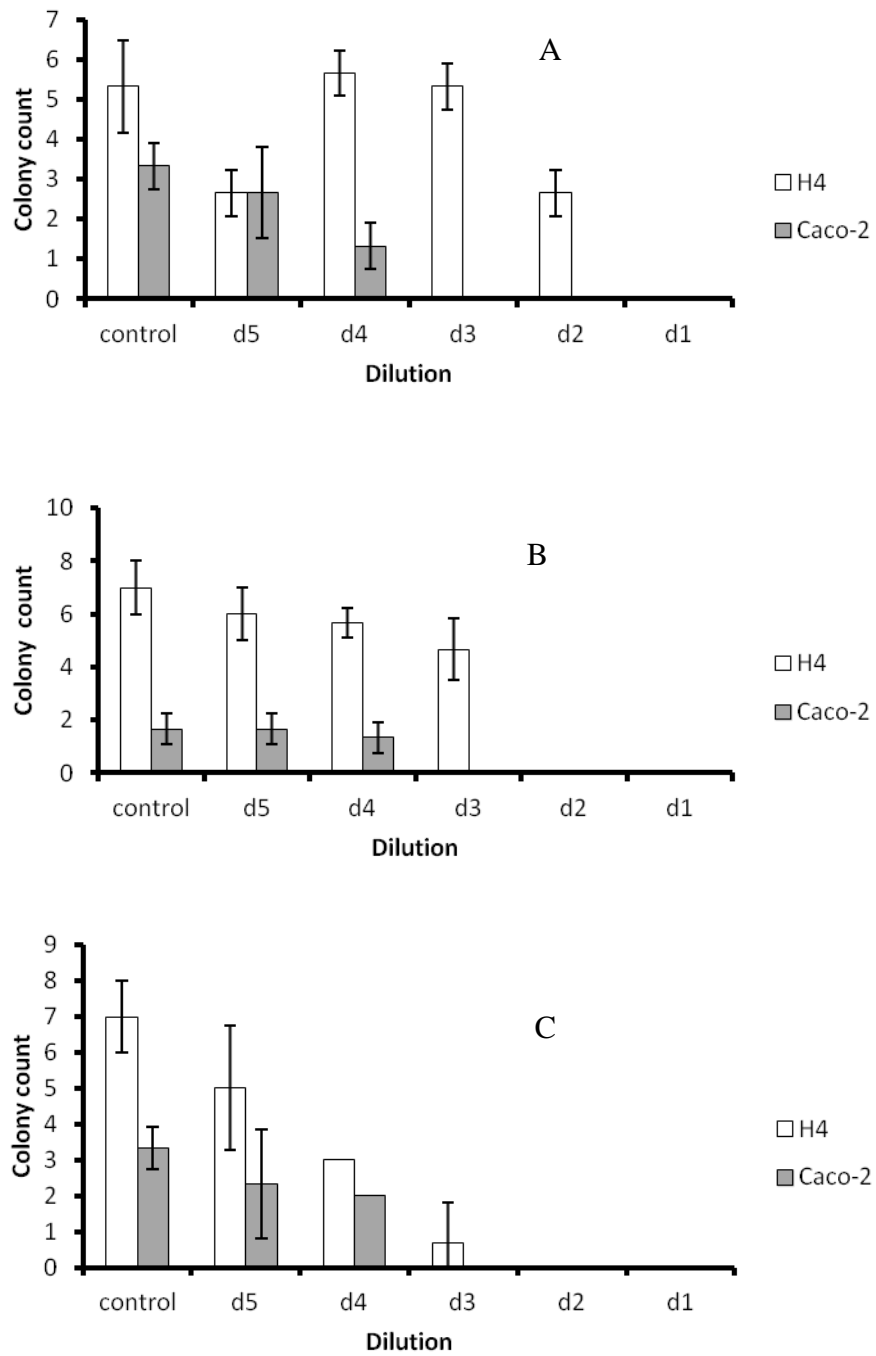
Figure 4.6: The proliferation of H4 and Caco-2 cell lines against GE (A), ZD (B) and HH (C).

Table 4.5: Plant extracts which showed selective toxicity towards Caco-2 cell line.

Plant	Hexane	Dichloromethane	Ethanol	Methanol
Z. mucronata	✘	✔	✔	✔
G. polycephala	✔	✔	✔	✔
H. ciliatum	✔	✘	✔	✔

4.3.3 MTT assay

4.3.3.1 Chinese hamster ovary cells

Water extracts of the three selected plants showed no activity at a concentration of 2.5 mg/ mL, while at a concentration of 5 mg/mL, GW and ZW showed inhibitory activity on the proliferation of CHO cell lines by 25% and 55% respectively (see **figure 4.7**).
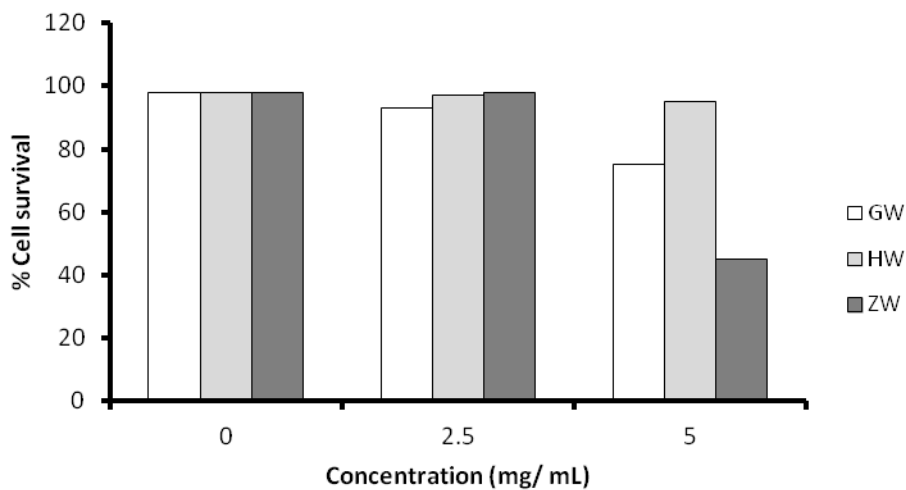
Figure 4.7: The effects of water extracts of selected traditional medicinal plants on the proliferation of CHO cells.

4.3.3.2 H4 cells

Plant extracts exhibited anti-proliferation activity in a dose dependent manner as illustrated by bar graphs in **figure 4.8.** A plot of concentration against % cell survival enabled the determination of the IC_{50}. HE and HH were the most active with IC_{50} of 116.80 µg/ mL and 119.34 µg/ mL respectively; whereas GE and ZE showed the least activity with IC_{50} values of 264.04 µg/ mL and 658.40 µg/ mL respectively (**table 4.6**).

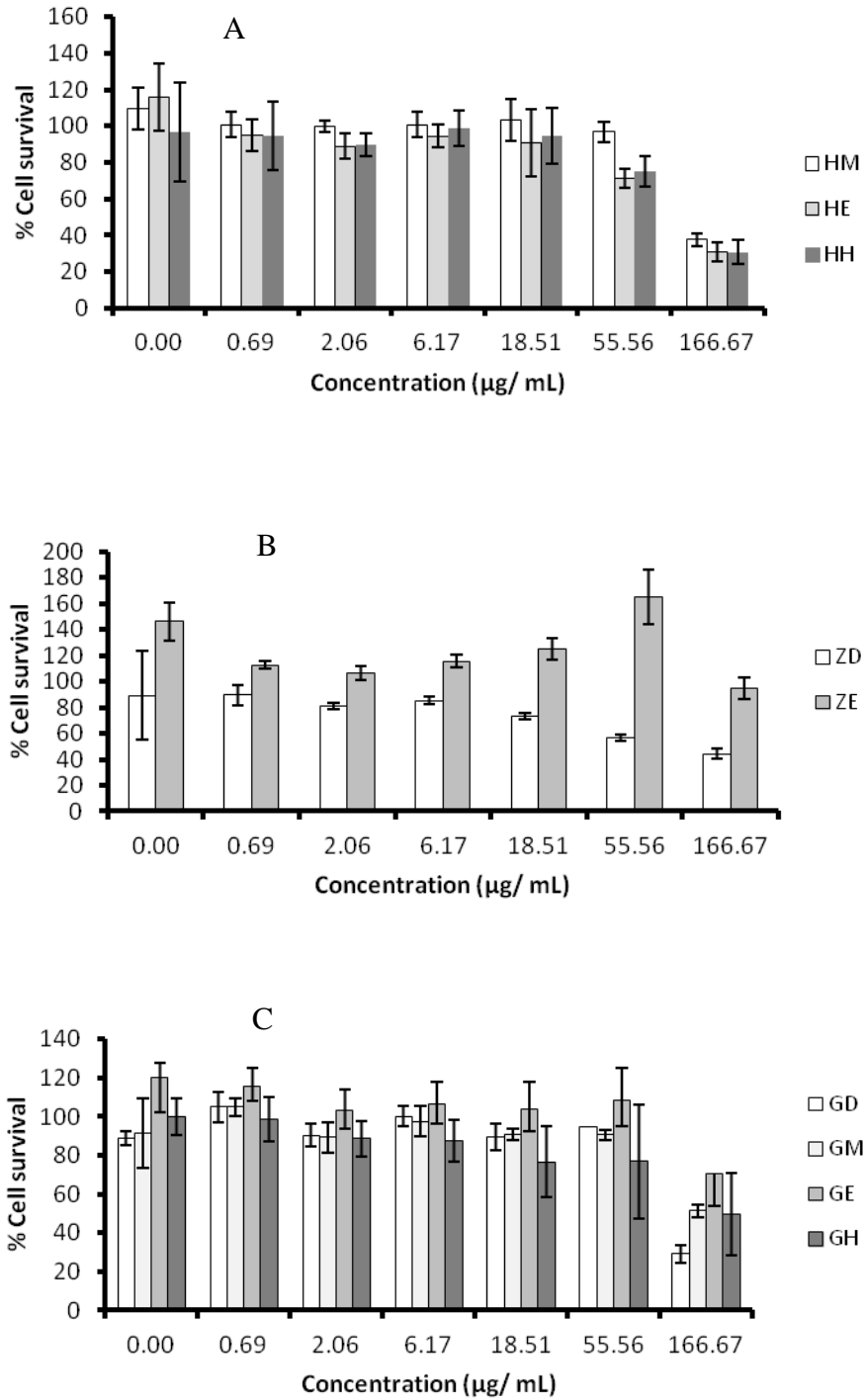**Figure 4.8:** The effects of different extracts on the proliferation of H4 cells, *H. ciliatum* (A), *Z. mucronata* (B) and *G. polycephala* (C).

Table 4.6: Anti-proliferation activity of selected plant extracts against H4 cells

Extract	IC_{50} (µg/ mL)
GM	180.91
GE	264.04
GD	126.95
GH	160.37
HM	145.73
HE	116.80
HH	119.34
ZE	658.40
ZD	126.73

4.3.4 NO assay

No significant amount of NO was produced by cells treated with different concentrations of plant extracts, except at the lowest dilution factor (**figure 4.9**).

Figure 4.9: The release of NO by H4 cells treated with different concentrations of plant extracts, extracts of *G. polycephala* (A) and *Z. mucronata* and *H. ciliatum* (B).

4.3.5 H$_2$O$_2$ assay

H4 cells treated with different concentrations of plant extracts produced no significant amount of H$_2$O$_2$ as the absorbances of cells treated with the extracts were comparable to that of the control, DMSO, except at the highest concentration tested (**figure 4.10**).
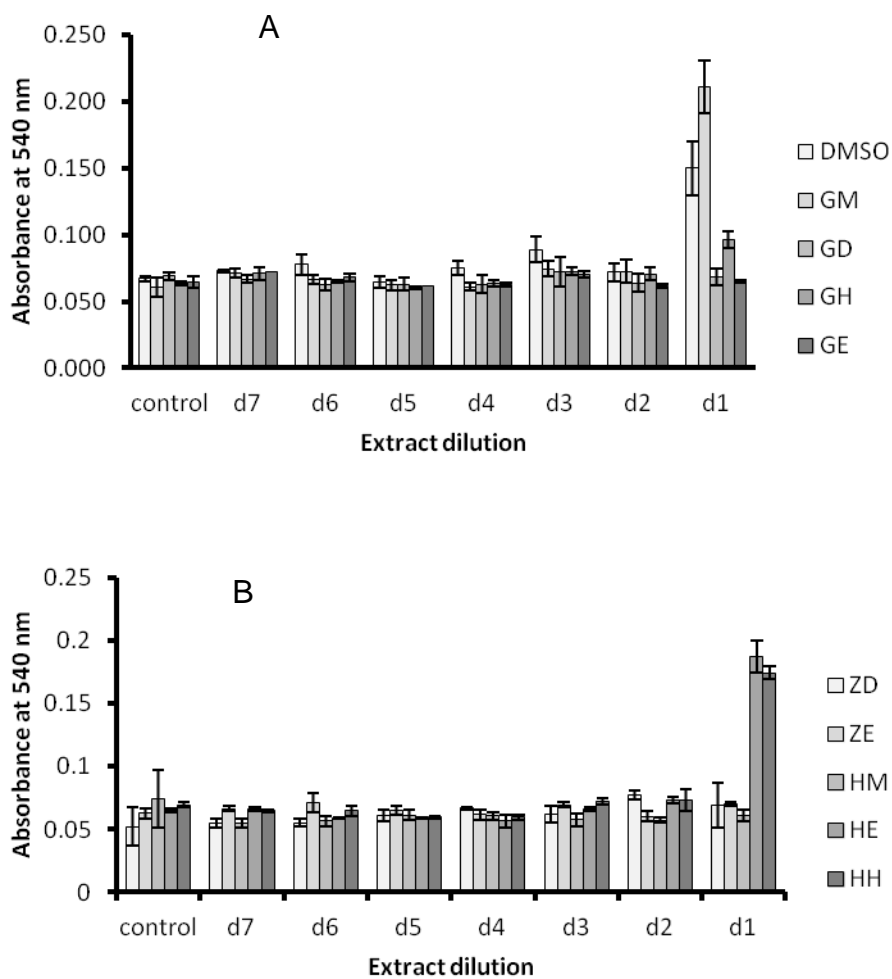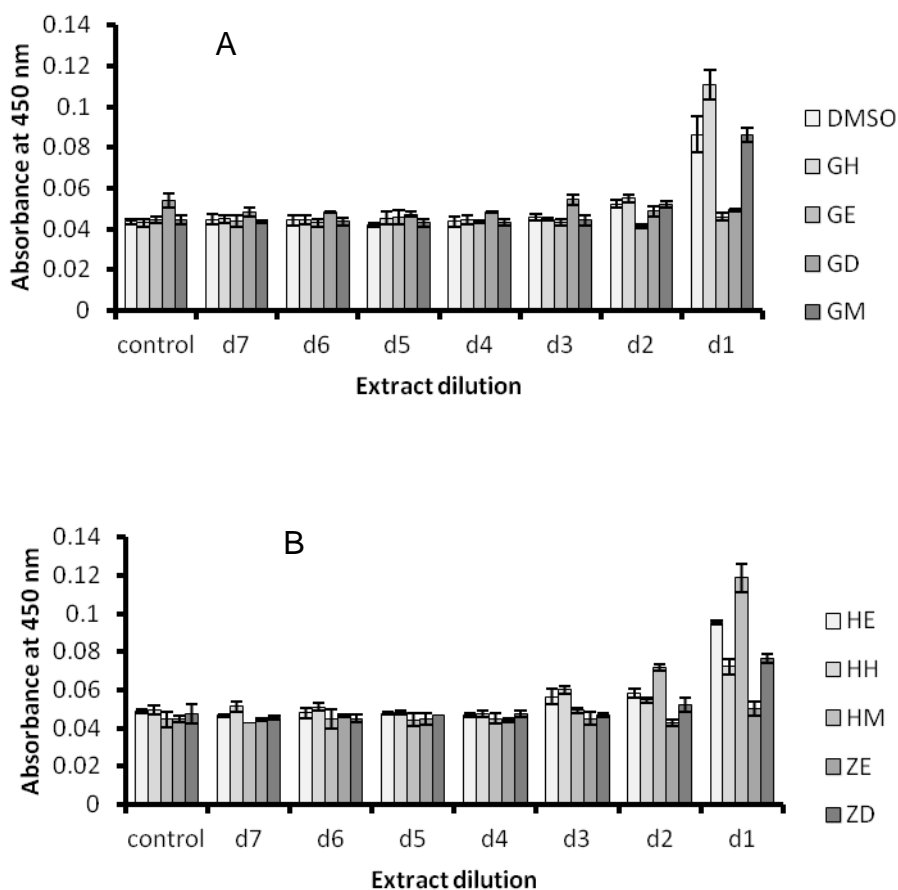

Figure 4.10: The release of H$_2$O$_2$ by H4 cells treated with different concentrations of plant extract, *G. polycephala* (A) and *Z. mucronata* and *H. ciliatum* (B).

4.3.6 APOP*ercantage*^{TM} assay

This assay was used to study the apoptotic effect of extracts of three traditional medicinal plants, and to evaluate its dose-response activity. Apoptosis also known as programmed cell death is a process characterised by cell shrinkage, membrane blebbing and nuclear condensation (Chinkwo, 2005). The morphological changes observed for cells treated with plant extracts include cell shrinkage and disintegration, compared to the cells in the control. Cells in the negative control were more intact and had shape as compared to cells treated with an apoptotic agent, ceramide. Comparing cells treated with plant extracts to the cells of the two control (positive and negative) reveals apoptotic activity of some of the tested extracts. An example of cell morphology observations is shown in **figure 4.11**.
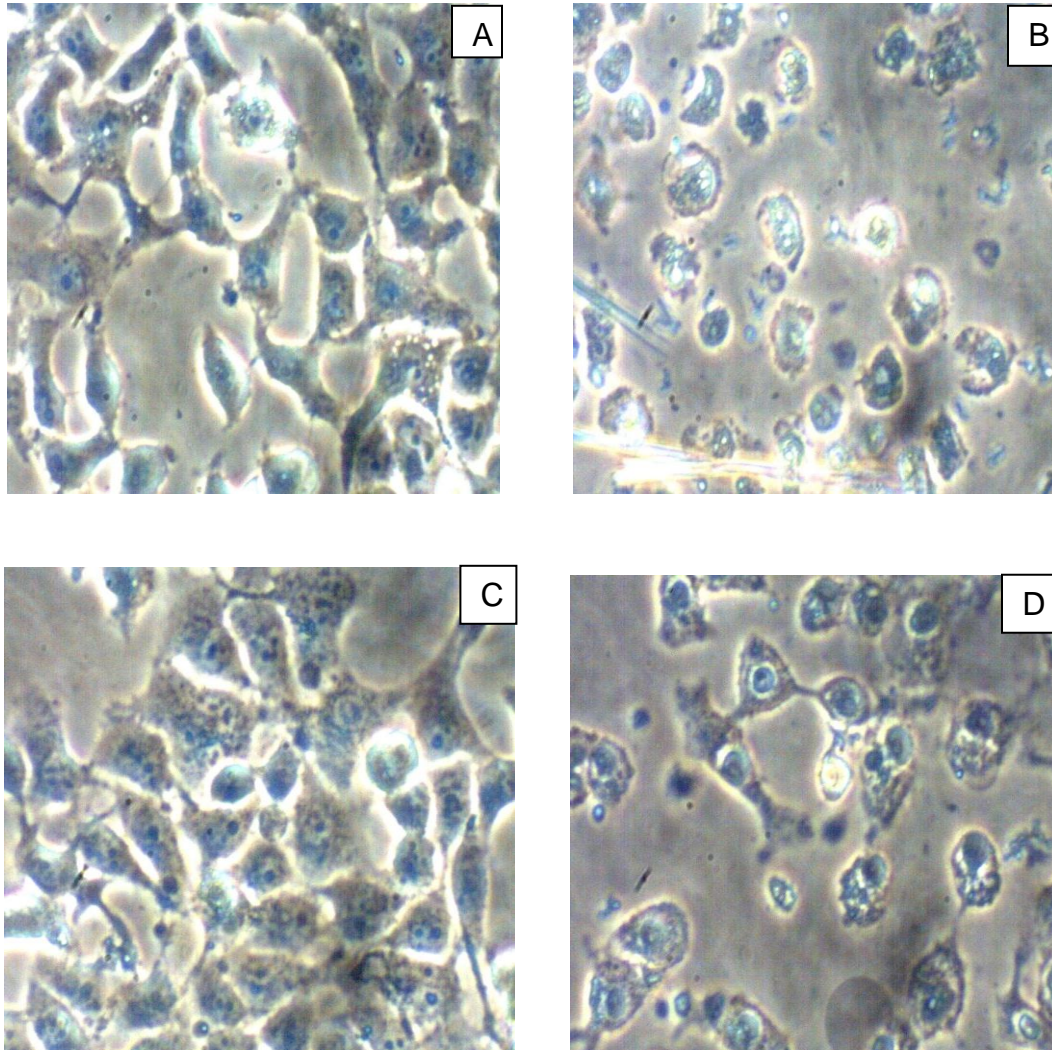
Figure 4.11: Morphological observations of HeLa cells treated with 2.5 mg/ mL plant extracts. Above, untreated control (A), cells treated with 150 µM ceramide (B), cells treated with ZH (C) and cells treated with ZM (D).

As stated in the materials and methods (**section 3.2.2.2.6.2**), acquisition in the APOP*ercentage*™ assay was done by setting forward scatter (FSC) and side scatter (SSC) on a log scale dot plot to differentiate population of cells and cellular debris. On a linear histogram dot plot, APOP*ercentage*™ (FL-3 channel) was measured against

relative cell numbers. Samples of the plot FSC and SSC as well as the histogram dot plot are shown in **figure 4.12**.

A
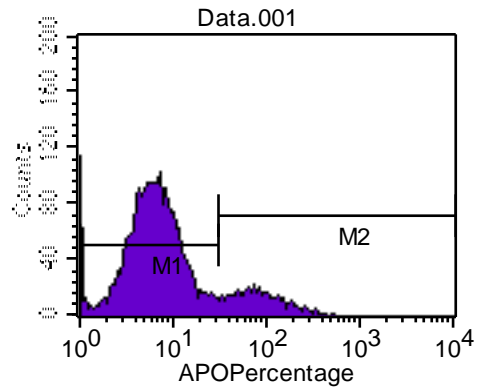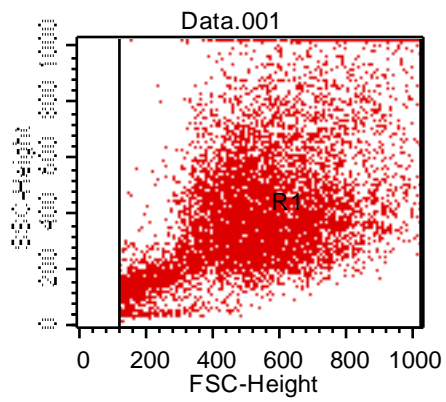
File: Data.001
Tube: Apop HeLa (-

Marker	% Gated	Mean	SD	CV
All	100.00	18.87	36.52	193.57
M1	85.78	7.29	4.99	68.52
M2	14.22	88.73	59.51	67.06

B
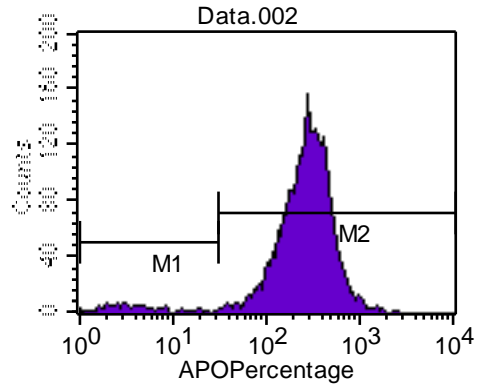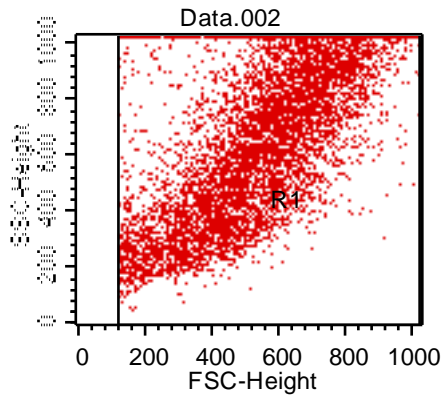
File: Data.002

Tube: Apop HeLa (Cera)+C-150µl

Marker	% Gated	Mean	SD	CV
All	100.00	271.75	166.16	61.14
M1	2.87	5.27	5.93	112.41
M2	97.13	279.62	162.06	57.96

C
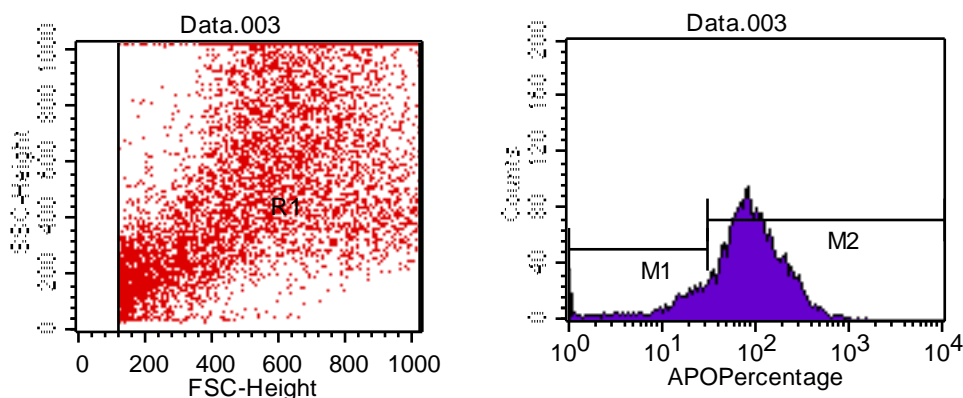
File: Data.003
Tube: Apop HeLa (GCM

Marker	% Gated	Mean	SD	CV
All	100.00	99.51	88.25	88.69
M1	15.06	16.43	8.72	53.07
M2	84.94	114.24	87.84	76.89

Figure 4.12: Forward and side scatter and histogram analysis of HeLa cells stained with APOP*ercentage*™ dye. Untreated cells/ control (A), cells treated with 150 μM ceramide (B) and cells treated with 2.5 mg/ mL of GM (C).

Generally, plant extracts exhibited apoptotic activity in a dose dependent manner and reacted differently towards different cell lines. Although HM was highly active at a concentration of 2.5 mg/ mL against H157 and KMST-6 cells causing percentage cell death of 99.15 % and 95.77 %, a higher percentage cell death was recorded at 5.0 mg/ mL (99.76 % and 99.06 % respectively). The percentage cell death caused by GM increased from 58.46 % against H157 and 61.54% against KMST-6 to 78.52 % and 66.30 % respectively when the concentration of the extract was increased to 5.0 mg/ mL (**figure 4.13 and 4.14**). The LC_{50} for the different extracts tested could not be determined as most extracts exhibited more than 50 % cell death at the lowest concentration tested, 2.5 mg/ mL. The water extracts for the three traditional medicinal

plants were the least active as they caused less than 10 % cell death at the highest concentration tested (**figure 4.15**). Testing the apoptotic activity of different extracts on HeLa cells revealed that, at a concentration of 2.5 mg/ mL, all extracts except GH, HD and ZH which gave apoptotic percentage of 20.62 %, 42.25 % and 11.06 % respectively, were highly apoptotic, killing more than 50 % of the cells, with some causing more than 90 % cell death, activity comparable to that of a conventional drug ceramide (150 µM) (**figure 4.16**).
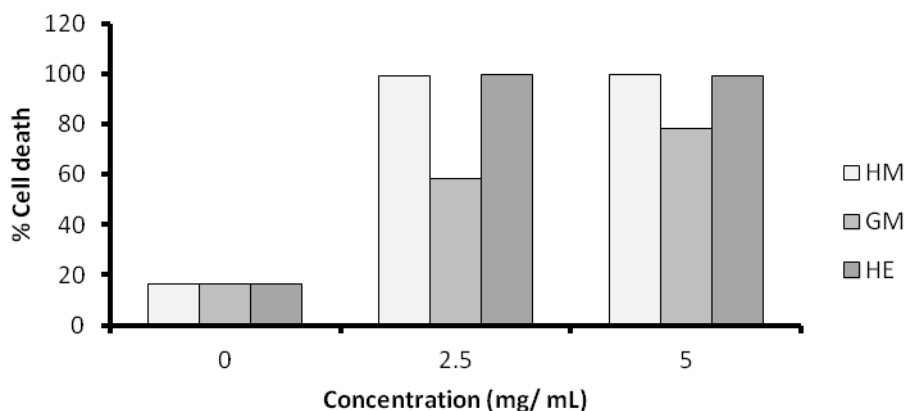

Figure 4.13: The effects of selected traditional medicinal plant extracts on H157 cells
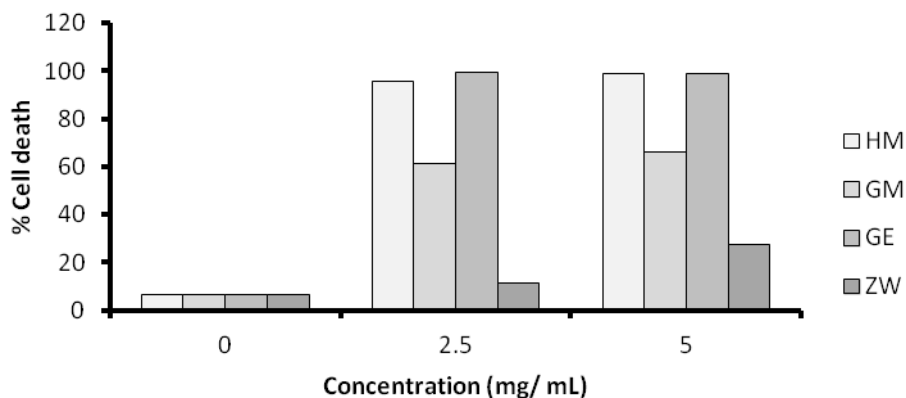
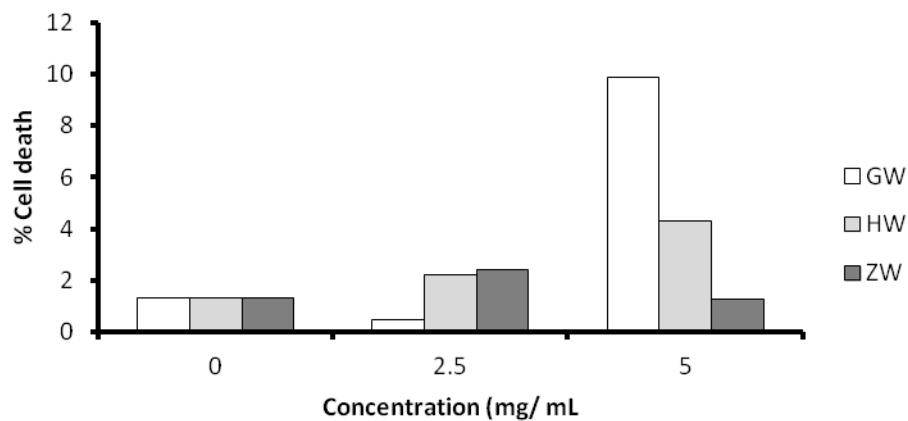**Figure 4.14:** The effects of selected traditional medicinal plant extracts on KMST-6

Figure 4.15: The effects of water extracts of traditional medicinal plants on the proliferation of CHO cells
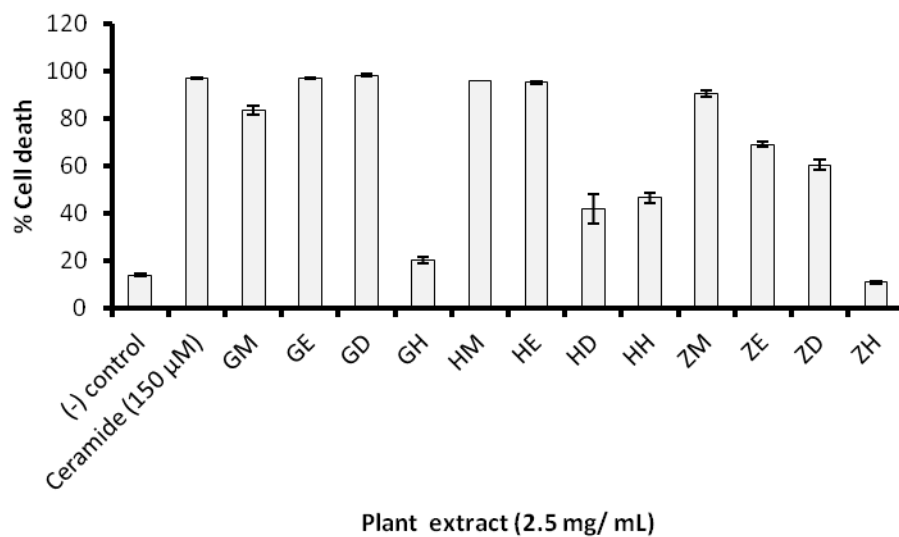

Figure 4.16: Apoptotic effects of different extracts on HeLa cells

4.3.7 Antimicrobial activity

The antimicrobial activity exhibited by the plant extracts were dose and length of exposure dependent, enabling the determination of the MIC of each extract against the pathogens tested. In addition, the antimicrobial activities exhibited by different plant extracts were pathogen specific as they exhibited different antimicrobial activity against different pathogens. The MIC values exhibited by different extracts against different pathogens are presented in **table 4.7**.

A comparison of the MIC of different plant extracts and those of the antibiotics, gentamycin and streptomycin, for the different test organisms revealed, that the plant extracts were generally less active. Some extracts, however, showed better antimicrobial activity than the conventional drug streptomycin against some of the microorganisms tested. Against *M. terrae*, plant extracts were less active than the two antibiotics. HH, HD and ZD (MIC < 0.156 mg/ mL) showed the highest activity against *M. terrae* and GE the least activity against this same microbe (MIC = 20 mg/ mL) compared to gentamycin and streptomycin (0.03125 and 0.07813 mg/ mL). Comparing with gentamycin and streptomycin (MIC < 0.03125 and 5.0 mg/ mL respectively), HM and GD (MIC 5.0 mg/ mL each) were comparable to streptomycin; ZE and ZM (MIC 2.5 mg/ mL each) were more effective than streptomycin. The rest of the extracts were less active, exhibiting MIC $\geq$ 10.0 mg/ mL.

Against *P. aeruginosa*, all extracts with the exception of HD, HE and HH showed better results than streptomycin, with all exhibiting MIC values $\leq$ 1.25 mg/ mL compared to 5.0 mg/ mL obtained for streptomycin. The same can be said about the extracts activity against *S. aureus* though some extracts activity was comparable to that of streptomycin. Against MRSA, all extracts showed better antimicrobial activity than streptomycin (MIC of $\leq$ 1.25 mg/ mL and 2.5 mg/mL respectively). ZD showed better activity than gentamycin (MIC of 0.078 and 0.5 mg/ mL respectively). All extracts showed some

antimicrobial activity against *C. tetani* and *S. epidermidis* with MIC values ≤ 2.5 mg/ mL though in all cases they were less effective than both gentamycin and streptomycin MIC < 0.03125 and < 0.07813 mg/ mL against *C. tetani* and *S. epidermidis* (**table 4.7**).

To ease interpretation, extracts were classified as being good, moderately good, moderate or poor antimicrobial agent using the following criteria (Gibbons, 2004; Ríos and Recio, 2005 in Suliman, 2010):

• MIC ≤ 1 mg/ml: good antimicrobial activity
• MIC > 1mg/ml or < 4 mg/ml: moderately good antimicrobial activity
• MIC = 4 mg/ml or < 6 mg/ml: moderate antimicrobial activity
• MIC ≥ 6 mg/ml: poor antimicrobial activity

ZD extract for example showed good antimicrobial activity against MRSA, *P. aeruginosa* and *S. aureus* and poor antimicrobial activity against *E. coli*. ZE showed good antimicrobial activity against *M. terrae*, MRSA and *C. tetani* and moderately good antimicrobial activity against all other pathogens. ZH showed good antimicrobial activity against all pathogens except for *M. terrae* and *E. coli* were it showed moderate and poor antimicrobial activity respectively. ZM showed good antimicrobial activity against MRSA and *C. tetani*; moderately good antimicrobial activity against *S. epidermidis*, *C. albican*s and *E. coli* and moderate activity against *M. terrae* (**table 4.7**). Generally, the antimicrobial activities of all extracts (except for ZD against MRSA, MIC 0.078 mg/ mL) against all microorganisms tested were less effective than the conventional antibiotic gentamycin.

Table 4.7: The minimum inhibition concentrations (MIC, mg/ mL) of different extracts of *Z. mucronata*, *H. ciliatum* and *G. polycephala* on different pathogens. Note: MT: *M. terrae*, EC: *E. coli,* PA*: P. aeruginosa,* SA: *Streptococcus A,* CA: *C. albicans,* MRSA: Methillicin Resistant *Staphylococcus aureus*, CT: *C. tetani,* SE: *S. epidermidis.*

Extract	MT	EC	PA	SA	CA	MRSA	CT	SE
ZD	<0.156	10	0.625	0.625	>5.0	0.078	0.313	0.313
ZE	0.313	2.5	1.25	1.25	2.5	0.625	0.625	1.25
ZH	5.0	10.0	1.25	1.25	1.25	1.25	1.25	1.25
ZM	5.0	2,5	1.25	1.25	2.5	0,625	0.625	1.25
GD	2.5	5.0	0.313	0.313	2.5	0.313	1.25	0.313
GE	20.0	10.0	0.313	0.313	2.5	0.313	0.625	0.625
GH	1.25	10.0	0.625	0.625	2.5	0.625	0.625	1.25
GM	2.5	10.0	1.25	1.25	2.5	1.25	1.25	0.625
HD	<0.156	10.0	>5.0	1.25	1.25	1.25	1.25	0.313
HE	1.25	10.0	>5.0	1.25	1.25	0.625	1.25	0.625
HH	<0.156	>20	>5.0	1.25	1.25	1.25	1.25	2.5
HM	2.5	5.0	0.625	1.25	2.5	>5.0	1.25	0.625
Gentamycin	<0.0313	<0.0313	0.25	0.25	1.0	0.5	<0.0313	<0.0313
Streptomycin	<0.0781	5.0	5.0	1.25	5.0	2.5	<0.0781	<0.0781

CHAPTER 5

5. DISCUSSION

5.1 Extract yield

Slightly higher extraction yields were obtained with the Soxhlet extraction method as compared to the cold extraction method though the yields were not statistically different. The elevated yield obtained with the former method is attributed to the increase in temperature of the extraction solvent compared to the temperature of the solvent in the cold extraction. This is because the solubility of solute increases with increased temperature (Chang, 1998). However, the risk of destroying valuable substances increases with temperature (Bhat, Nagasampagi, and Sivakumar, 2005).

5.2 Antioxidant activity (DPPH assay)

The DPPH assay was used to assess the antioxidant activity of the extracts of the tested indigenous traditional medicinal plants. DPPH is a stable free radical, which accepts an electron or hydrogen radical to form a stable diamagnetic molecule which is widely used to investigate radical scavenging activity. Antioxidant reacts with the DPPH radicals (Prasad *et al*., 2009). Positive results were obtained from this assay, with *Z. mucronata* exhibiting the most activity, followed by *G. polycephala* and *H. ciliatum* showed the least activity. In all cases, methanol extracts proved to be better antioxidants then the corresponding ethanol extracts (**figure 4.1-4.5**). A pattern of increasing antioxidant activity with increasing polarity has been reported (Goze *et al*., 2009). Because like dissolves like, the polar methanol solvent tends to extract polyphenols and phenolic compounds which are also polar. Polyphenols and phenolic compounds are good antioxidants (Goze *et al*., 2009). Flavonoids, a subclass of the phenolic compounds class have been tentatively identified from the leaves of *Z. mucronata* (Suliman, 2010), which justifies the high antioxidant activity reported for the methanol extract of *Z. mucronata*.

Generally, the presence of carbon-carbon double bond, carbonyl and free hydroxyl groups seems to enhance the free radical scavenging activities of scavengers, since they can donate either a hydrogen radical or an electron to other radicals. By so doing, the scavenger forms a more stable radical (Solomons and Fryhle, 2008).

5.3 Anticancer activity

A few assays were employed in the assessment of the anticancer activity of the extracts of the three traditional medicinal plants, namely: the MTT, BST, NO, H_2O_2 and APOP*ercentage*™ assay. These are common and widely used cytotoxic and anticancer assays employed in the preliminary assessment of toxicity, detection of toxins and evaluating anticancer potential of compounds or extracts. The assays are reliable and the results obtained are a true reflection of the potential of the test compounds as anticancer agents. The NO and H_2O_2 assays produced no significant activity for all the extracts tested (see **figure 4.9 and 4.10**). These results suggest that no NO and/ or H_2O_2 were released during cell death or that these compounds were released in low amounts at the concentrations of extracts tested. Comparing the performance of different extracts in the three anticancer tests BST, MTT and APOP*ercentage*™ assays, the following observations were made. ZD, HE and HM exhibited good anticancer activity in all the three tests performed. HD, ZW, GW and HW extracts were found to be inactive against cancer in all the three anticancer tests performed. GM, GD, ZM showed negative results in the BST test and, positive results in the other two tests. ZE, GE showed positive results only in the APOP*ercentage*™ assay. HH and ZH showed positive results for the BST and/ or MTT assay and negative results in the APOP*ercentage*™ assay (see **section 4.3.1, 4.3.3 and 4.3.6**).

Water extracts of the three traditional medicinal plants showed weak anticancer activity. The low cytotoxic potential of the aqueous extracts is of great significance for their

traditional use in the treatment of various disorders other than cancer (Uddin *et al*., 2011). Aqueous extracts are administered in traditional medicine. And since they are of low toxicity, their use in traditional medicine is justified as the extracts are able to provide toxicity enough to cure a certain ailment but not high enough to intoxicate the cells.

Anticancer activity was reported for at least one or more extracts of the three traditional medicinal plants in at least one anticancer test. Interestingly, antioxidant activity was also reported for some of these extracts. Plant anticancer and antioxidant compounds are secondary metabolites, compounds which according to Balandrin *et al*. (1985) can be restricted to certain taxonomic groups be it family, genus or species. Though no published work could be located for the three traditional medicinal plants on the exact work done in this study, reports on studies of other species in the same genus and on different topics regarding these plants exist. Some biological activity and chemical composition on species belonging to the same group as the plants under study were reported. Assuming the production of secondary metabolites is restricted to the genus level, some conclusion concerning the observations made in this study can be drawn.

The majority of plant based secondary metabolites are phenolic compounds, alkaloids, flavonoids and tannins (Uddin *et al*., 2011; Wong, Li, Cheng and Chen, 2006; Gupta, Mazumder, Kumar, Sivakumar and Vamsi, 2004). These natural products possess diverse pharmacological properties including cytotoxic and cancer chemopreventive effects. Flavonoids, triterpenoids and steroids particularly exert multiple biological effects due to their antioxidant and free radical scavenging abilities (Gupta *et al*., 2004). Studies have shown antioxidant and anticancer activity to be associated with a variety of classes such as polyphenols, flavonoids and catechins (Uddin *et al*., 2011).

The results of this study are supported by the literature. Numerous alkaloids, flavonoids and anthocyanins have been isolated from *Z. mucronata* (Suliman, 2010). Anticancer

activity of *Z. jujube* against HepG2 cells has also been reported (Huang, Kojima-Yuasa, Norikura, Kennedy, Hasuma and Matsui-Yuasa, 2007). Some triterpenoid acids isolated from *Ziziphus jujube* have shown moderate anticancer activity against some cell models, (HT-29, HepG-2 and NCI-H460) using the MTT method (Guo, Duan, Tang, Su, and Qian, 2011). Pyrrolizidine alkaloids and indicine-n-oxide was reported for *H. indicum* and other *Heliotropium* species (Spjut, 1985; Velasco *et al*., 2005). *In vivo* anticancer and antioxidant activity was also reported for methanol extracts of *H. zeylanicum* against Ehrlich ascites carcinoma cells in Swiss albino mice (Kandasamy *et al*., 2005). Diterpene esters and coumarins have also been reported for *Gnidia* species (van Wyk and Gericke, 2000). Antitumor activity has also been reported for *G. kraussiana* (van Wyk *et al*., 1997). All these are in support of the findings of this study.

The mechanism behind the exhibited biological activity is not known but speculations have been made. Polyphenolic compounds might inhibit cancer cells by xenobiotic metabolizing enzymes that alter metabolic activation of potential carcinogens, while some flavonoids could also alter hormone production and inhibit aromatase to prevent the development of cancer cells. The mechanism of action of anticancer activity of phenolics could be by disrupting cellular division during mitosis at the telephase stage. It was also reported that phenolics reduce the amount of cellular protein, mitotic index and colony formation during cell proliferation. The more the number of hydroxyl groups in the phenolics, the greater is the antioxidant activity. The presence of a 4-carbonyl group of the flavonoid molecule also contributes to the anticancer activity. In addition, the presence of 2, 3-double bond in the flavonoid molecules correlates with mitochondrial damage and cancer cell death (Prasad *et al*., 2009).

Some extracts of these traditional medicinal plants have the potential to yield useful antioxidant and anticancer drugs. However, further studies need to be conducted. This includes the isolation of potentially useful drugs from these extracts.

5.5 Antimicrobial activity

5.5.1 Sensitivity of the tested pathogens

An analysis method described by Suliman (2010) was used to assess the sensitivity of the tested organisms. This method entails two criteria: the first being the number of plant extracts that showed good antimicrobial activity i.e. MIC value of $\leq$ 1 mg/ mL and the second is the average MIC value obtained by the plant extracts against a particular pathogen. This data is shown in **table 5.1**.

Seven extracts of the twelve tested plant extracts obtained good antimicrobial activity (MIC value $\leq$ 1 mg/ mL) against MRSA and *S. epidermidis*, making the two pathogens the most sensitive of the eight tested pathogens. *P. aeruginosa* and *C. tetani* were the second sensitive as good antimicrobial activity (MIC value $\leq$ 1 mg/ mL) against these pathogens was obtained in five of the twelve extracts tested. *E. coli* and *C. albicans* were the least sensitive of the eight pathogens as no good antimicrobial activity was obtained against these pathogens by the plant extracts tested.

With regard to the average MIC values, the lowest average MIC value (0.91 mg/ mL) was obtained against *S. epidermidis*, followed by *C. tetani*, *Streptococcus* A and MRSA obtaining average MIC values of 0.96 mg/ mL, 0.99 mg/ mL and 1.10 mg/ mL respectively. *C. albicans*, *M. terrae* and *E. coli* were the least sensitive obtaining MIC values of 2.29 mg/ mL, 3.40 mg/ mL and 8.75 mg/ mL respectively (**table 5.1**).

Table 5.1: Summary of antimicrobial activity of plant extracts against wound pathogens

Pathogen	Number of extracts with MIC value ≤ 1 mg/ mL	Average MIC value (mg/ mL)
M. terrae	4	3.40
E. coli	0	8.75
P. aeruginosa	5	1.88

Streptococcus A	4	0.99
C. albicans	0	2.29
MRSA	7	1.10
C. tetani	5	0.96
S. epidermidis	7	0.91

5.5.2 Extract that showed the best antimicrobial activity

To determine which extract exhibited the best antimicrobial activity against the microorganisms tested, a method described by Suliman (2010) was used. This method makes use of two criteria: first, the lowest MIC value of each extract and the pathogen against which this MIC value was obtained and second, the number of pathogens against which the extract obtained an MIC value of ≤ 1 mg/ mL. This summarizes the pathogens against which the plant extracts obtained good antimicrobial activity.

Dichloromethane extracts of *Z. mucronata* (ZD) obtained the lowest MIC value (0.078 mg/ mL) among all extracts tested against MRSA. HD and HH obtained the second lowest MIC values (<0.156 mg/ mL) against *M. terrae*. The next lowest MIC value (0.3125 mg/ mL) was obtained for ZE, GE and GD against *M. terrae*, *P. aeruginosa*, *Streptococcus* A, MRSA or *S. epidermidis*. For all extracts tested, an MIC value ≤ 1 mg/ mL was obtained against at least one of the pathogens, except for ZH extract whose lowest MIC value was 1.25 mg/ mL. This makes the majority of the extracts screened good antimicrobial agents against pathogens for which these MIC values were obtained.

With regard to the second criterion which was the number of pathogens against which the plant extracts obtained MIC value ≤ 1 mg/ mL, ZD demonstrated the most broad-spectrum activity, exhibiting good antimicrobial activity against six of the eight test pathogens. GE followed , obtaining an MIC value ≤ 1 mg/ mL against five of the eight

tested pathogens followed by GD and GH, which both exhibited good antimicrobial activity against half (4) of the tested pathogens. ZH was the least active, not obtaining an MIC value of ≤ 1 mg/ mL against any of the tested pathogens (**table 5.2**).

Table 5.2: Lowest MIC values obtained per plant extracts and pathogens against which MIC values of ≤ 1 mg/ mL were obtained.

Plant extract	Lowest MIC value (mg/ mL)	Pathogen against which the lowest MIC value was obtained	Pathogens against which the extract obtained an MIC value of ≤ 1 mg/ mL
ZD	0.078	MRSA	*M. terrae*, *P. aeruginosa*, *Streptococcus* A, *C. tetani*, *S. epidermidis*, MRSA
ZE	0.3125	*M. terrae*	*M. terrae*, *C. tetani*, MRSA
ZH	1.25	*P. aeruginosa*, *Streptococcus* A, *C. tetani*, *S. epidermidis*, MRSA, *C. albicans*	None
ZM	0.625	*C. tetani*, MRSA	*C. tetani*, MRSA
GD	0.3125	*P. aeruginosa*, *Streptococcus* A, *S. epidermidis*, MRSA	MRSA, *P. aeruginosa*, *Streptococcus* A, *S. epidermidis*
GE	0.3125	*P. aeruginosa*, *Streptococcus* A, MRSA	MRSA, P. aeruginosa, *Streptococcus* A, *C. tetani*, *S. epidermidis*
GH	0.625	*P. aeruginosa*,	*C. tetani*, *P. aeruginosa*,

		Streptococcus A, MRSA, *C. tetani*	*Streptococcus* A, MRSA
GM	0.625	*S. epidermidis*	*S. epidermidis*
HD	<0.156	*M. terrae*	*S. epidermidis*, *M. terrae*
HE	0.625	MRSA, *S. epidermidis*	MRSA, *S. epidermidis*
HH	<0.156	*M. terrae*	*M. terrae*
HM	0.625	*P. aeruginosa*, *S. epidermidis*	*P. aeruginosa*, *S. epidermidis*

ZD, ZE, ZM, GD, GE, GH, GM, HD, HE, HH and HM all exhibited good antimicrobial activities against some of the pathogens tested. ZD, ZE and ZM exhibited good antimicrobial activity against *M. terrae*, *P. aeruginosa*, *Streptococcus* A, *C. tetani*, *S. epidermidis* or MRSA. Antimicrobial activity reported for extracts of *Z. mucronata* was not unexpected as antimicrobial activity of different extracts of *Z. mucronata* against different pathogens has been reported. In a study by Mthethwa (2009), good antimicrobial activity for methanol and acetone extract of both the leaves and bark of *Z. mucronata* against *S. aureus*, *Klebsiella pneumoniae* and *Bacillus subtillis* have been reported (MIC ≤ 6.25 mg/ mL). Antimicrobial activity of ethanol extracts of *Z. mucronata* against *S. aureus* and *E. coli* has also been reported (Adamu, Abayeh, Agho, Abdulahi, Uba, Dukku, *et al*., 2004). Olajuyigbe and Afolayan (2011) also reported antimicrobial activity of methanolic extracts of the bark of *Z. mucronata* against *E. coli* and *S. aureus*.

The good antimicrobial activity of some plant extracts against some of the pathogenic strains reported in this study also supports the use of these plant extracts in traditional

medicine. The traditional medicinal plants investigated in this study are all used in the treatment of wounds, and the antimicrobial activity reported for extracts of these plants supports the traditional use of these plants. According to reports, the prevention of microbial wound infections is one of the most essential factors in wound healing and wound management (Ayyanar and Ignacimuthu, 2009, Davis and Perez, 2009). However, more studies need to be done to confirm the role of the different natural products in the observed biological activity.

CONCLUSION

The yields per extraction of the two extraction methods were not significantly different, which suggests the use of the cold extraction method to lower the risk of destroying valuable compounds. High antioxidant activity was reported for methanol extracts of *Z. mucronata*. This could be attributed to flavonoids, alkaloids and terpenes, as identified in preceding reports. High apoptotic activity was reported for *H. ciliatum*. In the literature, *Heliotropium* species were reported to possess toxic pyrrolizidine alkaloids and indicine-n-oxide. Antitumor activity has also been reported in some *Heliotropium* species. Diterpenes have been reported in *Gnidia* species, which could explain the toxicity observed for *G. polycephala*, though further analysis is required to confirm this. Low toxicity was reported for the water extracts of the three plants. Water extracts are usually used in traditional medicine and the low toxicity observed here is of significance to their use in the treatment of other diseases and ailments other than cancer. The low toxicity could also suggest low concentration of the active compound since crude extracts were analysed. Antimicrobial activity of extracts against different wound pathogens was reported, which is not surprising as these plants are also used in the treatment of wounds.

However, a lot of aspects of this research need to be further investigated to confirm the observed activity. The simple assays used in this study gave good results about the potential of these plant extracts as antioxidant, anticancer and antimicrobial agents. To confirm the activity of the promising extracts, more in-depth studies are required. These includes the employing of other antioxidant assays such as the total antioxidant assay, total phenolic assay and the superoxide assay just to mention a few, and compare the antioxidant potential of the extracts through different methods. For anticancer assay, numerous cell lines were used, some of which were only used against some of the extracts and not others. In addition, for some tests such as the APOP*ercentage*™ assay, the test concentrations were high (2.5 and 5.0 mg/ mL) and as a result, it was not

possible to determine the IC_{50} concentrations. In future, the use of fewer cell lines and rather the test of all plant extracts at different concentrations against the cell lines is suggested as this will enable the comparison of the activity of different extracts against a particular cell line and also give their IC_{50} concentrations. An assay-guided fractionation and separation of the crude extracts is also recommended as this will give more information as to which fraction of the crude extract is active and the percentage activity as compared to the crude extract.

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