The therapeutic value of *Aloe ferox* Mill.

By

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Dissertation submitted in fulfilment of the requirements for the degree:

MAGISTER TECHNOLOGIAE: BIOMEDICAL TECHNOLOGY

in the

DEPARTMENT OF HEALTH SCIENCES

at the

CENTRAL UNIVERSITY OF TECHNOLOGY, FREE STATE

BLOEMFONTEIN

2014

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Declaration of independent work

I Refilwe Mhaladi, Identity number BN0009771 and Student number 208090177, do hereby declare that this research project submitted to the Central University of Technology, Free State for the Degree Magister Technologiae: Biomedical Technology, is my own independent work. It complies with the Code of Academic Integrity, as well as other relevant policies, procedures, rules and regulations of the Central University of Technology, Free State. It has not been submitted before to any institution by myself or any other person in fulfilment (or partial fulfilment) of the requirements for the attainment of any qualification.

13/11/2014

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Summary

The rising costs of health care, the outbreak of drug resistant organisms, health depleting lifestyles and the risky side effects of currently used drugs are world-wide problems. This has led to the search for novel drugs and drug leads. Traditional healers and other individuals across the globe possess unlimited knowledge on the healing powers of different plants that has been passed on through generations. This knowledge together with scientific investigations can lead to the eradication of most of the diseases either by treatment or prevention.

Aloe ferox Mill. is one of the plants that have gained a lot of interest from the pharmaceutical industry. The plant has over 80 documented medicinal uses. These include treatment of impotence and infertility, sexually transmitted infections, arthritis, hypertension, leukaemia, bacterial and fungal infections. It is also known as a blood purifier, widely used as a laxative and anti- inflammatory agent. More research is required to discover more about *A. ferox* and its benefits to health as well as to investigate its potential for the development of novel drugs.

The current study was focused at investigating the anti- cancer, anti- microbial anti-diabetic, cytotoxic activities and phytochemical composition of leaf extracts of *A. ferox*. Three cancer cell lines namely: breast (MCF7), colon (HCT116) and prostate (PC3) cancer cell lines were used to investigate the anticancer activity of the extracts using the Sulforhodamine B (SRB) method.

To determine the anti- diabetic activity of the plant extracts the C2C12 and Chang cell *in- vitro* models of glucose uptake were used. The micro- dilution technique was

used to evaluate the antibacterial and antifungal activity of the extract. The safety of these extracts against normal human foetal lung fibroblasts (W138), Chang and C2C12 cells was done by through the SRB and the MTT methods. To determine the phytochemical profile of *A. ferox* the DPPH radical scavenging and the Folin Ciocalteu methods were used to test the antioxidant activity and the total phenolic content of the different extracts respectively. Different methods were used to determine the presence of phytochemicals such as steroids, saponins, alkaloids, carbohydrates and flavonoids. LCMS was also done to detect the elemental composition of the plant extracts.

According to the CSIR criteria *A. ferox* was inactive against the cancer cell lines used. It however exhibited antioxidant activity even at low concentrations, with an EC50 of 0.865 ± 0.783. The methanol extract showed more phenolic content than the dichloromethane and aqueous extracts at a concentration of 5mg/ml. It is believed that the antioxidant activity correlates with the phenolic content and quality of the phenols present in the plant and more assays have to be done to prove this hypothesis. Other phytochemicals found in the extract included saponins, steroids, alkaloids as well as flavonoids. Both the methanol and aqueous extracts *of A. ferox* caused a significant increase in glucose uptake by C2C12 cells but caused a slightly decreased uptake by the Chang cells. The plant extracts inhibited the growth of *Staphylococcus epidermidis, Streptococcus pneumonia, Escherichia coli* and *Candida albicans* at a concentration of 15mg/ml extract. *Candida tropicalis* and *Escherichia faecalis* were resistant to *A. ferox* extracts. Finally the extracts showed no toxic activity against the normal foetal lung fibroblasts, Chang and C2C12 cells validating the safety of this plant for human use.

The results in conjunction with literature findings show *A. ferox* to be a promising source of drugs and therapeutic agents. Due to the fact that traditional healers already rely on it as treatment for different ailments, it is important that the safety of the plant for use has been validated though other studies and clinical trial still need to be done to fully confirm this. All the information gathered also showed this plant to be of great benefit against major health problems, responsible for millions of deaths each year such as cancer, cardiovascular and inflammatory diseases, and diabetes. There is however still a great need for more investigation to be done on this plant against a vast majority of organisms and diseases so as to fully benefit from it.

Acknowledgements

I would like to sincerely thank the following:

1. God Almighty for all His blessings and giving me the power and wisdom to go above and beyond, not just to progress but excel.
2. Prof S.S Mashele and Dr I.T Manduna for your guidance from the beginning of this project to the very end. Thank you, I have surely learnt a lot.
3. My dear departed mother Nchadi for all her hard work ensuring that I get the best life possible. She will forever be my inspiration.
4. My dear sister Tumie, thank you for keeping me on my toes, making me laugh even during the toughest times and giving me a purpose to strive for a better life always.
5. My dear friends and partners through the endless nights of research Mrs K Polori and Mr D. F. Onyeka Okolie. The struggle and stress was better with you around.
6. My family, the Mbayi's, words cannot describe how thankful and grateful I am to have you in my life. Your love and support pulls me through every time.
7. The Free State University Agriculture department (Prof JC Pretorius and Dr E van der Watt) for offering assistance where we were stranded without some equipment.
8. The Biomedical Technology programme colleagues for their support and advice.

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ABBREVIATIONS

Abbreviation	Meaning
AGEs	Advanced glycation end products
ATP	Adenosine tri-phosphate
BHA	Butylated hydroxy anisole
BHT	Butylated hydroxy toulene
C2C12	Muscle cell line
COX	Cyclooxeygenase
CSIR	Council for Scientific and Industrial Research
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DPPH	2, 2-dipheny-1- picrylhydrazyl
ECACC	European Collection of Cell Cultures
EMEM	Eagle' s Minimal Essential Medium
GAE	Gallic acid equivalents
GCMS	Gas chromatography/mass spectrometry
GI50	Growth inhibition (50%)
HCT116	Colon cancer cell line
HIV	Human immunodeficiency virus
IASC	International Aloe Science Council
INT	p-iodonotrotetrazolium violet
LCMS	Liquid chromatography/mass spectrometry
MCF7	Breast cancer cell line
MIC	Minimum Inhibitory Concentration

MRC	Medical Research Centre
MTT	Mossmann's Tetrazole Test
NCI	National Cancer Institute
NO	Nitric oxide
OH	Hydroxyl
PC3	Prostate cancer cell line
RNS	Reactive nitrogen species
RO_2	Peroxyl
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
SRB	Sulforhodamine B assay
STI	Sexually transmitted infections
TB	Tuberculosis
TCA	Trichloroacetic acid
TGI	Total growth inhibition
TLC	Thin layer chromatography
UV	Ultraviolet
W138	Normal human foetal lung fibroblast cell line
WHO	World Health Organization

CHAPTER 1

Background

The need for less expensive health care has led to an increased search for and use of medicinal plants. Different plant parts, such as leaves, bark, flowers, fruit, stems and roots, or the whole plant can be used to obtain active compounds for therapeutic use. Plants serve not only as novel sources of drugs, but also as drug leads for optimisation by medicinal and synthetic chemists (Balunas and Kinghorn, 2005). The therapeutic activity of plants has been explained as a result of the synergistic effects of phytochemicals in the different plants. Drug discovery from plants has evolved from the use of crude preparations to the isolation and identification of active compounds (Balunas and Kinghorn, 2005). Predetermined and novel molecular targets in diseases can be targeted through the identification and isolation of some compounds from particular plants, giving them the ability to have selective activity in eradicating the disease (Kramer and Cohen, 2004; Balunas and Kinghorn, 2005). Examples include indirubin, which selectively inhibits cyclin-dependent kinases (Hoessel *et al*., 1999; Eisenbrand *et al*., 2004) and β-lapachone, selectively targeting cancer cells over normal cells through checkpoint activation during the cell cycle (Li *et al*., 2003; Balunas and Kinghorn, 2005).

Some of the earliest drugs isolated from plants include digitoxin, codeine, cocaine, quinine and morphine, of which some are still in use (Newman *et al*., 2000; Butler, 2004; Samuelsson, 2004). Between 1981 and 2002 around 48% of all new chemical entities were obtained from natural products or their derivatives (Butler, 2004). Table

1.1 shows some of the newly identified, validated and approved plant-derived drugs that were introduced to the market.

The significant role of plants in drug discovery is most evident in the areas of cancer and infectious inflammatory diseases, where respectively 60% and 75% of the drugs used are of natural origin (Newman *et al*., 2003). These diseases are some of the main causes of mortality worldwide. However, other diseases, such as tuberculosis and diabetes, are also rapidly becoming part of the main causes of premature deaths worldwide, with a person dying every few seconds from these diseases or from the resulting complications.

Extensive research has shown a negative correlation between the consumption of a diet rich in fruit and vegetables and the risk of chronic diseases such as arthritis, cardiovascular diseases, chronic inflammation and cancer (Zhang *et al*., 2005). Plant-based sources of medication are a highly promising and untapped source of pharmaceutical drugs that are effective in the prevention and treatment of diseases in view of their abundant phenolic content, among other beneficial secondary compounds (Conforti *et al*., 2008). A great deal of progress has been made in areas of research to find effective and therapeutically valuable compounds from plants. However there is still a lot to be done to find and identify more plants with compounds or a combination thereof with fewer or no side effects on normal cells or physiological activities.

Table 1.1. Plant-derived drugs recently introduced to the market.

Drug name	Trade name	Plant name (Source)	Use	References
Arteether	Artemotil	*Artemisia annua* L.	Anti-malarial drug	Van Agtmael *et al*., 1999; Graul, 2001.
Galantamine	Reminyl	*Galanthus woronowii* L.	Treatment of Alzheimer's disease	Heinrich and Teoh, 2004; Pirttila *et al*., 2004.
Nitisinone	Orfadin	*Callistemon citrinus* Stapf.	Tyrosinaemia	Hall *et al*., 200lb; Mitchell *et al*., 2001.
Tiotropium	Spiriva	*Atropa belladonna* L. and other members of the Solanaceae family	Chronic obstructive pulmonary disease	Mundy and Kirkpatrick, 2004; Frantz, 2005.

(Balunas and Kinghorn, 2005)

Traditional medicine is the pivot of most of the current search for drugs and drug leads worldwide. There is no tribe or country that does not depend on plants and herbs for some form of therapy. Semenya *et al*. (2012) stated that about 80 - 85% of black South Africans in both urban and rural areas use traditional medicine from local healers and herbalists. Most of these plants are sold in local markets, but not all of them have been scientifically proven to work. Validating the use of these plants in the health care system requires scientific investigations on their effects, safety, quality as well as appropriate dosages (Masika and Afolayan, 2002). Only 10% of the higher plant species have been studied chemically and pharmacologically.

1.1. Literature review

Oxidative stress results from the overproduction of reactive oxygen species (ROS) and reactive nitrogen species (RNS), which are products of normal cellular metabolism (Valko *et al*., 2006). At high concentrations they are harmful and function by mediating the destruction of the cell structure, cellular lipids and proteins, as well as cell membrane and deoxyribonucleic acid (DNA).These species are the key players in the development of diseases, especially tumoural and inflammatory diseases such as cancer, atherosclerosis, tuberculosis, diabetes, cardiovascular and neurodegenerative disorders.

Antioxidant defences are just one of the mechanisms developed by organisms to protect themselves against free radicals (Cadenas, 1997; Valko *et al*., 2007). There are different types of antioxidants and different mechanisms through which they function. The following is a list of some of the different mechanisms of action:

1. Degradation of free radicals by enzymes such as superoxide dismutase, ascorbate peroxidase and catalase (Mittler, 2002).
2. Metals enhancing free radical production by proteins such as transferrin, antioxidants binding to these metals, preventing free radical production.
3. Free radical scavenging by antioxidants such as vitamin C and E, as well as glutathione (Pencofer *et al*., 2002).

The plant antioxidants studied most frequently are polyphenols, which are abundant in plants, hence those with the strongest antioxidant activity and total phenolic content are the most valuable (Larkins and Wynn, 2004). Some plant antioxidants include cinnamic acids, counarins, diterpenes, flavonoids, lignans, monoterpenes, phenylpropanoids, tannins and triterpenes (Larkins and Wynn, 2004).

Flavonoids are the most common group of plant phenols mainly acting as antioxidants that are involved in free radical scavenging (Urquiaga and Leighton, 2000). They can combat cancer by inhibiting initiation, promotion and progression of tumours. Polyphenols function through a number of biological effects such as protecting DNA from oxidative damage, hence inhibiting late age development of cancers and diabetes. In addition to the action of flavonoids, phenolic acids and hydrolysable tannins have anti-carcinogenic and antimutagenic effects (Urquiaga and Leighton, 2000). It is therefore highly advisable for individuals to indulge in a diet rich in fruit and vegetables, which can go a long way in preventing and delaying the onset of chronic diseases.

Cancer results from a disturbance within the cell cycle causing uncontrolled cell proliferation (Ruffa *et al*., 2002). The disease is caused by alterations in oncogenes, tumour suppressor genes as well as messenger ribonucleic acid genes (Carlo and Crose, 2008). These are molecular factors involved in the development and inhibition of cancer. The most frequently studied ones include p53, p21 and Bcl-2; p53 is a tumour-suppressor gene responsible for apoptosis, p21 a cell cycle regulator responsible when activated for causing arrest in the G1 phase of the cell cycle and Bcl-2 is an anti-apoptotic factor (Kuo *et al*., 2002). All the factors are involved in the regulation of the cell cycle; especially those that induce apoptosis are usually the main focus when investigating anti-cancer therapy (Fisher, 1994; Brown and Woulters, 1999).

In 2002 there were an estimated 10.9 million new cancer cases, 6.7 million cancer-related deaths and 24.6 million people living with cancer within five years of diagnosis world-wide (Parkin *et al.,* 2002). These numbers were a significant increase from the estimates made in 2000 of 22 million people living with cancer and 6 million deaths (Parkin *et al*., 2001). According to the statistics, lung cancer is the most common, followed by breast, stomach, liver, colon and rectal cancers respectively.

There is a correlation between the intake of non-steroidal anti-inflammatory drugs and the reduced incidence of certain cancers, including colon, rectum and stomach cancer (Langman *et al*., 2000). This is because a link has been established between cancer and inflammation (Balkwill and Mantovani, 2001). The inflammatory environment allows for easy proliferation, survival and metastasis of tumours through

the selectins, chemokines and receptors. ROS and RNS such as hydroxyl (OH), peroxyl (RO_2) and nitric oxide radical (NO) are observed in chronic inflammatory diseases such as rheumatoid arthritis and hepatitis, as well as in cancers and diabetes (Sabu and Kuttan, 2002). These entities have been experimentally shown to be mutagenic and carcinogenic and they play a key role in the development of inflammatory diseases and their progression to cancer (Wiseman and Halliwell, 1996). Their release leads to tissue damage and the release of cytokines such as interleukin-1 and 6, tumour necrosis factor and interferon-y (Conforti *et al*., 2008). Tumour-associated macrophages, tumour-infiltrating lymphocytes that produce pro-inflammatory cytokines, growth factors, chemokines, signal transducers and transcriptional activators are found on the inflammatory site of tumours. The above-mentioned substances promote growth and invasion by mechanisms such as inhibition of apoptosis, and DNA repair mechanisms (Neergheen *et al*., 2010). The relation between cancer and inflammation permits for the use of anti-inflammatory drugs in the prevention of and inhibition of the progression of cancer.

The current methods for cancer treatment include radiation, chemotherapy and surgery or a combination of two or all of them. There are currently several plant-derived agents that are in clinical use for cancer treatment. These include vinblastine, vicristine and camptothecin derivatives, topotecan, etoposide derived from epipodophyllotoxin and paclitaxed (Cragg and Newman, 2005). However, these treatments are not ideal in view of their toxicity and low survival rates. They have several more side effects that are sometimes just as harmful as the cancer itself, such as leukaemia and anaemia, they also destroy normal cells (McWhirter and Pennington, 1996). The search for selective anti-cancer drugs from plant sources

may lead to the discovery of new drugs that are less toxic and target cell division components, making them selective in their action (Mashele and Kolesnikova, 2010).

Current anti-cancer drugs function by inhibiting the enzymatic activity of certain oncogenes. They also target specific junctions within the cell cycle. Natural phytochemicals have a range of activities ranging from inhibition of genotoxic effects, increased antioxidant and anti-inflammatory activity, inhibition of proteases and cell proliferation to protection of apoptosis and signal transduction pathways (Neergheen *et al*., 2010).

The variety of signs and symptoms which are often unclear that results from cancer make it difficult to find specific antitumor agents (Steenkamp and Gouws, 2006), It is for that reason and the fact that cancer has only recently been considered a common problem that make it rare to get reports of specific anti- cancer agents. Plants and their derivatives that can be used to treat or prevent inflammatory diseases, bacterial, viral and parasitic infections are of great interest in the pharmaceutical industry, as the above-mentioned diseases cause a disease state similar to that of cancer (Cordell *et al*., 1991). Plants and their derivatives have played an important role in the development of numerous clinically useful anti-cancer agents.

Table 1.2 shows examples of plants that display high antioxidant activity, making them potential anti-cancer agents. Their anti-cancer activity and potential as chemo-preventative agents are explained as a result of the detoxifying enzymes and antioxidant enzymes (Neergheen *et al*., 2010). However, the activity may also be

due to the inhibition of the cyclooxygenase activity of COX-2, eg by *Platycodon grandiflorum* (Ahn *et al*., 2005; Neergheen *et al*., 2010) and therefore, the reduction of prostaglandin production.

Table 1.2. Potential plant sources of anti-cancer agents.

Family	Plant species	Antioxidant activity
Asteraceae	*Bacharis grisebachii*	Scavenger of superoxide radicals.
Campanulaceae	*Platycodon grandiflorum*	Inhibition of lipid peroxidation and a scavenger of DPPH.
Ebenaceae	*Diospsyros mellanida* *Diospsyros revaughanii* *Diospsyros neraudii*	Hyprochlorous acid. Hydroxyl and peroxyl free radical scavenging activity.
Fabaceae	*Bauhinia racemosa* *Acacia salicina*	Reduced lipid peroxidation and upregulation of the antioxidant levels. Scavenger of DPPH and inhibitor of superoxide radical production respectively.
Lamiaceae	*Rosmarinus officinalis*	Substantial trolox equivalent activity.
Myrtaceae	*Syzygium commersonii* *Syzygium glomeratum* *Eugenia pollicina*	Hypochlorous acid, hydroxyl radical and peroxyl radical scavenger

Source: (Neergheen *et al*., 2010)

Diabetes is an endocrine disorder characterised by hyperglycaemia caused by complete or relative insulin deficiency (Narkhede *et al*., 2011). By 2007 there were 3.5 million diabetes-related deaths worldwide, most of which resulted from cardiovascular complications (Das and Rai, 2008). By 2011 an estimated 25% of the world population was affected by this disease (Kavishanka *et al*., 2011). Other complications, such as glucose intolerance and hyperglycaemia, might manifest in cellular damage, renal and retinal, as well as neurological diseases (McCue *et al*., 2005). Previously termed “rich people’s disease”, as it was prevalent in developed societies, diabetes has since spread to other societies owing to changing lifestyles (Narkhede *et al*., 2011).

There are many different types of this disease; however, types 1 and 2 are most prevalent. Type 1 diabetes is autoimmune, characterised by destruction of the insulin-secreting beta cells of the pancreas, leading to insulin deficiency. Type 2 is characterised by insulin resistance or abnormal secretion of insulin (Shafrir, 1996; Semenya *et al*., 2012). Oxidative stress is seen in both types of diabetes (Nazirogilu *et al*., 2005) and leads to pathogenesis and resulting complications (Rahimi *et al*., 2005; Shih *et al*., 2002). Oxidative stress is due to increased glucose oxidation, leading to the formation of free radicals, a decrease in enzymes that remove the free radicals and the increased binding of advanced glycation end products (AGEs) to the AGE receptors. The binding to receptors can lead to the modification of cell signalling, leading to further free radical production causing more complications (Pencofer *et al*., 2002). Diabetes places affected individuals at high risk of contracting other diseases such as tuberculosis (TB); according to the World Health Organization (WHO) about 10% of all TB cases are linked to diabetes (WHO, 2011).

Phenolic- rich Chinese and Indian plants have long been used to treat diabetes (Grover *et al*., 2002). In addition, more plants that have anti-diabetic properties are being discovered, eg *Caesalpinia digyna* and other members of the *Caesalpinia* genus, such as *C. sappan* and *C. bonducella*.

Diabetes mellitus 2 can be managed through reduction of the postprandial blood glucose level by targeting and reducing the enzymes involved in carbohydrate metabolism alpha amylase and alpha glucosidase (Narkhede *et al*., 2011). Phytochemicals in medicinal plants are thought to function among other mechanisms through regeneration of the pancreatic beta cells leading to insulin release (Kavishankar *et al*., 2011). The Asphodelaceae family has lately generated a lot of interest in the pharmaceutical industry because of its rich phytochemical content and its local traditional use for ailments such as diabetes, hypertension, cancer, sexually transmitted diseases and wound healing, among others.

Aloe L. is a big genus comprising over 500 species (Frodin, 2004; Grace, 2011), with about 60 species of aloes documented in traditional medicinal use in Southern Africa (Grace *et al*., 2008; Grace *et al*., 2011). The most recorded species in use are *A. ferox, A. marculata, A. marlothii, A. micrantha, A. tenuior* and most of all *A. vera* (Williams *et al*., 2001). Two materials obtained from aloe leaves seem to be the basis of all aloe-derived products thus far. These are the leaf exudates, mostly used for their laxative properties, and the leaf mesophyll, used for general, skin and digestive problems (Grace, 2011).

More than half of the aloe species have well-documented medicinal uses as well as being used for general well-being worldwide (Glen and Hardy, 2000). Currently the main source of these products is *Aloe vera*. Its use has even led to the creation of a massive multi-billion dollar industry (International Aloe Science Council, 2004). This business uses even the gel, mainly in cosmetics and to make tonic drinks (O' Brien *et al*., 2011). The leaf pulp may be applied directly to the problem area without preparation to treat ring worm (Reynolds, 1950) or to dress wounds (Morton, 1961). The aloe-emodin as well as the anthraquinone content found in this species has been used as the main explanation for its therapeutic use as a laxative. Not many other aloe species have been extensively studied but *A. ferox* rapidly gained a lot of interest in the pharmaceutical industry after 1994 when its first gel was produced (Botha, 1994). The gel from *A. ferox* has been compared to that of *A. vera*, but some differences, mainly within the polysaccharides after hydrolysis, have been discovered (O' Brien *et al*., 2011). *A. vera* only yields mannose, whereas different *A. ferox* species can either produce glucose only or galactose-glucose in a ratio of 1:1 or 1:2 (O' Brien *et al*., 2011). *A. ferox* has become one of the most interesting and studied aloes after *A*. *vera* and South Africa remains the largest exporter of this aloe.

The name "aloe" is derived from the Greek word for its dried juice and "Ferox", meaning "fierce", is from the appearance of its leaves, which have spear-shaped edges. The other names of this plant include iKhala (Xhosa), iNhlaba (Zulu), bitteraalwyn (Afrikaans) and red aloe or bitter aloe (English) (O' Brien *et al*., 2011). It grows up to 2 - 3 metres in height and has dull green leaves that cover the entire stem. Even after drying the leaves remain intact, forming a "petticoat". The flowers are found in large numbers on a candelabra-like flower head, as shown on Figure

1.1. There are usually five to eight branches, each carrying a spike-like head of flowers whose colour varies from yellow-orange to bright red.

Figure 1.1. *Aloe ferox* Mill.

A. ferox is widely distributed on rocky hill slopes and is found from the South Western Cape through KwaZulu-Natal and the Free State to Southern Lesotho (Van

Wyk *et al.,* 1997). Although the species is widely distributed, uncontrolled harvesting for private and local commercial use places it under threat of extinction (Kambizi *et al.,* 2005). This plant has a well-documented history of use as medication in different formulations. Its history goes so far back that there are some San rock paintings showing the plant and its uses (Grace, 2011; Reynolds, 1950).

A. ferox has been reported to have therapeutic benefits and is widely used in the Cape, South Africa to treat some ailments (Shackleton and Gambiza, 2007). Traditional healers prescribe it as a water concoction to treat infertility in women and impotence in men (Grace *et al*., 2008). Although the discovery of its activity in absorbing ultraviolet (UV) light, especially UV B, was surrounded by controversy about addictiveness and hypersensitivity, these setbacks were dealt with. Lower concentrations of the gel compounds were used to test its safety and it was found to protect against solar radiation with no further problems (Grollier *et al*., 1987). The nectar from the flowers is often given to children to eat (Fox and Young, 1982) and it can be blended into fruit and other food supplements (Grace, 2011).

Even with the high demand for products from the aloe species, only a few are used commercially. *A. ferox* has been shown to have as many as 86 uses as a therapeutic agent (Kambizi *et al*., 2005). Kambizi *et al*. (2005) isolated and identified **1,** 1, 8-dihydroxy -3–hydroxymethyl-9, 10-anthracenedione (Aloe- emodin), **2,** 1, 8-dihydroxy-3-methyl-9, 10-anthracenedione (Chrysophanol) and **3,** 10-C-β-D-glucopyranosyl-1, 8-dihydrocymethyl-9-anthracenone (Aloin A) from *A.loe ferox.* It is therefore important to determine if there are any more compounds that may be extracted from this plant and to determine their bioactivity.

1.2. Motivation for the study

With a large number of organisms developing resistance to therapeutic agents in use, it is necessary to find effective and safer alternatives. Medicinal plants remain an important source of new drugs, new drug leads and new chemical entities. The therapeutic potential of Aloe *ferox* Mill. from the Asphodelaceae family has not been extensively studied, although this plant has been and is still being used locally to prevent, treat or manage different ailments such as arthritis, diabetes and sexually transmitted infections (STIs).

1.3. Aim and objectives

The aim of this study was to evaluate the potential of *A. ferox* Mill. as a source of novel therapeutic agents.

Objectives

The objectives of this project (Figure 1.2) were as follows;

1. To determine the anti-cancer activity of aqueous, methanol and dichloromethane extracts of *A. ferox*.
2. To determine the anti-microbial and anti-diabetic activity of the plant.
3. To evaluate the cytotoxic potential of the methanol and aqueous extracts of *A. ferox* on normal cell lines.
4. To separate and identify secondary metabolites from *A. ferox* by conducting qualitative and quantitative phytochemical tests.

1.4. Chapter overview

The following chapters report on the anti- cancer activity, phytochemical composition, anti- diabetic, anti- microbial and antioxidant activity of the leaf extracts of *A. ferox* based on the information gathered from local traditional healers who commend this plant for its strength and value in traditional medicine. The safety of the plant extracts was also investigated against normal cell lines. All this was done to validate the eventual use of this plant. Initially, three extracts (aqueous, methanol and dichloromethane) of *A. ferox* were investigated. However due to lack of enough plant material to extract and the fact that dichloromethane did not extract much from the plant the dichloromethane extract was not studied in other chapters.

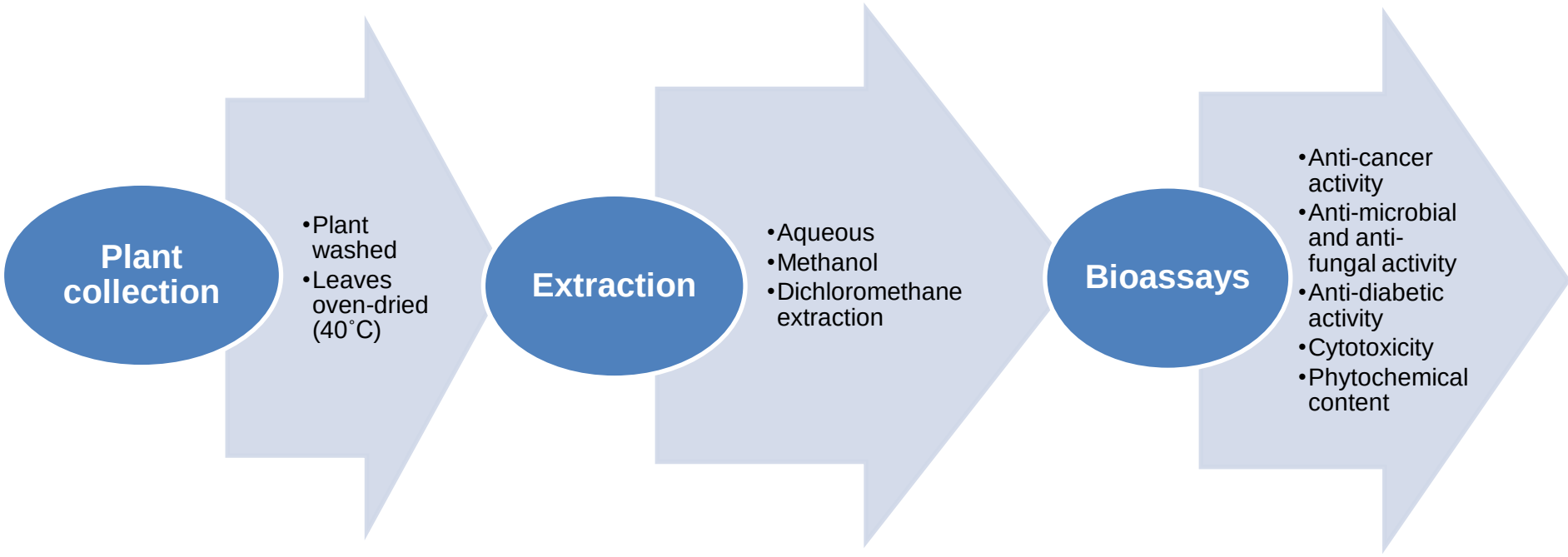

Figure 1.2. Flow diagram of the procedure to investigate the therapeutic potential of *A. ferox* leaf extracts.

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CHAPTER 2

In- vitro anti-cancer activity of *Aloe ferox* Mill.

2.1. Introduction

Since the isolation and modification of the anti-cancer compound camptothecin from the Chinese plant *Camptotheca acuminata* in the 1960s (Dancey and Eisenhauser, 1996; Wall and Wani, 1996), much progress has been made to improve cancer therapy. Camptothecin and its derivatives exerted their activity by targeting topoisomerase 1, an enzyme modulating DNA structure development (Dancey and Eisenhauer, 1996). Many synthetic and semi-synthetic derivatives of this compound have been developed and tested, and combinations of these derivatives were also investigated against cancer cell lines. Drugs such as tamofixen are believed to exert their effects through their antioxidant as well as anti-oestrogenic properties (Wiseman and Halliwell, 1996). Although they proved to have substantial activity, their toxicity remained a prime problem. More compounds have been discovered throughout the years since the first discovery, but their severe side effects such as anaemia, leukopenia and liver damage remain unchanged. Certain herbs have been suggested to reduce the side effects of current chemotherapy drugs and radiation therapy, perhaps by boosting immunity against cancer (Sakarkar and Deshmukh, 2011).

There are numerous ways in which drugs can function to avoid further damage or avoid damage by carcinogens in cancer therapy (Sakarkar and Deshmukh, 2011), some of which are listed below:

1. Stimulation of phagocytosis, enhancing natural killer cell activity.

2. Inhibition of blood flow to the cancerous tissue, leading to necrosis and inhibited translocation.
3. Promotion of apoptosis by increased production of elements leading up to the complement cascade.
4. Enhancing the production of platelets and leucocytes activating the haemopoietic function.
5. Promotion of metabolism, leading to inhibited metastasis and conversion of normal cells to tumour cells.
6. Pain relief, appetite increase and promotion of good quality sleep, leading to improved health of the patient.

It is therefore important to find alternatives or ways to overcome the toxicity side effect of the drugs used at present. Aloes have been found to contain the apoptosis-inducing compound aloe- emodin and have been used since ancient times, as far back as 500 B.C. in Egypt; they were also mentioned by Dioscorides in the first century A.D. (Castleman, 1991). Consequently aloe emodin makes this plant a potential source of anti-cancer drugs, as apoptosis is the main focus for cancer therapy (Chui *et al*., 2009). This chapter focuses on testing the aqueous, methanol and dichloromethane extracts of *A. ferox* Mill. for anti-cancer activity against selected breast, prostate and colon cancer cell lines.

2.2. Methods

2.2.1. Sample preparation

A. ferox was purchased from a nursery in Pretoria, South Africa. A voucher specimen (RM001) was kept for future reference in the Biomedical Technology

research laboratory at the Central University of Technology. The leaves were washed, oven-dried at 40ºC and then pulverised. Ten grams of the powdered plant material was then soaked separately in 150 ml of different solvents: purified water, methanol and dichloromethane, and shaken for 72 hours, after which the extracts were filtered through filter paper. The methanol and dichloromethane solvents were then removed through rotary evaporation at 35ºC and the water extracts were freeze- dried. Before screening for different activities, the extracts were re-dissolved into the appropriate solvents in accordance with the required concentrations and volumes for each assay. For anti-cancer activity screening the extracts were dissolved in dimethyl sulfoxide (DMSO).

2.2.2. *In- vitro* anti-cancer activity screening

The sulforhodamine B (SRB) assay method of Monks and colleagues (Monks *et al*., 1991) was used to test the cancer growth inhibitory effects of the *A. ferox* Mill. extracts against three cancer cell lines, namely MCF7 (breast cancer), HTC116 (colon cancer) and PC3 (prostate cancer). The human cancer cell lines were obtained from the National Cancer Institute (NCI) in the framework of a collaborative research programme between the Council for Scientific and Industrial Research (CSIR) and the NCI. The colon cancer cell line (HCT116) was obtained from the European Collection of Cell Cultures (ECACC). The cell lines were maintained in a monolayer cell culture at 37ºC, 5% CO_2, 95% air and 100% relative humidity in Roswell Park Memorial Institute (RPMI) medium containing 5% foetal bovine serum, 2 mM L-glutamine and 5 µg/ml gentamicin.

For the SRB assay, the cancer cells were inoculated on 96-well micro-plates at planting densities of 7 - 10 000 cells per well. The cells were then incubated at 37ºC for 24 hours, after which one plate was fixed with trichloroacetic acid (TCA). This plate was labelled the T0 and represented the measurement of cells inoculated in each plate before drug addition. The test extracts at different concentrations (6.25 – 100 µg/ml) as per protocol were added to the other plates (Ti). The plates were then incubated for 48 hours, after which cold 50% TCA was used to fix viable cells to the bottom of each well. The cells were then washed, dried and dyed with SRB. Optical density measurements on a multi-well spectrophotometer were used to calculate the net cell growth percentage at 540 nm. Cells without any drug/extract added were used as the control; the blank was complete medium without cells and etoposide was used as the standard. The samples were ran in triplicate.

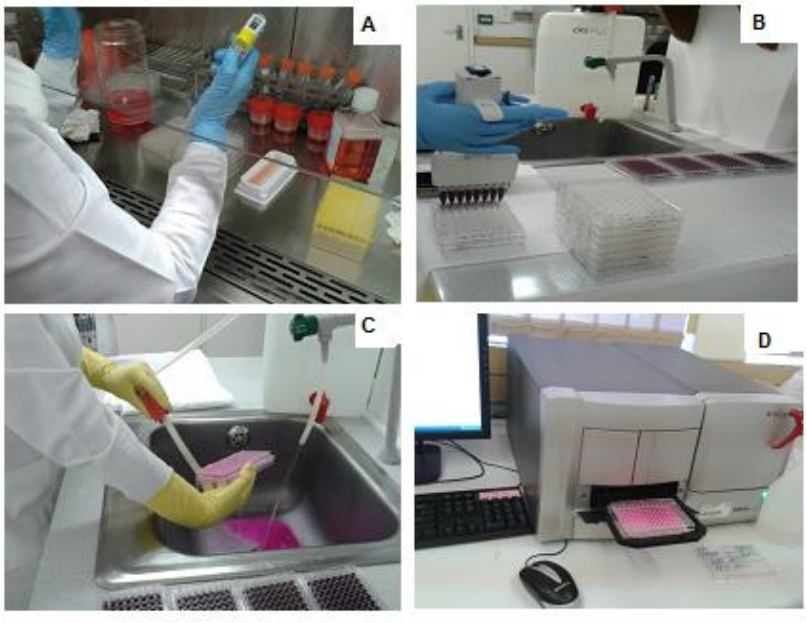

Figure 2.1. Inoculation of cancer cell lines on RPMI (A), the cells being stained with SRB after inoculation (B), washing the plates with 50% TCA and (C) optical density measurement with a spectrometer (D).

The optical density of the test well after a 48-hour period of exposure to the test drug is Ti, the optical density at time zero is T0, and the control optical density is C. The following equations were used to calculate the percentage cell growth:

[(Ti - T0)/(C - T0)] x 100 for concentrations at which Ti≥ T0.

[(Ti - T0)/ T0] x 100 for concentrations at which Ti<T0. (Monks *et al*., 1991).

The biological activities of the five doses tested were reported as total growth inhibition (TGI) and divided into four categories, as shown in Table 2.1.

Table 2.1.Criteria for biological activities of the extracts.

TGI	**Category**
> 50 µg/ml	Inactive
< 50 µg/ml>15 µg/ml	Weak Activity
< 15 µg/ml> 6.25 µg/ml	Moderate Activity
< 6.25 µg/ml	Potent Activity

2.3. Results and discussion

According to the CSIR criteria (Table 2.1), the tested extracts (aqueous, methanol and dichloromethane) are all inactive against all the cell lines, as indicated in Figure 2.2. They all had a TGI of more than 100 µg/ml against all the breast, colon and prostate cancer cell lines.

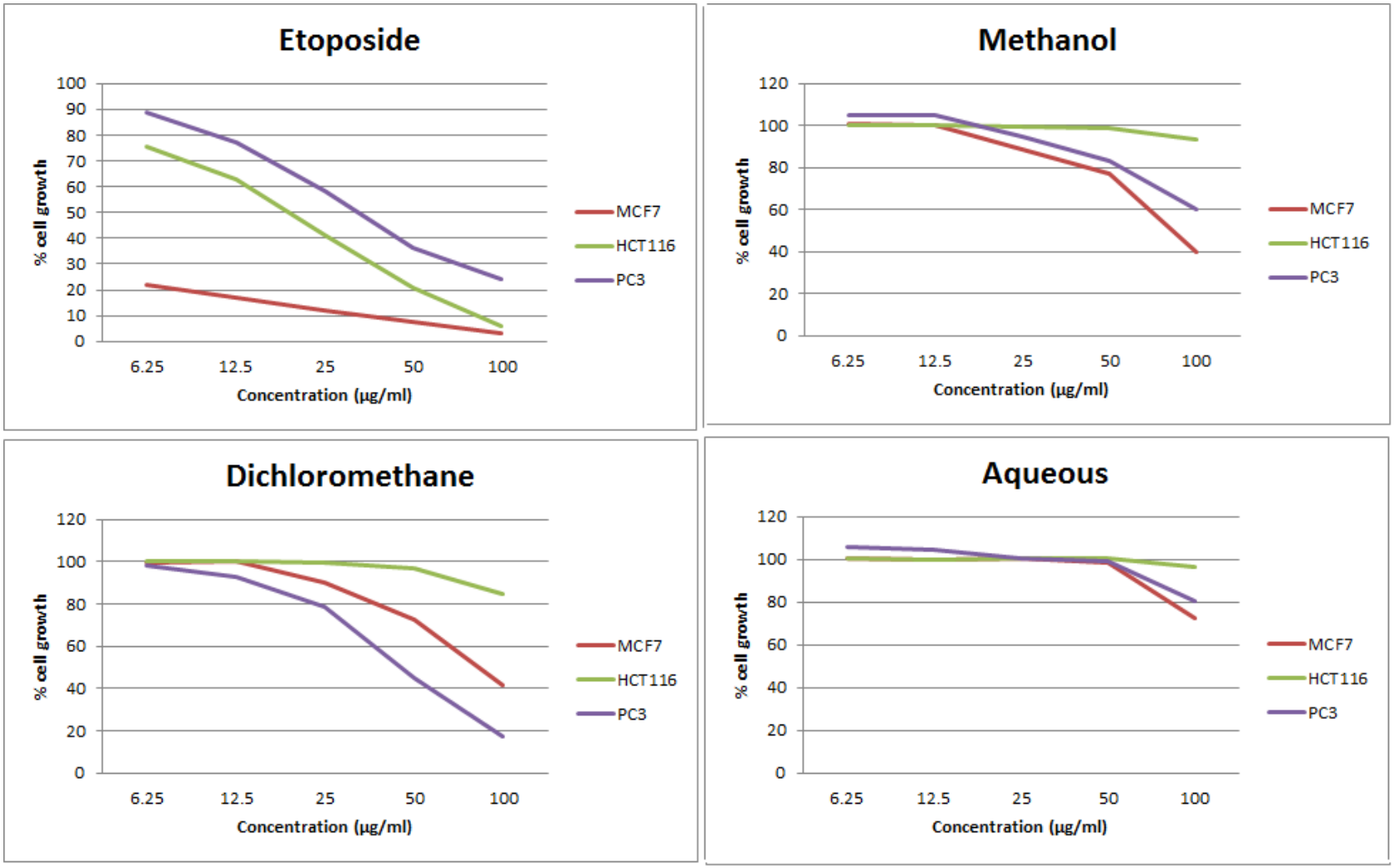

Figure 2.2. Activity of methanol, dichloromethane and aqueous extracts of *A. ferox* against the breast cancer (MCF7), colon cancer (HCT116) and prostate cancer (PC3) cell lines.

Table 2.2 shows the results of all the parameters measured. As indicated, the methanol extract did show a lower GI50 of86.56 µg/ml against the breast cancer cell line. The dichloromethane extract showed a 50% inhibitory concentration of 46.09 µg/ml against the prostate cancer cell line, which is much lower compared to 86.72 µg/ml needed to exert the same activity against the breast cancer cell line.

Table 2.2. The biological activities of *A. ferox* extracts against MCF7, HCT116 and PC3 cancer cell lines.

	Aqueous			**Methanol extract**			**Dichloromethane extract**		
Activities	**MCF7**	**HCT116**	**PC3**	**MCF7**	**HCT116**	**PC3**	**MCF7**	**HCT116**	**PC3**
GI50	>100	>100	>100	86.56	>100	>100	86.72	>100	46.09
TGI	>100	>100	>100	>100	>100	>100	>100	>100	>100
LC50	>100	>100	>100	>100	>100	>100	>100	>100	>100
LC100	>100	>100	>100	>100	>100	>100	>100	>100	>100

Aloin, chrysophanol and aloe-emodin were isolated and identified from ethyl acetate, n-hexane and water leaf extracts of *A. ferox* by Kambizi *et al*., (2004). This was done after the leaf extracts had been partitioned and fractionated. However, of all these compounds, aloe-emodin is studied most frequently because of its activity of promoting apoptosis and production of anti-tumour effects (Kim *et al*., 1999; Chiu *et al*., 2009). Since cancer is a disturbance within the cell cycle, apoptosis is the best way to control or even completely eradicate tumorous tissues. Thus apoptosis is the main focus whenever one is looking for anti-cancer agents (Chiu *et al*., 2009). In conjunction with cell death promotion, aloe-emodin has antiviral, antimicrobial and hepatoprotective activities (Lu *et al*., 2007; Eshun and He, 2004). Kuo *et al*. (2002) also studied the activity of aloe-emodin against HepG2 and Hep3B and found that it can induce its anti-cancer effects through either the p53 or p21 dependent apoptotic pathways. It also exhibited selective activity against neuro- ectodermal tumours and no activity against normal cells (Pecere *et al*., 2000). The emodin can be used against these tumours and also against Merkel cell carcinoma in conjuction with cisplatinol, a chemotherapeutic agent (Fenig *et al*., 2004). The anti-coagulant/anti-thrombotic activity of this plant in conjunction with the anti-cancer activity makes it an ideal choice for the treatment of cancer and also presents with a hyper-coagulable state at some stages of the disease (Grace, 2011).

The results from this investigation, however, do not correlate with the literature. The extracts were inactive against the cancer cell lines used, despite the fact that local traditional healers as well as other scientists believe that it can manage and even cure different cancers. There could be numerous reasons for the results being negative in this instance. Firstly, the cell lines were tested against impure crude

extracts at microgram concentrations. It is possible that if the extracts had been fractionated and tested against the cell lines separately, the results might have been positive and corresponded with other findings. Factors such as the age of the plant play a major role in the amounts of aloe polysaccharides and flavonoids available as well, which might also explain the difference in the results from what has previously been published (Hu *et al*., 2003).

2.4. Conclusions

A. ferox has many other health-boosting properties that have been studied and that may aid in the management of cancer. This may explain why traditional healers get good results after prescribing the crude extract to their patients. Thus the extracts may be functioning through other mechanisms that were not the focus of this study, such as improving metabolism, enhancing appetite, relieving pain and acting as a general immune boaster, minimising the symptoms and the pathology of the disease.

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CHAPTER 3

The *in- vitro* anti-diabetic activity of *Aloe ferox*

3.1. Introduction

Enzymatic and non-enzymatic antioxidant mechanisms in the body sometimes fail to reduce the reactive oxygen species that cause many degenerative diseases such as diabetes (Patel *et al*., 2012). Other mechanisms to rid the body of these harmful reactive oxygen species (ROS) are eagerly sought and should be well studied and identified. Collier *et al*. (1990) state that oxidative stress in diabetes coexists with a reduction in antioxidant concentrations. The accumulation of free radicals such as alloxan can lead to damage of the pancreas, furthering the complications. The damage would lead to reduced function and availability of the beta cells of the pancreas. With fewer beta cells there will be no binding of insulin and glucose, leading to hyperglycaemia. Thus antioxidant- rich diets can reduce oxidative stress and reduce the glycosylated haemoglobin in diabetic patients (Valko *et al*., 2007).

Recent research is targeted at discovering anti-diabetic agents that are safe, with fewer or no side effects. Current anti-diabetic therapies, such as acarbose and metformin, act on the liver and have severe negative side effects, such as hepatic dysfunction at high doses (McCue *et al*., 2005). Some ultimately lead to sorbitol accumulation, resulting in neuropathy and nephropathy (Lee, 2002). There are already many plants that are used across the world to treat and manage diabetes. Some of them are used traditionally, whereas others have already been made into formulations and sold as herbal preparations in pharmacies. Table 3.1 shows a list of plants that are used for diabetes. The listed plants are found across the world. Some

of them are native to India, such as *Andrographis paniculata* and *Allium sativum*, whereas others are from as far as Pakistan (*Azadirachta indica*) (Kavishankar *et al*., 2011). Medicinal plants can be cultivated or found in nature, but their importance remains universal. As one of the locally used plants as a way to manage diaetes, *Aloe ferox* has to be studied to establish its effectiveness regarding blood glucose reduction in diabetic patients. The glucose uptake models to be used in this chapter will establish whether *A. ferox* enhances, inhibits or has no effect on glucose uptake by cells *in- vitro* which is the mechanism through which blood glucose is reduced and utilized by muscle and other cells in the body.

Table 3.1. Medicinal plants used as treatment for diabetes.

Scientific name	Family	Parts used	References
Andrographis paniculata	Acanthaceae	Leaves	Dandu and Inamdar, 2009; Zhang and Tan, 2000.
Azadirachta indica	Meliaceae	Leaves	Wadood *et al*., 1989.
Prunus serotina	Roseaceae	Bark	McCune and Johns, 2007.
Aegle marmelos	Rutaceae	Leaves	Ponnnachan *et al*., 1993.
Aloe vera	Liliaceae	Leaves	Okyar *et al*., 2001.
Allium sativum	Lilliceae	Leaves	Eidi *et al*., 2005.
Solidago canadensis	Asteraceae	Bark	McCune and Johns, 2007.
Allium cepa	Alliaceae	Bulb onion	Mathew and Augusti, 1975.
Eclipta alba	Asteraceae	Leaves	Ananthi *et al*., 2003

Origanum vulgare	Lamiaceae	Leaves	Lemhandri *et al*., 2004.

3.2. Methods

3.2.1. Sample preparation

The plant extracts were prepared as described in Chapter 2 section (2.2.1).

3.2.2. The C2C12 *in- vitro* model of glucose uptake

The C2C12 muscle cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with 10% foetal bovine serum at 37ºC in humidified air with 5% CO_2. The cells were then placed in 96-well plates at a density of 4 000 cells per well and incubated for three days, after which 2% horse serum was supplemented into the cells after a further two days of differentiating the cells. The cells were then put in modified DMEM supplemented with 8 mM glucose and treated with methanol and water at 50 µg/ml, metformin (1µM), insulin (1µM) or solvent for the control. The plates were then incubated for three hours, after which the concentration of the remaining glucose was determined using a commercial fluorimetric kit. This was done to determine glucose uptake by the cells at a cell passage number of lower than 10.

3.2.3. The Chang cell *in- vitro* model of glucose uptake

Chang cells were grown and maintained in Eagle' s Minimal Essential Medium (EMEM) at 37ºC with 10% foetal bovine serum with 5% CO_2 humidified air. The cells were then planted into 96-well plates at planting densities of 6 000 cells per well and incubated for five days. The cells were then put in modified EMEM supplemented with 8 mM glucose and treated with the extracts (50 µg/ml), metformin (1 µM), insulin (1 µM) or solvent for the control. The plates were then incubated for three hours at 37ºC and the concentration of the remaining glucose was determined using a

commercial fluorimetric kit. This was done to determine glucose uptake by the cells at a cell passage number of lower than 10.

3.3. Results and discussion

After addition of the extracts, there was a greater increase in glucose levels in C2C12 cells than the vehicle control (Figure 3.1). The methanol extract raised the glucose levels from 100 µg/ml (control) to 109.57 µg/ml, whereas the aqueous extract raised it to 134.00 µg/ml. The extracts were, however, less effective with the Chang cells, where a slight decrease in glucose levels was observed after the extracts were added. The aqueous extract decreased glucose to around 90 and 98 for the methanol extract. The results showed that the extract was more effective in enhancing glucose uptake in muscle cells more than the Chang cells.

These results correlate with the statement by Van Wyk *et al*. (2009) that locally this plant is known as a blood purifier and is widely used by locals in cases of high blood pressure and acne, among other blood-related disorders. Other plants from the Asphodelaceae family are widely used to manage diabetes. The most common is *A. vera,* which has been formulated into drinks and pills. According to Hui (Hui *et al*., 2009) medicinal plants function through enhancement of insulin release, increasing glucose uptake by muscle and adipose cells.

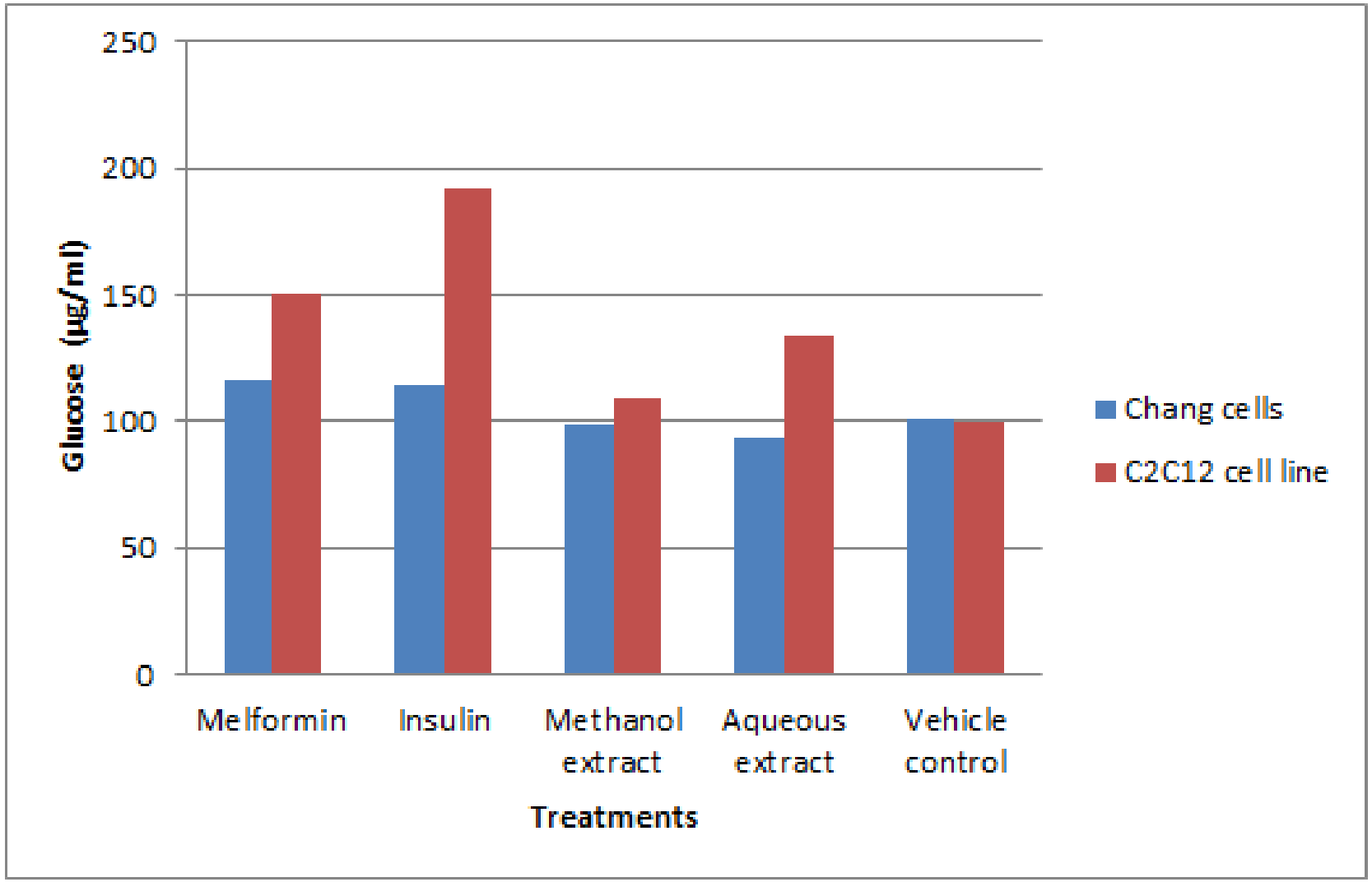

Figure 3.1. Glucose uptake of the two cell lines (C2C12 and Chang cells) after treatment with *A. ferox* extracts.

3.4. Conclusions

A. ferox is an encouraging possible source of a new anti-diabetic agent, as proven by the results. This finding is very encouraging, as it validates the use of this plant for diabetes, even more so as the aqueous extract proved to be more effective than the methanol extract. The local belief that it is a blood purifier has been proven correct. More investigations are highly recommended to determine the mechanism of action of these extracts. This may lead to a broad study of the plant and eventually the formulation of clinically approved drugs.

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CHAPTER 4

Anti-bacterial and anti-fungal activity of *Aloe ferox*

4.1. Introduction

Possible sources of antimicrobial drugs are those that can inhibit the growth of or kill pathogens with little or no toxicity towards host cells (Masoko *et al*., 2005). Synthetic drugs are continually becoming useless against pathogens, be these bacteria, fungi or viruses. They are increasingly developing resistance at a rate higher than the discovery of new alternatives (Marr *et al*., 1997; Dannaoui *et al*., 2001). Examples include the drug-resistant *Mycobacterium tuberculosis* strains, human immunodeficiency virus (HIV) and penicillin-resistant bacteria. There have been an increasing number of reports about the discovery of medicinal plants with antibacterial, antifungal and antiviral activity in recent years (Cowan, 1999). Most of the medicinal properties are found to be from higher plants (Masoko *et al*., 2005) and they are used worldwide to treat different ailments (Mitscher *et al*., 1987). They are prescribed by traditional healers and it is ideal for their traditional use to be supported by pharmacological and scientific proof (Locher *et al*., 1995).

Sexually transmitted infections, also known as venereal diseases, are the most common problem faced by South African society and are also regarded as the ailments that are most responsive to traditional treatment (Buwa and van Staden, 2006). The most commonly known venereal diseases (Buwa and van Staden, 2006) include gonorrhoea, syphilis and candidiasis. These have been implicated in the increased risk of acquiring and transmitting HIV (Buwa and van Staden, 2006). It is therefore important to treat these before they propel the HIV epidemic in Africa

further (Grosskurth *et al*., 1995). Because of their good responsiveness to traditional medicine, they are the ailments most commonly treated by traditional healers. *A. ferox*, as an infusion is the main plant prescribed by traditional healers for the treatment of such diseases, especially in the Eastern Cape Province.

It is therefore fitting that this chapter focuses on exploring the anti-bacterial and anti-fungal activities of *A. ferox* methanol extracts. The aqueous extract was not tested for these activities due to lack of test material. The ability of this extract to inhibit the growth of selected bacterial and fungal strains was evaluated, using the micro-dilution method.

4.2. Methods

4.2.1. Micro-dilution method

The methanol extract of *A. ferox* was prepared as mentioned in Chapter 2 (2.2.1.). The extract was screened for anti-bacterial and anti-fungal activity through the micro-dilution technique developed by Eloff (1998). Different concentrations of the test extract (15 mg/ml- 3 mg/ml) were prepared and 100 µl of each concentration was added to the 96-well micro-plates, as seen in figure 4.1. One hundred microlitres of the diluted (1: 100) three-hour-old culture of *Streptococcus pneumonia* (ATCC 49619), *Staphylococcus epidermidis* (ATCC 12228), *Enterococcus faecalis* (ATCC 89212), *Escherichia coli* (ATCC43888), *Candida albicans* (ATCC90028) and *Candida tropicalis* (ATCC750) in Muller Hinton broth was added to these 96-well micro-plates. Chloramphenicol was used as a standard drug for all organisms. The micro-plates were covered and incubated overnight at 37ºC. The tests were run in triplicate for each concentration.

To indicate growth after 24 hours of incubation, 40 µl of *p*- Iodonitrotetrazolium violet (INT) [Sigma] solution was added to each well. The plate was then incubated at 37ºC for 10 - 30 minutes. Growth was indicated by colour change ranging from pink to violet. Since fungi grow more slowly than bacteria, these were incubated for 36 hours before observing for growth after addition of the INT indicator. The MIC signifies the concentrations at which there was no bacterial growth observed.

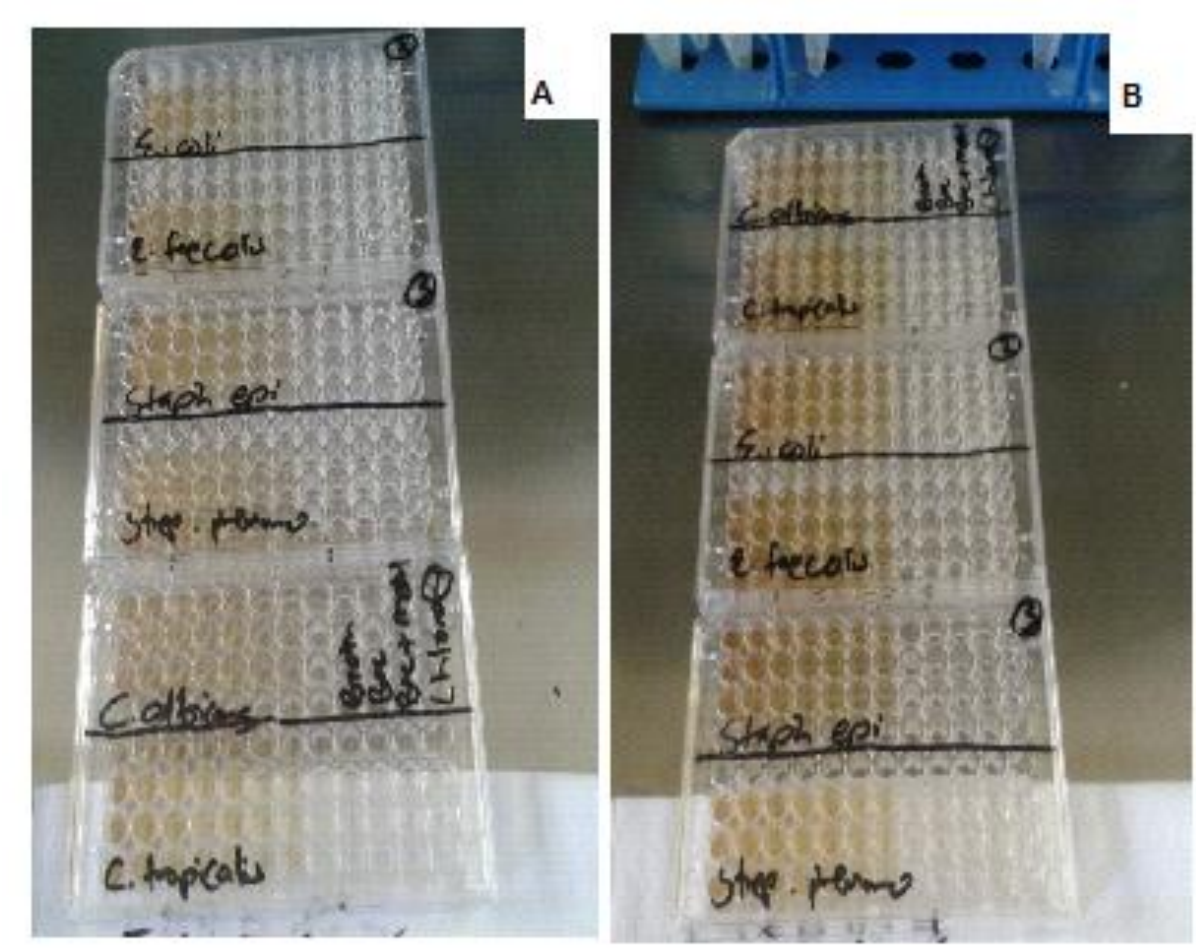

Figure 4.1. Bacteria inoculated into 96 microwell plates: (A) Plates with media and methanol extract only; (B) after inoculation of bacteria.

4.3. Results and discussion

As shown in Table 4.1, the tested extract did not completely inhibit the growth of the *E. faecalis* and *C. tropicalis* strains tested against, even at the highest concentration of 15 mg/ml. The violet colour (Figure 4.2) shows growth of the bacteria at different concentrations. It was however effective against *Staph epidermidis* and *C. albicans* at concentrations of 15 mg/ml and 13 mg/ml and only effective at 13 mg/ml against *Strep pneumonieae* and *E. coli*. These findings of activity, even if weak in most parts, correlate with the findings of Kambizi *et al., (*2004). *A. ferox* showed antimicrobial activity against *Bacillus cerus, Bacillus subtilis, Staphylococcus aureus, Staphylococcus epidermidis, Shigella sonnei and Escherichia coli.* This activity is important, as some bacteria, such as *E. coli,* have been associated with specific cancers such as colon cancer. It is through these kinds of results that the true value of this plant in the cosmetic industry was discovered. Some of the bacteria can be fatal in seemingly harmless infections such as septicaemia and other nosocomial infections in susceptible immune-compromised patients. The aloin that makes up most of the aloe leaf exudate is said to be important in determining the degree of activity against bacteria; its glycoside content enables it to invade bacterial cells easily (Niciforovic *et al*., 2007). Plants from the Aloe species are mainly used as treatment for STIs such as herpes, syphilis, gonorrhoea and candidiasis and also for the treatment of internal parasites (Grace *et al*., 2008).

Though concentrations as high as 15 mg/ml were required to inhibit bacterial growth, this could be explained as a result of the state of the extract used. Crude extracts usually contain only a low concentration of the active compounds and more of other materials that are inactive. It is possible that the extracts will exhibit a much stronger

activity after purification. That could be achieved by isolating the compounds separately and testing them for anti-microbial and anti-fungal activity.

Table 4.1. Minimum inhibitory concentration (MIC) of the *A. ferox* methanol extract against the bacterial and fungal strains.

Bacterial/ fungal strain	**MIC (mg/ml)**
Staphylococcus epidermidis	13
Streptococcus pneumonieae	15
Escherichia coli	15
Escherichia faecalis	>15
Candida albicans	15
Candida tropicalis	>15

Figure 4.2. Photograph showing fungal and bacterial growth after treatment with extracts at different concentrations and incubation **(A)** *Candida albicans* and *Candida tropicalis,* **(B)** *Escherichia coli* and *Escherichia Faecalis* and **(C)** *Staphylococcus epidermidis* and *Streptococcus Pneumonia*

4.4. Conclusions

The fact that this plant is effective against vastly different organisms, from viruses and bacteria to fungi, further stresses its value. The activity could be credited to compounds such as phenols, aloesin, alkaloids and steroids found in the extracts. These results, however, show that history and the knowledge passed on through generations are right, as the plant does inhibit growth, even if not in all the bacterial strains used.

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CHAPTER 5

In- vitro cytotoxicity of methanol and aqueous extracts of *Aloe ferox*

5.1. Introduction

According to Balick *et al*., (1996) the chance that an active ingredient in any drug is from plants is one in four. Although plants are rich in pharmaceutically relevant compounds, they can also be dangerously toxic to the human body. Since there is so much interest in medicinal plants, measures have to be put in place to ensure that they are effective and safe for use. Traditional healers prescribe these plants to the public with no scientific validation of their efficacy and safety.

Misidentification, incorrect preparation, administration and dosage are the main causes of poisoning and in some cases death from plants used for medicinal purposes (Stewart and Steenkamp, 2000; Fennell *et al*., 2004). There are currently no regulations in place for the prescription and use of traditional medicine in South Africa (Fennell *et al*., 2004). That could be the reason for the estimated 8 000 to 20 000 deaths per annum that result from poisonous plants (Thomson, 2000). For instance, between 1991 and 1995 about 43% or perhaps even more (Popat *et al*., 2001) of all reported poisoning cases in Johannesburg were due to supposedly medicinal plants (Stewart *et al*., 1999).

Plants such as *Iboza riparia* (Bodenstein, 1977; Fennell *et al*., 2004), *Cryptocarya latifolia*, *Synadenium cupulare* (Fennell *et al*., 2004) and *Callilepis laureola* (Wainwright and Schonland, 1977; Popat *et al*., 2001) have already been established as toxic to the human body. These are plants that were previously believed to be

beneficial in the treatment of different ailments. For example, *Callilepis laureola* was discovered after years of traditional use to be the cause of the high incidence of liver necrosis among most of the KwaZulu-Natal population in the 1970s (Wainwright and Schonland, 1977). Like many of the above-mentioned plants, *A. ferox* is well known and used often to treat different ailments. This can be done through self-administration, with no information on the health risks, proper mode of administration and more importantly, appropriate dosages. It is for that reason that the cytotoxic potential of *A. ferox* was studied.

This chapter focuses on testing the toxicity of *A. ferox* water and methanol extracts against normal cell lines, namely C1C12 muscle cells, Chang cells and the W138 normal human foetal lung fibroblast.

5.2. Methods

5.2.1. Sample preparation

Two extracts were selected and further tested for cytotoxicity against normal muscle and Chang cells. The methanol and aqueous extracts of *A. ferox* were prepared as described in Chapter 2 (2.2.1). They were then dissolved in their respective solvents, i.e. methanol and distilled water, to make up the required concentrations for the assays.

5.2.2. Mosmann Tetrazole Test (MTT) cytotoxicty activity assay on muscle and Chang cells

The MTT cytotoxicity test is based on the colour reaction of mitochondrial dehydrogenases from living cells (Valko *et al*., 2006). The assay was done with

modifications, as previously described (Mosmann, 1983). The C2C12 muscle cells were grown in DMEM with 10% foetal bovine serum at 37ºC in humidified air with 5% CO_2. The cells were then placed in 96-well plates at a density of 4 000 cells per well and incubated for three days, after which 2% horse serum was supplemented into the cells after a further two days of differentiating the cells. Simultaneously Chang cells were grown and maintained in EMEM at 37ºC with 10% foetal bovine serum with 5% CO_2 humidified air. The cells were then planted into 96-well plates at planting densities of 6 000 cells per well and incubated for five days. After incubation of both the C2C12 and Chang cells the methanol and water extracts at 5 µg/ml were added to the relevant wells. The covered plates were then incubated for 48 hours at 37ºC.

5.2.3. Sulforhodamine B assay

The SRB assay (Monks et al., 1991) was done to test the cytotoxic activity of *A. ferox* methanol and aqueous extracts against a selected normal cell line. W138 (normal human foetal lung fibroblast from ECACC) cell line was maintained as a monolayer at 37ºC, 5% CO_2 and 100% relative humidity in EMEM with 10% foetal bovine serum, 2 mM L-glutamine and 50 µg/ml gentamicin. The cells were then inoculated into 96- well plates (densities of 10 000 cells/well) and incubated for 24 hours at 37ºC. The cells were then treated with the test extracts at five different concentrations ranging from 6.25 - 100 µg/ml (5 x twofold serial dilutions). Etoposide was used as the standard drug, cells without drugs served as the control and medium with no cells as the blank. The plates were incubated for 48 hours at 37ºC, after which viable cells were fixed to the bottom of each well with cold 50% trichloroacetic acid, washed, dried and dyed with SRB. Tris (10 mM) was used to

remove the protein-bound dye for optical density determination at 540 nm using a multiwall spectrophotometer. The optical densities were used to determine the IC50 (µg/ml) for each extract.

5.3. Results and discussion

The test extracts had no toxic side effects on either the C1C12 or the Chang cells. The methanol extract caused an increase in MTT activity (percentage cell viability), whereas the aqueous extract only caused a slight and insignificant decrease in MTT activity on the Chang cells (Figure 5.1b) from 100 to 97.7. The methanol extract performed very well against the C1C12 cell line, as there was no significant change at all in the cell viability when compared to that of the positive control (Figure 5.1a). The aqueous extract did, however, cause a slight decrease in C2C12 cells from 100 to 90.1 activity. The decrease is considered insignificant, as it is less than 15%. It was noticed that in both C2C12 and Chang cells the methanol extract performed even better than insulin and metformin. To date there has not been any scientific report of adverse effects of aloes in humans. It is, however, possible that *A. ferox* may be toxic at high concentrations, especially when used regularly in young children. It is also safe to assume that regular use of this plant as a laxative is harmful, as it could lead to disruption of the normal functioning of the digestive system. This means that more research has to be done to validate each species.

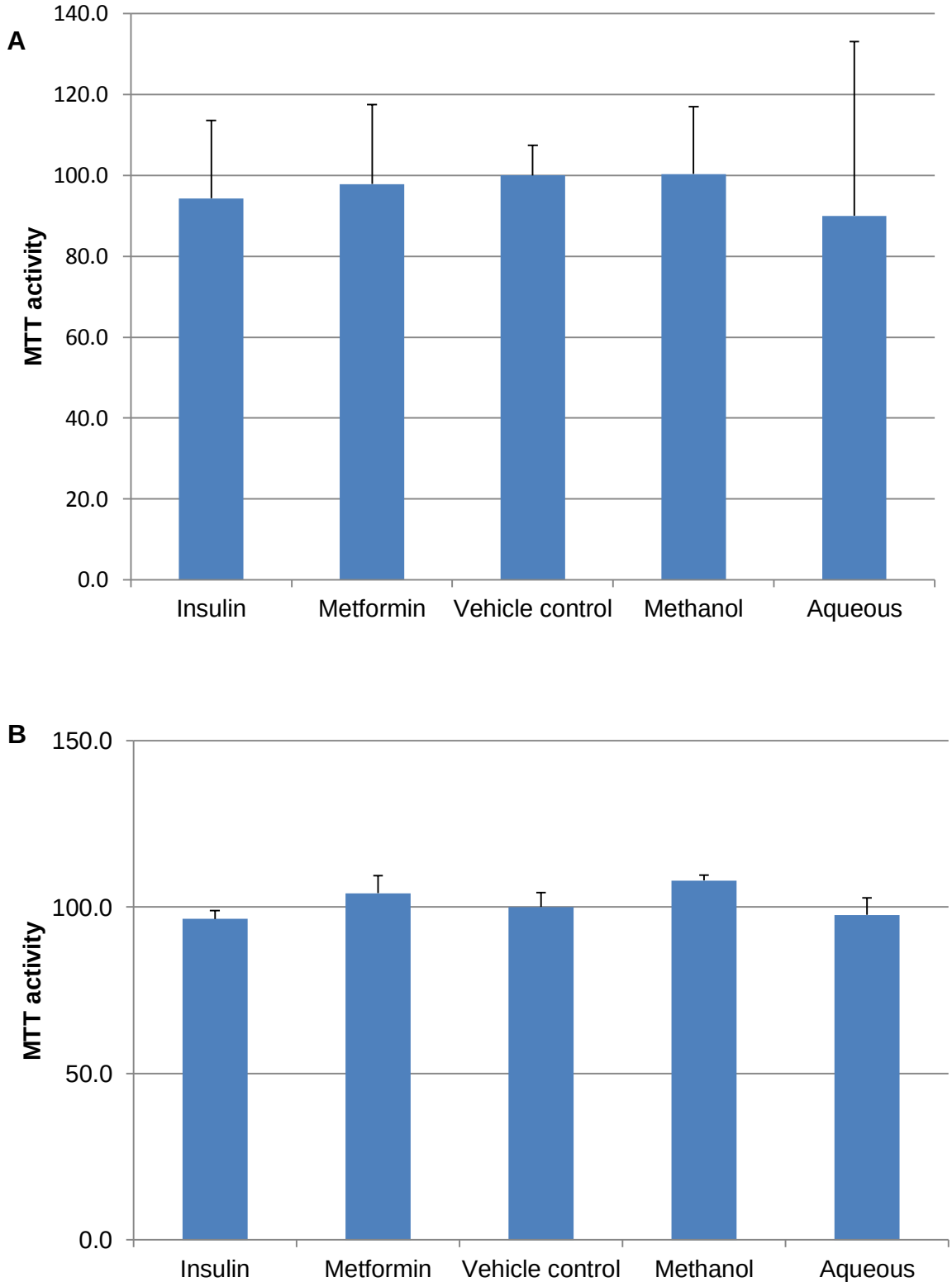

Figure 5.1. Cytotoxic activity (MTT) of the methanol and aqueous extracts of *A. ferox* Mill. against the C1C12 muscle cell line (A) and Chang cells (B).

Table 5.1. CSIR criteria for cell cytotoxicity and *A. ferox* extract results.

IC50 (µg/ml)	**Status**	**Methanol extract (*A. ferox*)**	**Aqueous extract (*A. ferox*)**
> 100	Low Hazard	>100	>100
< 100 > 30	Weak Hazard		
< 30 >5	Moderate Hazard		
<5	High Hazard		

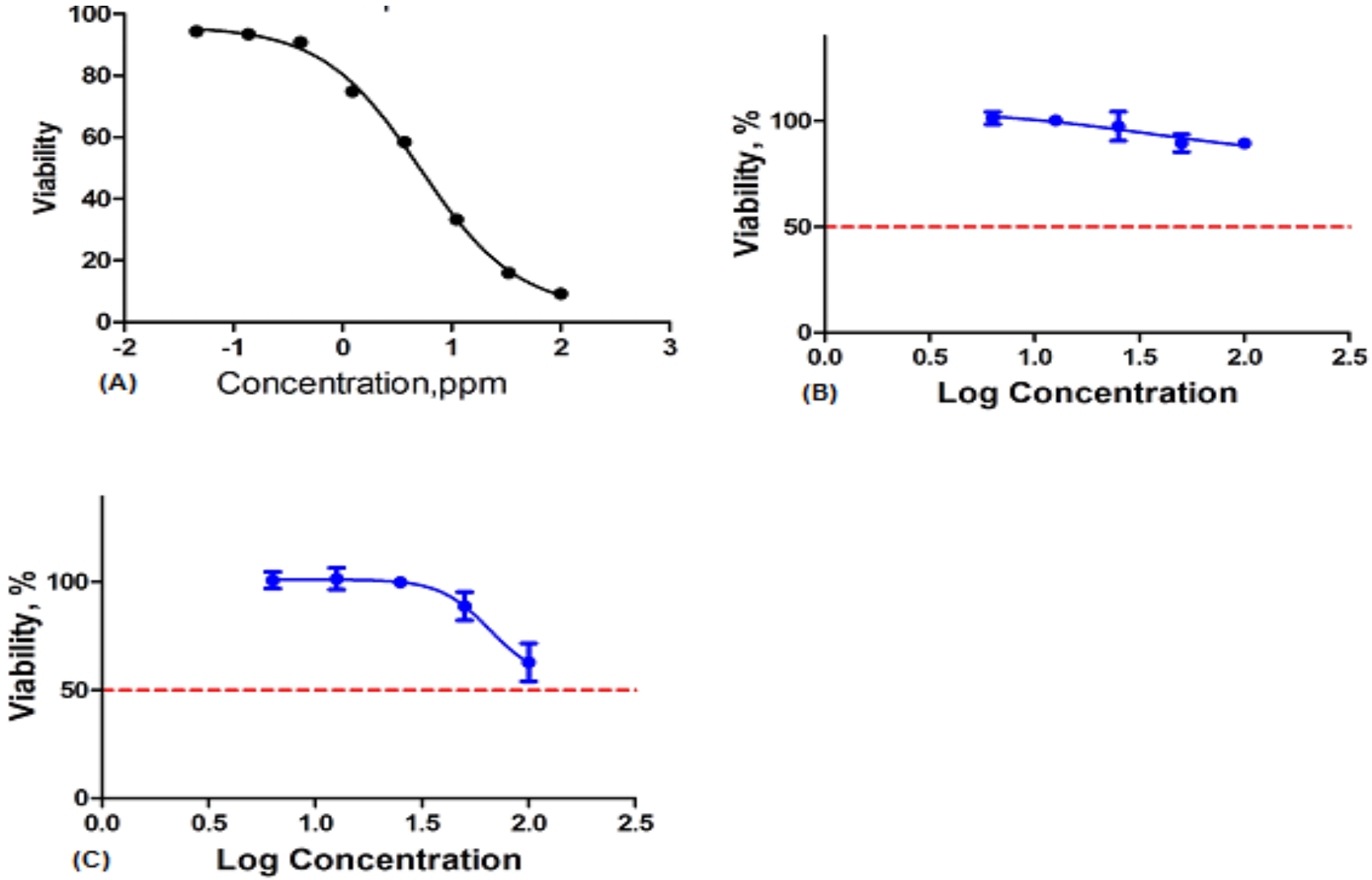

Figure 5.2. Cytotoxic effects of the methanol and aqueous extracts on viability of W138 cells. (A) represents the etoposide, (B) represents the *A. ferox* methanol extract and (C) represents the *A. ferox* aqueous extract.

According to the CSIR criteria, using the SRB method the two *A. ferox* extracts tested did not exhibit any toxicity against normal human foetal lung fibroblasts. The concentration required to reduce the percentage cell viability by 50% was more than 100 µg/ml (Table 5.1) for both the methanol and aqueous extracts. Although the aqueous extract (Figure 5.2 C) showed a higher cytotoxic activity than the methanol extract (Figure 5.2 B), it also failed to reduce the cell viability to 50% even at its highest concentration, still rendering it safe for use.

5.4. Conclusion

The investigation shows that the extracts are safe for use by humans, as they exhibited no toxicity against the cell lines. The results are very encouraging and validate the use of this plant by traditional healers. The *A. ferox* plant extracts performed very well as they exhibited no toxicity in a way close to that of insulin and metformin which are drugs that are currently clinically prescribed for diabetes. This is a preliminary investigation and more needs to be done to confirm the safety of the plant definitely and determine the lethal doses of both extracts.

5.5. References

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CHAPTER 6

Phytochemical content of *Aloe ferox* Mill.

6.1. Introduction

Secondary metabolites such as alkaloids, tannins, flavonoids and phenolic compounds constitute the most bioactive compounds in plants (Hill, 1952; Edeoga *et al*., 2005). They exert their pharmacological effects either separately or in a synergetic way. The largest class of secondary plant compounds is alkaloids (Barbosa- Filho *et al*., 2006). They all have the same basic structure, usually comprising one or more nitrogen atoms, often in combination as part of the cyclic system (Harbone, 1991; Barbosa- Filho *et al*., 2006).

Flavonoids, which were originally recognised as the pigments responsible for the different shades (yellow, orange, and red) in flowers and food, are found in all vascular plants. They have been proven to have anti-inflammatory, antioxidant, anti-allergic, hepatoproliferative, antithrombotic, anti-viral and anti-carcinogenic activities (Middleton *et al*., 2000). The other groups of compounds that are of great interest in the pharmaceutical industry are steroids and steroidal saponins. Because of the relationship between steroids and sex hormones, the steroidal structure could be the starting point for the synthesis of these hormones (Okwu, 2001). Sometimes steroids present themselves as saponins, which can be classified into triterpenoids and steroidal saponins, with both groups having important pharmacological effects on mammalian cells. Triterpene saponins have anti-inflammatory, anti- tussive, expectorant, cytotoxic and analgesic activity, whereas steroidal saponins deal mostly

with hormone synthesis as precursors for androgens, oestrogen and progestrins, as well as anti-inflammatory agents (Gurib- Fakim, 2006).

To maintain a healthy biological system there has to be a balance between oxidation and anti-oxidation (Hong and Liu, 2004; Katalinic *et al*., 2006; Pak *et al*., 2006). ROS and RNS are found in most of the disease states such as inflammatory infections, diabetes and cancer. Antioxidants directly remove ROS-mediated reactions, hence they can be used to prevent and treat these and other aging-related diseases. This inspires the search for antioxidant active drugs and drug leads; since all higher plants have some level of this activity, they are of great interest in this search.

Plants and herbs have been used for their therapeutic activity since ancient times and are still used in this way (Madsen and Bertelsen, 1995; Wangensteen *et al*., 2004). The therapeutic activity of such plants and herbs has been credited to the presence of compounds such as vitamins, terpenoids and polyphenols. These compounds function through different mechanisms, the most intensively studied being free radical scavenging (Suhaj, 2006; Albano and Miguel, 2010; Rice-Evans *et al*., 1996). Reports show that medicinal plants contain much more antioxidant compounds than dietary plants (Wong *et al*., 2006; Bouayed *et al*., 2006), which may explain their advanced therapeutic activities.

Most of the drugs currently used as treatment for inflammatory and chronic diseases have adverse effects and require long-term administration in order to take effect (Conforti *et al*., 2008). The common synthetic antioxidants such as butylated hydroxy toulene and Butylated hydroxy anisole have severe side effects, such as internal and

external haemorrhaging and carcinogenesis (Ito *et al*., 1986).It is for these reasons that the pharmaceutical industry is seeking natural sources for medicine and antioxidants. However, a diet rich in vegetables is still a common recommendation, as it goes a long way in maintaining a healthy balance and preventing diseases.

The Aloe genus is known for its rich anthraquinone compounds, such as chrysophanol, aloin, aloe- emodin and other aloe derivatives. These are very promising anti-cancer agent sources, as they cause cell death (Lu and Chang, 2009). *A. ferox* alone has over 80 recorded medicinal uses. Most of the information available was obtained from local healers. However, more has to be done to validate these uses and one of the most important steps is determining the individual compounds present in the plant. This chapter focuses on determining the phytochemical makeup and antioxidant activity of *A. ferox* methanol extracts through a number of techniques. The total phenolic content was determined for the methanol as well as aqueous extracts of *A. ferox*.

6.2. Methods

6.2.1. Sample preparation

The methanol extract was prepared in the same way as explained in Chapter 2 (2.2.1). However for the determination of the total phenolic content, the aqueous, methanol and dichloromethane extracts were all re-dissolved in the respective solvents that were used to extract them. Dichloromethane and water were not very effective in extracting compounds from the plant leaves hence they were not used in the rest of the chapter as this lead to shortage of extract material. The solvents were added in accordance with the protocol to prepare the specified concentrations.

6.2.2. Qualitative assays

Different qualitative methods adapted from De *et al*., (2010) were used to determine the phytochemical make-up of *A. ferox* extract and the antioxidant activity was also evaluated.

Test for carbohydrates: Fehling's test

To 2 ml of the sample, 2 ml of Fehling's reagent A and B was added. The solution was then shaken and boiled. The formation of a brick-red precipitate (cuprous oxide) indicated the presence of reducing sugars (De *et al*., 2010).

Test for steroids: Libermann- Buchard test

A few drops of acetic anhydride were added to treat the extract, the mixture was boiled and then cooled. Sulphuric acid was added to the mixture drop by drop down the sides of the test tube. The presence of steroids was indicated by the formation of a brown ring at the junction of the two layers with the upper layer turning green. Formation of a deep red colour at the upper layer indicated the presence of triterpenoids (De *et al*., 2010).

Test for saponins: The foam test

Two millilitres of the extract was mixed with 2 ml of distilled water and the solution was shaken vigorously. The formation of persistent foam was an indication of the presence of saponins in the extract (De *et al*., 2010).

Test for alkaloids: Dragendorff's test

The extract was mixed with a few millilitres of Dragendorff's reagent. Alkaloids give a reddish brown precipitate (De *et al*., 2010).

Test for flavonoids: Shinoda's test

Flavonoids give a pink, scarlet, green or green to blue colour after addition of a few fragments of magnesium ribbons and the drop-wise addition of concentrated hydrochloric acid (De *et al*., 2010).

6.3. Quantitative assays

6.3.1. Determination of total phenolic content: Folin Ciocalteu method

The total phenolic content of *A. ferox* extracts was determined using the Folin-Ciocalteu method according to Lowman and Box, (1983) with a few modifications. Gallic acid, which is a strong antioxidant, was used as the standard from which the phenolic concentration of the sample was determined and expressed as milligram Gallic acid equivalent (GAE). The methanol and aqueous extracts of *A. ferox* (0.1 ml) were mixed with 46 ml of distilled water in a 50 ml volumetric flask and 1 ml of Folin-Ciocalteu reagent was added. The mixture was then thoroughly mixed and allowed to react for three minutes, after which it was topped up to 50 ml with sodium carbonate solution (2%). Then the solutions were allowed to incubate for two hours at room temperature. The absorbance of the solutions was measured at 760 nm. The same procedure was followed for Gallic acid at different concentrations (0.039 - 2.5 mg/ml) to obtain the standard curve.

6.3.2. Determination of antioxidant activity: DPPH radical scavenging activity

The method described by Brand-Williams *et al*., (1995), with some modifications, was used to screen the methanol *A. ferox* extract for antioxidant activity. A stable radical 2, 2-dipheny-1-picrylhydrazyl (DPPH) at 0.1 mM was used to investigate the free radical scavenging activity of the extracts. This was compared to that of ascorbic acid, a known antioxidant. One hundred millilitres of methanol solutions of the extract at various concentrations (0.078, 0.156, 0.3125, 0.625, 1.25 and 2.5 mg/ml) was added to 2 ml of the DPPH solution. The mixtures were shaken vigorously and then incubated in the dark at room temperature for one hour. The absorbance (Abs) of the solution was then measured at 517 nm, and the percentage of antioxidant activity (AA) calculated using the following formula:

$AA\% = 100-\{[(Abs_{sample}–Abs_{blank}) \times 100]/Abs_{control}\}$ (Brand-Williams *et al*., 1995)

(Abs= absorbance)

A solution of methanol (0.1ml) and DPPH (2 ml) was used as the control and to blank methanol was used. The IC_{50} for the sample was determined from a plotted graph of DPPH disappearance as a function of sample concentration.

6.3.3. Liquid chromatography mass spectrometry (LCMS) analysis

The following set-up was used for the LCMS analysis of the *A. ferox* methanol extract:

1. A Waters Synapt G2 UPLC with a Waters UPLC BEH C18, 2.1 X 100 mm column.
2. A flow rate of 0.4 ml/min.

3. Use of (1) 0.1% formic acid to acetonitrile gradient and (2) water as eluting solvents.
4. Negative electrospray at a capillary voltage of 3 kV and cone voltage of 15 V.
5. Running time of 15 minutes.

6.3.4. Gas chromatography mass spectrometry (GCMS)

The following method/set-up was used for the GCMS analysis of the methanol extract of *A. ferox*:

1. The initial temperature was set at 50ºC for five minutes
2. Ramped at 3ºC per minute to 100ºC for 10 minutes.
3. The inlet temperature was 200ºC with splitless flow mode.
4. The carrier gas was helium at 1 ml/min flow rate.

6.4. Results and discussion

The aqueous plant extract showed the presence of carbohydrates, saponins, steroids, alkaloids as well as flavonoids. The presence of steroids and alkaloids strongly suggest that these methanol and aqueous extracts of *A. ferox* may have potential in the production of anti-inflammatory or even anti-cancer agents. Any agent that has steroids and alkaloids is a strong contender in the fight against cancer and other chronic inflammatory diseases, as most of these compounds have been found to have anti-inflammatory, free radical scavenging and apoptosis-inducing properties (Furusawa and Wu., 2007). The presence of saponins may also indicate the presence of steroids and triterpenoids, which is often present in the form of saponins (Haralampidis *et al*., 2002). The pharmacological effects of plants are a result of the activity of single compounds or a synergetic effect of the compounds

(Eloff *et al*., 2008). The presence of flavonoids also contributes to the antioxidant activity of plant extracts, as in addition to free radical scavenging, they also have vein-strengthening effects (Van Wyk *et al*., 2004). They have anti-inflammatory, anti-allergic, anti-bacterial and anti-viral effects (Middleton *et al*., 2000). Findings by Kambizi (Kambizi *et al*., 2004) are supported by the results of the present study. The above-mentioned findings correlate with those of Loots *et al*., (2007).

Table 6.1 shows the variation in the total phenolics of the three different extracts analysed at 5 mg/ml against Gallic acid. The phenolic content varied between the extracts with the methanol extract showing more polyphenols than the aqueous and dichloromethane extracts. The GAEs of the estimated phenolic concentrations range from 0.340 ± 0.057 to 0.726 ± 0.032 mg/ml GAE. These findings support those of Loots *et al*., (2007) that *A. ferox* is a rich source of different phenolic compounds. It can be postulated that their presence is part of the mechanisms that make this plant such a valued part of local herbal medicine.

Table 6.1. Total phenolic content of *A. ferox* extracts.

Sample	**Absorbance Mean**	**Polyphenol concentration (GAE)**
Aqueous extract 5 mg/ml	0.069	0.340 ± 0.057
Dichloromethane 5 mg/ml	0.109	0.550 ±0.042
Methanol extract 5 mg/ml	0.144	0.726 ± 0.032

The DPPH radical scavenging activity of the methanol extract of *A. ferox* is shown in Table 6.2. Only the methanol extract was evaluated for the antioxidant activity since it showed the best results with the highest phenolic content (Table 6.1). This was also done to preserve the aqueous extract as there was a shortage of the plant material for extraction. The methanol extract has potent antioxidant activity, which was compared to that of a known standard, ascorbic acid (Figure 6.1). The highest activity (101 ± 0.042%) was achieved at a concentration of 2.5 mg/ml. The EC50 of the extract was 0.865 mg/ml compared to that of ascorbic acid, which was 0.042 mg/ml. At a concentration of 1.25 mg/ml, more than 75% of DPPH was scavenged, which is massive activity that might be able to go a long way in delaying or preventing the disease process and restoring the oxidative balance.

Table 6.2. % DPPH scavenging activity of *A. ferox* extracts.

Concentration (mg/ml)	**% DPPH scavenging activity**
2.5	101.25 ± 0.042
1.25	75.87 ± 0.386
0.625	52.98 ± 0.416
0.3125	29.92 ± 0.699
0.156	21.38 ± 0.252
0.078	12.61 ± 0.682
EC50 (mg/ml)	**0.865 ± 0.783**
Ascorbic acid EC50 (mg/ml)	**0.042 ± 0.084**

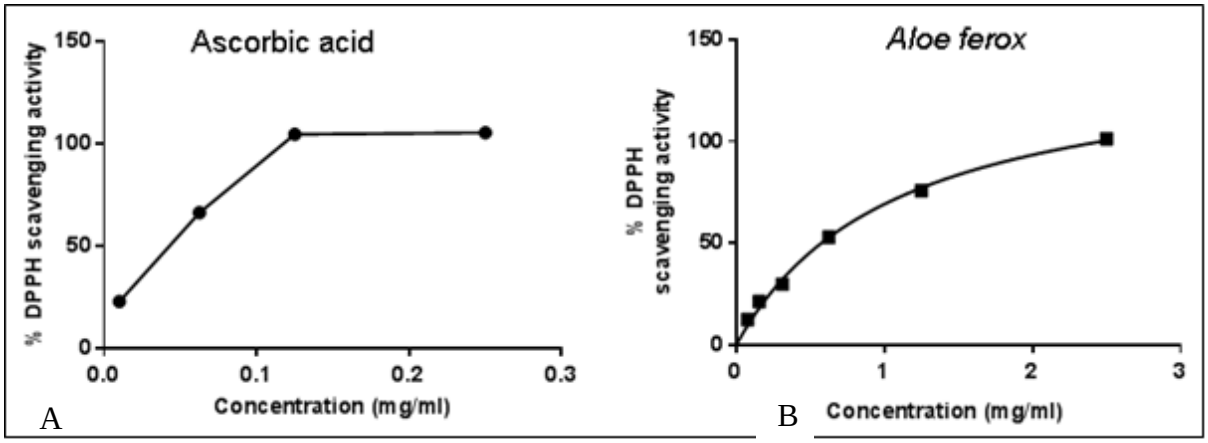


Figure 6.1. Free radical scavenging activity of methanol extract of *A. ferox* (B) compared to ascorbic acid (A) control.

Because of the presence of free radicals in almost all human disease states and the free radical scavenging activity of this plant is of great significance and importance. Compounds with free radical scavenging activity have been shown to prevent oxidative damage to tissues and significantly improve wound healing, among other effects (Svobodová *et al*., 2006). Therefore these results might validate the use and value of *A. ferox* by traditional healers for the treatment of different ailments such as diabetes and prevention of the development and progression of cancer.

Numerous studies have been done to investigate the relationship between the polyphenol content and antioxidant activity of different plants. It is clear from the findings that this relationship differs between plants (Conforti *et al*., 2008) and in some instances there is no relationship between the two. This is confirmed by the findings of Velioglu *et al*., (1998), who found a correlation between the two factors and Kahkonen *et al*., (1999) who found no correlation between polyphenolic content and antioxidant activity of the plants studied. Since phenolic compounds are the main contributors to the antioxidant properties of plants, it is believed that the quality

and quantity of the phenolic constituents are responsible for the difference in antioxidant activities (Shahidi and Marian, 2003). It is clear that water is a poor solvent for polyphenols, hence the lower value observed at a concentration similar to that of dichloromethane and methanol. This, however, does not discredit the use of aqueous extracts by traditional healers, as higher concentrations and volumes are usually used to obtain the desired effects.

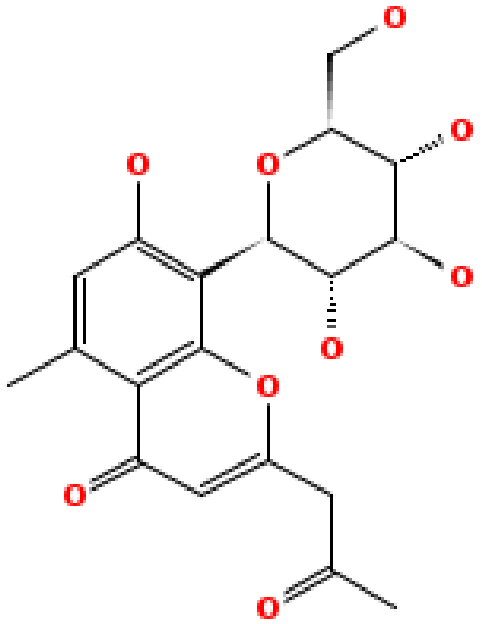
The LCMS analysis (Table 6.3) of the methanol *A. ferox* extract showed various peaks that were analysed, the elemental composition predicted and the molecular masses determined, to assist in the identification of the different compounds. The compounds were identified using three different online databases, namely KEGG, PubChem and ChemSpider. Aloesin, a chromone (Park *et al*., 2011) and promising anti-cancer agent as one of the most important phytochemicals found in aloes (Figure 6.2) was identified. Aloesin has apoptosis-stimulating activity and anti-inflammatory activity (Park *et al*., 2011; Speranza *et al*., 2005). As already mentioned in Chapter 1, anti-inflammatory agents are known to be used in cancer therapy since they ease the symptoms of cancer and improve the state of the patient. Different drugs and agents function in different mechanisms and the anti-inflammatory action of the aloesin could be the cause of the positive reviews of *A. ferox* by local healers.

The other identified compounds have not been studied for their therapeutic potential yet. However, retonone is a herbicide and pesticide that damages the mitochondria, hindering the respiratory chain (Vallin and Low, 1968; Valtierra, 2009). It has been shown to reduce adenosine triphosphate (ATP) synthesis in rats (Lu and Chang,

2009) and to cause retonone- induced neuronal cell death (Liu *et al*., 2012). The effects of these compounds can be studied further to investigate how they can be applied in disease control and treatment. It may be possible to apply their ability to disrupt cell life, whether through inhibited ATP production, cell death or in microbial treatment.

Table 6.3. Elemental composition predictions from LCMS spectra of the methanol extract of *Aloe ferox.*

	Mass	Calculated mass	Formula	Final formula $(M-H)^-$	Compound Name from (PubChem, ChemSpider and KEGG) databases
1	393.1183	393.1186	C19H21O9	C19H22O9	Aloesin
2		393.1127	C26H17O4	C26H18O4	5-methoxy-8-methyl-1,3-diphenyl-benzo[f]isobenzofuran-4,9-quinone
3		393.1244	C12H25O14	C12H25O14	Not found
4		393.1279	C30H17O1	C30H18O	4H, 4H-4,4'-Bicyclopenta[def] phenanthren-4-ol.
5		393.1033	C15H21O12	C15H22O12	propanoic acid [(2S)-2-hydroxy-2-[(2R)-5-hydroxy-3-oxo-4-[[(3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)-2-tetrahydropyranyl]oxy]-2-furyl]ethyl] ester
6		393.1338	C23H21O6	C23H22O6	Retonone
7	181.0501	181.0501	C9H9O4	C9H10O4	Homovanillate or Homovanillic acid



C19H22O9

Exact mass: 394.1264

Name: Aloesin

Figure 6.2. Aloesin

The GCMS analysis led to the identification of various (Figure 6.3) compounds via the computer library according to their retention indexes and masses. Each compound identified had a percentage probability above 20% **(bold**). These compounds are undecane (**24.14**), phosphoric acid trimethyl ether (**81.23**), p dioxane-2, 3 diol (**21.37**), butanedioc acid dimethyl ester (**89.11/ 81.23/ 41.38**), cyclotrisiloxane hexamethyl (**33.27**) and acetyl chloride (**28.93**). Most of the compounds have not been studied for their potential therapeutic activity as there is no evidence that they were previously isolated from *A. ferox*.

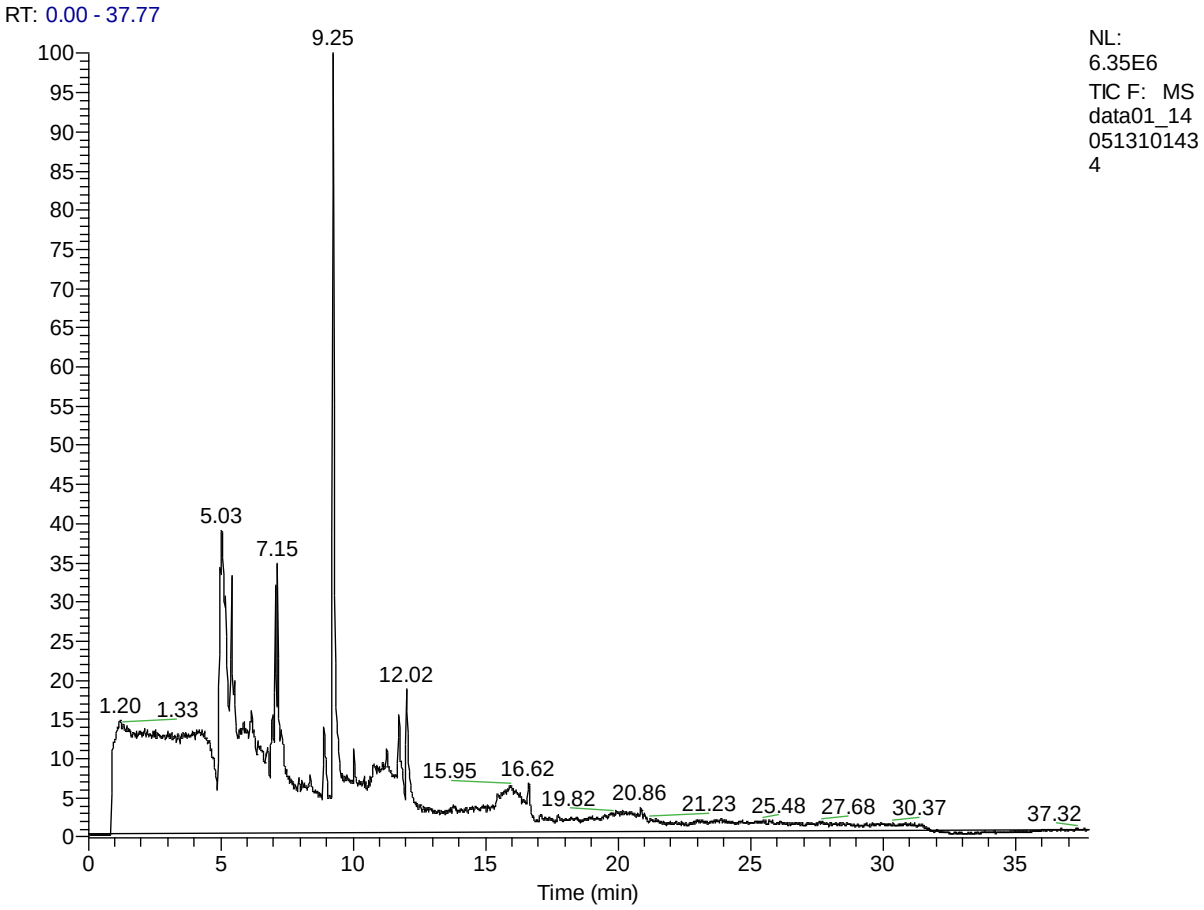


Figure 6.3. Gas chromatography mass spectrometry analysis of the methanol extract of *A. ferox*.

6.5. Conclusions

A. ferox is rich in compounds that are considered to be of major significance in the pharmaceutical industry. These compounds can serve as drug leads, part of drug combinations and sources of other derivatives. Compounds such as steroids, alkaloids, flavonoids and saponins, all of which are present in the extracts tested, are of major interest, as they form the starting point for the discovery and creation of new and improved drugs. Polyphenols have a range of activities that function in the prevention and treatment of diseases, such as antioxidant activities and other enzymatic activities. Antioxidant activity is very important in the treatment and

prevention of a wide range of diseases. The positive antioxidant activity of the *A. ferox* extract renders it very valuable therapeutically.

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CHAPTER 7

General discussion and conclusions

7.1. Discussion

Chapter 1 is an introduction to the world of medicinal plants and the current stand on natural sources for drugs. It also introduces *A. ferox* as a major contributor to local healthcare because of its phytochemical make up. Although it is most commonly known locally for its laxative properties*, A. ferox* Mill. has over 80 recorded medicinal uses. Some of these uses have been recorded in caves by the San people, showing just how far back recognition of the value of this plant goes (Grace, 2011; Reynolds, 1950). The benefits of this plants are vast and include treatment for infertility (Grace *et al*., 2008), solar radiation protection (Grollier *et al*., 1987) and even treatment for microbial and inflammatory infections caused by viruses, bacteria or fungi. This plant has generated great interest and its use is rapidly growing into a multi-billion dollar business (International Aloe Science Council, 2004).

The anti-cancer activity of *A. ferox* against selected cell lines (MCF7, HCT116 and PC3) was investigated using the SRB method (Chapter 2). According to the CSIR criteria, aqueous, methanol and dichloromethane *A. ferox* extracts were inactive against the three tested cancer cell lines, despite the fact that it contains aloe-emodin, which has apoptosis-promoting properties (Chiu *et al*., 2009). One of the recorded uses of *A. ferox* is as an anti-tumour agent; it was therefore expected that the extracts would show some degree of antioxidant activity.

Aloe species are widely known locally as treatment for STIs caused by bacteria, viruses, fungi and even parasitic infections. The glycoside compound found in the gel

and exudates of the plant leaves is credited with the ability to invade bacterial cells (Niciforovic *et al*., 2007). This may explain the positive results obtained when the extract was tested against a number of bacterial and fungal strains (Chapter 3). It was, however, inactive and unable to inhibit the growth of *C. tropicalis* and *E. faecalis* despite being able to inhibit *E. coli, Staph. epidermidis, Strep. pneumonieae* and *C. albicans* at the same concentration (15 mg/ml). It is possible that the extract showed inactivity at concentrations as high as 11 mg/ml owing to the fact that only crude extracts were used. The concentrations used were very high compared to those used by other researchers who obtained positive results for anti-bacterial and anti-fungal activity. Most of these researchers used fractionated compounds from the *A. ferox* leaf.

A. ferox plant extracts were able to increase glucose uptake by C2C12 cells, which are muscle cells, after treatment with the extracts (Chapter 4). There was, however, an insignificant decrease in glucose uptake by Chang cells after treatment with the extracts. The aqueous extract of *A. ferox* gave displayed higher anti-diabetic activity than the methanol extracts, which validates the belief in and use of this plant by local healers to manage diabetes. They usually prescribe it as a way to treat symptoms resulting from diabetes complications.

Since *A. ferox* is widely used locally to treat different ailments, sometimes with no prescriptions, it is important to determine whether the plant is in fact safe for human consumption and use. The safety of this plant against normal cells was investigated and the results are reported in Chapter 5. With two different methods used (MTT and SRB), the plant proved to be non-toxic to the selected cell lines (C2C12 and Chang

cells) *in- vivo*. The extracts also showed no cytotoxic activity against normal human foetal lung fibroblasts (W318). Thus it is safe to use, as it does not affect normal cells or cause any harm to them. In some instances the methanol extracts of *A .ferox* showed to be better than insulin and metformin as it cause less decrease to cell viability (MTT activity). These findings compound on the lack of any literature that reports *A. ferox* to have any toxic effects.

A number of qualitative and quantitative assays were used to detect, isolate and identify the phytochemical makeup of *A. ferox* Mill. and the findings were reported in Chapter 6. Phytochemicals such as steroids, phenols, alkaloids, flavonoids and saponins were found in the methanol and aqueous extracts. These are some of the most valuable phytochemicals, as they can be the starting point for a wide range of drugs and chemical entities. LCMS and GCMS assays were able to isolate and identify the major compounds in the methanol extract of *A. ferox*. The main and well-studied compound found was aloesin, a chromone with anti-inflammatory and apoptosis-inducing activity. It is part of compounds found in the gel of *A. ferox* and is responsible for most of the activity exhibited by the plant.

According to the results obtained, a lot of value can be placed on *A. ferox,* as it displays therapeutic effects. Although it proved inactive against the selected cancer cell lines, this does not mean it has no anti-cancer or anti-tumour activity and does not discredit its use by local traditional healers as treatment for cancer. It might function through other mechanisms that improve and strengthen the immune system and general well-being of individuals, which goes a long way in fighting cancer and other diseases. *A. ferox* extracts tested proved to have a wide range of compounds

that explain the therapeutic activity of the plant. The antioxidant activity of the plant means that it displays an array of activities. Anti-oxidation is required for the prevention and treatment of a wide range of diseases, such as diabetes, cancer, inflammation, arthritis, cardiovascular disorders etc. These findings emphasise the importance of plants in human health. Plants are able to supply the body with substances that it fails to obtain from the diet or that it lacks because of the lifestyle of individuals. Plants remain an untapped source of therapeutic agents and remedies that greatly improve and in some instances maintain human life.

It is recommended that this plant be investigated in more detail, especially its anti-cancer potential. More should be done, using purified compounds isolated from the extracts, as the crude extract may not show activity because of the low content of active compounds compared to inactive ones. The anti-diabetic activity of this plant should also be extensively studied and the mechanism of action determined. *A. ferox* may prove to be a good source of an alternative drug from metformin. As a natural source it may present fewer side effects compared to other therapies and drugs in use today.

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Annexure

Peer reviewed **ORIGINAL ARTICLE**

THE ANTICANCER, ANTIOXIDANT ACTIVITY AND TOTAL PHENOLIC CONCENTRATION OF *ALOE FEROX* MILL. LEAF EXTRACTS

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This project was made possible through financial assistance from the Innovation Fund awarded by the Central University of Technology, Free State.

ABSTRACT

Aloe ferox is widely used in South Africa for its anti-inflammatory, laxative, antibacterial and antifungal activity. This study was done to screen *A. ferox* leaves for anticancer properties ,antioxidant activity and to determine its phenolic content in gallic acid equivalents (GAE). The sulforhodamine B (SRB) assay method was used to screen methanolic, and dichloromethane extracts against the MC7 (breast cancer), HCT116 (colon cancer) and PC3 (prostate cancer) cell lines for anticancer activity, using etoposide as the control. The stable radical 2, 2- diphenylpicrylhydrazyl (DPPH) was used to test the radical scavenging (antioxidant) activity of the plant extracts and compared to that of ascorbic acid. The Folin Ciocalteu method was used to determine the phenolic content of the methanol, dichloromethane and aqueous extracts against a gallic acid standard. The methanolic and dichloromethane *A. ferox* extracts showed inactivity against all the cancer cell lines with a TGI of more than 100 µg/ml. However, *Aloe ferox* extracts showed antioxidant capacity with an EC50 of 0.865 mg/ml and thus is a potential source of natural antioxidants. The methanol extract (5 mg/ml) of *Aloe ferox* had the highest polyphenolic content with a GAE of 0.726.

KEYWORDS

Aloe ferox, anticancer activity, antioxidant, phenolic content.

INTRODUCTION

There is a growing interest across the world for the discovery of new drugs, especially from natural sources such as plants. They present a less expensive and non-toxic option for health care. The increased use of plants worldwide can be credited to high unemployment rates, expensive healthcare and life in general[1,2]. The development of resistance to synthetic drugs by different organisms as well as the toxicity of these drugs also contributes to the major interest in plants as potential sources of different therapeutic drugs. Due to the alarming high rates of uncontrolled harvesting, some of the South African plant species stay threatened and may become extinct. This can happen before their potential as useful sources of drugs can be discovered[2].

Chronic diseases such as cancer, diabetes, cardiovascular and neurodegenerative diseases fuelled by the ways of life of the modern generation are affecting the entire world. Most of the common diseases except, bacterial and fungal diseases result from oxidative stress damage from reactive oxygen species (ROS) and reactive nitrogen species (RNS)[3,4]. These ROS and RNS entities can only be eradicated by antioxidant compounds found in high quantities in fruits, vegetables and other plants. The antioxidants present in plants can be used to replace the possibly carcinogenic and harmful synthetic antioxidants such as butylatedhydroxytoluene (BHT) and butylatedhydroxyanisole (BHA)[5,4]. Polyphenols which also have antimutagenic and anticancer activity are the most commonly studied antioxidants[6]. It is due to this activity and their abundance in medicinal plants that ethnobotany has become the centre of focus for most researchers.

The Asphodelaceae family has polyphenol- rich species. Only a few of these species have been investigated[7] and most of the scientific evaluation has only been done on *Aloe vera* which is highly used in cosmetics. *Aloe vera* has antiseptic, wound healing, antitumor and antidiabetic properties. Due to the diversity of the genus and their abundance in South Africa, the local communities have decided to use which ever species is available in their region[8].

Although plant species such as *Aloe ferox* Mill. are widely used and have many documented medicinal uses, their use, role as well as efficiency is still to be studied and validated. *Aloe ferox* is locally used for its antibacterial, antifungal as well as laxative properties[7]. The *Aloe ferox* leaf gel alone has been shown to contain polyphenols, indoles and alkaloids which all have antioxidant capacity[8]. These findings make *Aloe ferox* a possible source of antitumor agents. In this study different extracts of the *Aloe ferox* leaf were screened for anticancer and antioxidant activities. The phytochemical screening and total polyphenol determination were also carried out on the extracts.

MATERIALS AND METHODS

Sample preparation

Aloe ferox was purchased from a nursery in Pretoria, South Africa. A voucher specimen (RM001) was kept for future reference in the Biomedical Technology research laboratory at the Central University of Technology. The leaves were washed, oven dried at 40ºC, pulverized. Ten grams of the powdered plant material was then soaked separately in 150 ml of different solvents; purified water, methanol, and dichloromethane and shaken for 72 hours, after which the extracts were filtered. The methanol and dichloromethane solvents were then removed through rotary evaporation at 35ºC and the water extracts were lyophilized. Before screening for different activities, the extracts were redissolved into the appropriate solvents in accordance to the required concentrations and volumes for each assay.

Phytochemical screening

Different qualitative methods from De *et al.*[9] were used to determine the phytochemical make up of *Aloe ferox* extract. The extracts were screened for carbohydrates, proteins, steroids, saponins, alkaloids and flavonoids.

Test for carbohydrates: Fehling's test

To 2 ml of the sample, 2 ml of Fehling's reagent A and B were added. The solution was then shaken and boiled. The formation of a brick- red precipitate (cuprous oxide) indicates the presence of reducing sugars.

Test for steroids: Libermann- Buchard test

A few drops of acetic anhydride were added to treat the extract, the mixture boiled and then cooled. Sulphuric acid was added to the mixture drop by drop to the sides of the test tube. The presence of steroids was indicated by formation of a brown ring at the junction of the two layers with the upper layer turning green. Formation of a deep red colour at the upper layer indicated the presence of triterpenoids.

Test for saponins: The foam test

Two millilitres of the extract were mixed with 2 ml of distilled water and the solution was shaken vigorously. The formation of persistent foam was an indication for the presence of saponins in the extract.

Test for alkaloids: Dragendorff's test

The extract was mixed with a few ml of Dragendorff's reagent. Alkaloids give a reddish brown precipitate.

Test for flavonoids: Shinoda's test

Flavonoids give a pink scarlet, green or a green to blue colour after addition of a few fragments of magnesium ribbons and a drop-wise addition of concentrated hydrochloric acid.

Anticancer activity screening

The human cancer cell lines MCF7 (breast cancer) and PC3 (prostate cancer) were obtained from the National Cancer Institute (NCI) in the frame work of collaborative research programme between Council for Scientific and Industrial Research (CSIR) and NCI in Pretoria, South Africa. The colon cancer cell line (HCT116) was obtained from European Collection of Cell Cultures (ECACC). The cell lines were maintained in a monolayer cell culture at 37ºC, 5% CO2, 95% AIR and 100% relative humidity in RPMI containing 5% fetal bovine serum, 2mM L-glutamine and 5 µg/ml gentamicin.

The Sulforhodamine B (SRB) assay method by Monks and collegues[10] was used to test the cancer growth inhibitory effects of the *Aloe ferox* extracts against 3 cancer cell lines. The cells were inoculated on 96 well microtiter plates at plating densities of 7- 10 000 cells per well. They were then incubated at 37ºC for 24 hours after which one plate was fixed with TCA. This plate was labelled the (T0) and represented the measurement of cells inoculated in each plate before drug addition. The test extracts at different concentrations (6.25- 100µg/ml) were added to the other plates (Ti). The plates were then incubated for 48 h after which cold 50% trichloroacetic acid was used to fix viable cells to the bottom of each well. The cells were then washed, dried and dyed with SRB. Optical density measurements on a multiwell spectrophotometer were used to calculate the net cell growth percentage at 540nm. The following equations were used to calculate the percentage cell growth:

[(Ti- T0)/(C- T0)] x 100 for concentrations at which Ti≥ T0.

[(Ti- T0)/ T0] x 100 for concentrations at which Ti<T0.

The biological activities of the extracts were reported as Total Growth Inhibition (TGI) and divided into 4 categories as shown in Table 1. Cells without any drug/ extract added were used as the control, the blank was complete medium without any cells and the Etoposide was used as the standard.

Antioxidant activity screening

The method described by Brand- Williams[11] with some modifications was used to screen the plant extracts for antioxidant activity. A stable radical 2, 2- dipheny- 1- picrylhydrazyl (DPPH) at 0.1mM was used to investigate the free radical scavenging activity of the extracts. Ascorbic acid, an antioxidant was used as a standard. One hundred millilitres of methanolic solutions of the extract at various concentrations (0.078-2.5 mg/ml) was added to 2 ml of the DPPH solution. The mixtures were shaken vigorously and then incubated in the dark at room temperature for 1 hour. The absorbance of the solution was then measured with a Helios Epsilon, CAT: 9423UVE1000E ThermoSpectronic spectrometer at 517nm, and the percentage of antioxidant activity (AA) calculated using the following formula:

$$AA\% = 100-\{[(Abs_{sample}–Abs_{blank}) \times 100]/Abs_{control}\}$$

A solution of methanol (0.1 ml) and DPPH (2 ml) was used as the control and for the blank, methanol (1ml) was used. The EC50 for the sample was determined from a plotted graph of DPPH disappearance as a function of sample concentration.

Total phenolic content

The total phenolic content of the plant extracts was determined using the Folin- Ciocalteu method according to Waterhouse[12] with a few modifications. Gallic acid which is a strong antioxidant was used as the standard from which the phenolic concentration of the sample was determined and expressed as mg gallic acid equivalent (GAE). The sample (0.1 ml) was mixed with 46ml of distilled water in 50 ml volumetric flask and 1ml of 2N Folin- Ciocalteu reagent added. The mixture was then thoroughly mixed and allowed to react for 3 minutes after which it was topped up to 50 ml with sodium carbonate solution (2%). Then the solutions were allowed to incubate for 2 hours at room temperature, after which the absorbance of the solutions was measured at 760nm with the Helios Epsilon, CAT: 9423UVE1000E ThermoSpectronic spectrometer. This procedure was also followed for gallic acid to obtain the standard curve.

RESULTS AND DISCUSSION

Phytochemical screening

The aqueous plant extract showed no presence of proteins but was positive for carbohydrates, saponins, steroids, alkaloids as well as flavonoids. The presence of steroids and alkaloids strongly suggest that these extracts may have a potential in the production of anti- inflammatory or even anticancer agents. Any agent that has steroids and alkaloids is a strong contender

Table 1: Categories of anticancer activity (CSIR criteria).

TGI	Category
>50 µg/ml	Inactive
<50 µg/ml > 15 µg/ml	Weak activity
<15 µg/ml > 6.25 µg/ml	Moderate activity
<6.25 µg/ml	Potent activity

Table 2: The biological activities of the extracts against the MCF7, HCT116 and PC3 cancer cell lines.

	Etoposide			Methanol extract			Dichloromethane extract		
Activities	**MCF7**	**HCT116**	**PC3**	**MCF7**	**HCT116**	**PC3**	**MCF7**	**HCT116**	**PC3**
GI50	<6.25	19.96	34.64	86.56	>100	>100	86.72	>100	46.09
TGI	>100	>100	>100	>100	>100	>100	>100	>100	>100
LC50	>100	>100	>100	>100	>100	>100	>100	>100	>100
LC100	>100	>100	>100	>100	>100	>100	>100	>100	>100

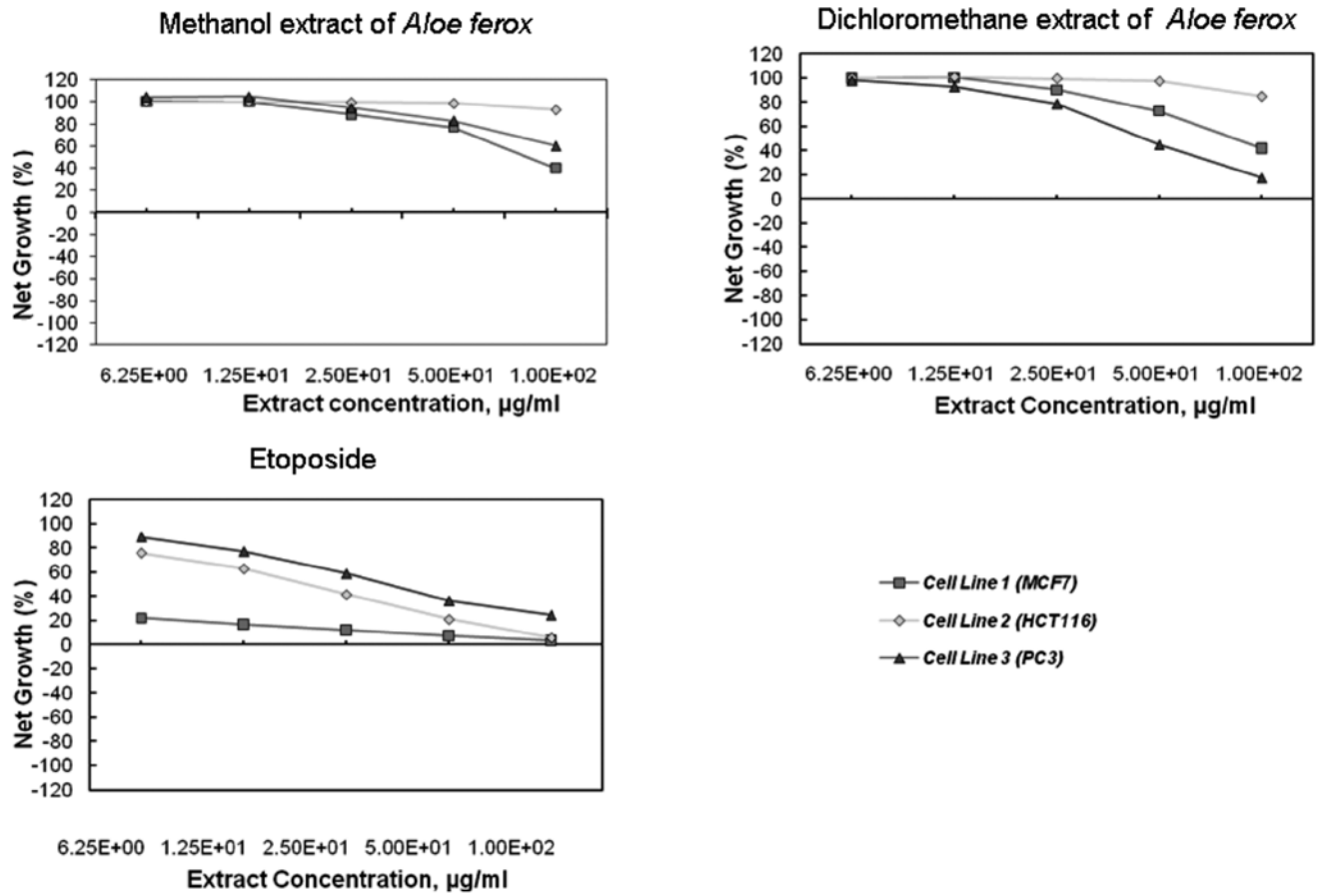

Figure 1: Effect of Methanol and dichloromethane extracts of *Aloe ferox* against the breast cancer (MCF7), colon cancer (HCT116) and prostate cancer (PC3) cell lines.

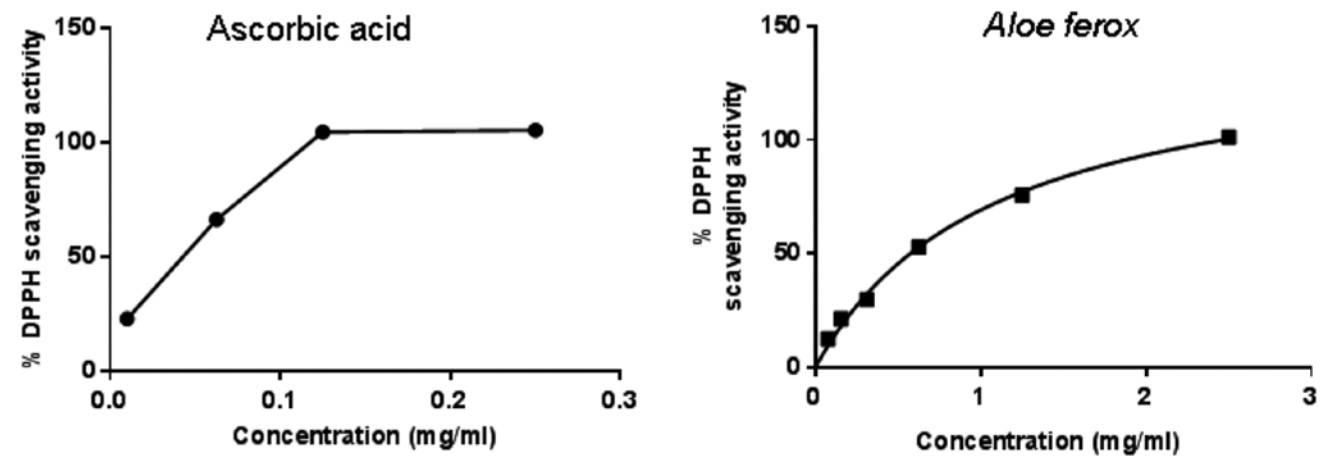

Figure 2: Free radical scavenging activity of the methanolic *A. ferox* extract.

Table 3: Antioxidant activity of *Aloe ferox* leaf methanol extracts.

Concentration (mg/ml)	% DPPH scavenging activity
2.5	101.25 ± 0.042
1.25	75.87 ± 0.386
0.625	52.98 ± 0.416
0.3125	29.92 ± 0.699
0.156	21.38 ± 0.252
0.078	12.61 ± 0.682
EC50	**0.865 ± 0.783**
Ascorbic acid EC50	**0.042 ± 0.084**

Table 4: Total phenolic content of *A. ferox* extracts.

Sample	Absorbance Mean	Polyphenol concentration (mg/ml GAE)
Water extract		
5mg/ml	0.069	0.340 ± 0.057
Dichloromethane extract		
5mg/ml	0.109	0.550 ± 0.042
Methanol extract		
5mg/ml	0.144	0.726 ± 0.032

in the fight against cancer and other chronic inflammatory diseases as majority of these compounds have been found to have anti- inflammatory, free radical scavenging and apoptosis inducing properties[13]. The presence of saponins may also further indicate the presence of steroids and triterpenoids which often present in the form of saponins[14]. The pharmacological effects of plants are a result of the activity of single compounds or a synergetic effect of the compounds[15]. The presence of flavonoids also contributes to the antioxidant activity of the plant extracts as in addition to free radical scavenging; they also have vein strengthening effects[16]. They have anti- inflammatory, antiallergic, antibacterial and antiviral effects[17]. The results of the present study show compounds that may support the findings by Kambizi[18].

Anticancer activity screening

According to the CSIR criteria (Table 1), the tested extracts (methanol and dichloromethane) are inactive against all the cell lines as indicated in Figures 1. The methanol extract exhibited inactivity against the colon, breast and the prostate cancer cell lines with the TGI of more than 100 µg/ml for all of them. Table 2 shows the results of all the parameters measured. As shown the extract did show a lower GI50 of 86.56 µg/ml against the breast cancer cell line. The dichloromethane extract also showed no activity against the three cell lines with a TGI of more than 100 µg/ml. However the extract showed a 50% inhibitory concentration of 46.09 µg/ml against the prostate cancer cell line which is much lower compared to the 86.72 µg/ml needed to exert the same activity against the breast cancer cell line. The inactivity of the extracts is not discouraging as the etoposide was also inactive against the cell lines as shown on Figure 1. It required a concentration of more than 6.25 µg/ml to have GI50 against the prostate and colon cancer cell lines. The inactivity of the extracts against these certain cell lines does not mean the plant has no anti- cancer activity as more evaluation against other cancer cell lines needs to be done. The inactivity may also mean the concentrations used were low for both the etoposide and the test extract.

Antioxidant activity

The DPPH radical scavenging activity of the methanolic extract is shown in Table 3. The methanolic extract was able to reduce the stable free radical DPPH to a yellow coloured 1,1- diphenyl-2-picrylhydarzyl and this activity was thought to be a result of the hydrogen- donating ability of the extract compounds. The extracts have potent antioxidant activity which was compared to that of the standard (ascorbic acid), shown in Figure 2. The highest activity (101 ± 0.042%) was achieved at extract concentration of 2.5 mg/ml. The EC50 of the extract was 0.865 mg/ml compared to that of ascorbic acid which was 0.042 mg/ml.

Total phenolic content

Table 4 reports the results of total phenolics of the different extracts. The extracts were analysed at 5mg/ml against gallic acid. The phenolic content varied between the extracts. Methanol extracted more polyphenols than water or dichloromethane. The gallic acid equivalents of the estimated phenolic concentrations are arranged in ascending order in Table 4 and range from 0.340 ± 0.057 to 0.726 ± 0.032 mg/ml GAE.

CONCLUSION

In conclusion, the results of this study show that A. ferox has antioxidant capabilities which may be due to its polyphenolic composition. Methanol extracts more polyphenols than water and dichloromethane. The presence of alkaloids, steroids and flavonoids as proved by the phytochemical screening show that this plant has the potential to prevent development of symptoms of cardiovascular diseases, diabetes, cancer and other inflammatory diseases. They are the main constituents of most of the drugs currently in use for these diseases. Further testing especially using different cell lines for anticancer screening should be done to validate the plant's use for cancer treatment. The lack of activity against cancer cells could also mean that the plant functions against cancer through other mechanisms that do not affect the cancer cells directly. Some of these mechanisms could be by easing pain, inflammation and other symptoms.

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