UNIVERSITY OF ZIMBABWE

Isolation, purification and characterisation of antiproliferative phytochemicals from *Dolichos kilimandscharicus* (Fabaceae)

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Faculty of Science

DECLARATION

I, Simbarashe Sithole, hereby declare that the experimental work described in this report was carried out in the Biomolecular Interactions Analyses Laboratory, in the Department of Biochemistry, at the University of Zimbabwe from November 2016 to May 2017. This is my original work and has not been reproduced from someone else's work. The work was not previously submitted and where other people's work has been used it acknowledged in the text.

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Signature **Date**

Supervisor: _____

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Signature **Date**

Associate Supervisor: _____

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Signature **Date**

DEDICATION

This work I dedicate to my wonderful, supportive and ever-inspiring wife, **Sharon Patricia Sithole**. You have had to put up with sleepless nights and lonely days as I have been working on this program, thank you for never complaining but continuously encouraging me. To my two sons **Seth and Sean**, I also dedicate this work and say you will never achieve any greater goals than those that you set yourselves. Make God your pillar of strength and you will overcome any and all circumstances.

ACKNOWLEDGEMENTS

Thank you, GOD, this has been a long time in coming but I realise Your plan is always the best! Words fail me in trying to show my endless gratitude to my supervisor **Professor S. Mukanganyama** for his continuous support and provision of intellectual guidance in ensuring the completion of this research work. The project would not have been successful without the contributions of Dr L. Nhamo, Ms W. Muzorandi, Ms M. Mombeshora, Mrs R. Mautsa and Miss M. V. Masuku. I am indeed appreciative of their assistance and support. I want to thank members of the Biochemistry and Chemistry departments for their help as I worked on this project. My special thanks and gratitude also go out to my exceptional friend Sharon Patricia Sithole and all my lecturers and classmates for making the journey through the Masters in Chemistry worthwhile and for their help and motivation throughout the entire duration of this project.

ABSTRACT

Plants are a source of over a quarter of the prescription drugs currently in use worldwide. Coupled with human perception that they provide a safe disease treatment regime, plant derived compounds have become the mainstay sources for human health products. *Dolichos kilimandischaricus* has previously been studied and shown to have antiproliferative activity against cancer cells. This study was set up to determine the chemical composition of *D. kilimandischaricus*. Isolated extracts of *D. kilimandischaricus* were screened for their antiproliferative effect on mouse peritoneal cells. GC-MS, LC-MS and Fourier Transform Infra-Red (FT-IR) spectral analysis of crude extract and purified components were carried out in order to predict various active functional groups which are responsible for their biological activities. Extraction with Dichloromethane: Methanol (1:1) had a yield of 2.64 % while serial exhaustive extraction, carried out with seven solvents of differing polarity, had a yield of 7.95 %. The methanol extract had the highest percentage yield of 2.54 %, with the hexane extract having the least yield of 0.27 %. The Methanol and Dichloromethane extracts showed a similar phytochemical compounds profile, though the Methanol extract possessing flavonoids, steroids and terpenoids while the Dichloromethane extract did not. FT-IR spectral analysis data of the methanolic extract revealed the presence of multiple functional groups. Spectral data of this extract confirmed the presence of bioactive functional groups such as –OH, –NH, –CHO, –COOH and –COOR. The methanol extract was shown to be the most potent extract in the inhibition of the growth of mouse peritoneal cells though the effects were less when compared to the cells incubated with the anticancer drug camptothecin. Fractions that were pooled together after TLC and pools P10, P12, P50, P51 and P52 exhibited antiproliferative effects against the mouse peritoneal cells at concentration of 0.1 mg/ml. GC-MS managed to identify 3,8-bis(2',2'-Dimethylpropionyloxy)-1-ethoxy-6-methoxy-2-methyl-9,10-anthraquinone, 1,4,5,6,7,11b-Hexahydro-10-chloro-4-(4'-chlorophenyl)-3,11b-bis[trifluoromethyl)imidazo[1,2-a] pyrazolo [4,3-b] indole, 1-(4-isopropylphenyl)-2-methylpropyl acetate, Dodecanamide, Methyl stearate, 7-Acetyl-4,5-epoxy-18,19-dihydro-3,6-dimethoxy-6,14-ethenomorphinane, 5-Benzoyl-1,3-bisethoxymethyl-1H-pyrimidin-2,4-dione, Decanamide that are responsible for the antiproliferative effects on mouse peritoneal cells in *D. kilimandischaricus* methanolic extract.

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ANOVA- Analysis of variance

DCM- dichloromethane

DMSO- dimethyl sulfoxide

FBS- foetal bovine serum

FTIR- Fourier Transform Infrared Spectroscopy

GC-MS- Gas Chromatography-Mass Spectrometer

LC-MS- Liquid Chromatography-Mass Spectrometry

PBS- phosphate-buffered saline

RBC- red blood cells

RPMI- Roswell Park Memorial Institute medium

TLC- Thin Layer Chromatography

TABLE OF CONTENTS

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 PLANTS AS SOURCES OF PHARMACEUTICALS

In the recent past, the pharmaceutical industry has undergone immense growth due to technological advancement in medicinal and combinatorial chemistry (Newman and Craig, 2007). There has also been an increase in the knowledge of molecular biology, the process of drug discovery has undergone rapid evolution (Newman *et al*., 2003). Chemical constituents sourced from natural products are characterised by having high chemical diversity, biochemical specificity and other molecular properties (Koehn and Carter, 2005). Plants as sources of therapeutic agents, however, remain at the forefront in combating existing, emerging and re-emerging diseases. Plant products have been used as medicinal plants with varying success to cure and prevent diseases from as early as 5000 years ago (Swerdlow, 2000). The plant kingdom possesses a high number of potential drugs. There has been an increased awareness in recent years to the importance of plants as sources of medicine (Newman *et al*., 2003). Drugs from the plants are easily available, less expensive, considerably safer, and more efficient and with the rare side effects (Newman and Craig, 2007). There is need for studies into the efficacy of medicinal plants traditionally accepted, thereby integrating the traditional medicine into conventional medical care systems. Plants are a source of over a quarter of the prescription drugs currently in use worldwide (Rates, 2001). There is a general perception among man that plants provide a safe disease treatment regime so much that plants have become the mainstay source for human health products (Raskin *et al*., 2002).

It is estimated that over fifty-percent of the medicines developed since 1980 have been natural products, their derivatives, or their analogs, with approximately 25% of those currently

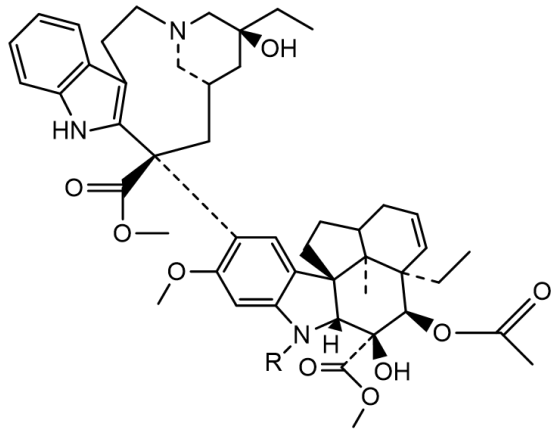
used as modern medicines derived from plants (Newman and Craig, 2007). Traditional medicine has remained the most affordable and easily accessible source of treatment in the primary healthcare system of poor communities including some in Zimbabwe (Rauf *et al*., 2017). However, from all the 600 000-plant species, a mere two percent has been studied for their pharmaceutical activity and chemical composition (Ladmier, 1984). In Zimbabwe, it has been reported that of the 5 000-plant species growing in the country, about 10 percent have medicinal properties as well as being used as traditional medicines (Gelfand, 1985). The use of traditional medicine is extensive but the knowledge needs to be recorded because if not then indications are that this knowledge will be lost with succeeding generations. The recovery of knowledge and practices associated with medicinal plants can be viewed as an important part of conserving of biodiversity. The discovery of new medicines will also improve the quality of life for the rural communities. People, particularly in developing countries, need to develop and invest in research programs that validate and standardise phytochemical products. Pharmaceutical companies in the United States, such as Merck, CIBA, Glaxo, Boehringer and Syntex, are studying new drugs from natural sources (Reid *et al*., 1993).

The current world estimations are that 80 % of the population depend on traditional medicine for the treatment of diseases. This dependence is due to ease to access, affordability and acceptability on socio-cultural grounds (Krause *et al*., 1993). The extensive use of plants as medicine is mainly based on folklore knowledge that has been passed along generations. This knowledge has provided the primary hint for scientific studies that has led to development of plant-based pharmaceuticals.

The use of plants as drugs has also been propagated by the inefficiency of conventional medicine due to, the abuse and/or incorrect use of synthetic drugs. In addition to there is general

inaccessibility to conventional treatment as well as development of multi-drug resistant strains (Rates, 2001). Drug resistance has developed with a number of bacteria, viruses, fungi, human cancers and parasites through multi-drug resistant strains caused by indiscriminant use of drugs (Ozben, 2006). Thirteen natural-product-related drugs were approved from 2005 to 2007, with over half of them being found to obey Lipinski's Rule of Five for orally available compounds. On average natural products are more readily absorbed than synthetic drugs (Harvey, 2008).

There have been varied studies in which many beneficial biological activities of plants such as anticancer, antimicrobial, antioxidant, antidiarrheal, analgesic and wound healing activity have been found (Newman and Craig, 2007). Vinca alkaloids, vinblastine and vincristine were the first to advance into clinical use marking the dawn of the use of plant material as anticancer agents (**Figure 1.1**). These compounds isolated from the Madagascar periwinkle, *Catharanthus roseus* G. Don. (Apocynaceae) were initially used for the treatment of diabetes (Gueritte and Fahy, 2005).



Vinblastine; R = CH_3
Vincristine; R = CHO

Figure 1.1 Vinblastine and Vincristine isolated from *Catharanthus roseus*

Extensive research into chemotherapeutic drugs has led to the development of a number of plant derived compounds as clinically effective agents which are used in the treatment of various cancers (Cragg and Newman, 2005). These include but are not limited to podophyllotoxin from *Podophyllum peltatum* and *Podophyllum emodii*, taxanes from *Taxus brevifolia*, camptothecin from *Camptotheca acuminata*, homoharringtonine from *Cephalotaxus harringtonia*, and elliptinum isolated from species of the Apocynaceae family (Cragg and Newman, 2005). The chemical structures are shown in **Figure 1.2**.

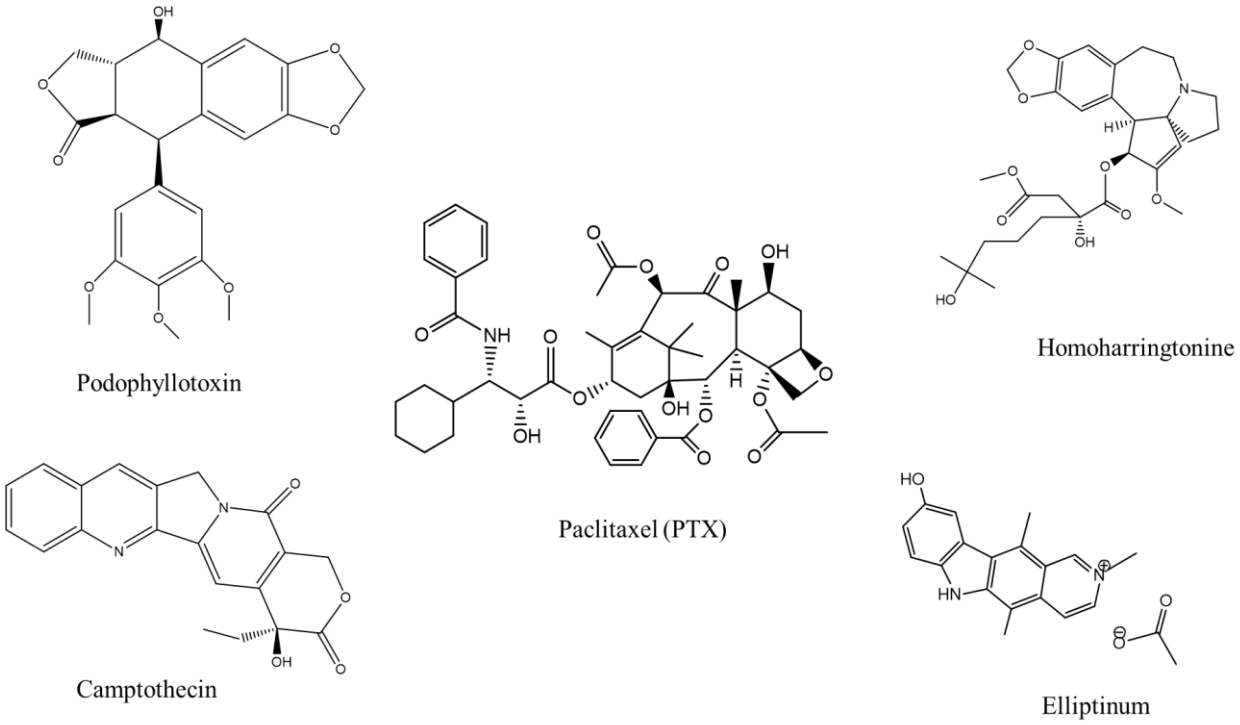


Figure 1.2 Examples of cancer drugs derived from plants

It has become pivotal to cancer research to identify plant compounds with antiproliferative properties. Some 60 % of anticancer and anti-infectious drugs on the market or undergoing clinical trials have been derived from natural products or plants (Hamburger and Hostettmann, 1991). There are over 100 natural-product-derived compounds that are undergoing clinical trials with most derived from leads from plants and are predominantly being studied for use in cancer, as well as many other therapeutic areas (Harvey *et al*., 2008). The traditional knowledge possessed by a small number of people in medicinal plants has led to the increased interest into the investigation of plants. There is development of documentation of the traditional uses of medicinal plants in Zimbabwe in order to provide documentation while identifying research gaps as well as suggesting viewpoints for future research.

Cytotoxicity can be defined as the ability of a compound to cause cell death (Wyllie, 1987), through apoptosis or necrosis. Apoptosis is a carried out in a regulated process which is part of the cell development cycle, which occurs in multicellular organisms (Kumar *et al*., 2016). A number of cells can be used in *in vitro* cytotoxicity or antiproliferative studies such as liver, blood, peritoneal cells, bacterial, fungal and yeast cells (Ivask *et al*., 2014). The cells inhabiting the peritoneal cavity are comparable to a lymph node in that some cells reside in the cavity, whereas others are transient, passing through on their way to other targeted tissues. In the peritoneal cavity, there are immune interactions that are geared up to respond rapidly to bacteria that may be released by intestinal spillage (Bazhanov *et al*., 2016).

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, is an *in vitro* assay for the measurement of cytotoxicity, cell proliferation or, when metabolic events lead to apoptosis or necrosis, a reduction in cell viability (Denizot and Lang, 1986). It is a colorimetric assay that measures the reduction of yellow MTT (a yellow tetrazole) by

mitochondrial succinate dehydrogenase in the presence of NADH and NADPH, the equation shown in **Figure 1.3**. Viable, metabolic active cells, produce in the respiratory chain, NADH and NADPH, which in turn become responsible for the cleavage of MTT. The cleavage of MTT produces a purple formazan only in living cells which is directly proportional to the number of cells alive (Vinjamuri *et al.*, 2015). The cells are then solubilised with an organic solvent (e.g. isopropanol, dimethyl sulfoxide) and the released, solubilised formazan reagent is measured spectrophotometrically.

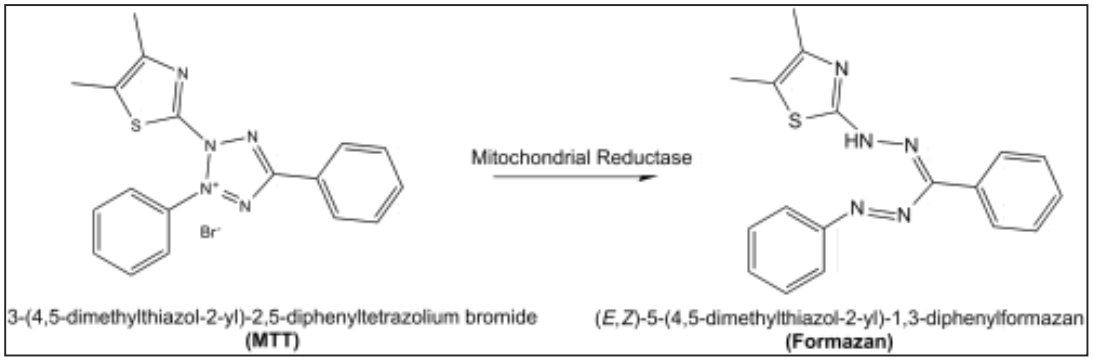


Figure 1.3 The reduction of the yellow tetrazolium (MTT) salt in the presence of viable cells to formazan, a purple coloured compound. Accessed 12 February 2017. http://labiq.iq.usp.br/kfile_arquivo.php?id=1629

Extraction is the separation of medicinally active portions of plant tissues using selective solvents through standard procedures with the products obtained being mixtures of metabolites, in liquid or semisolid state or in dry powder form. During the extraction process the solvents mix with the solid plant material, thereby solubilizing compounds with the same polarity (Ncube *et al.*, 2008). The extraction process leads to an extract with desired chemical components from the plant materials suited for further separation and characterization. Biologically-active compounds can then be isolated from medicinal plants by bioassay-guided fractionation procedures, in which various screening methods are employed to locate the desired activities in

the crude extracts and in the fractions. The quality of the extract has been shown to depend on the plant part used, solvent used for extraction and the extraction procedure (Ncube *et al.*, 2008). The principle is to grind the plant material (dry or wet), thereby increasing the surface area for extraction in turn increasing the rate of extraction, with a solvent to sample ratio of 10:1 (v/w) having been reported to be ideal (Das *et al.*, 2010).

The choice of solvent used in the extraction procedure greatly determines the type of biologically active compounds extracted from the plant material, bearing in mind that the end product will contain residual solvent, the solvent should be non-toxic and should not interfere with the bioassay (Ncube *et al.*, 2008). The various solvents that are used in the extraction procedures are:

Water: This is a universal solvent, used to extract plant products. Traditional healers use primarily water.

Acetone: Acetone dissolves many hydrophilic and lipophilic components from the two plants used, is miscible with water, is volatile and has a low toxicity to the bioassay used, it is a very useful extractant, tannins have been shown to be extracted more effectively by aqueous acetone than by aqueous methanol (Eloff, 1998).

Alcohol: Ethanolic extracts have been reported to possess higher antiproliferative activity due to the presence of polyphenols when compared to aqueous extracts (Eloff, 1998). Flavonoids are largely detected in 70 % ethanol as it possesses a higher polarity to pure ethanol, as addition of water increases the polarity of solvent (Bimakr, 2010). Most of the identified components obtained through ethanol or methanol (which is more polar than ethanol) extraction from plants with antimicrobial activity are aromatic or saturated organic compounds.

Several extraction procedures can be used and since the target compounds may be non-polar to polar and thermally labile, the suitability of the methods of extraction must be taken into consideration. These methods include:

Serial exhaustive extraction: is a method of extraction involves the successive extraction of solid material with solvents of increasing polarity to guarantee extraction of a wide polarity range of compound.

Soxhlet extraction: is used to extract a compound which has a limited solubility in the solvent. This method allows for one batch of solvent to be recycled instead of using many portions of warm solvent but it cannot be used for thermolabile compounds as prolonged heating may lead to degradation of compounds (Nikhal *et al.*, 2010).

Maceration: in maceration (for fluid extract), whole or coarsely powdered plant is kept in contact with the solvent in a stoppered container for a defined period with frequent agitation until soluble matter is dissolved.

Decoction: this method is used for the extraction of the water soluble and heat stable constituents from crude drug by boiling it in water for 15 minutes, cooling, straining and passing sufficient cold water through the drug to produce the required volume (Remington, 2005).

Infusion: It is a dilute solution of the readily soluble components of the crude drugs. Fresh infusions are prepared by macerating the solids for a short period of time with either cold or boiling water (Remington, 2005).

Digestion: is a kind of maceration in which gentle heat is applied during the maceration extraction process. It is used when moderately elevated temperature is not objectionable and the solvent efficiency of the menstrum is increased thereby (Remington, 2005).

Sonication: involves the use of ultrasound with frequencies ranging from 20 kHz to 2000 kHz; this increases the permeability of cell walls and produces cavitation (Remington, 2005).

It has been shown that natural products play an important role in the discovery of leads for the development of drugs for the treatment of human diseases (Newman and Cragg, 2007). The screening of plant species with the purpose of discovering new bioactive compounds is done in many laboratories from random collections or from leads supplied by local healers. The plant roots are being used extensively compared to other parts in the search for bioactive compounds (Ncube *et al.*, 2008). Natural products have also led to developments in organic chemistry in developing synthetic methodologies and the making of analogues for the original lead compound with improved pharmacological or pharmaceutical properties (Harvey, 2008).

1.2 PHYTOCHEMICALS WITH ANTICANCER PROPERTIES FROM PLANTS

Plants contain a wide variety of molecules which are commonly known as phytochemicals. A number of studies have been carried out on Chinese medicinal plants with a number of natural bioactive compounds with anticancer effects being identified (Xiao *et al*., 2000). These bioactive compounds belong to different classes depending on their chemical structures with the commonly active components being terpenes, alkaloids, coumarins, lignans, quinones, flavonoids, and tannins (Cai *et al*., 2004).

1.2.1. Terpenes:

The fragrance of plants is carried in the so called quinta essentia, or essential oil fraction which possesses compounds primarily based on an isoprene structure. They are commonly termed terpenes with a general chemical structure of $C_{10}H_{16}$, and they can occur as diterpenes, triterpenes, and tetraterpenes (C_{20}, C_{30}, and C_{40}). Terpenoids are terpenes which have additional elements such as oxygen (Cowan, 1999). Terpenes or terpenoids have been shown to be active against bacteria (Akpata cited in Cowan, 1999) and fungi (Ayafor cited in Cowan, 1999), with 60% of essential oil derivatives having inhibitory effects towards fungi while 30% inhibited bacteria (Chaurasia and Vyas, 1977).

1.2.2. Alkaloids:

Alkaloids are considered as one of the largest and well-known groups of secondary metabolites and they are heterocyclic nitrogen compounds (Cowan, 1999). Alkaloids are synthesized from amino acid whose hydrogen atoms in the peptide ring is replaced by radicals that mostly contain oxygen. Alkaloids have basic properties, readily soluble in alcohol but are partialy soluble in water (Doughari, 2012). These alkaloids function in the defence of plants

against herbivores and pathogens, and have become heavily applied as pharmaceuticals, stimulants, narcotics, and poisons (Madziga *et al*., 2010). Alkaloids are found mainly in the seeds and roots of plants and often in combination with vegetable acids. The initial medically useful alkaloid was morphine which was isolated from poppy *Papaver somniferum* (Wright, 2011). Another example is Codeine which is an opiate of plant origin used for its analgesic, antitussive and antidiarrheal properties (Van Wyk *et al*., 1997). Alkaloids have been shown to possess *in vitro* antifungal effects against fungi such as *Candida albicans* (Freile *et al*., 2003). Many drugs currently used clinically are alkaloid in nature, with early examples of the antileukemic alkaloids including vinblastine and vincristine which were both obtained from the *Catharanthus roseus* (Cragg and Newman, 2005).

1.2.3. Coumarins:

Another example of phenolic substances are coumarins which made of attached benzene and α-pyrone rings that give hay its characteristic odour (Cowan 1999). Coumarins can occur either as free or combined with the sugar glucose and are found in several plants, including tonka beans, lavender, licorice, strawberries and apricot. Coumarins have been shown to possess antithrombotic, anti-inflammatory, and vasodilatory, but albeit weak antibacterial activities (Namba *et al*., 1988; Piler, 1975; Ojala *et al*., 2000). There are hundreds of coumarins that have been reported as naturally occurring compounds present in traditional Chinese medicinal herbs associated with anticancer (Xiao *et al*., 2000).

1.2.4. Lignans:

Lignans in vegetables, fruits, spices, and medicinal herbs are assumed to have antioxidant activity which aids in the prevention of cancer (Yang *et al*., 2001). Lignan derivatives have been used for the clinical treatment of refractory testicular tumour and cancer (Kumar *et al*., 2012),

with the introduction of double bond in C-ring lowering the anticancer activity of the lignans. Even though the biological activity of lignans is generally unclear, they are known to have potent properties for antimicrobial, antifungal, antiviral, antioxidant, insecticidal and anti-feeding (Smeds *et al*., 2007).

1.2.5. Quinones:

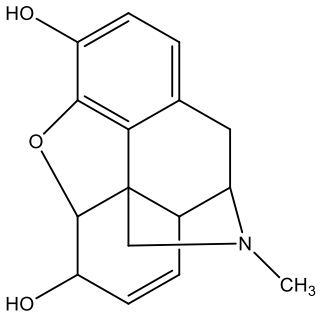
Quinones are highly reactive and ubiquitous in nature possessing aromatic rings with two ketone substitutions (Cowan, 1999). Quinones form a class of toxic metabolites generated as a result of the metabolism of phenols and related compounds, with anthraquinones the most common in the medicinal herbs. The quinones isolated from an alcoholic extract of the stem bark of *Tabebuia cassinoides* exhibited *in vivo*, slight but reproducible activity (Rao and Kingston, 1982).

1.2.6. Flavonoids:

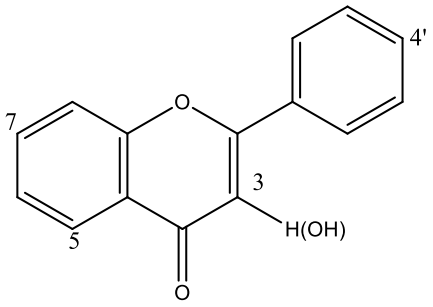
Flavones are phenolic structures containing one carbonyl group with the addition of a 3-hydroxyl group yielding flavonol, while flavonoids are also hydroxylated phenolic substances which occur as a C_6-C_3 unit linked to an aromatic ring (Cowan, 1999). Flavonoids are mainly introduced into the human diet through the consumption of fruits and vegetables and have been shown to possess antioxidant activity as well as an ability to relieve hay fever, eczema, sinusitis and asthma (Ogunleye and Ibitoye, 2003). Three flavonoids namely quercetin, kaempferol and quercitrin are present in nearly 70% of plants (Doughari, 2012). Flavopiridol isolated from *Dysoxylum binectariferum* is responsible for anti-inflammatory and immunomodulatory activity (Sausville *et al*., 1999). Several flavonoids in Africa and India reported in the treatment of "cancer" have been isolated (Pinney *et al*., 2005).

1.2.7. Tannins:

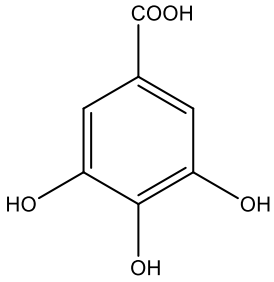
Tannins can be generally defined as naturally occurring polymeric phenolic compounds with molecular weights ranging from 500 to 3,000, that can form complexes with proteins of animal hides and prevent their decaying, thus capable of tanning leather or precipitating gelatin from solution (Cowan, 1999). Tannins are found as hydrolysable tannins, that are based on gallic acid frequently as multiple esters with β-D-glucose, while the more numerous condensed tannins are derived from flavonoid monomers (Bravo, 1998). Tannins have lately received a great deal of consideration as it has been suggested that the consumption of tannin-containing green teas and red wines, can cure or prevent diseases. Tannins have been isolated from various medicinal plants with biological and pharmacological activities having been found for several of these polyphenolic compounds. It was found that tannins isolated from *Pimenta dioica* leaves are cytotoxic against solid tumour cancer cells and induced the proliferation of T-lymphocytes and macrophages (Marzouk *et al*., 2007).



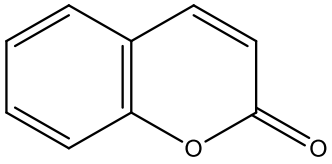
Alkaloid – Morphine



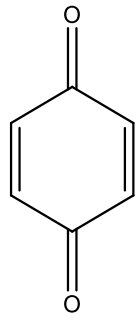
Flavone (Flavonol)



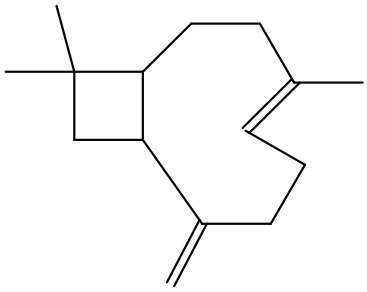
Tannins – Gallic acid



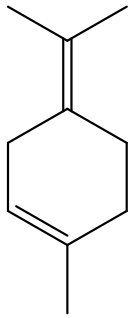
Coumarin



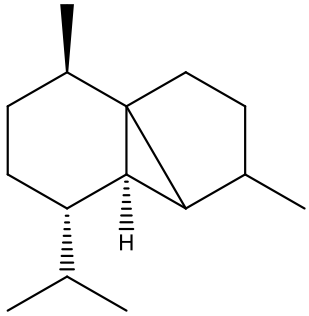
Quinone



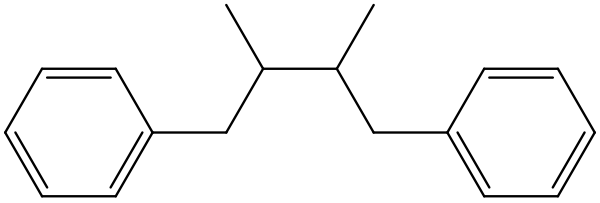
Terpenes – β-caryophyllene



Terpenolen



α-Cubebene



Lignans

Figure 1.4 Basic structures of some pharmacologically important plant derived compounds

1.3 PRINCIPLES OF FRACTIONATION, ISOLATION AND IDENTIFICATION OF COMPOUNDS

Natural product extracts contain complex undetermined chemical entities that require proper characterization. The use of Thin Layer Chromatography (TLC) in the separation and identification of plant constituents is now a common technique. TLC is employed since it allows for the quick analysis of herbal extracts with largely limited sample clean-up and also that it provides qualitative and semi quantitative information of the resolved compounds. The advantages of TLC in the routine analysis of plant phytochemicals include: use of minimum apparatus, it is relatively cheap, simple to use, minimum laboratory space required, it is fast, excellent resolution of components and it is highly sensitive to drug constituents (Poole, 2003). Chromatographic fractionation of plant extracts can be achieved by use of liquid-solid column chromatography (LSC), this procedure will yield purified components in gram amounts (Harborne, 1998). Solid adsorbents such as silica gel and alumina are used as stationary phases while various organic solvents are used as mobile phases. This technique uses gradient elution which involves changing the mobile phase composition in a stepwise manner. The mobile phase initially comprises of the less polar solvent which separates the low-polarity components. Elution continues with the increase of the more polar solvent(s) thus enabling elution of the more strongly adsorbed sample components (Njogu *et al*., 2011). The eluate is collected as a series of fractions which are then examined appropriately for the presence of compounds using TLC. Those fractions showing similar profile are pooled and subjected to laboratory manipulation aimed at isolation of pure compounds. Crystalline compounds can then be purified by recrystallization from a suitable solvent or mixture of solvents. The combination of high-performance liquid chromatography (HPLC) with on-line Ultra-Violet, Mass Spectrophotometry,

and Nuclear Magnetic Resonance detection has become a very valuable tool in the analysis of phytochemicals in plant extracts and as such in drug development (Butler, 2004). Gas chromatography (GC) equipment can be directly interfaced with rapid scan mass spectrometry (MS) of various types consequently speeding up the separation and structural elucidation processes. The column output of the GS has a low flow rate which allows for the output to be directly fed into the ionization chamber of MS. Hyphenated GC-MS systems have found many application in the identification of hundreds of components present in natural and biological systems (Oleszek and Marston, 2000).

1.4 *Dolichos kilimandischaricus* (Harms) ex Taub. Fabaceae

Figure 1.6 Dolichos kilimandscharicus shrub Location: Centenary (17° S, 31° E)

Dolichos kilimandscharicus also known as *Dolichos lupiniflorus*, is commonly known as Veld lupin. This is an erect perennial herb that grows up to a metre arising from a perennial woody rootstock, usually flowering when the leaves are absent or under-developed with silky hair covered leaves that trifoliolate (Hyde *et al*., 2013). The stems arise from a large turnip-shaped root. *D. kilimandscharicus* is widely distributed in Africa southwards of Ethiopia and it is native to several African countries namely Angola, Ethiopia, Kenya, Malawi, Mozambique, Rwanda, Sudan, Tanzania, Zambia. In Zimbabwe, it is particularly common in the central parts occurring in Brachystegia woodland (Hyde *et al*., 2013). *D. kilimandscharicus* is being used as a natural fungicide in small scale farming, and as a non-vertebrate poison main application in fishing (Neuwinger, 2004). The root slurry of *D. kilimandscharicus* is used in Ethopia to treat sorghum smuts and the methanolic crude extracts from *D. kilimandscharicus* were shown to

possess *in vitro* antifungal activity with *Botyritis cinereal* (Tegegne and Pretorius, 2007). The roots are used for the treatment of aching limbs (Hyde *et al*., 2013). In Kenya, Uganda and Tanzania, the leaves are boiled together with other plants for the treatment of colic, dysentery, gonorrhoea and syphilis (Moshi *et al*., 2012). The plant is used as a pesticide in Zambia and Malawi (Nyirenda *et al*., 2010) as well as for the treatment of malaria by traditional healers in Cameroon though the practice is not completely recognised by modern medicine (Bahekar and Kale, 2013).

There have been few studies in which the phytochemical properties of *D.* species have been found with the methanolic extracts of the roots possessing molluscicidal and antifungal activity (Hostettmann *et al*., 2000). The flavonolglycoside isolated from the seeds of *D. lablab* showed antibacterial activity against *Bacillus coagulas, Staphylococcus aureus, Escherichia coli* and *Psedomonas aeruginosa* (Jain, 2014). The methanolic extract of *D. biflorus* was shown to possess antioxidant activity when evaluated using *in vitro* techniques (Muthu, 2011). A prenylated dihydroflavonoid obtained from the root of *D. tenuicaulis* inhibited human cancer cells' growth for 10 cell lines *in vitro* (Peng, 2007). Shava *et al*., (2016) confirmed the presence saponins in the extracts of *D. kilimandischaricus*. The authors also tested for antibacterial and antiproliferative effects of the saponins and DCM extracts. The growth of *S. aureus* was inhibited by a saponin rich fraction at low concentration. The DCM extract had dose- and time-dependent anti-proliferative effects on HL60 cells.

1.5 RATIONALE OF THE STUDY

Plants are a source of over a quarter of the prescription drugs currently in use worldwide, coupled with human perception that they provide a safe disease treatment regime; plants have become the mainstay sources for human health products. The use of plants as sources of drugs has also been propagated by the inefficiency of conventional medicine due to abuse and/or incorrect use of synthetic drugs, as well as inaccessibility to conventional treatment in developing countries such as Zimbabwe. There have been a few documented ethnomedicinal uses of *D. kilimandischaricus*. *D. kilimandischaricus* needs to studied and tested for antiproliferative activity against cancer cells which pose a threat to human life. The present study is designed to characterize bioactive compounds *D. kilimandischaricus*. Encouraged by the reported anticancer properties of *D. kilimandischaricus* (Shava *et al*., 2016) and prompted by the usage of root extracts in traditional medicine as treatment for cancer, it is essential to isolate, purify and analyse of phytochemicals of *D. kilimandischaricus*. GC-MS, LC-MS and Fourier Transform Infra-Red (FT-IR) spectral analysis of crude extract and purified components were carried out in order to predict the various active functional groups which are responsible for biological activities in this plant.

CHAPTER 2:

2.1 HYPOTHESIS

1. One or more extract(s) of the roots of *D. kilimandscharicus* (Fabaceae) possess compounds with an antiproliferative effect on cells.
2. The observed antiproliferative effect is attributable to one or more chemical compound(s) that can be identified from fractions of *D. kilimandischaricus*.

2.2 RESEARCH QUESTION

What phytochemicals from *D. kilimandscharicus* are responsible for the antiproliferative effects on peritoneal cells from Balb/c mice?

2.3 MAIN OBJECTIVE

The aim of the study was to isolate, purify and characterise compounds from *D. kilimandscharicus* (Fabaceae) that might have antiproliferative effects on murine peritoneal cells.

The specific objectives of this study were:

a) To obtain extracts from *D. kilimandscharicus* through total extraction and serial exhaustive extraction.

b) To screen the n-hexane, dichloromethane (DCM), acetone, ethyl acetate, methanol, ethanol, water and DCM: Methanol (1:1) extracts of *D. kilimandischaricus* (Fabaceae) root extracts for their antiproliferative effect on murine peritoneal cells.
c) To determine the components, present in the extracts by thin layer chromatography.
d) To carry out phytochemical screening of secondary metabolites using qualitative tests
e) To isolate by chromatographic fractionation and purify compounds from root extracts using a chromatographic technique.

2.4 STATEMENT OF THE PROBLEM

Universal accessibility to affordable health care is one of the Sustainable Development Goals set by the United Nations in 2016 to be achieved by the year 2030 (United Nations, 2015). In Zimbabwe, efforts towards provision of affordable health care have been hindered by lack of drugs in the public health care facilities which cater for the health needs of the majority of the citizenry. There is, therefore, a need to devise cost-effective solutions to improve delivery of health care services especially for cancer patients. One such solution is the integration of affordable, safe and efficacious traditional medicine with the conventional health care system. Drugs from the plants are easily available, less expensive, considerably safer, and more efficient and with rare side effects. Regardless of the traditional reputation for the use of plants such as *D. kilimandscharicus* in traditional medicine systems, there is need for scientific documentation of their pharmacological actions and active constituents (Njogu *et al*., 2011). Few biological activity studies and isolated compounds have been reported from *D. kilimandscharicus*. This justifies the need to study the plant. In a study by Shava *et al*. (2016), the dichloromethane extract of *D. kilimandischaricus* was shown to be the most potent against HL60 cell line.

However, in that study all the extracts presented some level of antiproliferative effects on the human Caucasian promyelocytic leukaemia cell line. This study focuses on the search for phytochemicals with antiproliferative activity.

CHAPTER 3: MATERIALS AND METHODS

3.1 MATERIALS

The chemicals used in this study were all obtained from Sigma Aldrich Chemicals Company in Steinheim, Germany. All the solvents, n-hexane, DCM, acetone, ethyl acetate, methanol, and ethanol, of reagent grade were purchased locally, while all other chemicals of analytical grade were purchased from Sigma-Aldrich (Steinheim, Germany. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was obtained from Sigma Chemical Company (Steinheim, Germany). Analytical normal phase thin layer chromatography (TLC) was performed on fluorescent EMD Millipore silica gel F254 plates (Sigma, Germany).

3.2 PLANT COLLECTION AND PREPARATION OF EXTRACTS

The plants used in this work were collected from a provincial locality of Zimbabwe, namely Centenary (17° S, 31° E) in December 2016 and January 2017. The plant was authenticated by Mr Christopher Chapano, a taxonomist at the National Botanic Gardens, (Harare, Zimbabwe). Herbarium samples were kept at the Department of Biochemistry, University of Zimbabwe. The collected roots of *D. kilimandischaricus* were cut into small pieces and dried at room temperature. The dried roots were then ground into fine powder and kept in a labelled plastic container until used. Powdered dried roots of *D. kilimandischaricus* (100 g) were extracted in dichloromethane: methanol (50:50) mixture (1000), a ratio of 10:1 solvent to powder, over 72 hours. All filtrations were done using Whatman filter paper. The solvent was evaporated using a rotavapour (Rotavapor II, Büchi, Switzerland) with extra solvent

left to evaporate in the fume hood with air stream until a constant dry weight of the extract was obtained. Another portion of the roots (200 g) was extracted successively in seven solvents (hexane, dichloromethane, ethyl acetate, acetone, methanol, 70% ethanol, and water), in a ratio of 10:1 solvent to powder, over 72 hours. The solvent was then evaporated using a rotavapour with extra solvent left to evaporate in the fume hood with air stream until a constant dry weight of the extract was obtained.

3.3 TEST FOR ANTI-PROLIFERATIVE ACTIVITY

3.3.1 Induction and harvesting of immune cells

The induction and harvesting of murine peritoneal cells was done according to a method described by Ray and Dittel (2010). Briefly, a volume of 1 ml of 20 % starch solution was injected intraperitoneally into two male BALB/c mice (collected from the Animal House at the University of Zimbabwe). The mice were left for 48 hours in order to increase peritoneal cells yield. Each mouse was euthanized by cervical dislocation. The mouse was sprayed with 70 % ethanol and mounted on a Styrofoam board, on its back. The outer skin was cut to expose the inner peritoneal skin. A volume of 5 ml of cold PBS containing 3 % FBS was injected into the peritoneal cavity taking care not to puncture any organs. Cells were withdrawn using a 10-ml syringe and collected cells were put in tubes and kept on ice. The collected cell suspension was centrifuged, the supernatant discarded and the cells resuspended in RPMI 1640. Cells containing blood were thrown away as they had been contaminated.

These mouse peritoneal cells were maintained in RPMI 1640 media supplemented with 10% heat-nactivated FBS, and 1 % penicillin, streptomycin and neomycin solution at 37 ºC in a humidified atmosphere of 95% air and 5% CO_2 in a Shel Lab incubator (Sheldon Manufacturing,

Cornelius OR, USA). A cell suspension was prepared and 100 µl was mixed with equal volume of 0.4% trypan blue and the cells were checked for viability manually using a haemocytometer counting chamber under a Celestron digital light microscope (Celestron, Los-Angeles, USA), where viable cells appeared unstained and non-viable cells were stained blue.

$$\text{Viable cells (\%)} = \frac{\text{Total number of viable cells (unstained)}}{\text{Total number of cells (stained and unstained)}} \times 100 \text{ / ml of aliquot}$$

3.3.2 Growth inhibition assays

Murine peritoneal cell growth inhibition was measured using the MTT assay (Denizot and Lang, 1986). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) was prepared as a 5 mg/ml stock in phosphate-buffered saline. The MTT was diluted before use in RPMI to 1 mg/ml. Briefly, 2.0 x 10^4 cells were seeded into 96-well microtitre plates and 20 µl of compound solutions dissolved in 1 % DMSO were then added at a concentration of 0.1 mg/ml and left in contact for 72 hours. Control wells were treated in the same way over each 72-hour period. Each experiment was performed using four replicated wells for each extract as shown in **Figure 3.1**. To each well 50 µl of a 1 mg/ml solution of MTT in was added and the tray was shaken gently then incubated for 3 h at 37 °C after which 50 µl of propanol was added to each well. The plate was then shaken (Microshaker II, Dynatech; speed setting 7, 30 s) to solubilise the blue formazan. The optical density of each well was measured using an automatic Tecan Microplate reader (Tecan Group Ltd Männedorf, Switzerland) with a 650-nm 590-nm measurement wavelength.

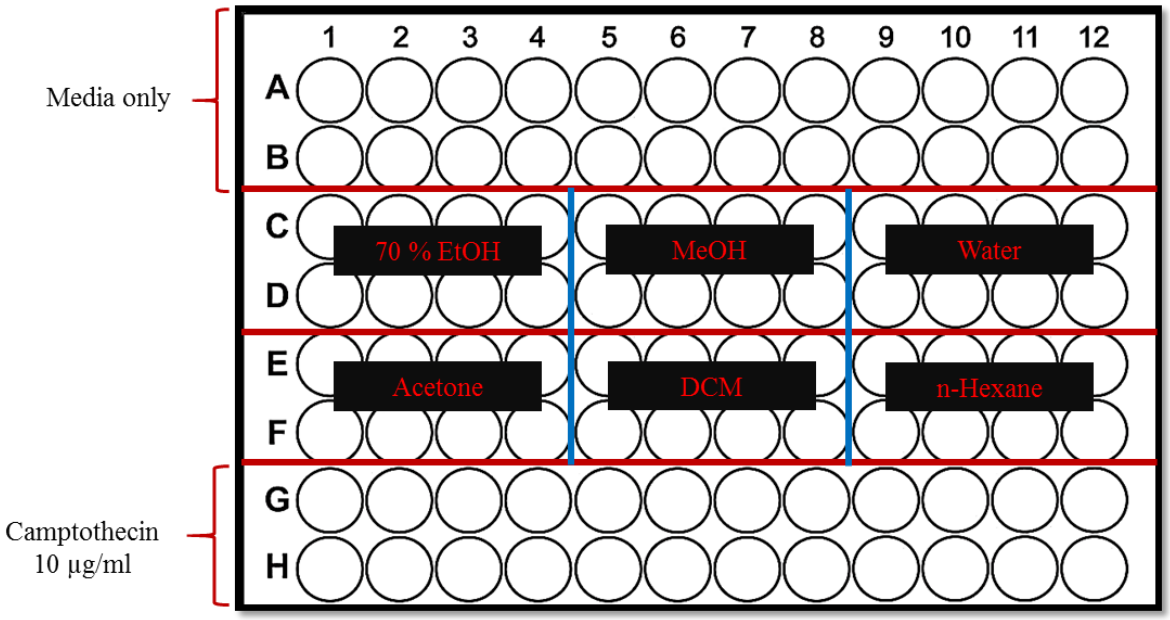


Figure 3.1 A 96-well template for toxicity testing. Camptothecin and plant extracts had a fixed volume of 20 µl. volume of peritoneal cells and RPMI was dependent upon the cell count.

3.4 PHYTOCHEMICAL SCREENING

The methanolic extract which had the highest yield, as well as the dichloromethane extract which has shown antiproliferative effects (Shava *et al*., 2016) from previous studies, were both screened for the presence of alkaloids, saponins, tannins, anthraquinones, cardiac glycosides, flavonoids and phlobatannins according to the methods described by (Edeoga *et al*., 2005).

3.4.1 Test for alkaloids: About 0.5 g of the extract was digested with 2 M HCl, and the acidic filtrate was mixed with:

Wagner's reagent (Iodine in Potassium iodide) test: A volume of 1 ml of Wagner's reagent was added drop by drop to 1 ml of filtrate. Formation of a reddish-brown precipitate would indicate the presence of alkaloids.

Dragendorff's reagent (Potassium bismuth iodide solution) test: A volume of 1 ml of Dragendorff's reagent was added drop by drop to 1 ml of filtrate. Formation of a brick red precipitate would indicate the presence of alkaloids.

3.4.2 Test for tannins: About 0.5 g of the extract was boiled in 20 ml of water in a boiling tube and then filtered. Two or three drops of 0.1% ferric chloride were added and a brownish-green or a blue-black colouration was observed.

3.4.3 Test for phlobatannins: About 0.5g of each extract was boiled with 1% aqueous hydrochloric acid a red precipitate was taken as evidence for the presence of phlobatannins.

3.4.4 Test for saponins: About 1 g of the extract was boiled 20 ml of distilled water and filtered. Half of the was mixed with 5 ml of distilled water and shaken vigorously for a stable persistent froth. The froth was mixed with 3 drops of olive oil and shaken vigorously, then observed for the formation of emulsion.

3.4.5 Test for flavonoids: About 5 ml of dilute ammonia solution were added to a portion of the extract followed by concentrated H_2S0_4. The presence of flavonoids was shown by a yellow colouration.

A portion of the extract was heated with 10 ml of ethyl acetate over a steam bath for 3 min. The mixture was filtered and 4 ml of the filtrate was shaken with 1 ml of dilute ammonia solution. The presence of flavonoids was shown by a yellow colouration, which becomes colourless upon addition of concentrated hydrochloric acid.

3.4.6 Test for steroids: Two ml of acetic anhydride was added to 0.5 g extract with 2 ml H_2S0_4. A colour change of violet to blue or green in some samples indicated the presence of steroids.

3.4.7 Test for terpenoids (Salkowski test): Five ml of each extract was mixed in 2 ml of chloroform, then concentrated H_2S0_4 (3 ml) was carefully added to form a layer. The occurrence of a reddish brown colouration on the interface showed the presence of terpenoids.

3.4.8 Test for cardiac glycosides (Keller-Killani test): Five ml of each extract was treated with 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This was underlayed with 1 ml of concentrated sulphuric acid. A brown ring on the interface indicated a deoxy-sugar, while a violet ring appears below the brown ring.

3.5 INFRA-RED SPECTROSCOPY OF CRUDE SAMPLES

Infra-red spectra of the crude samples were recorded to detect various functional groups responsible for antiproliferative activities. Dried powder of the extracts was placed on the sample chamber of FT-IR spectrophotometer and the spectra were recorded in the range of 3600–600 cm^{-1} on a Nicolet Avatar 330 FTIR spectrometer (White Bear Lake, Minnesota, USA). Important absorption frequencies appearing in functional group region as well as fingerprint region of the spectra were noted down.

3.6 ISOLATION OF COMPOUNDS

3.6.1 Fractionation of Methanol Root Extract

Crude extracts were tested in the preliminary detection of different classes of compounds. The adsorbent, silica gel (492 g, 35 – 70 µm) was carefully packed using the wet slurry method. Methanol extract, 123 g, was loaded on to packed adsorbent and allowed to stabilize, the chromatographic column was run by gradient elution using n-hexane, ethyl acetate, and methanol (elution profile shown in **Appendix 1**). In the chromatographic procedures, the eluate was

collected in 250 ml fractions. **Appendix 2** shows *D. kilimandischaricus* root methanol extract undergoing chromatographic fractionation. Each fraction was concentrated using a rotavapor and evaporated in a tube. The tubes of the fractions were profiled by thin-layer chromatography using n-hexane, ethyl acetate, chloroform and methanol. The root methanol extract was included as a reference. The spots in the developed chromatograms were located by observing under both short and long ultraviolet (A425, London, United Kingdom) light (short UV and long UV at 254 nm and 366 nm respectively), then spraying with 10 % w/v H_2SO_4 and drying at 100 ºC. The chromatograms were analysed and those fractions showing similar profile pooled. From the 480 fractions collected, 53 pooled fractions labelled P1 – P53 were obtained. The pooled fractions were reduced to dryness and their yields determined. Each pooled fraction was then kept at room temperature.

3.6.2 Gas Chromatography-Mass Spectrometer

The pooled fractions with the highest yield that is P10, P50 and P51 were analysed by a Gas Chromatography-Mass Spectrometer with the assistance of Ms Vimbai M. Masuku at EMA, on an Agilent 7890A coupled to Agilent 5975c VL MSD. Before analysis the sample was dissolved in methanol: chloroform (1:1) and each sample concentrated using a rotary vacuum evaporator. The pooled fractions were injected into the instrument. The instrument temperature conditions used were programmed as follows an initial temp 70 °C (holding time 3mins), final temp was 300 °C, the flow rate was 10 °C /min (for 9 mins), helium flow rate was 1.5mL/min, ionization mass spectrometer was operating at 70 eV, split mode 10:1, ion source temp was 230 °C, interface temp was 240 °C, the detector temp was 280 °C. The overall run time was 36

minutes for each sample. The NIST library in the system was then used to give information on the name of the compounds analysed and also giving their retention times and quantities.

3.7 STATISTICAL ANALYSES

Graphical and statistical analyses was carried out using GraphPad Prism Version 5. All data were expressed as mean ± standard deviation of the mean. Statistically significant differences between the mean of the controls and the tests were analysed using one way ANOVA with Dunnett's Multiple Comparison Post Test with p value being 0.05.

CHAPTER 4: RESULTS

4.1 TOTAL AND SERIAL EXHAUSTIVE EXTRACTION

Extraction of dried roots of the plant (100 g) was extracted in dichloromethane: methanol (1:1) mixture, and serial extraction on the roots was extracted successively in seven solvents, in a ratio of 10:1 solvent to powder for 72 hours. The serial exhaustive extraction gave a higher combined percentage extract yield than the total extraction method. Total extraction had a percentage yield of 2.64 % and serial exhaustive extraction had a total percentage yield of 7.95 %. Methanol extract had the highest percentage yield, with the hexane extract having the least yield. The colour, consistency after drying and percentage yield are noted in **Table 4.1**.

Table 4.1. Yields of extracts from *D. kilimandischaricus* (Fabaceae) root

Extraction Solvent	Colour and consistency after drying	Final Mass	Percentage Yield
n-Hexane	Greenish-brown, viscous solid	0.6900	0.35
Dichloromethane	Yellowish-orange, solid	0.5377	0.27
Ethyl acetate	Greenish brown, viscous solid	0.4210	0.21
Methanol	Dark brown, solid	5.0752	2.54
Acetone	Dark brown, solid	1.8684	0.93
Distilled water	Red-brown, solid	4.1764	2.09
70 % Ethanol	Dark brown, solid	3.1100	1.56
DCM: MeOH (1:1)	Dark brown, solid	2.6370	2.64

4.2 PHYTOCHEMICAL CONFIRMATORY TESTS

The methanolic extract which had the highest yield and the dichloromethane extract which showed antiproliferative effects (Shava *et al*., 2016) in previous studies, were screened for the presence of alkaloids, saponins, tannins, anthraquinones, cardiac glycosides, flavonoids and phlobatannins to confirm the presence of respective phytochemicals in the extracts. The results obtained are shown in **Figure 4.1** and **Table 4.2**.

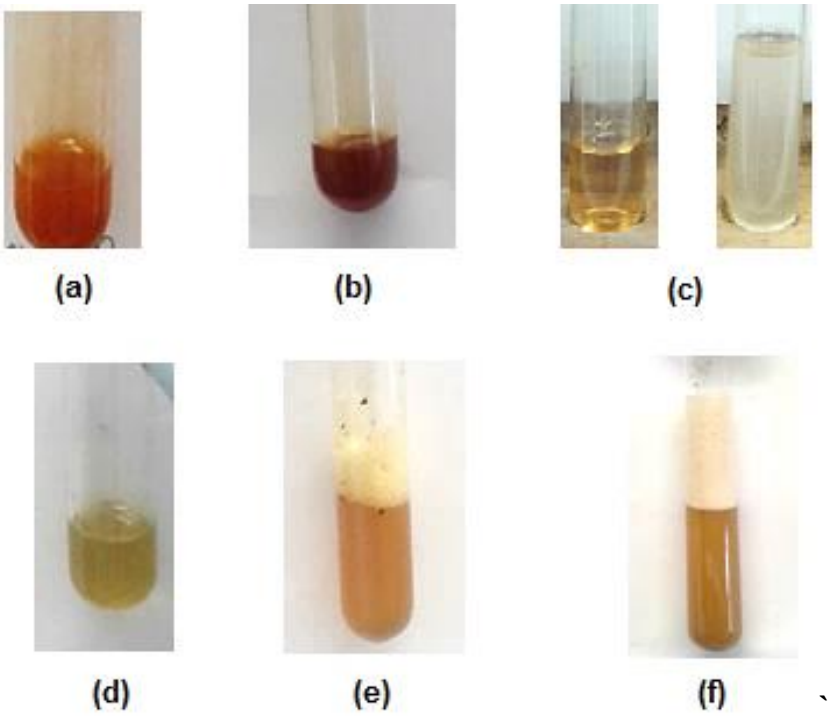


Figure 4.1 Selected qualitative analysis results from methanolic extract for alkaloids: - (a) Dragendorff's reagent test, (b) Wagner's reagent test. Tests for flavonoids- (c) Sodium hydroxide test, (d) Ferric chloride test, (e) Ammonia solution test. Test for saponins- (f) Frothing test.

Table 4.2 Phytochemical confirmatory tests of fractions isolated from the methanolic extract.

Phytochemical	**Methanolic extract**	**DCM extract**
Alkaloids	+	+
Flavonoids	+	-
Saponins	+	+
Anthracenes	-	-
Glycosides	-	+
Anthraquinone	-	-
Tannins	+	+
Phlobatannins	+	+
Steroids	+	-
Terpenoids	+	-
Cardiac glycosides	-	+
Saponin glycosides	+	+

The two extracts showed a similar phytochemical compounds profile apart from glycosides, cardiac glycosides, steroids, terpenoids and flavonoids. The MeOH extract possessed flavonoids, steroids and terpenoids while the DCM extract did not. The DCM extract however showed presence of glycosides and cardiac glycosides which were absent in the MeOH extract.

4.3 FT-IR SPECTRAL ANALYSIS

FT-IR spectral analysis data of the methanolic extract showed the presence of multiple functional groups. Spectral data of this extract confirmed the presence of bioactive functional groups such as –OH, –NH, –CHO, –COOH and –COOR. The extract exhibited the presence of a broad peak for hydrogen bonded –OH stretching in functional group region. Important IR absorption frequencies are tabulated in **Table 4.3** and FT-IR spectra of the methanol extract are shown in **Figure 4.2**.

Table 4.3 Major bands observed in the FT-IR spectra of the methanol extract.

Extract	**IR υ max (cm-1) (Vibration mode)**	**Probable phytochemicals**
Methanol	3487.5 (–N–H stretching, amides) 3412.5 (H-bonded O–H stretching; broad or –N–H stretching), 3262.5 (–N–H stretching) 2925 (aldehydic –C–H stretching), 2850 (–C=O high polar i.e. sp^2 C of –CHO), 1705.0 (aldehyde/ketone –C=O stretching), 1687.5 (–C=C–stretching), 1612.5 (–C=C–stretching), 1425.0 (–NH bending), 1387.5 ($–CH_3$ bending), 1125.0 (amine C–N stretching), 1050.0 [–C–C(O)–C (acetate) ester stretching]	**Alkaloids, flavonoids, polyphenols, carboxylic acid containing phytochemicals etc.**

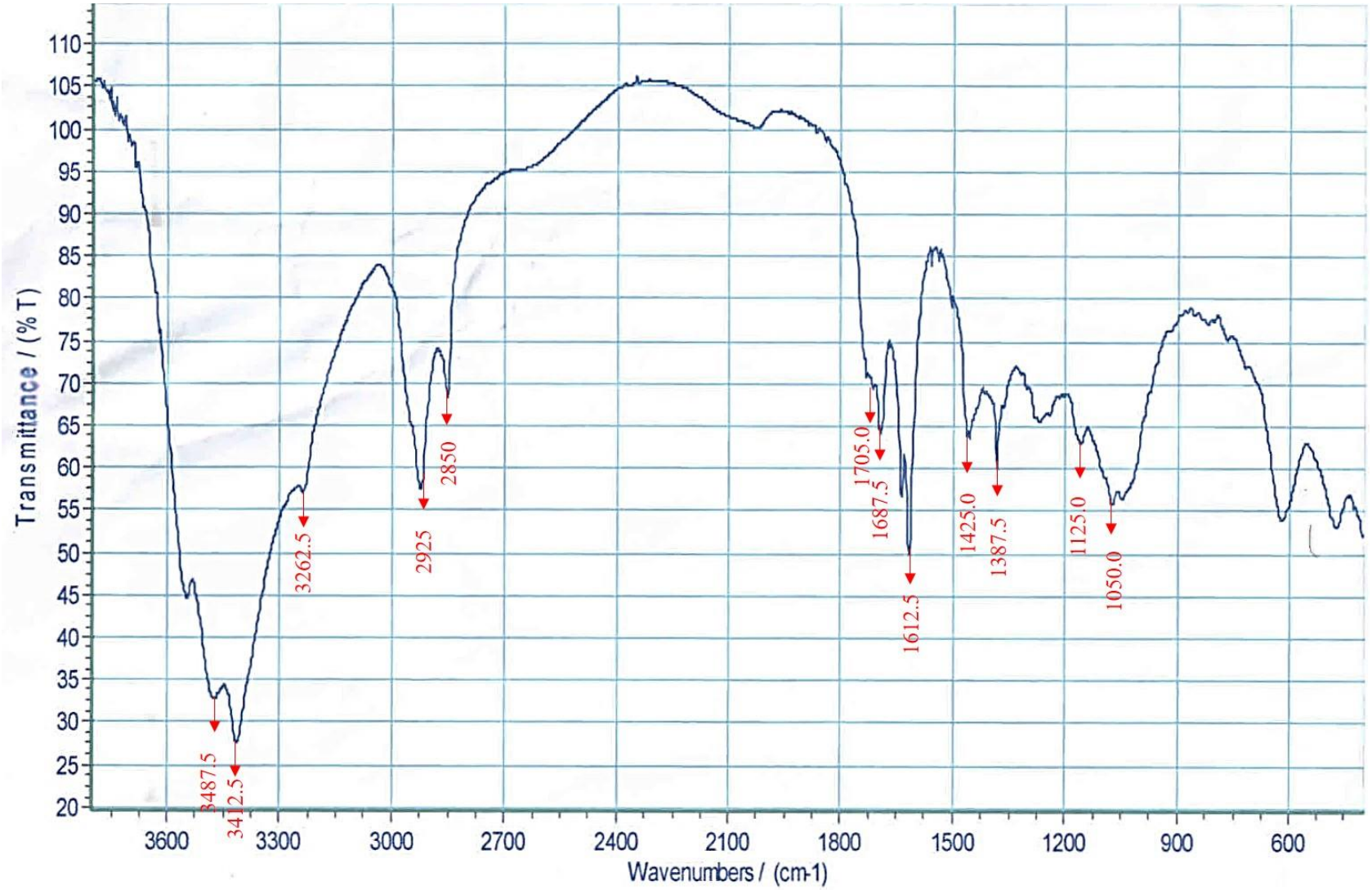


Figure 4.2 FT-IR spectrum of methanol extract.

4.4 ANTIPROLIFERATIVE ACTIVITY OF *D. kilimandischaricus* EXTRACTS

The results for the investigation into the antiproliferative effects of six extracts of *D. kilimandischaricus* against mouse peritoneal cells at 0.1 mg/ml are shown in **Figure 4.3**. These were investigated by incubating the mouse peritoneal cells with the 70 % EtOH, MeOH, Water, Acetone, DCM and n-hexane extracts as well as various pooled fractions in 96-well plates for 72 hours. Percentage cell viability was expressed in comparison to untreated cells (Cells only). The results showed that the tested microorganisms were more sensitive to the dichloromethane and methanol extracts than the rest of the extracts. The methanol extract was shown to be the most potent extract in the inhibition of the growth of mouse peritoneal cells though the effects were less when compared to the cells incubated with the anticancer drug, Camptothecin. The results indicated that water root extract exerted the least antiproliferative effect on the mouse peritoneal cells. The effect of various pooled fractions of the most potent methanolic root extract against the mouse peritoneal cells was observed using the MTT assay. The fractions pooled together and labelled P10, P12, P50, P51 and P52 exhibited antiproliferative effects against the mouse peritoneal cells at concentration of 0.1 mg/ml shown in **Figure 4.4**. Pools 14 and 18 showed proliferative effects when incubated with mouse peritoneal cells over the same 72-hour period.

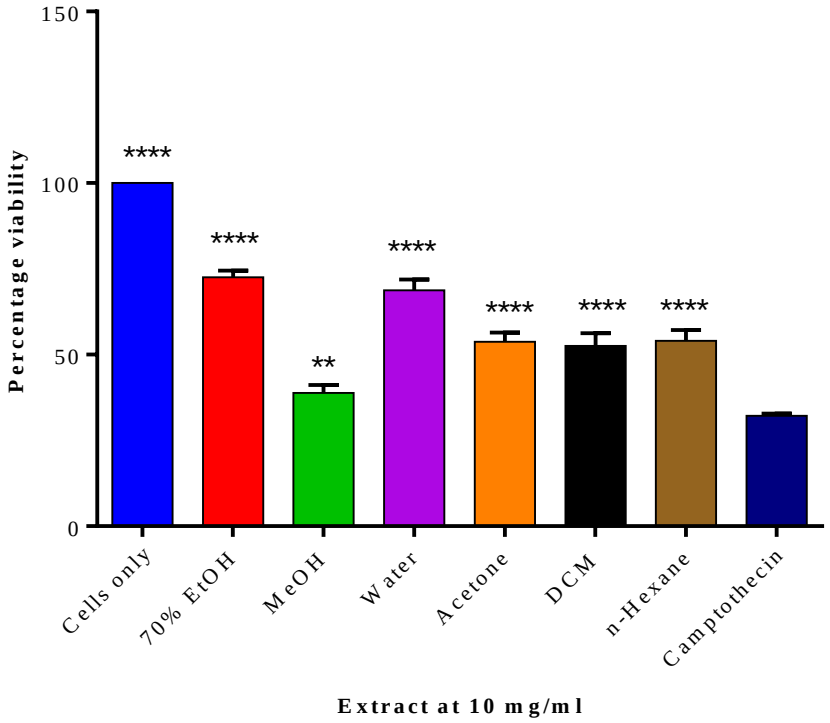


Figure 4.3 Antiproliferative effects of extracts of *D. kilimandischaricus* against mouse peritoneal cells at 0.1 mg/ml, investigated by incubating mouse peritoneal cells with the 70 % EtOH, MeOH, Water, Acetone, DCM and n-hexane extracts in 96-well plates for 72 hours. Percentage cell viability was expressed in comparison to untreated cells (Cells only). Each value is the mean ± SD of four replicates. Statistical one-way ANOVA (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ vs. cells treated with BCNU).

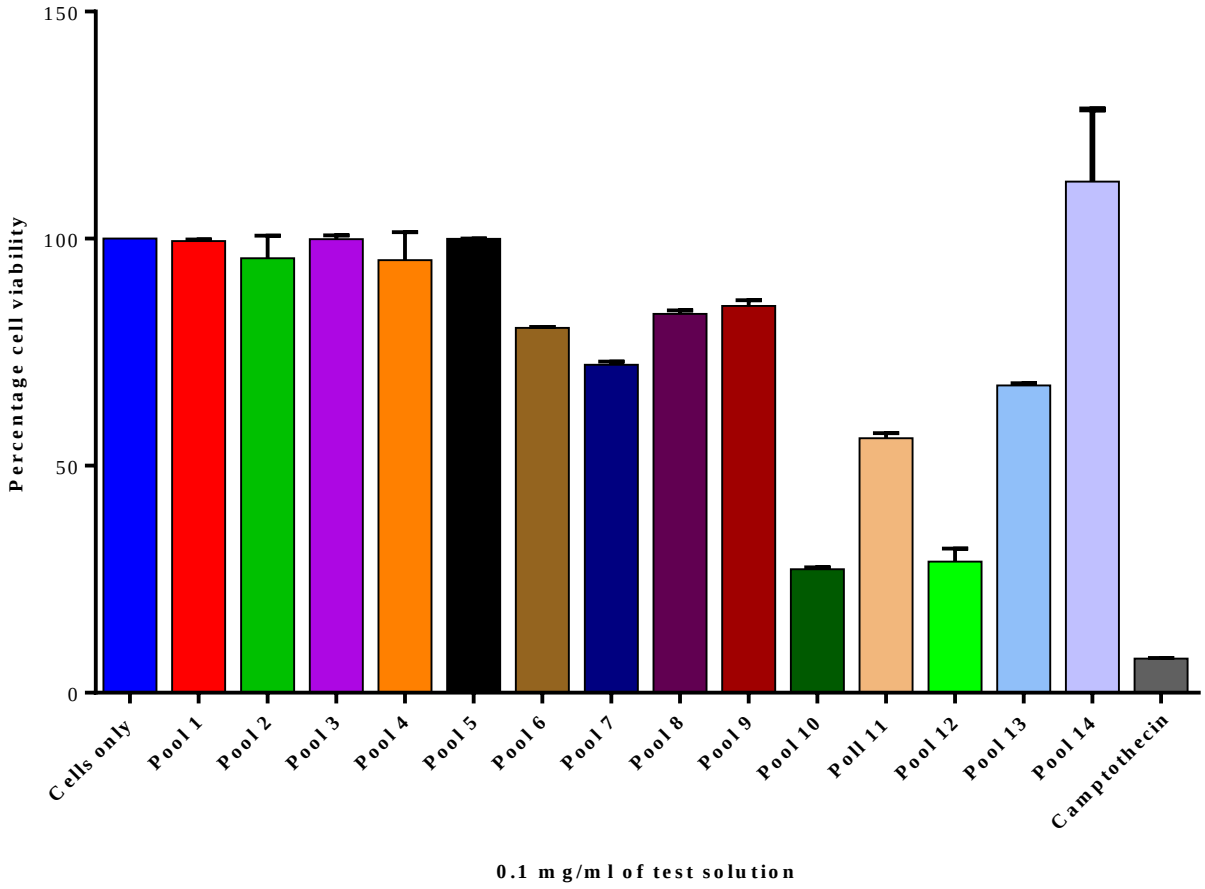


Figure 4.4 Antiproliferative effect of methanolic pooled fractions of *D. kilimandischaricus* Pools 1-14 on mouse peritoneal cells. Mouse peritoneal cells were treated with a concentration of 0.1 mg/ml of the various pools for 72 hrs, and the cell viability was detected using an MTT assay. Results are expressed as the mean ± standard deviation.

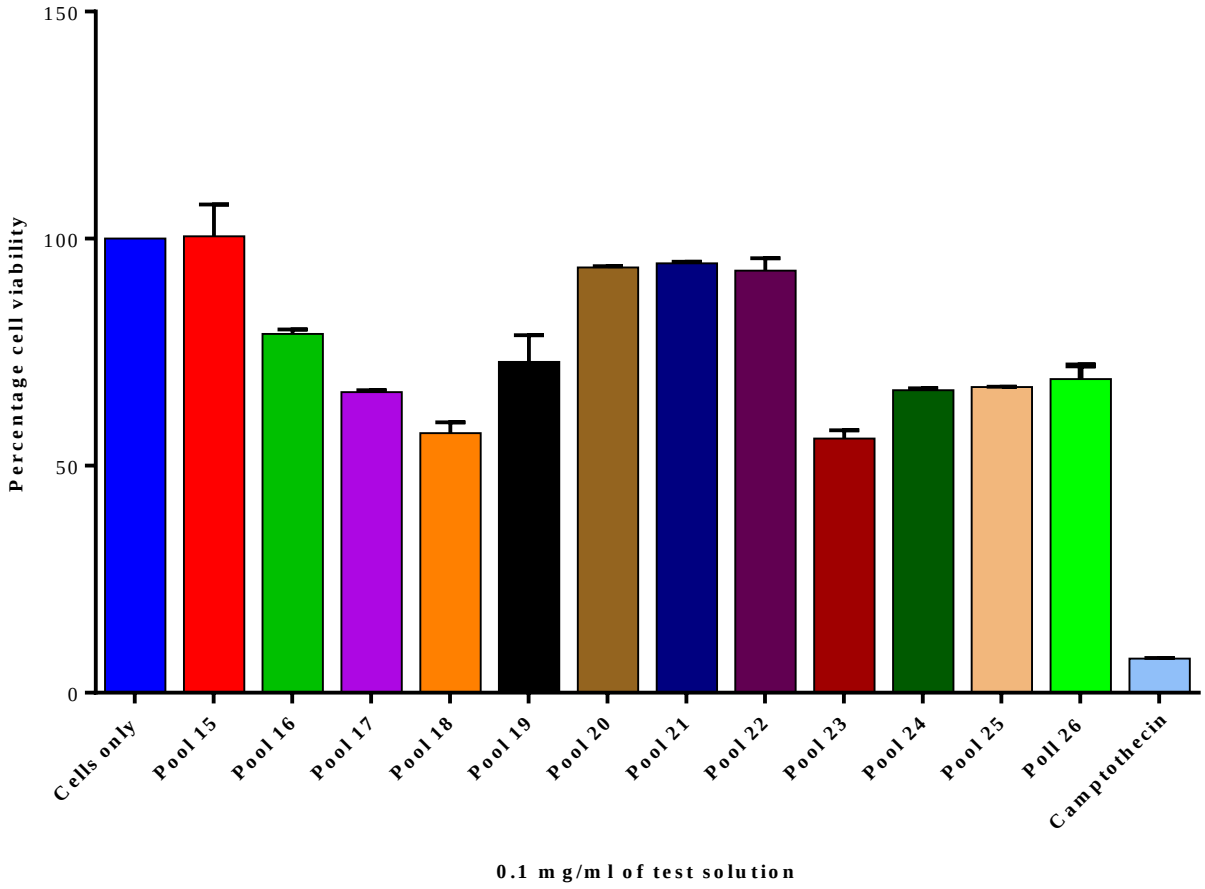


Figure 4.5 Antiproliferative effect of methanolic pooled fractions of *D. kilimandischaricus* Pools 15-26 on mouse peritoneal cells. Mouse peritoneal cells were treated with a concentration of 0.1 mg/ml of the various pools for 72 hrs, and the cell viability was detected using an MTT assay. Results are expressed as the mean ± standard deviation.

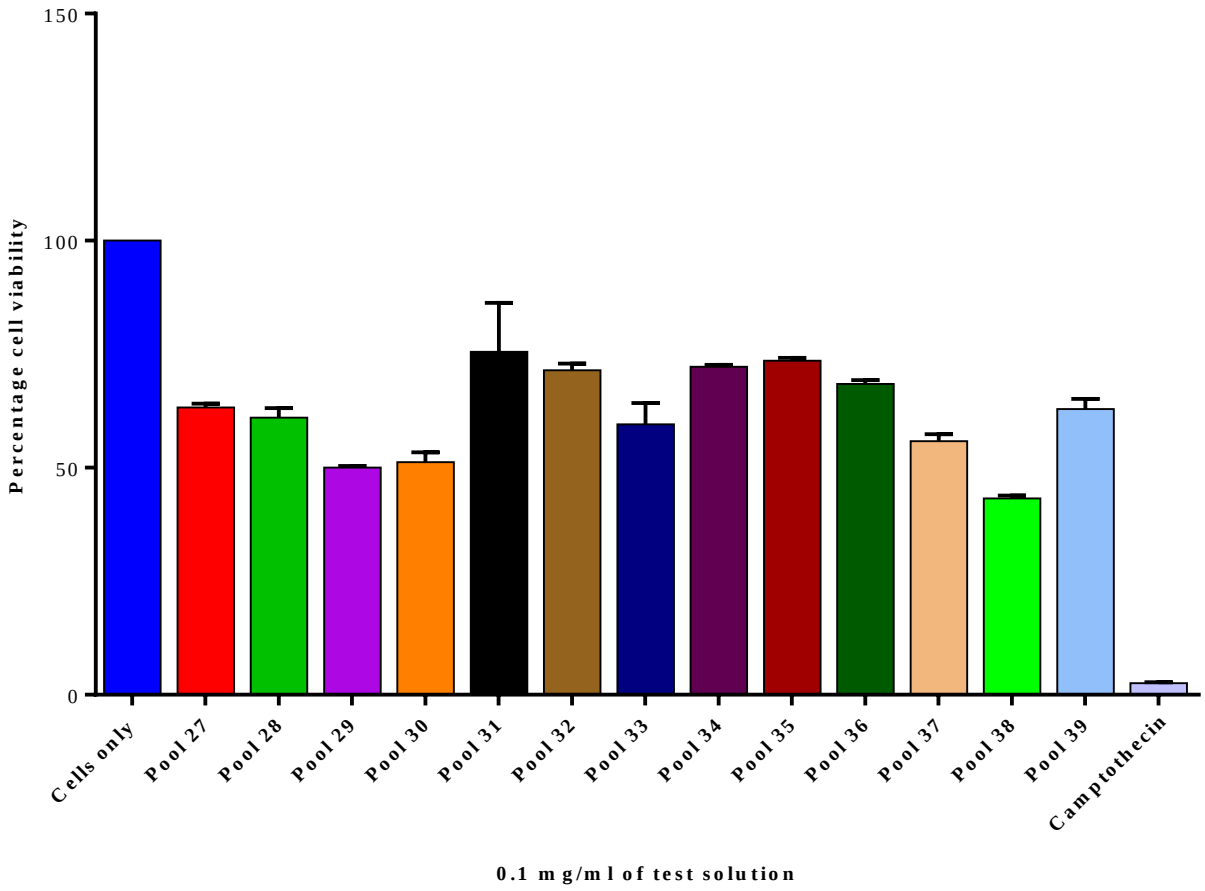


Figure 4.6 Antiproliferative effect of methanolic pooled fractions of *D. kilimandischaricus* Pools 27-39 on mouse peritoneal cells. Mouse peritoneal cells were treated with a concentration of 0.1 mg/ml of the various pools for 72 hrs, and the cell viability was detected using an MTT assay. Results are expressed as the mean ± standard deviation.

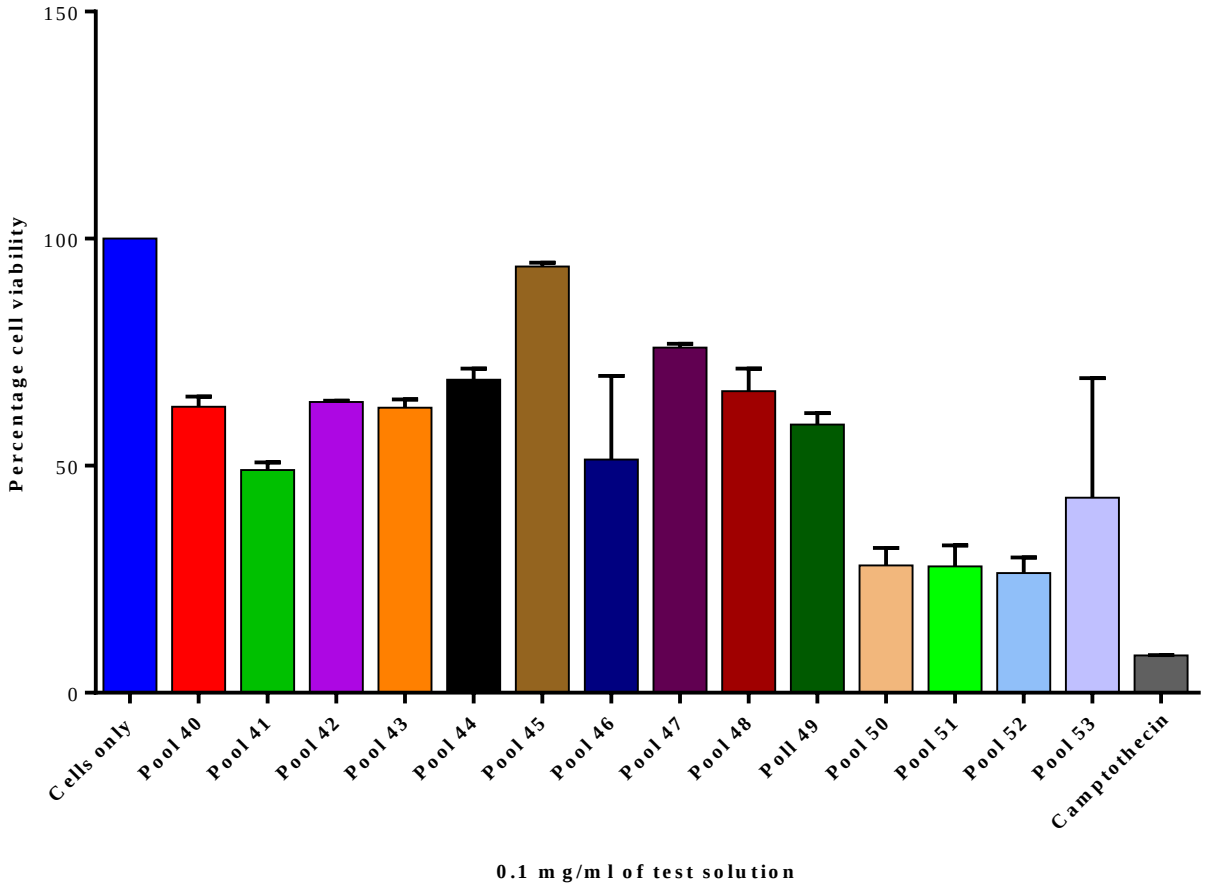


Figure 4.7 Antiproliferative effect of methanolic pooled fractions of *D. kilimandischaricus* Pools 40-53 on mouse peritoneal cells. Mouse peritoneal cells were treated with a concentration of 0.1 mg/ml of the various pools for 72 hrs, and the cell viability was detected using an MTT assay. Results are expressed as the mean ± standard deviation.

4.5 COLUMN CHROMATOGRAPHIC SEPARATION OF *D. kilimandischaricus*

The elution of *D. kilimandischaricus* methanol root extract by silica gel column chromatography and the resulting fractions were analysed by TLC on silica gel. The results shown in **Table 4.4**, show the gradient eluted fractions examined.

Table 4.4 Chromatographic separation of *D. kilimandischaricus*

Number of fractions	Eluent Solvent(s)
1-24	100 % hexane
25-48	97.5 hexane: 2.5 EA
49-72	95 hexane: 5 EA
73-96	92.5 hexane: 7.5 EA
97-120	90 hexane:10 EA
121-144	87.5 hexane:12.5 EA
145-168	85 hexane: 15 EA
167-192	82.5 hexane: 17.5 EA
193-216	80 hexane:20 EA
217-240	75 hexane:25 EA
241-264	70 hexane:30 EA
265-288	65 hexane:35 EA
289-312	60 hexane:40 EA
313-336	50 hexane:50 EA
337-360	40 hexane:60 EA
361-384	25 hexane:75 EA
385-408	10 hexane: 90 EA
409-432	100% EA
433-456	95% EA: 5% MeOH
457-480	90% EA:10 % MeOH

Column chromatographic fractions that showed similar TLC profiles were combined. Each of these fractions was evaluated by TLC and those with the same R_f values were pooled and dried. The TLC plates were developed in a tank and detection of compounds was done under UV light at 254 and 365 nm. The results shown in **Table 4.5** show that Pools 10, 11, 37, 48, 50 and 51 showed the highest yields.

Table 4.5 TLC profiles of phytochemicals of *D. kilimandischaricus*

Pool Name	Pools formed	Mass of pool /g
P1	40-43	0.0507
P2	44-48	0.0009
P3	49-56	0.0011
P4	57-70	0.1978
P5	73-80	0.0892
P6	81-90	0.0696
P7	92-98	0.0412
P8	99-102	0.0250
P9	103-107	0.0861
P10	108-118	0.6627
P11	121-127	0.4603
P12	128	0.0391
P13	129-136	0.0745
P14	137-144	0.0361
P15	145-157	0.0711
P16	158	0.0110
P17	159-168	0.0426
P18	169-174	0.0240
P19	175-179	0.0398
P20	180-190	0.0638

Pool Name	Pools formed	Mass of pool /g
P28	257-263	0.0697
P29	264-269	0.0559
P30	270-276	0.0846
P31	277-285	0.0708
P32	286-289	0.0214
P33	290-299	0.1472
P34	300-312	0.2088
P35	313-323	0.2241
P36	324-336	0.1483
P37	337-355	0.3482
P38	356-372	0.2490
P39	373-380	0.0735
P40	381-387	0.0679
P41	388-394	0.1653
P42	395-409	0.1394
P43	410-414	0.1485
P44	415-418	0.1231
P45	419	0.0234
P46	420-432	0.1614
P47	433-439	0.1031

P21	191-197	0.0863
P22	198-216	0.1560
P23	217-220	0.0395
P24	221-223	0.0430
P25	224-237	0.1105
P26	238-247	0.1005
P27	248-256	0.0909

P48	440-441	0.4029
P49	442-448	0.1478
P50	449-455	0.6688
P51	456-466	0.5827
P52	467-476	0.1713
P53	477-480	0.2018

The gas chromatograms of the methanolic extracts of *D. kilimandischaricus* roots are shown in **Figures 4.5**, **4.6** and **4.7** while the compounds and their corresponding identities are shown in **Table 4.6**, the retention/ acquisition times (R_t) were reported in minutes. The major compounds identified in Pool 10 of the methanol extract were, 3,8-bis(2',2'-Dimethylpropionyloxy)-1-ethoxy-6-methoxy-2-methyl-9,10-anthraquinone (19.837, 60.3 %) and 1,4,5,6,7,11b-Hexahydro-10-chloro-4-(4'-chlorophenyl)-3,11b-bis[trifluoromethyl)imidazo[1,2-a] pyrazolo [4,3-b] indole (19.837, 65.5 %). In Pool 50, there were 8 major constituents identified namely, 1-(4-isopropylphenyl)-2-methylpropyl acetate (15.635, 71.1 %), Bicyclo[9.3.1]pentadecan-15-one, 12-hydroxy-1-nitro- (16.907, 54.8 %), Dodecanamide (18.785, 82.0 %), Methyl stearate (20.167, 91.5 %), 5-Benzoyl-1,3-bisethoxymethyl-1H-pyrimidin-2,4-dione (21.388, 55.7 %), Decanamide (22.779, 76.0 %), Ethyl 6,7-dimethoxy-2-pivaloyl-1,2,3,4-tetrahydroisoquinoline-1-carboxylate (25.023, 76.5 %), and (22R)-23,23-Difluoro-22-hydroxy-24-methyl-24-oxo-5.alpha.-cholane-3.alpha.,6.alpha.-diyl diacetate (27.680, 50.7 %). In Pool 51, the major chemical constituent was 7-Acetyl-4,5-epoxy-18,19-dihydro-3,6-dimethoxy-6,14-ethenomorphinane (23.728, 72.3 %) also known as thevinone. The chemical structures of methanol root extract-derived compounds are shown in **Figure 4.11**.

Table 4.6 Chemical components identified in *D. kilimandischaricus* pooled fractions of the methanolic extract by GC-MS

No.	Component	Molecular Formula
1	3,8-bis(2',2'-Dimethylpropionyloxy)-1-ethoxy-6-methoxy-2-methyl-9,10-anthraquinone	$C_{28}H_{32}O_8$
2	1,4,5,6,7,11b-Hexahydro-10-chloro-4-(4'-chlorophenyl)-3,11b-bis[trifluoromethyl)imidazo[1,2-a] pyrazolo [4,3-b] indole	$C_{19}H_{12}Cl_2F_6N_4$
3	1-(4-isopropylphenyl)-2-methylpropyl acetate	$C_{15}H_{22}O_2$
4	Bicyclo[9.3.1]pentadecan-15-one, 12-hydroxy-1-nitro-	$C_{15}H_{25}NO_4$
5	Dodecanamide	$C_{10}H_{21}NO$
6	Methyl stearate	$C_{19}H_{38}O_2$
7	5-Benzoyl-1,3-bisethoxymethyl-1H-pyrimidin-2,4-dione	$C_{19}H_{27}NO_5$
8	Decanamide	$C_{29}H_{44}F_2O_6$
9	Ethyl 6,7-dimethoxy-2-pivaloyl-1,2,3,4-tetrahydroisoquinoline-1-carboxylate	$C_{22}H_{27}NO_4$
10	(22R)-23,23-Difluoro-22-hydroxy-24-methyl-24-oxo-5. alpha. -cholane-3. alpha.,6. alpha. -diyl diacetate	$C_{17}H_{20}N_2O_5$
11	7-Acetyl-4,5-epoxy-18,19-dihydro-3,6-dimethoxy-6,14-ethenomorphinane	$C_{10}H_{21}NO$

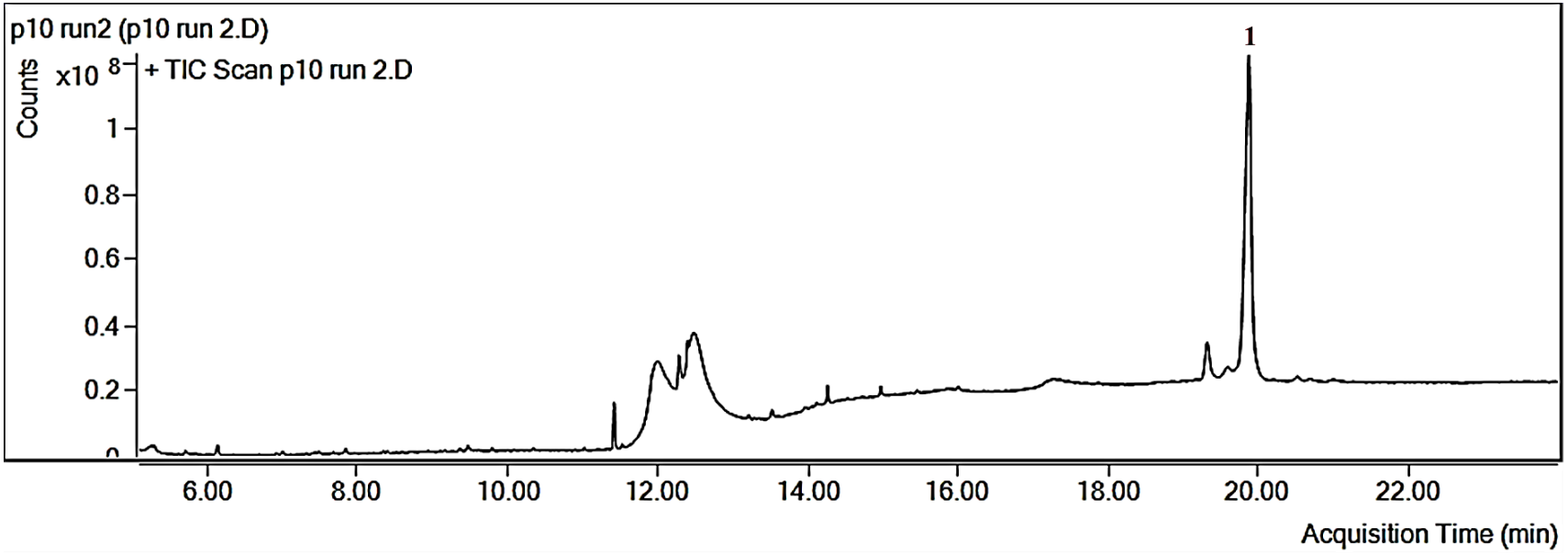


Figure 4.8 Gas chromatogram of the Methanolic extracts of Pool 10 of the methanolic extracts of *D. kilimandischaricus* roots

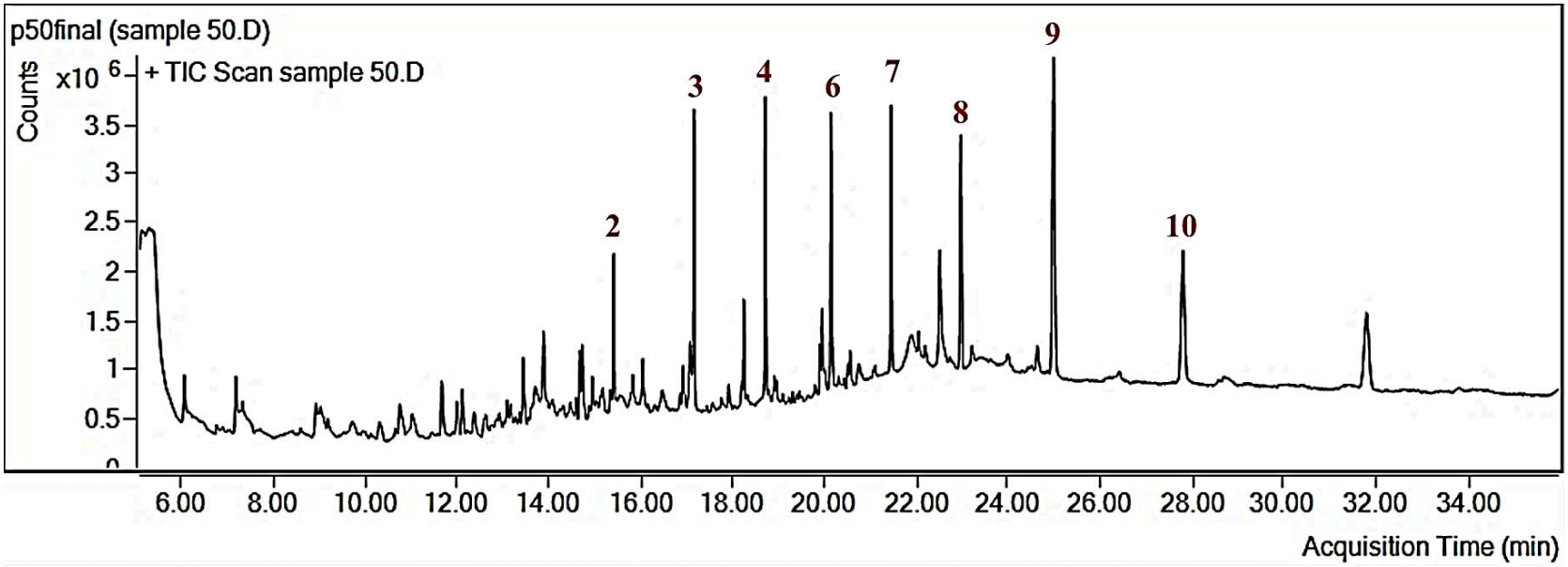


Figure 4.9 Gas chromatogram of the Methanolic extracts of Pool 50 of the methanolic extracts of *D. kilimandischaricus* roots

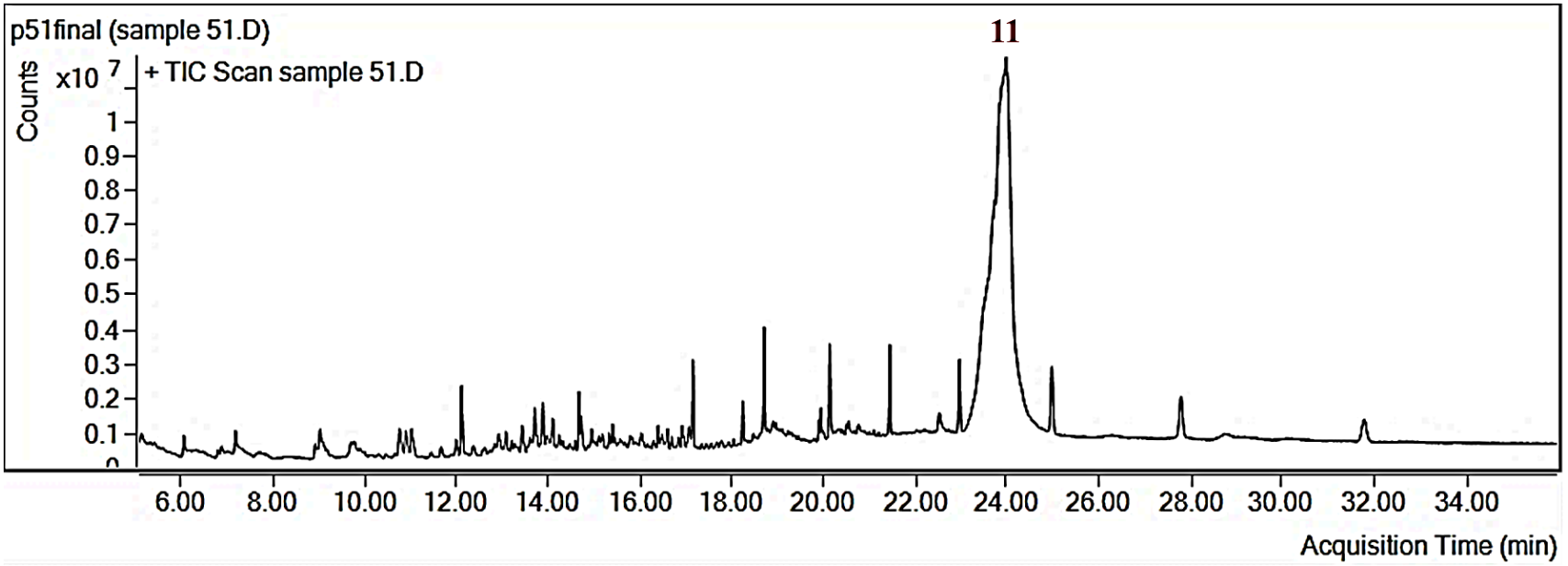
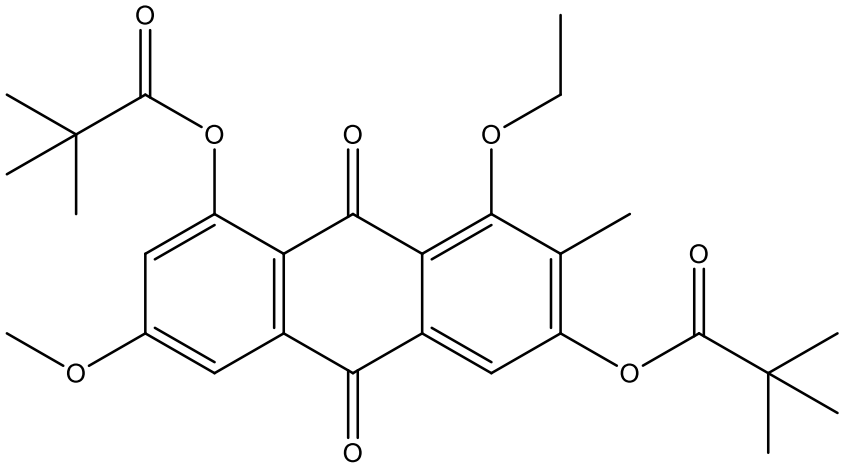
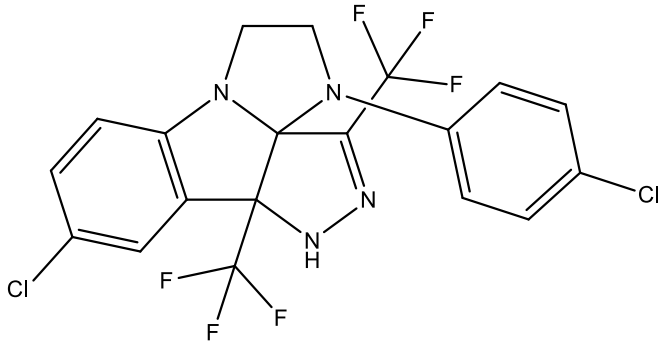


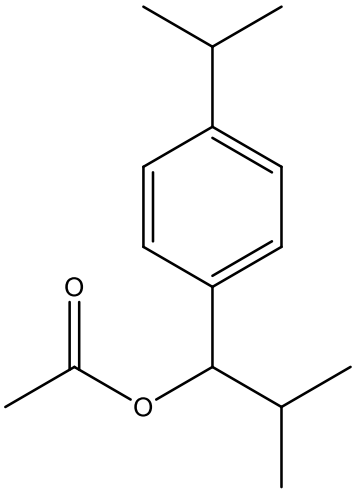
Figure 4.10 Gas chromatogram of the Methanolic extracts of Pool 51 of the methanolic extracts of *D. kilimandischaricus* roots



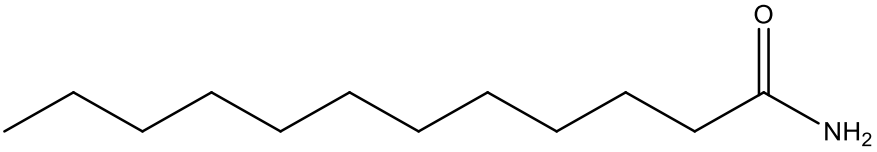
3,8-bis(2',2'-Dimethylpropionyloxy)-1-ethoxy-6-methoxy-2-methyl-9,10-anthraquinone



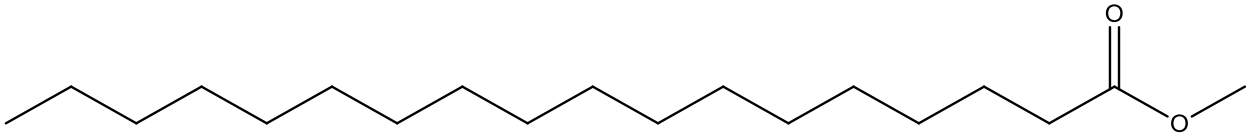
1,4,5,6,7,11b-Hexahydro-10-chloro-4-(4'-chlorophenyl)-3,11b-bis(trifluoromethyl)imidazo[1,2-a]pyrazolo[4,3-b] indole



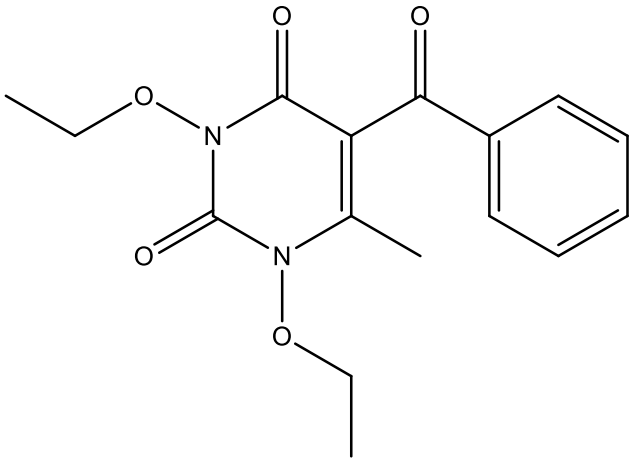
1-(4-isopropylphenyl)-2-methylpropyl acetate



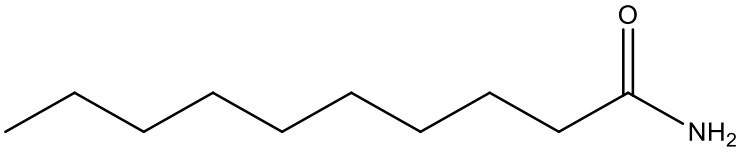
Dodecanamide



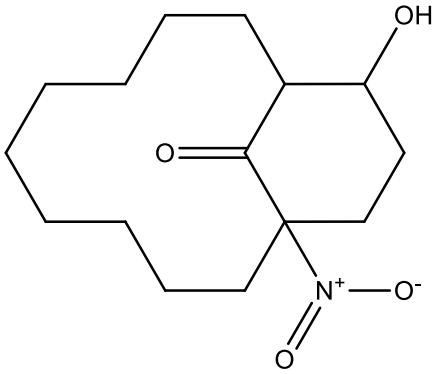
Methyl stearate



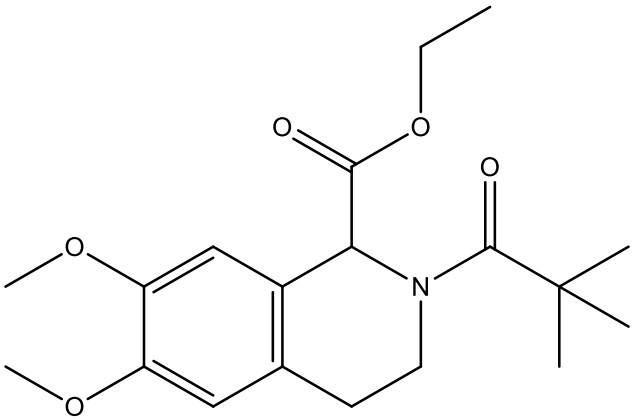
5-benzoyl-1,3-bisethoxylmethyl-1h-pyrimidin-2,4-dione



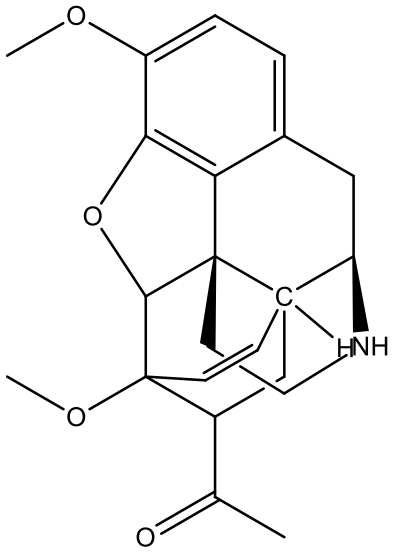
Decanamide



Bicyclo[9.3.1]pentadecan-15-one, 12-hydroxy-1-nitro-



Ethyl 6,7-dimethoxy-2-pivaloyl-1,2,3,4-tetrahydroisoquinoline-1-carboxylate



7-Acetyl-4,5-epoxy-18,19-dihydro-3,6-dimethoxy-6,14-ethenomorphinane

Figure 4.11 Chemical structures of methanol root extract-derived compounds

CHAPTER 5: DISCUSSION

In developing countries, the use of natural plant products in the fight against many ailments is quite widespread as most of the population are in the low-income category such that plants remain the primary source of medical care for many, as in most situations commercial drugs are unaffordable or unavailable. In the fight against cancer, use of natural products has gained immense ground on synthetic alternatives with as much as 60 % of the currently used anticancer agents having been derived in one way or another from natural sources (Newman and Cragg, 2007). The World Health Organization has over the years encouraged countries to look into traditional medicine with a view to identifying and exploiting products that provide safe and effective remedies for ailments (World Health Organization, 1978).

Several major groups of phytochemicals with antiproliferative capacity have been identified, with the current isolation and purification methods including solvent extraction processes that utilize solvent polarity as a major separation technique (Lapornik *et al*., 2005). The present study focused on the analytical methodology, namely the extraction, of the phytochemical ingredients in *D. kilimandischaricus* powdered roots. As extraction is the most important step in the analysis of constituents present in plants, the strengths and weaknesses of different extraction solvents needed to be assessed. Care should be taken to assure that potential phytochemicals are not lost, distorted or destroyed during the extraction of plant samples. They are different solvent systems that can be used in the extraction of bioactive compounds from natural sources, with polar compounds extracted using solvents such as methanol, ethanol or ethyl-acetate while non-polar compounds are extracted using dichloromethane (Cos *et al*., 2006). The methanol extract had the highest percentage yield showing a larger presence of polar compounds in the root as opposed to the hexane extract having the least yield which extracted

non-polar compounds. Methanol is frequently used to extract specific bioactive ingredients from various natural products giving higher yields of extractions of specific functional ingredients from natural products such as polyphenols and antioxidants (Chon *et al*., 2009). Methanol is commonly used as an organic solvent of choice in extraction as it has wide solubility properties for low molecular weight as well as moderately polar substances (Khalil *et al*., 2013).

The activity of the root extracts of *D. kilimandischaricus* on the antiproliferative activity of mouse peritoneal cells was evaluated using a colorimetric assay. Based on the observations made by Shava *et al*. (2016), that various extracts demonstrated antiproliferative activity on Human promyelocytic leukaemia cells (HL60 Cell line), this study was set up to investigate the phytochemicals present in *D. kilimandischaricus* that are responsible for the root extracts' antiproliferative property. The results obtained by the MTT assay showed that the methanol root extract of *D. kilimandischaricus* was shown to decrease the cell viability with the greatest potency when compared to the other extracts. The presence of cytotoxicity may be indicative of anticancer action of the constituents of the methanolic extract. Studies have shown that natural compounds possessed by many plants and herbs have antioxidant capabilities thereby reducing oxidative stress in cells (Chon *et al*., 2009). The different bioactivity of the extracts of varied polarity against mouse peritoneal cells can be attributed to the specificity of a phytochemical group extracted by the solvent (Jain, 2014). It was concluded in various studies that the methanol extract can be used for its medicinal properties. The authors showed that the methanol had antioxidant capacity, can act as an antibacterial agent as well as inhibiting tumour cell growth (Karri *et al*., 2011; Jain, 2014; Peng *et al*., 2007).

The most potent extract determined by the MTT bioassay was subjected to silica gel chromatography and the molecular structures of the compounds present in the isolated fractions

were elucidated using GC-MS analysis. In the search of the bioactive components of a plant it is important to know the chemical constituents of the plant. The present study sought to evaluate the presence of different phytochemicals along with GC-MS and FTIR investigations of methanolic root extracts. The qualitative screening of phytochemical compounds in *D. kilimandischaricus* methanolic extracts showed the presence of alkaloids, saponins and phenolic compounds (flavonoids, steroids, terpenoids, and tannins). Saponins have been isolated from *Dolichos* species, with those isolated from *D. falcatus* shown to possess analgesic and antitumor activities while three with molluscicidal and fungicidal activities were isolated from *D. kilimandischaricus* (Mastorn *et al*., 1988). Alkaloids identified as being present in the plant could give *D. kilimandischaricus* antimalaria, analgesic, antispasmodic, antibacterial and antioxidants activity (Khalil *et al*., 2013). Polyphenols such as tannins have been shown to reduce the risk of chronic diseases related to oxidative stress as they possess antioxidant activity and thus promote general health benefits. Phytochemical screening has indicated that plants rich in tannins and saponins followed by terpenoids and flavonoids are most likely to possess antimicrobial and antioxidant activity while having no observable toxicity (Mbwambo *et al*., 2013). Tannins present in this plant show that *D. kilimandischaricus* tannins suggests the ability of this plant to play a major role as an antidiarrhoec and antihaemorrhagic agent (Asquith and Butler, 1986).

The methanol root extract of *D. kilimandischaricus* have been shown to possess antifungal activity both *in vitro* and *in vivo* (Tegegne and Pretorious, 2007). Few plants of the *D.* genus have been studied as extensively as *D. biflorus* with the plant being used widely as a medicinal plant in the cure of various ailments (Mansoor *et al*., 2014). The methanol extract of the seeds of *D. biflorus* have been shown to possess considerable cytotoxicity with *Artemia*

salina eggs (Jayaweera and Jayasinghe, 2007). A number of chemical constituents have been isolated from this plant, in particular streptogenin, β-sitosterol, bulbiformin, linoleic acid, polyphenols, oxalates and crude fibre. Isoflavones have also been isolated from the leaves (Keen *et al*., 1980). The seeds as well as leaves are the main plant parts that were studied to yield 5-hydroxy-7,3,4-trimethoxy-8-methylisoflavone-5-neohesperidoside, genistein, B-sitosterol and 5-o-α-Lrhamnopyranosyl (1-2)-β-D-glucopyranoside, dolichin A and dolichin B (Mansoor *et al*., 2014), these compounds showed analgesic, antidiuretic and anti-inflammatory activities.

The infrared spectra of the methanol extracts were taken and compared to those found in literature. FTIR spectrum of the methanol only root extract of *D. kilimandischaricus* showed the main characteristic absorption broad bands appearing at 3487.5 cm^{-1}, 3412.5 cm^{-1} and 3262.5 cm^{-1} that were assigned to the presence of (O–H/N–H) group. Fourier transformed infrared spectra revealed the presence of the methyl ($–CH_3$) and methylene ($–CH_2$) aliphatic saturated (C–H) sharp asymmetric and symmetric stretching vibration band which was observed as a strong band at 2925 cm^{-1} indicating the presence of an aliphatic compound (Rasool *et al*., 2010). The (C=O) stretching band is observed at 1705.0 cm^{-1}; it is due to the presence of carboxylic acid/ketones groups, it is also indicative of an ester, a saturated aliphatic ester. The band corresponding to C-H bending of methylene vibration in which all of the methylene groups vibrate in phase occurred at 1387.5 cm^{-1} characteristics of a straight chain hydrocarbon of seven or more carbon atoms (Rasool *et al*., 2010; Zhang *et al*., 2010). The band at 1612.5 cm^{-1} shows the presence of aromatic ring (–C=C–stretching). The presence of alkaloids was supported by using FTIR studies. The FTIR results showed the presence of aromatic rings (C=C stretching bands) and the O-Me group appeared from 1387.5 cm^{-1}. Furthermore, a broad band of the FTIR spectrum showed the presence of N-H groups (Kim, 2009).

A successful strategy for investigating plants for biologically active compounds has become easier due to rapid advancement of complex chromatographic and spectroscopic systems of analysis (Bohlin and Bruhn, 1999). Hyphenated chromatographic and spectroscopic techniques are powerful analytical tools used in the generation of chemical structures of the bioactive compounds isolated from plants (Oleszek and Marston, 2000). These techniques include LC–UV–MS, LC–UV– NMR, LC–UV–ES–MS and GC–MS for the separation and structure determination of bioactive compounds from plants. Gas chromatography-mass spectrometry (GC-MS) analysis of pooled fractions (P10, P50 and P51) led to the identification of 11 components from the three pooled fractions. It is of importance to note that all the identified compounds from the pooled fractions contained nitrogen, oxygen and/or π-electrons in their molecules.

In this study the enzyme inhibitor 1,4,5,6,7,11b-Hexahydro-10-chloro-4-(4'-chlorophenyl)-3,11b-bis[trifluoromethyl)imidazo[1,2-a] pyrazolo [4,3-b] indole was found to be predominant in Pool 10. Emerging *in vitro* data shows that enzyme inhibitors selectively inhibit growth of cancer cells without affecting normal cells by generating hydrogen peroxide (Padayatty *et al*., 2006). Similar results were obtained in studies with enzyme inhibitors such as hydroxyurea and 5-fluorouracil which affect the proliferation of L1210 leukemic cells (Chou and Talalay, 1984). The presence of 1,4,5,6,7,11b-Hexahydro-10-chloro-4-(4'-chlorophenyl)-3,11b-bis[trifluoromethyl)imidazo[1,2-a] pyrazolo [4,3-b] indole can be considered a contributor to the antiproliferative effects of the methanol root extract of *D. kilimandischaricus*.

An anthraquinone, 3,8-bis(2',2'-Dimethylpropionyloxy)-1-ethoxy-6-methoxy-2-methyl-9,10-anthraquinone, was found to be present in Pool 50 of the isolate. Anthraquinones are aromatic compounds which can be viewed as quinone derivatives (Cowan, 1999). Previous

phytochemical investigation of *Xanthoria* species reported the isolation of anthraquinones which were shown to possess antibacterial and antifungal activity (Manojlovic *et* al., 2000). The anthraquinones isolated from *Morinda citrifolia* have previously been shown to possess antiproliferative activity against SKBR3 human cell line (Moongkarndi *et al*., 2004). More recently, Hou *et al*., (2009), reported the antiproliferative and antimalarial activities of extracts of three anthraquinones from the roots of *Scutia myrtina*. According to these authors, all three anthraquinones inhibited the growth of A2780 human ovarian cancer cell line. The authors also suggested the possibility of using anthraquinones as antimalarial agents in humans. The results of previous studies give further proof of the antiproliferative effects of anthraquinones present in plants and possibly those in *D. kilimandischaricus*.

The derivative of the ester 2-methylpropyl acetate, 1-(4-isopropylphenyl)-2-methylpropyl acetate and methyl stearate were identified in this study. In a study carried out by Cao *et al*., (2011) the derivative of 2-methylpropyl acetate isolated from *Cyphostemma greveana* showed activity when tested with A2780 ovarian cancer line. Antiproliferative screening tests of 1-methyl stearate from *Cedrus libani* against K562 suggested that an ester group in the compound was responsible for the antitumor activity (Guerrini, 2011). There have been studies showing the antiproliferative effects of naphthoquinone esters isolated from *Rhicanthus nasutus*, which inhibit the growth of cancer cells both *in vitro* and *in vivo* (Siripong *et al*., 2006). The esters isolated from the leaves and barks of seven different plants selectively inhibited growth of B16F10 murine melanoma cells (Nagao *et al*., 2001), giving further evidence that the ester 1-(4-isopropylphenyl)-2-methylpropyl acetate from methanolic root extracts *D. kilimandischaricus* has the ability to inhibit cell proliferation.

Dodecanamide, identified in the GC-MS of Pool 50. Dodecanamide found in the extracts of *Juglans regia* has been shown to possess antioxidant and antiproliferative activities (Anjum *et al*., 2017). Dodecanamide has previously been shown to have selective antiproliferative effects against different cell lines other than the one tested here (Hayashi *et al*., 2009). The major chemical constituent of pool 51 was found to be 7-Acetyl-4,5-epoxy-18,19-dihydro-3,6-dimethoxy-6,14-ethenomorphinane (23.728, 72.3 %) also known as thevinone. Thevione has been synthesised based on bioisosterism and found to possess antifungal activity (Zou *et al*, 2002). The chemical constituent 5-Benzoyl-1,3-bisethoxymethyl-1H-pyrimidin-2,4-dione is a biologically active compound widely distributed in pool 51. Cytotoxic activities of synthetic diones were assessed in three cultured tumour cell models. The diones were found to possess antiproliferative activity at low concentrations (Temburnikar *et al*., 2015).

The activity of a compounds against microorganisms was shown to depend to a large extent on the structure of the compound (Manojlovic *et* al., 2000). However, the highly complex chemical compositions of the pooled fractions make it rather quite difficult to assign the inhibitive effect to one particular compound present in plants extracts unless pure compounds are isolated and their structure elucidated.

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

FT-IR spectral analysis of the methanol root extracts of *D. kilimandischaricus* showed the presence of phytochemicals carrying hydrogen bonded –NH functional group. It is well established that amine functionality is an integral part of alkaloids. This study also suggests 70 % ethanol and methanol are suitable solvents for extraction of bioactive components from the roots of *D. kilimandischaricus*. The GC/MS technique revealed the presence of 3,8-bis(2',2'-Dimethylpropionyloxy)-1-ethoxy-6-methoxy-2-methyl-9,10-anthraquinone, 1,4,5,6,7,11b-Hexahydro-10-chloro-4-(4'-chlorophenyl)-3,11b-bis[trifluoromethyl)imidazo[1,2-a] pyrazolo [4,3-b] indole, 1-(4-isopropylphenyl)-2-methylpropyl acetate, Dodecanamide, Methyl stearate, 7-Acetyl-4,5-epoxy-18,19-dihydro-3,6-dimethoxy-6,14-ethenomorphinane, 5-Benzoyl-1,3-bisethoxymethyl-1H-pyrimidin-2,4-dione, Decanamide and others. The DCM extract as well as the methanol extract showed antiproliferative activity on mouse peritoneal cells. The present study managed to identify the chemical constituents that are responsible for the antiproliferative effects on mouse peritoneal cells in *D. kilimandischaricus*. Further investigations should be carried out with human cancer cell lines to explore the human health benefits of the methanol extract. The chemical constituents identified in this study need to be further purified and analysed by other spectrophotometric techniques so as to elucidate their structures.

CHAPTER 7: REFERENCES

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APPENDIX 1

ELUTION PROFILE FOR SAMPLE FROM A COLUMN USING HEXANE, ETHYL ACETATE AND METHANOL MIXTURE

Sample No.	Number of fractions	Solvent(s)	Volume	Mobile phase for TLC
1	0ne-24	100 % hexane	4 L	97.5:2.5 (HEX: EA)
2	25-48	97.5 hexane: 2.5 EA	"	95:5(HEX: EA)
3	49-72	95 hexane: 5 EA	"	92.5:7.5 (HEX: EA)
4	73-96	92.5 hexane: 7.5 EA	"	90:10 (HEX: EA)
5	97-120	90 hexane:10 EA	"	85:15 (HEX: EA)
6	121-144	87.5 hexane:12.5 EA	"	80:20 (HEX: EA)
7	145-168	85 hexane: 15 EA	"	chloroform
8	167-192	82.5 hexane: 17.5 EA	"	chloroform
9	193-216	80 hexane:20 EA	"	chloroform
10	217-240	75 hexane:25 EA	"	97.5 chloroform:2.5 MeOH
11	241-264	70 hexane:30 EA	"	97.5 chloroform:2.5 MeOH
12	265-288	65 hexane:35 EA	"	97.5 chloroform:2.5 MeOH
13	289-312	60 hexane:40 EA	"	97.5 chloroform:2.5 MeOH
14	313-336	50 hexane:50 EA	"	97.5 chloroform:2.5 MeOH
15	337-360	40 hexane:60 EA	"	97.5 chloroform:2.5 MeOH
16	361-384	25 hexane:75 EA	"	90 chloroform:10 MeOH
17	385-408	10 hexane: 90 EA	"	90 chloroform:10 MeOH
18	409-432	100% EA	"	90 chloroform:10 MeOH
19	433-456	95% EA: 5% MeOH	"	75 chloroform:25 MeOH
20	457-480	90% EA:10 % MeOH	"	75 chloroform:25 MeOH

Appendix 2

D. kilimandischaricus root methanol extract undergoing chromatographic fractionation