EVALUATION OF PHYTOCONSTITUENTS, ANTIOXIDANTS POTENTIAL, CYTOTOXIC, ANTIMICROBIAL ACTIVITIES AND MINERAL COMPOSITION OF *VIGNA SUBTERRANEA* (L) VERDIC. EXTRACTS

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Evaluation of Phytoconstituents, Antioxidants Potential, Cytotoxic, Antimicrobial Activities and Mineral Composition of *Vigna subterranea* (L) Verdic. Extracts

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university

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DEDICATION

This work is first and foremost dedicated to my supervisors for their unreserved support and motivation in all aspects throughout the study period, my dear parents and friends. All this would not have been possible without the boundless grace from the Almighty God.

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LIST OF ABBREVIATION

AIDs	Acquired immunodeficiency syndrome
CDK2	Cyclin dependent Kinase-2
CHD	Cardic Heart Disease
CTMDR	Centre for Traditional Medicine and Drug Research
DMSO	Dimethyl sulfoxide
DU 145	**P**rostate carcinoma cell line
ERC	Ethical Review Committee
FBS	Fetal Bovine Serum
FDA	Food and drug administration
GC	Gas chromatography
GC-MS	Gas chromatography- Mass spectroscopy
IC	Inhibition concentration
JKUAT	Jomo Kenyatta University of Agriculture and Technology
KEMRI	Kenya Medical Research Institute
MCF- 7	Michigan Cancer Foundation-7
MEM	Eagle's Minimun Essential Medium
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide,Triazol
NIST	National institute of standards and Technology
P21	Protein 21
PG	Prostaglandins
ROS	Reactive oxygen species/ Reactive Nitrogen Species
RPMI- 1640	Roswell Park Memorial Institute medium-1640
RT	Retention Time
RF	Retention Factor
SPSS	Statistical Package for Social Science
TLC	Thin layer Chromatography

ABSTRACT

Vigna subterranea is a leguminous drought tolerant crop of African origin which has food, nutrition and medicinal value. Nuts were investigated for their phytochemicals, cytotoxicity, antioxidants, antimicrobial activity and mineral composition.Nut landraces were selected from Western part of Kenya. Extraction of the six nut cultivars was done using single extraction method of 1:1 ratio of methanol: dichloromethane solvent. Extracts were analyzed for their phytochemical composition using gas chromatography-mass spectroscopy, cytotoxic activity by MTT assay against two human cancer cells (DU 145 & Hep 2) and a non-cancerous cell (Vero), antioxidant potential against DPPH, antibacterial activity to evaluate against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas auriginosa* using the Kirby-Baurer diffusion test and antifungal activity to assay against the *Candida albicans* and mineral composition analysis for macro and micro elements. Data for cytotoxicity and antioxidant activities were expressed as mean of three-independent experiments. The analysis was performed using one way ANOVA and two-tailed students t-test from SPSS program. With the aid of NIST library the peaks from GC-MS analyses were compared and identified. Terpenoids, saponins, flavonoids and alkaloids were identified. The dark brown variety extract exhibited cytotoxicity against Hep 2 with IC_{50} value of 15.5 µg/ml but showed less (41.1 µg/ml) for DU 145 cell line. The extracts exhibited cytotoxicity against Vero cells on dilution by giving IC_{50} values with dark brown varieties at 19.2 µg/ml. Furthermore, the extracts of *Vigna subterranea* varieties of dark brown and cream spotted had the highest cytotoxicity against Hep 2 cancer cell line with IC_{50} of 15.5 and 17.9 µg/ml respectively. Additionally, manganese, magnesium and potassium, sodium, iron and calcium were identified in the extracts. Extracts inhibitory potentials towards the isolated strains were varied with *E. coli* showing zone of inhibition at 27±0.7 mm, *S. aureus;* 25.3±0.2 mm, and *P. aeruginosa* at 25.1±0.2 mm compared to 37.0±0.5, 41.3±0.9 and 42.3± 0.9 respectively for ceftriaxone. The black variety extracts had the greatest (91.1 %) inhibitory effect on mycelial growth of *C. albicans* at 4 µg/ml as compared to

Clotrimazole (95.3%) at the same concentration with insignificant values from negative control. The antioxidant potential on comparison of ascorbic acid and cultivars of *Vigna subterrenea* against DPPH scavenging assay method gave IC_{50} values at 10.1 and 25± 1.4 µg/ml respectively. GC-MS analysis of extracts led to identification of several phytocomponents viz. farnesyl alcohol, 2-chloroethyl linoleate, 7-octadecenoic acid methyl ester, methyl behenate, trans-2- methyl-4-n-butylthiane- s,s –dioxide, methyl arachate, (-Z)-octadecenyl ester, 9,17-decadecadienal (Z) and palmitic acid. These results indicate the possible potential medicinal use of nuts because of the biological activities it exhibits. Use of GC-MS analysis is the first step towards understanding the nature of phytocomponents in this nut. The isolation of individual bioactive components and modifications are necessary for future studies and to help find new and better drugs.

CHAPTER ONE

INTRODUCTION

1.1 Background

The exploitation of medicinal plants presents a very significant aspect of the traditional medicine which is entrenched in the culture of people of developing countries (Rungsung, 2015). In many developing countries including Kenya, medicinal plants have not been well studied, tested, or documented. Most of the information is still in the hands of traditional therapists or people with apprenticeship and this knowledge is either lost or passed to next generation by the word of mouth (Tamene *et al.,* 2000). The magnitude of the knowledge of traditional medicine practice based on medicinal plants should be documented through botanical surveys. Botanical collection and documentation of the associated ethnobotanical knowledge should be carried out before such rich heritages are lost due to various anthropogenic and other natural causes (Giday *et al.,* 2003) and also development of modern medicine with the introduction of modern drugs produced by pharmaceutical companies, which is dealing harshly with traditional medicine which have been accused of being inefficient, laborious in preparation and unavailable due to scarcity of raw material. Fortunately, nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs (morphine and codeine from opium poppy). Quinine and its diastereomer from cinchona plant, artemether an artemisinin derivative from *Artemisia annua* L., digoxin from *digitalis purpura* etc. have been isolated from natural source. Searching for plant substances that are capable for being used to develop new therapeutic drugs against illnesses such as antimicrobial infections, cancer, diabetes and acquired immunodeficiency syndrome (AIDs) is one of the main topics that researchers around the world have been focusing on. Attention towards traditional natural products has improved on a larger scale (Dallahi *et al.,* 2016). Additionally, minerals have been appreciated by scientists and researchers in their role of decreasing incidences of neurodegenerative disorders and having an effect on microorganisms and cancer. Of

interest is the postulation that mineral deficiency may activate certain procarcinogens and thereby influence oncogenesis (Yousefi *et al*., 2014).

Vigna subterranea (L.) Verdc. originated in West Africa but has become widely distributed throughout the semi-arid zone of sub-saharan Africa. Sharing a high nutritive value with other widely consumed legumes, *Vigna subterrenea* has an appealing flavour which is reflected in demand from small local and niche markets. In Kenya, *Vigna subterrenea* groundnut is a minor crop and is used as a traditional food only by the Luhya, Giriama and Kambe at the coast, and to a lesser extent by the Luo (Ngugi, 1995). *Vigna subterrenea* nut is a leguminous drought tolerant crop which has food, feed, nutrition and medicinal value (Mkandawire, 2007). The plant *Vigna subterranea* species is not well known for its pharmacological properties and ethnobotanical use.

Potentially valuable treasures in this nut remain extensively unexplored as the groundnut six cultivars (dark brown, cream spotted, brown, black, and dark-brown spotted, red and light spotted) are among such treasures as their compounds have not been fully investigated with few reports being put forward. Report by Mbagwu.*et al*. (2011) indicate that the seeds of *Vigna subterrenea* are nutritious and contain phytochemicals such as alkaloids (0.40 %), saponins (0.43 %) and flavonoids (0.29 %). These results of the phytochemical screening and quantitative estimation compared favorably with those reported from some medicinal plants found in Nigeria (Sofowara., 1993). Research has shown that *Vigna subterrenea* groundnut landraces with low levels of condensed tannins have beneficial health effects on human nutrition (Akindahunsi & Salawu, 2005). For instance, landraces containing relatively high levels of soluble fibers are believed to reduce incidences of heart disease, colon cancer and diarrhea (Goli *et al.*, 1991). Black seeded landraces are reported to cure impotence in men, nausea and morning sickness in women in Botswana. These results of the phytochemical screening and quantitative estimation compared favorably with those reported from some medicinal plants found in Nigeria by Sofowara, 1993. Though the yields of the alkaloids are low, they are useful in prolonging the action of several hormones and acting as stimulants especially *V. subterranea* which has the highest value of alkaloid. Flavonoids are capable of treating

certain physiological disorder and diseases. They are potent water soluble, super anti-oxidant and free radical scavenger which prevents oxidative cell damage and have strong anti-cancer activity which adds protection against all stages of carcinogenesis (Enwere & Hung, 1996). Saponins have cholesterol binding properties, and help in hemolytic activities (Enwere & Hung, 1996).The fermented *Vigna subterrenea* groundnut seed was reported to have significant ($P < 0.05$) reducing power, free radical-scavenging ability (DPPH, $ABTS^{\bullet}$ and $OH^{\bullet}$ radicals) and Fe^{2+} chelating ability than the unfermented seed (Ademiluyi & Oboh,2011).

Furthermore, infectious diseases are the leading causes of death throughout the world, accounting for nearly one half of all deaths in the tropical countries, which are also becoming a serious problem in developed countries (Patel *et al.,* 2010). Failure of chemotherapy and multiple drug disease resistance that has been developed due mutation or gene transfer have led to organisms that can be drug tolerant, drug destroying and drug impermeability leaving questions on the antibiotics efficacy. In addition to this problem, antibiotics are sometimes associated with several problems that arise with the use of conventional medicines on the host including toxicity, hypersensitivity reaction, suprainfection, nutritional deficiencies and masking of infections. This problem has forced scientists to search for new antimicrobial substances. Given the alarming incidence of antibiotic resistance bacteria of medical importance, there is a constant need for new and effective therapeutic agents. Therefore, there is a need to develop alternative antimicrobial drugs for the treatment of infectious diseases from medicinal plants (Ngugi *et al.*, 1995). Antimicrobials of plant origin have enormous therapeutic potential. They are effective in the treatment of infectious diseases while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials (http://www.hbci.com/-wenonah/new/pnutskin.htm) *Vigna subterrenea* is a highly nutritious legume. Its seed consist of 49%–63.5% carbohydrate, 15%–25% protein, 4.5%–7.4% fat, 5.2%–6.4% fiber, 3.2%–4.4% ash and 2% mineral compared to whole fresh cow milk 88% moisture, 4.8% carbohydrate, 3.2% proteins, 3.4% fat, 0.7% ash, and 0.01% cholesterol. Its chemical composition is comparable to

that of soy bean. *Vigna subterrenea* nut has been reported to be a potential crop, owing to its nutritional composition, functional properties, antioxidant potential, and a drought resistant crop. Probiotics have been defined as live microorganisms which when administered in adequate amount confer a health benefit on the host. These benefits have been reported to be therapeutic, suppressing the growth and activity in conditions like infectious diarrhea, irritable bowel syndrome, and inflammatory bowel disease. The nutritional profile of *Vigna subterrenea* nut is high enough to sustain the growth of probiotics (Yvonne *et al.*, 2013).

However, the use of *Vigna subterrenea* nut extracts for the management of microbes and toxicity towards cancer has not been validated. Therefore the study was designed to determine the cytotoxicity and antimicrobial properties and also establish the minerals profile in *Vigna subterrenea* nuts obtained from Kenya. The study is for the first time reporting on the cytotoxic, phytocostituents, mineral composition, antimicrobial potency and antioxidant properties of *Vigna subterrenea* nut found in Kenya.

1.2 Statement of the problem

Increase in neurodegerative disorders, cancers, infections and aging process have led to high mortality rates. In Kenya, cancer for instance is one of the five leading causes of death contributing to 7% of the total national mortality where it's estimated that the annual incidence is about 28000 cases and the annual mortality is over 22000. This growing trend indicates the deficiency in the current cancer therapies, which include surgery, radiotherapy, and chemotherapy. Anticancer chemotherapeutic agents used to eliminate cancer cells also affect the entire body, not just a specific part. It works by targeting rapidly multiplying cancer cells.

Moreover, the search for newer source of antimicrobial is a global challenge preoccupying research institutions, pharmaceutical companies and academia, since many infectious agents are becoming resistant to synthetic drugs. Despite the enormous efforts to search for prevention/cure, some of the problems still remain a challenge for global

public health. There is at present a question for new generations of antifungal compounds due to certain limitations with side effects as toxicity and emergence of resistant strains. Some antifungal agents suffer from a number of limitations that can render their use complicated; for example, dose-limiting nephrotoxicity associated with Amphotericin B, rapid development of resistance with flucytosine, drug-drug interactions and resistance development with the azoles. Change in life styles, cost of medicines, polypharmacy, noncompliance to drugs, hygiene, drugs use, alcohol abuse, diet, HIV/AIDS and environmental changes and oxidants are some of other factors thought to contribute towards the rise of these disorders and diseases.

Research data through ethnomedicine have revealed that medicinal plants could be suitable for preventive and therapeutic purposes in several diseases (*e.g*. atherosclerosis, inflammatory injury, cancer and cardiovascular diseases). For the treatment of infectious diseases, different medicinal plants have been mentioned by many phytotherapy manuals because of their reduced toxicity, uncomplicated availability, and fewer side effects. One way also to prevent antibiotic resistance of pathogenic species is by using new compounds that are not based on existing synthetic antimicrobial agents. Problem of resistance with irrational use of orthodox medicines have necessitated renewed interest in nature as a source of effective and safer alternatives in the management of human infections.

There is also need for innovative ways in selecting therapeutic agents and finding lead compounds towards fighting these problems. Identification of alternative substances that are: i) more affordable ii) more closely corresponds to the patient's values or beliefs; iii) less paternalistic than is allopathic medicine; and iv) effective v) well tolerated by the body, vi) only target certain cells is necessary. Currently, no detailed biochemical and pharmacological studies have been published on effectiveness, toxicity and potential adverse effects associated with the medicinal usage of *Vigna subterrenea* groundnut. Since most of drug agents currently in use are in some way derived from natural sources, evaluating the potential of *Vigna subterrenea* as a source of alternative agent is imperative.

1.3 Rationale of the study

Medicinal plants either –through systematic screening programs or by serendipity - possess an important position in the drug discovery and many modern drugs have their origin in traditional medicine of different cultures. Hence, despite the advantages of the synthetic and combinatorial chemistry as well as molecular modeling, medicinal plants remain an important source of new drugs, new drug leads and new chemical entities. Several scientific publications have point out the fact that many groups of metabolites from vegetal origin show antioxidant and hepatoprotective activity.

The areas of cancer and infectious diseases have a leading position in utilization of medicinal plants as a source of drug discovery. Among food and drug adminstration approved anticancer and anti-infectious preparations drugs of natural origin have a share of 60% and 75% respectively. Drug unearthing from medicinal plants has led to the development of drugs such as codeine, digitoxin, colchicine, quinine, and morphine, which are still in use (Pan *et al*., 2013 ; Sahreen *et al*., 2010). Some phytocomponents differently exhibits anticancer, chemopreventive, and antifungal activities. Phytocomponents utilization lowers the risk of cardiovascular heart disease (CHD), which may be partly explained by the cholesterol-lowering effect. The favorable fatty acid composition and lipid lowering effect of nuts have been demonstrated in experimental studies with almonds and peanuts Kumar *et al*. (2013). Recently, oil seeds have been thoroughly investigated taking into account especially the phytochemicals representing the minor components (like phenolic compounds). This interest is connected with the activity of such compounds against CHD. Many researchers are constantly turning to natural products for alternative source of medicines. Above all, there is paucity of documented evidence of the potential of *V. subterrenea* to provide its pharmacological effect. More attention should therefore be given to it, if this nut is to make a greater contribution to improve health of communities in Africa.

Information on activities of *Vigna subterrenea* will form a basis for further research and development as leads for therapeutic agents. The potential value of the research results

for improving the health of the community with due regard to the increasing incidence of cancer and the feasibility of using accessible natural recourses as alternative treatment cannot be overemphasized. Although some limited research has been undertaken, no documented information on health benefits of *Vigna subterrenea* nut has been attempted in Kenya. This project was aimed to address this need.

1.4 Null Hypothesis

The phytochemicals in *Vigna subterrenea* do not have pharmacologic effects.

1.5 Objectives

1.5.1 General objective

To determine phytochemical, mineral composition, antioxidant and biological activities of extracts from *Vigna subterranea* nut

1.5.2 Specific objectives

1. To determine the phytochemical composition of *Vigna subterranea* nut extracts
2. To analyze for mineral composition of the *Vigna subterranea* nut extracts
3. To screen for the cytotoxic activity of *Vigna subterranea* nut extracts against two human cancer cells (DU 145 & Hep 2) and a non-cancerous cell (Vero)
4. To determine the antioxidant of DPPH radical against the *Vigna subterranea* nut extracts
5. To determine the antimicrobial activity of the *Vigna subterranea* nut extracts against *S. aureus, E. coli, P. auriginosa* and *Candida albicans*

1.6 Scope of the study

The preliminary study focused on determining the various phytochemicals present in the *Vigna subterranea* (L.) Verdc extracts. The antioxidant potential was achieved using DPPH and cytotoxic activity against two human cancer cells (DU 145 & Hep 2) and a

non-cancerous cell (Vero) of the *Vigna subterranea* against various cell lines were analyzed using the MTT Assay. Mineral composition analysis helped in determining micro and macro minerals that were present. Finally, the antimicrobial study was obtained using antimicrobial sensitivity tests.

1.7 Limitation to the study

One major limitation of this experimental research was that it was typically conducted in contrived or artificial laboratory settings. Such results may not generalize or extrapolate to external settings. Facility and equipment acquisition was a great challenge too.

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction

Medicinal plants have had a critical role in human culture and civilization. Their importance and the contribution of phytomedicine to the well-being of a significant number of the world's population, has attracted interest from a variety of disciplines and cultures (Pan *et al*., 2013). They are well-known natural sources of remedies for the treatment of various diseases since the antiquity. The use of medicinal plants in the world, and especially in Kenya contributes significantly to primary health care and have been used as natural medicines by local populations in the treatment of tropical diseases, including neurodegenerative diseases, malaria, cancers, fungal and bacterial infections (Alves *et al*., 2000 ; Duarte *et al*., 2005). Various types of herbology and other medical practices referred to as complementary or alternative medicine are increasingly used in both developing and developed countries. Recent studies are involved in the identification and isolation of new therapeutic compounds of medicinal importance from the plants for specific diseases (Kumar *et al*., 2013).

Studies to date have demonstrated that phytochemicals in common fruits and vegetables can have complementary and overlapping mechanisms of action ,including antioxidant activity; regulation of gene expression in cell proliferation, cell differentiation, oncogenes, and tumor suppressor genes; induction of cell-cycle arrest and apoptosis; modulation of enzyme activities in detoxification, oxidation, and reduction; stimulation of the immune system; regulation of hormone metabolism; and antibacterial and antiviral effects (Egwaikhidi, 2007; Wang *et al*., ; 2005 ; Yvonne *et al*., 2013).

Vigna subterranea is an agricultural plant distributed in some parts of Western and Coastal Kenya. The nut in its cooked and raw state has acted as food or therapy to combat/ reduce incidences of heart disease, and diarrhea (Goli *et al.,* 1991). Black seeded landraces are reported to cure impotence in men, nausea and morning sickness in women in Botswana. The nutritive capabilities of *Vigna subterrenea* groundnut have been documented. The seed is regarded as a balanced food because when compared to most food legumes, it is rich in iron and the protein contains high lysine and methionine (AduDapaah & Sangwan, 2004). In addition, it is known to contain 63 % carbohydrates, 18 % oil and the fatty acid content is predominantly linoleic, palmitic and linolenic acids (Minka & Bruneteau, 2000). It is reported also that it is richer than groundnut in essential amino acids such as leucine, isoleucine, lysine, methionine, phenylalanine, threonine and valine (Ihekoronye & Ngoddy, 1985).

Soils of medium or low fertility, with a pH of 5.0 - 6.5 will produce satisfactory crops. Yields of *Vigna subterrenea* groundnut on low - fertility soils are generally higher than those of groundnut grown on similar soils. Bambara groundnut will often yield well in environments that may be too hostile for more favoured legumes (Collinson *et al*., 1996). An evenly distributed rainfall in the range 600-1000 mm encourages optimum growth but satisfactory yield can be obtained in areas with pronounced dry season since the crop is relatively drought resistant. It is tolerant to periods of heavy rainfall except during the flowering period. Studies have strongly established the central role of duration of flooding and temperature and their interaction in determining the effect of flooding stress on germination and establishment of seedlings. Massawe *et al.* (1999) reported that pre-swing hydration reduced final germination percentages significantly as the duration of soaking increases from 2 to 8 days and a complete loss in germination occurs when seeds are soaked for 6 days at 35^{o}C and 8 days at all temperatures.

It has been scientifically declared that *Vigna subterrenea* bean is high in protein quotient, particularly in methionine which makes its protein more complete than any other bean. The proximate composition of the bambara groundnut was reported to be 9.7 % moisture, 16.6 % protein, 5.9 % fat, 2.9 % ash, 4.9 % crude fibre and 64.9 %

carbohydrate (Enwere & Hung, 1996). Moreover, as in research by Pasquet *et al*., (1999), using isozyme analysis, found high genetic identities between wild and domesticated bambara groundnut accessions and concluded that the wild bambara is the progenitor of the domesticated form, both being characterized by low total genetic diversity.

The high concentration of soluble fibre than any other bean also makes it one step ahead of other beans. This further enhances its quality as nutritious food which reduces the incidence of heart disease and certain types of cancer. Also, bambara beans being nitrogen fixers themselves and along with providing the soil with essential nutrients do not require any artificial fertilizer. The use of artificial flavours or preservatives during the food processing is greatly discarded. (Olaleye, 2010).

Bambara groundnut was reported to have been fairly well supplied with calcium and iron although poor in phosphorus. It contains thiamine, riboflavin, niacin and carotene but very low in ascorbic acid (Mkandawire, 2007). The study of the microstructure of the raw flour and seed showed that they contained differently shaped and sized starch granules and protein materials within the cell wall in the cotyledon. Milling disorganized the arrangement of these components in the cotyledons (Enwere & Hung, 1996). It is a non-oily leguminous seed which contains only about 6% of ether extract. It contains an appreciable amount of lysine (Mkandawire, 2007).

Several other reports have been made on bambara nut. The swelling capacity increases with increase in temperature (Adebowale *et al*., 2002). Bambara bean is higher in water absorption capacity than that of great Northern bean (Sathe & Salunkhe, 1981).

The digestion and bioavailability of the nutrients in the bambara seeds for animals and human nutrition is limited by antinutrients such as trypsin inhibitors and condensed tannins (Apata & Ologhobo, 1997). Condensed tannins are polyphenolic substances widely distributed in plants, especially in legumes and due to their large structure are known to inhibit protein digestibility by forming irreversible complexes with protein,

thereby reducing the bioavailability of amino acids. However, recent research has also indicated that condensed tannins in low concentrations have beneficial effects in animal and human nutrition and health (Akindahunsi & Salawu, 2005).

The chemical studies about these species (*www.daff.gov.za*) have shown seven types of Bambara groundnut varieties i.e.

- **Black**: Early maturing, usually small to medium-sized kernels. Mainly one-seeded
- **Red**: Late maturing. Kernels are large. A good yielder, however, it is prone to rotting onsite
- **Cream/black eye**: A large kernel and a good yielder
- **Cream/brown eye**: A moderate kernel and a good yielder
- **Cream/no eye**: Very small pods and kernels. It mainly produces one seed and yields are lower.
- **Speckled/flecked/spotted**: Purple colour predominates. Kernels are small and pods are mainly one-seeded.
- **Brown**: Continuous variation between light and dark brown. Kernels are of medium to large size.

The nuts come in a variety of colours (**Figure 2.1**) carrying different names (**Table 2.1**) depending on the location it's cultivated (www.ecprov.*gov.za*/department.php?index=1) as shown below.

Figure 2.1: Vigna subterranea varieties

Table 2.1: List of vernacular names that are assigned to Bambara groundnut according to the countries it is grown.

Country	Vernacular names used by different communities in Africa
Kenya(Luhya)	Dzimbande
Kenya (Swahili)	Njugu mawe
Madagascar	Madagascar groundnut, earth pea, baffin pea, Jugo, njugo bean
South Africa (Sepedi)	Ditloo
South Africa(Tshivenda)	Phonda voandzou, nzama
Malawi	Indhlubu and underground bean
Congo	Groundnut, Congo goober,

2.2 Phytochemicals in the Vigna subterrenea nuts

Vigna subterrenea, like other medicinal plants contains phytochemicals. Mbagwu *et al*. (2011) reported that their seeds contain phytochemicals such as alkaloids (0.40 %), saponin (0.43 %) and flavonoids (0.29 %). These results of the phytochemical screening

and quantitative estimation compared favorably with those reported from some medicinal plants found in Nigeria (Sofowara, 1993). Though the yields of the alkaloids are low, they are useful in prolonging the action of several hormones and acting as stimulants especially *V. subterranea*, which has the highest value of alkaloid.

Additionally, other phytochemistry studies have been done on *Vigna subterreanea* and *Vigna unguiculata* (cowpea). Pale *et al.* (1997) investigated the anthocyanins present in nuts through column and preparative thin-layer chromatography. Three anthocyanins (delphinidin 3-O-β-glucoside, petunidin 3-O-β-glucoside and malvidin 3-O-β-glucoside) were identified. Anthocyanins have many beneficial effects on health, and further investigation into the health properties associated with *Vigna subterreanea* consumption is needed. In a study by Onyilagha *et al*. (2009), eleven species of Vigna were surveyed for canavanine, proanthocyanidin and flavonoid profiles. Canavanine, delphinidinand cyanidin were absent in *Vigna subterreanea* seeds. The absence of canavanine is consistent in the species of Vigna. The flavonoid profiles revealed that the four *Vigna subterreanea* varieties studied accumulated four types of kaempferol glycosides. In all Vigna species, the prevalent flavonoid appears to be kaempferol. Kaempferol-3-O-glucoside-7-rhamnoside seemed to be restricted to *Vigna subterreanea*. As a polyphenol antioxidant, kaempferol imparts many health benefits and reduces the risk of many chronic illnesses such as cancer (Chen *et al*., 2013).

A recently published article by Ujowundu *et al*. (2013) also reveals the possible components in *Vigna subterreanea* which could have beneficial effects on health in their study on the effects of gas flaring on the African breadfruit and *Vigna subterreanea*. Valuable information on the phytochemical properties of *Vigna subterreanea* was found with high concentrations in the unpolluted samples for oxalate (0.38 ± 0.04%), saponin (0.24 ± 0.02%); vitamin E (3.18 ± 0.15 mg/100 g), vitamin C (1.17 ± 0.20 mg/100 g), vitamin A (26.05 ± 0.14 mg/100 g) and niacin (2.10 ± 0.06 mg/ 100 g). The concentrations of oxalate, saponin, alkaloid and flavonoid were increased by gas flaring, whilst the concentrations of vitamins were significantly [$p < 0.05$] reduced. Vitamin A which is important for maintaining good eye-sight and preventing eye diseases

according to Nwaogu & Ujowundu, (2010) were significantly higher [$p < 0.05$] in the *Vigna subterreanea* seeds as compared to the other vitamins detected.

Generally, phytochemicals may either be used as chemotherapeutic or chemo preventive agents with chemoprevention referring to the use of agents to inhibit, reverse, or retard tumorigenesis. In this sense chemo preventive phytochemicals are applicable to cancer therapy, since molecular mechanisms may be common to both chemoprevention and cancer therapy (D'Incalci *et al*., 2005; Sarkar & Li, 2006). Plant extracts and essential oils may exhibit different modes of action against bacterial strains, such as interference with the phospholipids bilayer of the cell membrane which has as a consequence a permeability increase and loss of cellular constituents, damage of the enzymes involved in the production of cellular energy and synthesis of structural components, and destruction or inactivation of genetic material. In general, the mechanism of action is considered to be the disturbance of the cytoplasmic membrane, disrupting the proton motive force, electron flow, active transport, and coagulation of cell contents (Kotzekidou *et al*., 2008). The information available on phytochemical components of *Vigna subterreanea* seeds is promising, and should be further investigated to determine and highlight their specific effects on human health, which could greatly influence the current underutilised status of this crop.

2.3 Cytotoxic activity of medicinal plant extracts

Research has shown that phytochemicals have been associated with cytotoxic and/or antioxidant properties. These properties have been reported in some African plants (Wang *et al*., 2005 ; Cos *et al*., 2006). For example, South African plants (Bisi-Johnson *et al.*, 2011 ; Fouche *et al.*, 2008), Ethiopian plants (Cragg & Newman, 2005), and Egypt (Nassr-Allah, 2009).

Many phytochemicals have demonstrated antitumor efficacy in preclinical animal models of cancer (Aoki & Wada, 2003). Within recent years cardiac glycosides reported in plants are suggested to possess valuable cytotoxic activity where compounds were

prepared and evaluated for their potencies to inhibit the Na^+, K^+-ATPase and for their cytotoxic effect on cancerous MCF-7 cells (Nassr-Allah *et al.*, 2009). Terpenes are a large family of compounds synthesized by *Vigna subterranea* that have a common chemical structure. Terpenes are metabolites of isopentenyl pyrophosphate oligomers and represent the largest group of phytochemicals. Terpenes are biosynthesized in plants by the cyclization of squalene, a terpene hydrocarbon and precursor of all steroids (Sharma & Kumar, 2009). Although terpenes were considered to be biologically inactive for a long period of time, accumulating evidence on their broad spectrum pharmacological activities coupled with a low toxicity profile has sparked renewed interest with regard to human health and disease. Terpenes are used for medicinal purposes in many Asian countries for anti-inflammatory, analgesic, antipyretic, hepatoprotective, cardiotonic, sedative and tonic effects (Lahlou, 2007).

Recent studies have not only confirmed some of the aforementioned pharmacological properties of several terpenoids, but also identified a variety of additional biological activities including antioxidant, antimicrobial, antiviral, antiallergic, antipruritic, antiangiogenic and spasmolytic activity (Iranshah *et al*., 2009). An increasing number of triterpenoids have been reported to exhibit cytotoxicity against a variety of cancer cells without manifesting any toxicity in normal cells (Ferguson *et al*., 2004). They also demonstrate antitumor efficacy in preclinical animal models of cancer (Iranshah *et al*., 2009). A large number of triterpenoids have been synthesized by structural modification of natural compounds for optimization of bioactivity, and some of these semi-synthetic analogs are considered to be the most potent antiinflammatory and anticarcinogenic triterpenoids known to mankind. The antitumor efficacy of several terpenoids are currently being evaluated in phase I clinical trials (Ferguson *et al*., 2004).

Research has shown that *V. subterrenea* landraces with low levels of condensed tannins have beneficial health effects on human health (Miguel, 2010). They have astringent property for healing of wounds and inflamed mucous membrane associated with cancer and this is also observed in a species of the same genus- *Vigna uniguiculata* -that has highest value of tannins (Nedi, 2004). Increasing evidence suggests that interaction

between the chemo attractant CXCL 12 / stoma cell-derived factor-1α and its receptor CXCR4 plays a pivotal role in the metastasis of various tumors. The anti-CXCL 12/ CXCR 4 activity of a commercial tannic acid has potential to inhibit tumor cell migration and angiogenesis *in vitro* (Iranshahi *et al*., 2009).

In pancreatic adenocarcinoma cells, farnesol induces G0/G1 phase cell cycle arrest, down-regulates expression of cyclin A, cyclin B1, and CDK2, and increases expression of p21 and p27. It has also been proposed that fatty acids have potential antibacterial and antifungal principle for clinical application (Altieri, *et al*., 2008). Triterpene-fatty acid esters and free fatty acids including long chain C16-C20 unsaturated fatty acids were suggested to be responsible for the anti-inflammatory activity as it gives rise to PG1 (Li *et al*., 2004).

Oleic acid has been found to be fungistatic against a wide spectrum of moulds and yeasts (Sheba, *et al*., 1999). Oleic acid regulates the activity of adrenoreceptor signaling pathways which direct the adrenergic receptors (α- and β-adrenoceptors) that help regulate blood pressure (Teres *et al*., 2008). Lim *et al*. (2013) found that oleic acid increases the expression of genes involved in fat burning. A recent study found a 6.2% decline in oleic acid in the postmortem brains of patients who had been suffering from major depressive disorder when compared to a normal brain (Hamazaki *et al*., 2012). People and rats with heart failure are unable to process or store fats for use as fuel. This causes the heart to become energy starved and muscles start to break down. Lahey *et al*. (2014) reported that diseased rat's hearts perfused with oleic acid behaved more like healthy rat hearts.

2.4 Antioxidant activity of medicinal plants

Antioxidants guard cells against the destructive effects of reactive oxygen species otherwise called, free radicals such as singlet oxygen, super oxide, peroxyl radicals, hydroxyl radicals and peroxynite which results in oxidative stress leading to cellular damage (Mattson & Cheng, 2006). Natural antioxidants play a key role in health

maintenance and prevention of the chronic and degenerative diseases, such as atherosclerosis, cardiac and cerebral ischema, carcinogenesis, neurodegenerative disorders, diabetic pregnancy, rheumatic disorder, DNA damage and ageing (Uddin *et al*., 2008; Jayasri *et al*., 2009).

Antioxidants exert their activity by scavenging the 'free-oxygen radicals' thereby giving rise to a fairly 'stable radical'. The free radicals are metastable chemical species, which tend to trap electrons from the molecules in the immediate surroundings. These radicals if not scavenged effectively in time, they may damage crucial bio molecules like lipids, proteins including those present in all membranes, mitochondria and, the DNA resulting in abnormalities leading to disease conditions (Uddin *et al*., 2008; Ahn *et al*., 2007). Thus, free radicals are involved in a number of diseases including: tumour inflammation, hemorrhagic shock, atherosclerosis, diabetes, infertility, gastrointestinal ulcerogenesis, asthma, rheumatoid arthritis, cardiovascular disorders, cystic fibrosis, neurodegenerative diseases (e.g. parkinsonism, Alzheimer's diseases), AIDS and even early senescence (Chen *et al*., 2006; Uddin *et al*., 2008; Okoh,. 2010).

The human body produces insufficient amount of antioxidants which are essential for preventing oxidative stress. Free radicals generated in the body can be removed by the body's own natural antioxidant defences such as glutathione or catalases (Sen, 1995). Therefore this deficiency had to be compensated by making use of natural exogenous antioxidants, such as vitamin C, vitamin E, flavones, carotene and natural products in plants (Madsen & Bertelsen, 1995; Rice Evans *et al*., 1997; Diplock *et al*., 1998).

Generally the antioxidants delay autoxidation by inhibiting formation of free radicals or by interrupting propagation of the free radical by one (or more) of several mechanisms: (a) scavenging species that initiate peroxidation, (b) chelating metal ions such that they are unable to generate reactive species or decompose lipid peroxides, (c) quenching $^{\bullet}O_2^-$ preventing formation of peroxides, (d) breaking the autoxidative chain reaction, and/or (e) reducing localized O_2 concentrations (Nawar, 1996). Chain-breaking antioxidants differ in their antioxidative effectiveness depending on their chemical characteristics and

physical location within a food (proximity to membrane phospholipids, emulsion interfaces, or in the aqueous phase). The chemical potency of an antioxidant and solubility in oil influence its accessibility to peroxy radicals especially in membrane, micellar and emulsion systems, and the amphiphilic character required for effectiveness in these systems (Wanatabe *et al*.,2010). Antioxidant effectiveness is related to activation energy, rate constants, oxidation–reduction potential, ease with which the antioxidant is lost or destroyed (volatility and heat susceptibility), and antioxidant solubility (Nawar, 1996). In addition, inhibitor and chain propagation reactions are both exothermic. As the A**:**H and R**:**H bond dissociation energies increase, the activation increases and the antioxidant efficiency decreases. Conversely, as these bond energies decrease, the antioxidant efficiency increases.

Plants contain a wide variety of free radicals scavenging molecules including phenols, flavonoids, vitamins, terpenoids that are rich in antioxidant activity (Madsen & Bertelsen, 1995; Cai & Sun, 2003). The fermented *V. subterranea* was reported to have significant ($P < 0.05$) reducing power, free radical-scavenging ability (DPPH, $ABTS^{\bullet}$ and $OH^{\bullet}$ radicals) and Fe^{2+} chelating ability than the unfermented seed (Ademiluyi & Oboh, 2011).

Vigna subterrenea like other plants have the phytocomponents with the antioxidant power. Significant antioxidant properties have been recorded in phytochemicals that are necessary for the reduction in the occurrence of many diseases (Hertog & Feskens, 1993; Anderson & Teuber, 2001). Many dietary polyphenolic constituents derived from plants are more effective antioxidants *in vitro* than vitamins E or C, and thus might contribute significantly to protective effects *in vivo* (Rice-Evans & Miller, 1997; Jayasri *et al*., 2009). Methanol extract of Cinnamon contains a number of antioxidant compounds which can effectively scavenge reactive oxygen species including superoxide anions and hydroxyl radicals as well as other free radicals *in vitro*. The fruit of Cinnamon, an under-utilized and unconventional part of the plant, contains a good amount of phenolic antioxidants to counteract the damaging effects of free radicals and may protect against mutagenesis.

Antioxidants are often added to foods to prevent the radical chain reactions of oxidation, and they act by inhibiting the initiation and propagation step leading to the termination of the reaction and delay the oxidation process. Due to safety concerns of synthetic compounds, food industries have focused on finding natural antioxidants to replace synthetic compounds. In addition, there is growing trend in consumer preferences for natural antioxidants, all of which has given more impetus to explore natural sources of antioxidants.

2.5 Antimicrobial Activity

Commercial anti-microbial drugs against human pathogenic microorganisms have increased extensively in recent years. Effective antimicrobials have been developed over the past years, however several reports on development of antibiotic resistance of human pathogens to available antibiotics is increasing (Umthong *et al*., 2010; Bharathi *et al*., 2012). Due to the cost effectiveness, safety, increasing failure of chemotherapy and antibiotic resistance, search for plant resources has been increased for their potential antimicrobial activity. The new compounds from plant sources are not based on the obtainable synthetic antimicrobial agents, and also phytochemicals from plants have different structures from microbial derived antibiotics and typical modes of action (Fabricants *et al*., 2001) antibiotic resistance can be much reduced.

Nevertheless, infectious diseases are still the leading causes of death throughout the world, accounting for nearly one half of all death in the tropical countries. This is becoming a serious problem in developed countries (Demissew & Dange, 2001). Nowadays multiple drug disease resistance has been developed due to the indiscriminate, use of commercial antimicrobial drugs commonly used in the treatment of infectious diseases. In addition to this problem, antibiotics are sometimes associated with adverse effects on the host including hypersensitivity, immuno suppression and allergic reactions. This problem has forced scientists to search for new antimicrobial substances. Given the alarming incidence of antibiotic resistance bacteria of medical importance, there is a constant need for new and effective therapeutic agents. Therefore,

it is a need to develop alternative antimicrobial drugs for the treatment of infectious diseases from medicinal plants (Agarwal *et al.,* 1996). Antimicrobial of plants origin have enormous therapeutic potential. They are effective in the treatment of infectious diseases while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials **(**Joshi & Edington, 1990 ; Manandhar 1987).

Researchers are now engaged in developing new molecules with potent activity and greater stability for resolving the problem of antimicrobial-resistance phenomenon in the pathogens. Plant derived antimicrobials have been proposed their suitability for developing new active drugs. On this note, research have shown that terpenes e.g. farnesol, a *Candida albicans* cell-cell signaling molecule that participates in the control of morphology, has an additional role in protection of the fungus against oxidative stress (Arthington-Skaggs *et al*., 2002; Bahn *et al*., 2007). This is also shows that although farnesol induces the accumulation of intracellular reactive oxygen species (ROS), ROS generation is not necessary for the induction of catalase (Cat1)-mediated oxidative-stress resistance. Two antioxidants, α-tocopherol and, to a lesser extent, ascorbic acid effectively reduced intracellular ROS generation by farnesol but did not alter farnesol-induced oxidative-stress resistance (Burke *et al*., 2002). Farnesol inhibits the Ras1-adenylate cyclase (Cyr1) signaling pathway to achieve its effects on morphology under hypha-inducing conditions, and this is demonstrated that farnesol induces oxidative-stress resistance by a similar mechanism. Strains lacking either Ras1 or Cyr1 no longer exhibited increased protection against hydrogen peroxide upon preincubation with farnesol (Chauhan *et al*., 2006). While this is observed the previously reported increase in the phosphorylation level of Hog1, a known regulator of oxidative-stress resistance, in the presence of farnesol, the *hog1*/*hog1* mutant did not differ from wild-type strains in terms of farnesol-induced oxidative-stress resistance. Analysis of Hog1 levels and its phosphorylation states in different mutant backgrounds indicate that mutation of the components of the Ras1-adenylate cyclase pathway is sufficient to cause an increase of Hog1 phosphorylation even in the absence of farnesol or other exogenous sources of oxidative stress (Eisman *et al*., 2006 ; Enjalbert *et al*., 2006).

2.6 Critique of the Existing Literature Relevant to the Study

Despite there being screening of a number of plants having been done to correlate their activity and further expand their scope for drug development (Akter *et al*., 2014) much has stopped at the preliminary stage. Drugs that have been there with their purported side effects still exist on the market. No newer drug with special drug related characteristics or improved characteristics has been synthesized. Owing to potential benefits of plant based drugs for chemotherapeutic treatment, the use of newer drugs should be increasingly growing across the globe but its unfortunate the task has been left in the hands of herbalist (Barnes *et al*., 2004). Chemotherapeutic benefits associated with natural plant derivatives demand extensive scientific screening and clinical experimentations for the development of improved/synthesized drugs has not come to pass. Since bioactive compounds occurring in plant material consist of multi-component mixtures, their separation and determination still creates problems. Practically, most of them have to be purified by the combination of several chromatographic techniques and various other purification methods to isolate bioactive compound(s), but it's unfortunate some steps are undone because of the unavailability of some equipments. In addition, some considerations are overlooked during extraction; as target compounds may be non-polar to polar and thermally labile, the suitability of the methods of extraction must be considered. Various methods, such as sonification, heating under reflux, soxhlet extraction and others are commonly used (United States Pharmacopeia and National Formulary, 2002; Pharmacopoeia of the People's Republic of China, 2000; The Japanese Pharmacopeia, 2001) for the plant samples extraction might not be reachable. If the plant was selected on the basis of traditional uses (Fabricant & Farnsworth, 2001), then it is needed to prepare the extract as described by the traditional healer in order to mimic as closely as possible the traditional 'herbal' drug. This is overlooked by certain scientists.

2.7 Research Summary

Natural products and their derivatives have been recognized for many years as a source of therapeutic agents and of structural diversity (Patwardhan *et al*., 2004). Recently the

scientific world has experienced an upsurge of interest in the therapeutic potential of these natural products as sources of promising chemotherapeutic agents. However, the application of plant-based compounds for the treatment of infections and diseases can be traced back to 1950s. Some of the very first chemotherapeutic agents derived from plants are alkaloids, vinblastine, vincristine, and cytotoxic podophyllotoxins. Statistical data suggest that several plant-derived chemotherapeutic drugs have been subjected to clinical trials thus far (Belayachi *et al*., 2013). However, in addition to their chemical structure diversity and their biodiversity, the development of new technologies has revolutionized the screening of natural products in discovering new drugs. Applying these technologies compensates for the inherent limitations of natural products and offers a unique opportunity to re-establish natural products as a major source for drug discovery. Plants used for traditional medicine contain a wide range of substances that can be used to treat chronic as well as infectious diseases (Duraipandiyan *et al*., 2006). Landmarks of clinical trials on several extracts and compounds, isolated from various plants and which have been documented to have less toxicity than conventional chemotherapeutic drugs (Saklani & Kutty, 2008) has picked up. These discoveries have propelled the scientific interest of various research groups in the discovery of new chemotherapeutic agents from all-natural product sources, inclusive of plant secondary metabolites. The emerging importance of natural chemotherapeutic agents demands more research and experimentation in order to develop successful natural therapeutic options for these disease and infections. This review focuses on the phytochemical aspect of the potential chemotherapeutic medicinal plant, *Vigna subterrenea* with data gathered from the scientific literature of the PubMed database. Their secondary metabolites have not been studied extensively especially the cytotoxic, antimicrobial, antioxidant and componential elucidation of its bioactive substances.

2.8 Research Gaps

Despite the enormous efforts to search for prevention/cure, some of the diseases/infections still remains a challenge for global public health. Poor survival rate of patients in developing countries is attributed to the lack of timely diagnosis and

limited treatment medications/facilities. Change in life styles, drugs and alcohol abuse, diet, HIV/AIDS and environmental changes and oxidants are some of the factors thought to contribute towards the rise of these infections and diseases (http://www.ipcrc.net/pdfs/intl_programs/Final-Draft-of-the-Kenya-Cancer-Control-Strategy-April-2011.pdf). There is a great need to address this epidemic disease and infections with more effective therapeutic and preventive strategies, which could be possible with the use of natural compounds. Currently, no detailed biochemical and pharmacological studies have been published on effectiveness, toxicity and potential adverse effects associated with the medicinal usage of *V. subterrenea.*

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study site

The study was carried out in the Centre for Traditional Medicine and Drug Research (CTDMR), at Kenya Medical Research Institute (KEMRI), Chemistry laboratories at Jomo Kenyatta University of Agriculture and Technology and at Kenya Medical Training College in Nyeri County, Central Kenya.

3.2 Plant materials

Vigna subterranea nuts under study were collected from Bungoma, Busia and Kakamega Counties, Western Kenya and from Kenya agriculture and livestock research organization (KALRO) in April, 2014 where six cultivars: dark brown, cream spotted, brown, black, and dark-brown spotted, red and light spotted were obtained. Samples were coded as KEMRI/DBMM/6, KEMRI/CMM/5, KEMRI/BBT/7, KEMRI/BMT/7 KARI/BN/BK-1, KARI/BN/BN-2, KARI/BN/BK-3 and KARI/BN/BK-4 and were deposited in the herbarium for future reference.

3.3 Study Design

Experimental design was employed where effects were measured by comparing the outcome of the test in the experimental category versus the outcome of another test in the control category to provide answers to the research questions and allow valid statistical analysis to be carried out in the resulting data.

3.4 Preparation of extracts

3.4.1 Extraction and Screening for Phytochemicals

Nuts were air dried at room temperature to a constant weight, milled mechanically using a miller, ground (each cultivar 800.0 g) and stored in labeled airtight bags prior to use. The powdered materials from the six cultivars (800g each) were extracted separately with methanol-dichloromethane (1:1, 0.5 L) for 48 hours at room temperature. The marc was filtered with a 3mm Whatman filter paper. The filtrates were dried *in vacuo* using rotary evaporator at 64.5^{o} C to yield yellowish sticky solids with black (8.0 g), dark brown (13.0 g), cream spotted (15g), brown (11.0 g), dark brown (9.0 g) and spotted red (6 g). The extracts were transferred to the test-tube and then covered them using aluminium foil and preserved in a fridge at 4^{o}C. The six extracts were qualitatively analyzed for the presence of phytochemicals. Drangendoff's reagent was used to determine the presence of alkaloids (Adetuyi & Popoola, 2001 ; Egwaikhidi, 2007). The extract was dissolved in 2N -hydrochloric acid on a water bath, then shaken and filtered.

The filtrates were extracted with chloroform to remove undesirable matters. Finally the pH of the acidic aqueous layer was adjusted with ammonia followed by extracting the alkaloid bases with chloroform. Thin layer chromatography was carried out and then sprayed with Drangendoff's reagent. The persistent frothing test (Siddiqui & Ali, 1997) was used to determine presence of saponins. To a 1g of each extract, 30mL of tap water was added. The mixture was vigorously shaken for 30 seconds and left to stand for 30 minutes, and heated to boil. Tannins were also determined as described by Trease and Evans, (1989). Briefly, 0.5 g powdered extract was dissolved in 5 mL of distilled water, then boiled gently and cooled. 1mL of this solution was put in a test tube and 3 drops of Ferric Chloride solution added.

Determination of flavonoids in the nut was done according to the method described by (Ujowundu *et al,* 2013). In brief, 5 mL of diluted ammonia solution was added to a portion of the aqueous filtrate of the extract, followed by addition of concentrated sulphuric acid. The Salkowski test was used to determine terpenoids (Mariyan *et al*., 2016) where 5mL/ of extract was mixed in 2mL of chloroform, and 3mL concentrated sulphuric acid was carefully added to see color changes.

3.4.2 Fractionation of Extracts

Since oils in the nut extracts are a mixtures of products with a wide range of chemical and physical properties, it is usually reasonable to perform some pre-fractionations through Column chromatography and then Thin Layer Chromatography (TLC) to validate the complexity of the mixture before using gas chromatography-mass spectrophotometric technique as the complexity of the sample can often offer a real challenge. Column chromatography was carried out on silica gel 60 where 6.0 g of dark brown portion was fractionated eluting with a mixture of methanol and dichloromethane with the gradient 0:100, 10:90, 15:85, 30:70, 50:50, 75:25, 80:20, and 100:0. Then the seven column elutes were collected and kept in separate containers and named as F1, F2, F3, F4, F5, F6, and F7. TLC was run and was visualized under UV light, 254 or 366 nm, followed by spraying with 1% vanillin dissolved in sulphuric acid. Ethyl acetate: petroleum ether at a ratio of 3:7 was also used to check the separation of the components. After 30 minutes the distinct spots/dots were observed from dark brown cultivars for F2 and F6 hence retention factors were calculated (see appendix B**)**. The GC-MS analysis of pre-cleaned fractions (F2 &F6) was quantitatively performed.

About 1μL of the methanol extract of F2 and F6 were injected into the GC-MS using a micro syringe and the scanning was done for 12 minutes. The time from when the injection was made (Initial time) to when elution occurred is referred to as the Retention time (RT) (Jassim *et al*., 2015 ; Hadi *et al*., 2016). While the instrument was run, the computer generated graphs from the signal called Chromatogram. Each of the peaks in the chromatogram represented the signal created when a compound eluted from the Gas

chromatography column into the detector. The m/z (mass / charge) ratio obtained was calibrated from the graph obtained, which was called as the Mass spectrum graph which is the fingerprint of a molecule. Before analyzing the pre-cleaned extracts using gas chromatography and mass spectroscopy, the temperature of the oven, the flow rate of the gas used and the electron gun were programmed initially. GC-MS had a number of specifications; Hp5-Ms column was used, high pure helium was the carrier gas and the flow rate was 1 mL/min, temperature at the front inlet was 220°C and at the oven was 50°C to 250°C, gradually raised at 10°C/min. Ion chamber and GC interface temperature was maintained at 250°C. The technique was used to determine molecular weights and structural formula (from the fragmentation patterns). The identity of the components in the extracts was assigned by the comparison of their retention indices and mass spectra fragmentation patterns with those stored on the computer library and also with published literatures. Compounds were identified by comparing their spectra to those of NIST spectral libraries (Hussein *et al*., 2016).

3.5 Determination of Minerals

Qualitative analysis of macro and micro cations from all the extracts were done. The cations included K^+, Na^+, Ca^{2+} Fe^{3+}, Zn^{2+}, Mn^{2+}, and Al^{3+} ions were analyzed to verify their presence. To test for K^+ and Na^+, 4 test tubes were used where 5-8 drops of 0.1 M solutions of KCI, NaCl and 3 M HCl, and bambara extracts were placed into separate test tubes. A wire loop was used to check for the presence of this ion on a hot flame of a bunsen burner. Colors of the ions were observed. The presence of Na^+ and K^+ ions in the extracts was confirmed by the flame test. Presence of Mg^{2+} ions required diluting the extracts with ammonia solution.

$$Mg^{2+}{}_{(aq)} + 2NH_{3(aq)} + 2H_2O_{(l)} \rightarrow Mg(OH)_{2\,(s)} + 2NH_4^{+}{}_{(aq)} \text{---------- Equation 1}$$

$$Mg(OH)_{2\,(s)} + 2NH_4Cl \rightarrow MgCl_{2\,(aq)} + 2NH_4OH_{(aq)} \text{ ----------Equation 2}$$

When disodium hydrogen phosphate solution was added to the product ($2NH_4OH$) above, with cooling and scratching the inside of the test tubes with a glass rod, a white crystalline precipitate of magnesium phosphate was formed as follows.

$$Mg^{2+}_{(aq)} + 2NH_{3(aq)} + HPO_4^{2-}{}_{(aq)} \rightarrow Mg(NH_4)PO_{4(s)} \quad \text{---------- Equation 3}$$

Ca^{2+} ions present in extracts formed precipitate with ammonium carbonate in the presence of ammonium chloride in neutral medium. The precipitating solution was buffered at pH 9.5 with NH_4^+ ions and NH_3 molecules. $(NH_4)_2CO_3$ was used at 0.2 M with a neutral or slightly basic pH. Confirmation of cations of Al^{3+}, Fe^{3+} and Mn^{2+} in the extracts was also carried out. They initially formed sulfide precipitate in the presence of high S^{2-} ion concentration in basic solution. Furthermore, Al^{3+} ions precipitated as hydroxides rather than as sulfides together with Fe^{3+} which formed both hydroxides and sulfides. These ions precipitated in 0.1 M hydrogen sulphide solution at pH 8-9. A buffer was used to maintain this pH. In these conditions, H^+ ion concentration was reduced to a very low concentration (10^{-9}M) and the solution saturated with H_2S. Under these conditions, the equilibrium shifted to the right.

$$H_2S_{(aq)} \rightleftharpoons 2H^+_{(aq)} + S^{2-}_{(aq)} \quad \text{---------- Equation 4}$$

The concentration of S^{2-} was high enough to precipitate FeS and MnS. The presence of ammonia converted Mn^{2+} into the amine complexes. The cations of Al^{3+}, Fe^{3+} and Mn^{2+} formed sulfide which precipitated in the presence of high S^{2-} ion concentration in basic solution.

3.6 DPPH Radical Scavenging Assay

The 2, 2-Diphenyl-1-picryl hydrazyl (DPPH, Sigma-Aldrich) radical scavenging assay is based on the reduction of DPPH, a stable free radical (**Figure 3.1**). The free radical scavenging activity was tested according to Mensor *et al*. (2001). The organic extracts were re-dissolved in methanol and different concentrations (5, 10, 15, 20, 25 and 50 µg/ml) of each extract were used. Similar concentrations of ascorbic acid were used as

positive control. The assay mixture was contained in a total volume of 1 ml; 50 µl of the extract, 12.5 µl prepared DPPH (1 mM in methanol) and 937.5 µl solvent (methanol or 5% ethanol). After 30 min incubation at 25°C, the decrease in absorbance or optical density (OD) of the solution was measured spectrophotometrically at 517 nm (purple color) against the corresponding blank solution. The assay was performed in triplicates. Percentage inhibition of free radical DPPH was calculated based on control reading by following equation (Abdul *et al*., 2013).

$$\text{Inhibition (\%)} = \frac{(OD_{control} - OD_{sample})}{OD_{control}} \times 100$$

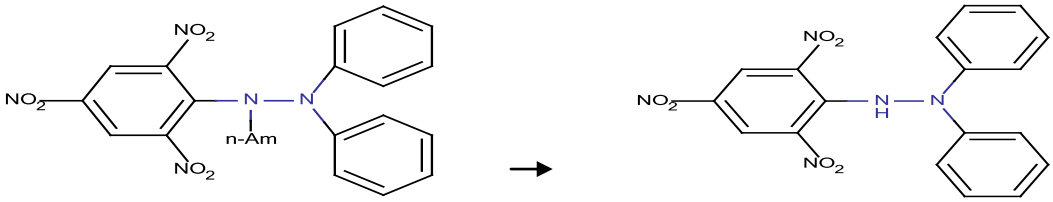

Figure 3.1 Reactions of DPPH (free radical) to DPPH (Non-radical). Where n-Am represents a dot/ odd electron.

3.7 Cytotoxic Activity

3.7.1 Cell Culture

The mammalian cells used included human cancer cell line Hep-2 (human hepatic carcinoma) and DU 145 (prostate cancer cell line) and noncancerous Vero cell line (African green monkey kidney fibroblast cells). The cells were cultured in 75-cm^3 culture flasks in Eagles Minimum essential medium (MEM, Gibco) supplemented with Fetal Bovine serum GiBco BRL (10%) and amikacin (60 mg/liter), at 37°C in an atmosphere of 95% humidity, 5% CO_2. The culture medium was changed twice a week.

3.7.2 Cytotoxic Assay

The cytotoxic activities of Bambara extract was determined using rapid colorimetric assay based on the fact that mitochondrial oxidoreductase enzymes are capable of reducing the tetrazolium indicator MTT 3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium bromide)(see appendix A) to its insoluble formazan, which has a purple color. The cellular oxidoreductase enzymes may, under defined conditions, reflect the number of viable cells present (Rios *et al*., 2005). A cell density of 2.0×10^4 cells per well in 100 µL were seeded on 96-well plates and incubated for 12 hours at 37 °C and 5% CO_2 to attach to the surface. Extracts were added to the cultured cells in rows H-B over a concentration range of 0.14 to 100 µg/mL, whereas wells 1–8 of row A serve d as untreated controls and wells 9–12 as blank (1% Dimethyl sulfoxide (DMSO), v/v). The plates were incubated for 48 hours at 37 °C and 5% CO_2, followed by an addition of 10 µL MTT viability indicator reagent. The plates were then incubated for additional 4 h at the same conditions. All the media was removed from the plates and 100 µL DMSO was added to dissolve the formazan crystals (**Figure 3.2**).

Figure 3.2: Cell line

The plates were read on a scanning multi-well spectrophotometer at 562 nm. The results were recorded as optical density (OD) per well at each drug concentration. Results were

expressed as the mean ± standard error. The percentage of cell viability was calculated by the formula: % Viability = Corrected OD of sample /Control OD ×100 %; while the growth inhibition = 100 - % viability. The concentration of the extract causing 50% inhibition of cancer cell growth was considered as IC_{50}. Statistical analyses were performed using a two-tailed Student's *t*-test and $P < 0.05$ was considered to be statistically significant.

3.8 Antibacterial activity

The extracts from the Bambara nuts with varied concentration were evaluated for antibacterial agents against the growth of *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* using the Kirby-Baurer diffusion test (Wang, 2005). MIC's were determined by serial dilutions (5 µl, 7 µl, 8 µl, 12.5 µl, 25 µl, 50 µl and 75 µl) of extracts in test tubes with standard amount of broth (1 ml). Following a period of incubation (24 hours), the test tubes were examined for growth. Nutrient agar plates were prepared and inoculated with test organisms by a spread plate method (Wang, 2005). The sterilized filter paper disc of 5 mm diameter (Whatmann's No. 1 filter paper) was used. Furthermore, sterile impregnated disc with extracts were dried and placed on the agar surface with forceps and pressed gently down to ensure complete contact of the disc on the agar surface. All the plates were incubated at 37°C for 24 hours. Clear inhibition zone around the well indicated the antimicrobial activity of the corresponding extracts and the diameters of the zone were measured in millimeters. Ceftriaxone (see appendix A) was used as positive control against the tested bacteria to produce effects close to what was expected thus validating the experimental procedures. The experiment was done in triplicate, and the means +/- standard deviations were reported.

3.9 Antifungal Activity

The antifungal activity was assayed against the growth of *Candida albicans*. The diffusion method (Egwaikhidi, 2007) in sabouraud dextrose agar (SDA) was used to determine the antifungal activity of the extract at 1, 2 and 4 µg/ml

concentrations. Extract was dissolved in dimethyl sulfoxide (DMSO) and added to sabouraud dextrose agar medium immediately before it was poured into petri dishes (9 cm diameter) at 40–45°C to obtain a series of concentrations above. Negative control plates were treated with DMSO alone, and three replicates per treatment were used. 10 mg of Clotrimazole (British pharmacopoeia, batch number 15-03023) was used as a positive control (Visnja *et al*., 2015). Plates were incubated at 25°C. Colony growth diameter was measured after the fungal growth in the control treatment had completely covered petri dishes. The percent inhibition of mycelial growth, in terms of fungitoxicity of the extracts, was calculated (Rios, 2005):

$$\% \text{ inhibition} = \{(Mc-Mt)/Mc\} \times 100$$

Where Mc is the average increase in the mycelial growth in control and Mt is the average increase in the mycelial growth in treatment. The experiment was performed in triplicate.

3.10 Data Management

The experiments were done in triplicate and data recorded in a notebook then keyed in excel spreadsheet. Data analysis was done using SPSS 2010 version and the results were expressed as percentage decrease with respect to control values and compared by one-way ANOVA. Means, standard deviations and coefficients of variation of antioxidant activity in percentage (AA %) of the tested extracts was obtained. The t-test was used to test for statistical significance with a p-value <0.05 deemed as significant (p is probability). With the aid of National Institute of Standards and Technology library the peaks were compared and identified in regard to GC-MS analyses.

3.11 Ethical Consideration

This study commenced after CTMDR Centre Scientific Committee (CSC), Scientific Steering Committee (SSC no. 2860) and the Ethical Review Committee-KEMRI/RES/7/3/1 granted permission. No animal or human subjects were used in the project.

CHAPTER FOUR

RESEARCH FINDINGS

4.1 Qualitative Phytochemical Analysis

Table 4.1 shows the results of the phytochemical screening for the *Vigna subterranea* nut extracts.

Table 4.1: Phytochemicals present in the nut extract analysis

Test	Compounds tested	Results	Inference
Liebmans-Burchard reagent	Terpenoids	Reddish-brown precipitate	Terpenoid present
Dragendorff's reagent	Alkaloids	Orange colouration of spot on TLC	Alkaloid present
Froth foam test	Saponins	Stable froth	Saponins present
Magnesium chloride reagent	Flavonoids	White precipitate	Flavonoids absent
Ferric chloride reagent	Tannins	Blue Black precipitates	Tannins present

4.2 Thin layer chromatography and Gas chromatography-mass spectrometry (GC-MS) analysis

A dark brown cultivar extracts on subjection to column chromatography two fractions. A good resolution was observed in the fraction 2 and 6 having distinct retention factors of 0.70 and 0.46 respectively (Appendix B).The compound(s) present in the pre-cleaned DCM: methanol nut extract of *V.subterranea* were identified by GC-MS. GC-MS separated the volatile nut components of complex mixtures and also recorded a mass spectrum of each component. This hybrid instrument provided two separate dimensions

of information about the components in the sample; GC retention times and electron ionization (EI) mass spectra. GC retention time is related to specific chemical properties of the molecules in question (e.g. volatility, polarity, presence of specific functional groups) while molecular weight (derived from the mass spectrum) is indicative of atomic composition. Chromatographic techniques separate mixtures of species based on their interactions with a stationary phase and a mobile phase.

The components with their retention time (RT), molecular formula, and molecular weight (MW) in the DCM: Methanol extract is presented in Table 4.2. The molecular weights of ions in the spectra were determined at resolving powers in a 10% valley definition by the usual "peak-matching" technique with NIST structures as references. The determinations gave molecular weights unique, within 2 ppm or less, for a particular compounds composition in the experimental samples

Table 4.2: Compounds 1–9 identified through GC-MS analysis from Vigna subterranea

Peak No.	Chemical Compound and molecular formular	Retention time	Molecular weight	Applications
1.	Farnesyl Alcohol, (C15H26O)	7.40	222	• Cytotoxic/antiproliferative • Anticancer (colon cancer) • Antifungal, • Antibacterial. • regulates the volatility of odorants in perfumes
2.	Methyl Arachate, (C21H42O2)	9.63	326	• Production of detergents • Manufacture of pharmaceuticals • Lubricant • Emulsifier
3.	(-Z)-Octadecenyl ester/oleic acid, (C18H34O2)	8.88	282	• Fungistatic against a wide spectrum of moulds and yeasts. • Reduces blood pressure • increases fat burning to help with weight loss, • protects cells from free radical damage, • may prevent type 2 diabetes, • prevents ulcerative colitis • Generates brain myelin.
4.	Hexadecanoic acid / palmitic acid, (C16H32O2)	7.50	256	• Produce soaps, cosmetics, and release agents. • A drug paliperidone palmitate used in the treatment of schizophrenia • Clindamycin palmitate
5.	7-octadecenoic acid methyl ester	9.63	326	• Anti-inflammatory • Anti-androgenic • Anticancer • Antileukotriene • Insectfuge • Flavor
6.	1, 2-chloroethyl Linoleate; (C20H35O2Cl)	9.72	342	• Selectively inhibits the Fab I enzyme in staphylococcus aureus and *Escherichia coli* (Waller *et al.*, 2008)
7.	Methyl behenate, (N-docosanoic acid), (C23H46O2)	10.40	354	• Is a cholesterol-raising fatty acid in humans • Give hair conditioners and moisturizers their smoothing properties
8.	trans-2- methyl-4-n-butylthiane- s,s – dioxide (C10H20SO2)	5.2	204	• No research on it encountered
9.	9,17-octadecadienal (Z), (C18H32O)	8.68	264	• No research on it encountered

The activity of phytocomponents identified in *V.subterranea* by GC-MS is based on Dr. Duke's phytochemical and ethnobotanical database. The spectral uniqueness of the pre-cleaned fractions and the respective compounds and their fragmentation were illustrated in appendices C1&C2 and D1-9 where on comparison with NIST spectrum considering m/z and relative intensity for both cases led to identification of 10 compounds (**Table 4.2)**.

For example, the mass spectrum of the compound with retention time 11.67 minutes gave 12 peaks with four prominent to help in the identification of the compound as methyl behenate. Its peak corresponds to mass spectra with molecular mass 354 gm/mol., which is same as the molecular mass of methyl behenate (**Figure 4.1**)(http://www. numericana. com/ answer /culture.htm).

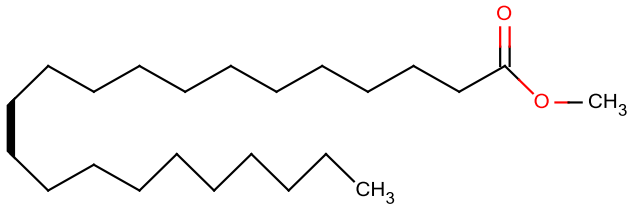

Figure 4.1: Structural formula of methyl behenate

The formed molecular ions; $[C_{23}H_{46}O_2]^+$, was thus not steady hence it underwent rapid disintegration to more stable fragments. The main fragmented ions gave structural information which were found at m/z of 143 [M-211] $^+$, $[C_{11}H_{20}]^{+\cdot}$; m/z 87[M-267]$^+$,$[C_7H_{10}]^+$; m/z 73.9 [M-280]$^+$,$[C_3O_2H_6]^+$,[base peak] and 55 [M-299]$^+$,$[C_4H_9]^+$Daltons. The m/z = 73.9 [M-280]$^+$ is base peak and is plotted to 100% of the spectrum scale. A prevalent peak and often the base peak [M-280]$^+$ which is a straight chain esters is discernable and it resulted from the familiar McLafferty rearrangement and hydrocarbon ions loss due to lysis of fragments containing the carbonyl group by homolytic cleavage between respective carbons with additional rearrangements of hydrogens. The intensity of the peaks tends to decrease as the fragment masses get larger. Larger fragments are less likely to survive the 10^{-5} second trip to the detector.

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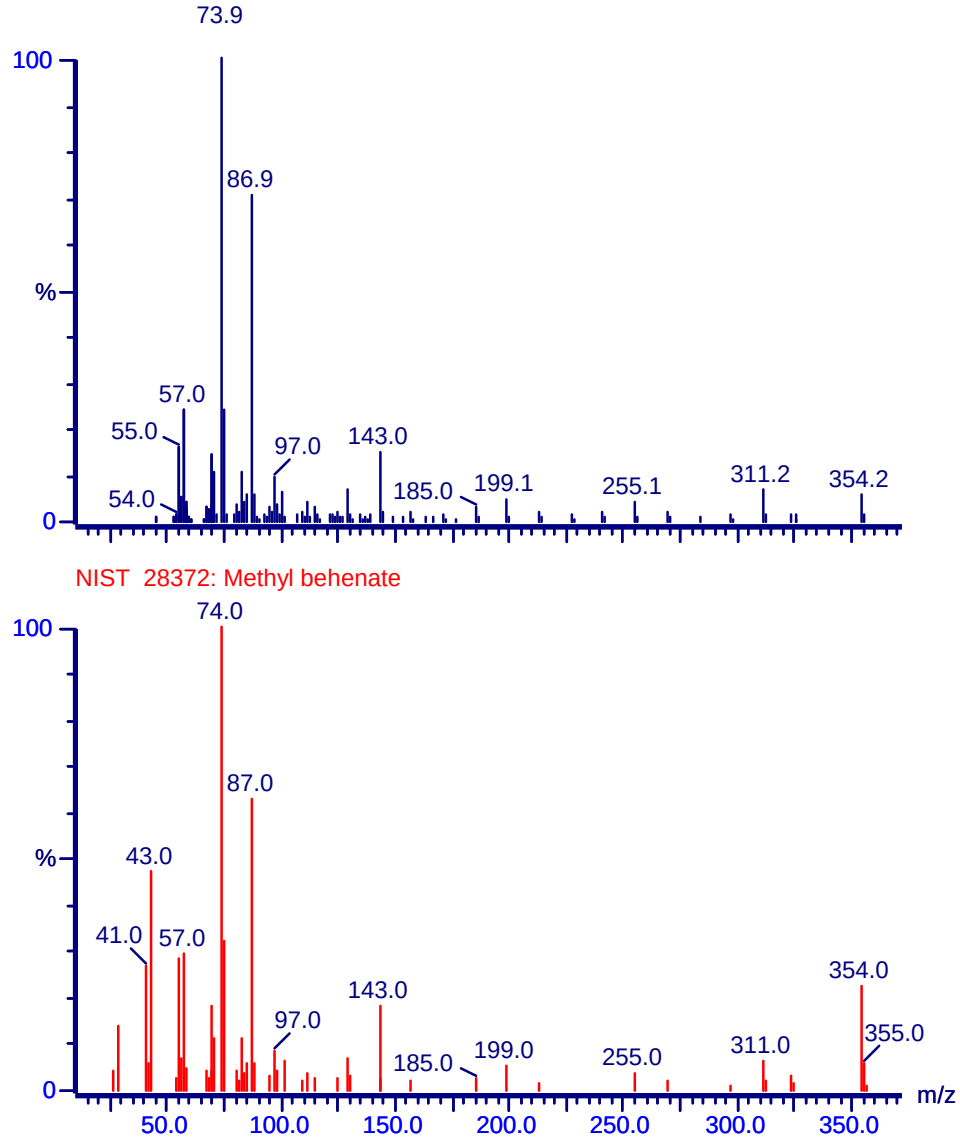

Figure 4.2: Mass spectrum of Methyl Behenate. The intensities of ions are shown relative to the ion at m/z 73.9 which had a relative intensity of 100 % (base peak).

4.3 Determination of Minerals

All the extracts on subjecting to qualitative analysis gave similar results. The extracts on flame test gave a characteristic yellow flame color for Na^+ with a pale–violet flame color for K^+. Furthermore, Mg^{2+} ions formed a white gelatinous precipitate of Mg $(OH)_2$ in dilute ammonia solution, where then on ammonium chloride solution addition to the precipitate, and the pH lowered, the Mg $(OH)_2$ precipitate dissolved. Ca^{2+} ions formed precipitates with ammonium carbonate in the presence of ammonium chloride in neutral medium. A white precipitate indicated the presence of Ca^{2+}. Cations of iron (III), manganese (II) and aluminium (III) on analyzing of the extract did not react with dilute hydrochloric acid. However they formed precipitates with ammonium sulphide in neutral medium i.e. they formed sulfide precipitates in the presence of high S^{2-} ion concentration in basic solution. Also, Al^{3+} ions precipitated as hydroxides rather than as sulfides together with Fe^{3+} which formed both hydroxides and sulfides. Al $(OH)_3$ formed gelatinous solids where Al $(OH)_3$ was white. The concentration of S^{2-} became high enough to precipitate FeS and MnS. FeS was black as MnS, like Mn^{2+} ion was pale pink

4.4 DPPH• Scavenging Activity

Comparison of the antioxidant activity of the DCM:Methanol extract and ascorbic acid is shown in (**Table 4.3**). One way ANOVA on the six cultivars revealed that the F-statistic was lower (0.67) compared to the F critical value (3.11). At a concentration of 25 µg/ml the students t test revealed t value of 11.25 as compared to computed values of 2.73. The effect size after rejecting the null hypothesis was calculated and was large (17.28). The extract of *Vigna subteranea* exhibited a significant dose-dependent inhibition of DPPH activity, with a 50% inhibition (IC_{50}) determination.

Table 4.3: Scavenging of 1, 1-diphenyl-2-picrylhydrazyl (DPPH)

	Percent inhibition						
Concentration µg/ml	Red Cultivar.	Black cultivar	Dark brown cultivar	Cream spotted Cultivar	Brown Variety Cultivar	Dark brown spotted cultivar	Ascorbic acid
5	1.6± 2.1	0.1± 0.4	1.4± 2.5	0.5± 4.4	0.1± 3.7	1.7± 1.3	2.8±9.1
10	24.4± 3.0	23.4± 1.0	23.5± 2.1	23.8± 4.6	26.4± 5.0	25.4± 3.0	39.7±7.3
15	29.5± 1.5	30.2±2.3	29.7± 9.1	29.3± 4.6	30.4± 1.1	30.1± 1.9	79.8±2.6
20	34.6± 3.4	35.7± 5.4	35.9± 6.3	36.2 ± 6.4	38.4 ± 5.3	34.7 ± 5.2	84.0±7.1
25	41.3± 1.2	42.0± 7.7	40.1± 1.4	41.4± 2.3	41.3± 9.1	40.9± 1.6	84.1±2.5
50	50.6 ± 3.5	49.6± 9.5	48.5± 4.3	48.8 ± 6.5	46.7± 4.4	43.1± 1.5	84.0±3.3

The nut extract showed relative antioxidant potential when compared to ascorbic acid by DPPH scavenging assay method (**Figure 4.3**). The IC_{50} values obtained were 10.1 and 25± 1.4 µg/ml for ascorbic acid and cultivars of *Vigna subterrenea* respectively.

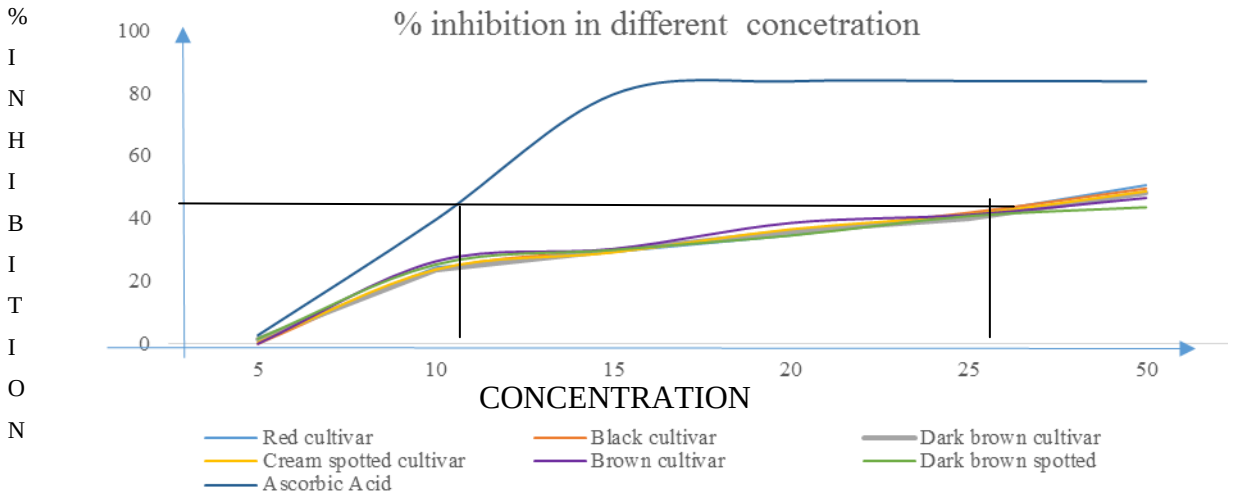

Figure 4.3: Concentration of standard and extracts against Percent Inhibition

Reducing power of *Vigna subterrenea* and ascorbic acid is measured spectrophotometrically with each value as mean of three replicates demonstrating relativity. Free radical scavenging activity of the extract also increased with increasing concentration in the range of 10–50µg/ml.

4.5 Cytotoxic activity

Cytotoxicity of the extracts was studied by means of a colorimetric assay (MTT assay) against three cell lines; Hep 2, DU 145 and Vero, yielding varying IC_{50} values. Considering one way ANOVA, the F statistic was lower (1.305) as compared to the computed values (5.786). There is no significant difference in the groups within the mean in relation to the treatment in the study. All extracts were able to cause cytotoxicity against the cancer cells (DU 145, Hep2) and the normal Vero cell lines but at varying degrees. It was observed that the dark brown extracts was most cytotoxic with IC_{50} of 15.5 µg/ml closely followed by cream spotted cultivar with IC_{50} of 17.9 µg/ml against Hep 2 cell lines (Table.4.4).

Table 4.4 Cytotoxic activity of Vigna subterrenea cultivar against two cancer cell lines and one non-cancerous cell line (n=3) IC50 values (µg/ml).

Drug cultivar	Hep 2 IC_{50}	DU 145 IC_{50}	Vero IC_{50}
Black	50.5± 2.3	60.1± 6.0	35.2± 3.1
Dark brown	15.5± 4.1	41.1± 1.2	19.2± 6.5
Cream spotted	17.9± 1.6	34.0± 3.1	28.9± 9.0
Brown	39.8± 4.9	35.0± 5.7	33.7± 1.6
Dark brown spotted	44.4± 7.6	40.2± 0.1	40.1± 6.4
Red	39.7± 3.0	39.9± 2.6	43.3± 7.1

Hep 2 cancer cell lines were more sensitive to the extract at values 15.5± 4.1 µg/ml in comparison to DU 145 cell lines which showed higher values to various *Vigna subterrenea* varieties as indicated by the IC_{50} against the cell line. These data are of

interest, as it suggests that the extracts are more toxic to the cancer cells than normal cells. Cogitating the overall activity of the extracts, it was exhumed that the dark brown extracts could be considered as potential cytotoxic drugs.

4.6 Antibacterial activity

Table 4.5: Antibacterial activity of dark brown cultivar extract of *Vigna subterrenea*

Tested Microorganism	Concentration and Zones of Inhibition (mm) of extract					MIC (in µg/ml) for the extracts
	25 µg/ml	50 µg/ml	75 µg/ml	100 µg/ml	Ceftriaxone at 100 µg/ml	
Escherichia coli	12.5±0.1	20.2±0.4	21.0±0.3	27.1±0.7	37.6±0.5	7.72± 0.5
Pseudomonas aeruginosa	12.0±0.7	12.6±0.5	21.0±0.3	25.1±0.2	41.3±0.8	7.95± 0.1
Staphylococcus aureus	10.3±0.2	13.1±0.6	23.3±0.8	25.2±0.4	42.4±0.9	12.5± 0.3

*Values are represented as mean ± SD of triplicate experiments; MIC means Minimum Inhibitory Concentration (**µg/ml**)*

The extract of *Vigna subterrenea* inhibited all the bacterial strains as in (Table 4.5). The results showed that increase in concentration of extract increased the zone of inhibition against all the tested microbial strains (Mkandawire, 2008). At 100 µg/ml for example, the displayed inhibitory effect towards the strains gave zone of inhibition as *E. coli* = 27.0± 0.7 mm, *S. aureus* = 25.3± 0.4 mm and *P. aeruginosa* = 25.1± 0.2 mm compared to 37.0±0.5, 41.3±0.9 and 42.3± 0.9 respectively for ceftriaxone.

The MIC obtained against *E. coli* at 7.0± 0.4 µg/ml, *S. aureus* at 12.5± 0.3 µg/ml and *P. aeruginosa* at 7.0± 0.1 µg/ml (Table 4.5). At 100 µg/ml, the extracts showed varied means as compared to ceftriaxone, where on t test analysis, the calculated values were;

20,30, and 30.25 for *E.coli, P.auriginosa* and *S. auriginosa* respectively. The values were much higher than the tabulated values (2.73). The activities of extracts were less on comparison to those of ceftriaxone (Table 4.5). The zone margin was taken as the area showing no obvious, visible growth that can be detected with the unaided eye. Faint growth of tiny colonies, which was detected only with a magnifying lens at the edge of the zone of inhibited growth were ignored.

4.7 Antifungal activity

The extract of *Vigna subterrenea* led to reduced mycelial growth of *Candida albicans* by 62.44%, 71.6%, and 91.1% at the concentrations of 1, 2, and 4 µg/ml compared to clotrimazole whose inhibition was 71.2%, 81.4%, and 95.3% respectively at the same concentrations (Table 4.6).

Table 4.6: Effect of Methanol-Dichloromethane extract on mycelial growth of tested fungi (mean ± S.D) using disc diffusion method

	Percent Reduction In Mycelial Growth for *Candida albicans* at Three Concentrations		
	1 µg/ml	2 µg/ml	4 µg/ml
Vigna subterrenea extract	62.4± 4.3	71.6± 2.4	91.1±0.2
Clotrimazole	71.2 ± 3.2	81.4± 7.1	95.3± 9.1
Dimethylsulfoxide	-	-	-

Statistical differences ($p \leq 0.05$) with negative controls indicated that DMSO did not influence the results of biological evaluation but clotrimazole had impact. The extracts showed significant antifungal activity *in vitro* that was comparable to the standard drug clotrimazole.

CHAPTER FIVE

DISCUSSION

5.1.1 Qualitative Phytochemical Analysis

It could be observed that terpenoids, alkaloids, tannins and saponins are present in the *Vigna subterranea* extracts used for this study, while flavonoids were absent in the extracts. Similar results of different phytochemical content though quantitavely by Ujowundu's *et al*., (2013) did verify phytochemicals in the nut as tannins, saponin, flavonoids and alkaloids. According to them, tannins were low at 0.09-0.84 mg/100 g, saponins at 1.01-1.38 g/100 g, flavonoids at 0.34-0.79 g/100g as alkaloid were at 0.14 - 0.39 g/100 g. Adeyeye, (2011) report also showed that tannins weighed between 0.09-0.84 mg /100 g for *Vigna subterrenea* extracts. These phytochemicals are used as medication in herbal and homeopathic sector.

Several phytochemicals isolated from plants generally exhibit varied pharmacological use. Some alkaloids for example show anticancer and anti-metastasis on various types of cancers both *in vitro* and *in vivo.* Alkaloids such as camptothecin, vinblastine, berberine, evodiamine, matrine, piperine, sanguinarine, tendratrine have shown some activity and mechanism against been successfully developed into anticancer drugs (Jin-jian Lu *et al*., 2012). Moreover, some alkaloids have analgesic, antispasmodic, and also have bactericidal properties (Enwere & Hung, 1996). Some tannins are recognized for their anti-oxidant and anti-microbial properties, soothing relief, skin regeneration, as anti-inflammatory and diuretics (Takuo & Hideyuki, 2011). Some saponins reduces the cholesterol quantity; can be used as diabetic drug and anti-carcinogenic properties (Asadi, 2015; Enwere & Hung, 1996). Furthermore, saponins aglycones show pharmacological effects as expectorants, cough suppressants and also have haemolytic activities (Sofowora, 1993; Baltina 2003). Some terpenes or terpenoids have anti-hepatoxic properties, they equally have anti-microbial or anti-septic properties (Asadi *et*

al., 2015). These nuts contain phytocomponents that might help in research possibly drug research and its development.

5.1.2 Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

In the present study the chemical profile of *V.subterranea* was characterized using GC-MS analysis. The chromatograms showed the relative concentration of various compounds getting eluted as a function of retention time. The comparison of retention times is what gives GC its analytical usefulness. The heights of the peak indicate the relative concentrations of the components present in the nut. The mass spectrometer analyses the compound eluted at different times to identify the nature and structure of the compounds. The large compound in the gaseous phase fragments into small compounds giving rise to appearance of peaks at different *m/z* ratios. These mass spectra are fingerprint of that compound which can be identified from the data library.

Fractions F2 and F6 contained 4 and 5 peaks respectively (appendices C1 and C2) consisting in totality ten compounds viz: farnesyl alcohol, 2-chloroethyl linoleate, methyl behenate, trans-2- methyl-4-n-butylthiane- s,s –dioxide, Methyl arachate, (-Z)-octadecenyl ester, 7-octadecenoic acid methyl ester, 9,17-decadecadienal (Z) and palmitic acid. The separation which was monitored by GC based on RT and the fragmentation pattern provided by MS was useful evidence for the structure of the compounds where fragments were put together to form a picture of the complete molecule. Full analysis of the mass spectrum was use of the mass difference between the peaks to identify the pieces which had fallen off. The GC-MS analysis of the dichloromethane: methanol extract resulted to compounds which have diverse use (table 4.3**)**. Compounds having anti-inflammatory, antioxidant, antibacterial, antifungal, cytotoxic, skin conditioning properties have been identified. Other compounds like farnesyl alcohol, a sesquiterpene, has an anticancer, antioxidant, antitumor and antimicrobial activity. It regulates the volatility of odorants in perfumes and chemopreventive properties (Akindahunsi & Salawu,; 2005 & Goli *et al*., 1991). The most useful information from the MS is the molecular weight (the M+ peak), which can

indicate what the formula is. The formula provides the degree of unsaturation, which gives important clues to the possible structures (rings and pi bonds). Fragment peaks that are detected provide hints as to the nature of the carbon skeleton, heteroatoms and functional groups present.

5.1.3 Determination of Minerals

Through qualitative analysis in the study, *Vigna subterranea* nuts contains both micro and macro minerals i.e K^{+}, Na^{+}, Ca^{2+} Fe^{3+}, Zn^{2+}, Mn^{2+}, and Al^{3+} ions. The characteristic of elements present in the sample were based on flame test and precipitation reactions. The qualitative chemical analysis can ensure that the extract meets certain specifications in case it is taken to other steps of drug production. The analysis done was in agreement with other qualitative analyses on *Vigna subterranea* to determine the presence of these minerals (Ujowondu *et al*., 2013; Benkendorff *et al*., 2005; Golam *et al.,* 2011; James *et al.,* 1995; N'Dri *et al*., 2015; Amarteifio *et al*., 2006).

The existence of such elements in nut is as imperative as they help in body functions such as producing energy, growing, and healing. Minerals are required for fluid balance, blood and bone development, maintaining a healthy nervous system, and regulating muscles, including heart muscles. Some like magnesium and zinc aid in reducing incidence of cancer and even in the management of the cancer patient. Other elements with some anticancer activity include zinc and magnesium (Weisinger & Bellorin, 1998; Ma *et al.,* 2010; Deheinzelin D. *et al,* 2000; Ujowundu *et al*., 2013; Olaleye 2010; Adeyeye, 2011)

5.1.4 DPPH• Scavenging Activity

In relation to one way ANOVA, the study rejected the null hypothesis, that there is no difference in activity among the six cultivars hence there is no significant difference among them. However, the t test shows the calculated P-value as large (11.5) than the computed (2.73) at alpha= 0.05 enabling null hypothesis to be rejected and accepting the

alternative. The effect size was equally large. There is a significant ($P < 0.05$) decrease in the percent inhibition of DPPH radicals due to the scavenging ability of the extracts and standard.

The 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) is a stable free radical, which has an unpaired valence electron at one atom of nitrogen bridge and is the most accurate method to investigate the antioxidant potential of various natural components *in vitro*. The effects of antioxidants on DPPH radical scavenging is thought to be due to their hydrogen-donating ability. DPPH is a free radical that accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The absorption maximum of a stable DPPH radical in methanol was 517 nm. The decrease in absorbance of DPPH radical caused by antioxidants, because of the reaction between antioxidant molecules and radical progressed, results in the scavenging of the radicals by hydrogen donation. The scavenging of DPPH free radical is the basis of the popular DPPH antioxidant assay, which is among the most frequently employed and most accurate methods to investigate the antioxidant potential of various natural components *in vitro*. It is visually noticeable that there is a change in color from purple to yellow. The nut extract that can lower the initial absorbance of DPPH solution by 50% was chosen as the endpoint for measuring the antioxidant activity. Hence, DPPH is usually used as a substrate to evaluate the antioxidant activity of antioxidants (Bichra *et al.,* 2013). This change was compared to the change induced by ascorbic acid, the reference standard, and the antioxidant activity of the extract. The phytochemicals present in the extract have an indication of some antioxidant activity. It has been reported that oxidative stress, which occurs when free radical formation exceeds the body's ability to protect itself, forms the biological basis of chronic conditions such as arteriosclerosis (Iqbal *et al.*, 2012). Based on the data obtained from this study, all the extracts gave appreciably effective scavenging, as well as a primary antioxidant that reacts with free radicals, which may limit free radical damage occurring in the human body.

5.1.5 Cytotoxic activity

The *Vigna subterranea* extracts showed appreciable degree of cytotoxicity against Vero cell line at IC_{50} = 19.3 µg/ml. This could be attributed to saponin, tannins, terpenoids, and alkaloids that were demonstrated to be present in the extract. The dark brown *Vigna subterrenea* cultivar was more cytotoxic at IC_{50} = 15.5 µg/ml followed by the cream spotted cultivar at IC_{50} = 17.9 µg/ml against the Hep 2 cell line (Table 4.5). The activity of the extracts against the DU 145 human cancer cell line was assessed and all the six cultivars showed lower activity as compared to their activity against Hep 2 cell lines which were more sensitive to the extract. United States National Cancer Institute (US-NCI) establishes that a crude extract that shows an IC_{50} value of less than 100 µg/mL is considered active (Boik, 2001). When the IC_{50} value is lower than 30 µg/mL, the US-NCI considers a crude extract promising for purification and a biological activity study. IC_{50} values below this stringent point were noted with two extracts in at least two of the three studied cell lines, the lowest (15.5 µg/ml and 17.9 µg/ml) being obtained with dark brown and cream spotted *Vigna subterranea* extracts in Hep 2 cancer cell lines and 19.2 µg/ml in vero cell lines (Table 4.5). These data suggests that the dark brown and cream spotted *Vigna subterrenea* nut extracts are more cytoxic to cancer cells than vero normal cells lines. Therefore, according to study, extracts from dark brown and cream spotted could be considered as favourable candidates for potential cytotoxic herbal drugs.

Vigna subterrenea nut extracts have phytochemicals that include terpenoids, saponins, flavonoids, and alkaloids that may be responsible for the cytotoxic effects against all these cell lines. Phytochemicals from medicinal extracts have been shown to exhibit cytotoxicity through the succeeding down-regulation of numerous toxicity and anti-apoptosis gene products (Aggarwal *et al*., 2011). Several cytotoxicity reports on cell lines reveals the importance of chemical constituents as potent cytotoxic agents against different cell lines.

The MTT assay quantifies metabolically viable cells by their ability to reduce MTT. The advantages of this MTT assay included rapid semi-automated reading, objective

assessment, comparative low cost, high reproducibility, low number of cells required, and the facility to quantify cells grown on monolayer (Tonder, *et al.*,.2015). However, there are 2 specific limitations with the MTT assay: (a) as previously reported by other groups, it is that the absorbance produced by a particular cell number can be greatly influenced both by the concentration of MTT used and by the time of incubation with MTT; (b) the relationship between cell number and absorbance over a wide range of cell numbers is not linear. Surviving fraction cannot be calculated by comparing absorbance as has been reported previously (Wang *et al.*, 2011) except when low cell numbers are used that fall on the more linear part of the calibration curve. There are reaction that occurs between the medium and a solution of MTT formazan in DMSO which changes the absorbance spectrum of the solution. The resulting optical density is not however greatly dependent upon the volume of the added medium in the range of 1-10 microliters.

5.1.6 Antibacterial activity

The sizes of the zones of inhibition were interpreted by referring to zone diameter interpretative standards and equivalent minimum inhibitory concentration breakpoints of the National Committee for Clinical Laboratory Standards (NCCLS) M100-S12: Performance Standards for Antimicrobial Susceptibility Testing: Twelfth Informational Supplement. The extract was found to be most effective against *Escherichia coli* showing the maximum zone of inhibition (27.0±0.7 mm) followed by *Staphylococcus aureus* (25.3±0.4) whereas low activity for *Pseudomonas auriginosa* (25.1±0.2mm) at 100 μg/ml of the extract (table 4.6). T test results revealed that there is a significant relationship at $\propto=0.05$ hence rejecting null hypothesis and accepting alternative

hypothesis.that the phytochemicals in *V. subtarranea* contains biologic activity.

All the tested organisms were affected moderately hence intermediate inhibition with the MeOH-CH_2Cl_2 nut extracts. The extract also showed good activity against both gram positive and gram negative tested microorganisms though with varied zones of inhibition

at the tested concentrations. This effect is in agreement with the antibacterial studies and may be attributed to the presence of extracts phytochemicals in the nut (Prescott *et al* 2002 ; Sathya *et al*., 2012).

In the study, a micro dilution test, a quantitative test that allows for higher accuracy and reproducibility, was used to determine MIC value (Carson & Riley, 1995 ; Bisi-Johnson *et al*., 2011). MIC (in μg/ml) for the extracts indicated too, the presence of the activity in the nut extract. MIC was expressed as the lowest dilution, which inhibited growth judged by lack of turbidity in the tube.

5.1.7 Antifungal activities

Nut extracts showed antifungal activity against the fungal *Candida albicans*. However, fungal sensitivity did not vary according to the nut cultivars. The extract significantly reduced the mycelial growth of the tested fungi from 62.4% to 75.6% to 91.1% at the concentration of 1 μg/ml through 4 μg/ml as compared to control drug (clotrimazole) which showed inhibition at 71.2% to 81.4% to 95.3% (table 4.7) The concentration of the extract was directly proportional to the activity on mycelial growth. T test results revealed that there is a significant relationship at $\propto=0.05$ hence rejecting null hypothesis

and accepting alternative hypothesis.that the phytochemicals in *V. subtarranea* contains biologic activity.

In general, there was a positive relationship between the concentrations of the MeOH-CH_2Cl_2 extracts and the inhibition rate on mycelia growth of the tested fungi activities. This shows that the nuts are the reservoirs of valuable phytochemicals and fats/oils signifying that they possess a good activity against *Candida albicans*. Previous studies reported that other plant extracts inhibit the Ras-cyclic AMP (cAMP)-protein kinase A (PKA) cascade, thereby inhibiting hyphal growth and inducing the expression of the catalase-encoding gene *CAT1*. In addition to its effect on *C. albicans* signaling pathways, extracts can induce ROS accumulation within *C. albicans* cells, which may

protect against subsequent OX stress. The cause of ROS generation in response to extracts is poorly understood though (Cosentino *et al*, 1999; Carson & Riley, 1995).

5.2 Conclusions

In the current study the biological potential of *Vigna subterranea* was studied in detail and the chemical compositions of nut extract were identified. Findings of this study revealed that *V. subterranea* extracts as promising sources of natural antioxidants. Extracts from different cultivar showed varying degrees of free radical scavenging power. Antioxidant properties of natural extracts are generally ascribed to redox reactions with some bioproducts present in the extracts, including phenolic compounds. It is envisaged from the extracts that the phytochemicals containing functional groups OH and N–H bond are likely to exhibit interesting antioxidant properties. From GC-MS analysis, components having such functional group and are potential contributors to such antioxidant activity might be farnesyl alcohol, methyl behenate, and oleic acid. However, it's prudent to focusing on the active principle in the extract which contributes to such activity. The findings suggest that *Vigna subterrenea* could be a potential source of natural antioxidant that could have great importance as therapeutic agent.

Furthermore, the nut extracts can be used for the treatment of infections caused by the strains of the test microbial organisms. The nuts secondary metabolites are promising sources of preventive agents in the pathogenesis and competent of microbial diseases, the examined components demonstrates moderate antibacterial and antifungal activities elucidated by the growth inhibitory response against clinically problematic microorganisms.

Additionally, the study indicates that *Vigna subterranea* nut extract showed cytotoxic activity against Hep 2, and Vero cell lines but less to DU 145. More specifically, cytotoxic activity against Hep 2 cells might affirm its traditional use to treating liver cancer. It may be worthwhile to investigate antimetastasis and other anticancer activities together with exploring the chemical compounds, which are responsible for cytotoxic

action and their mechanism of actions. More so, there are minerals that were verified to be present though this goes with soils the plant is growing.

5.3 Recommendations

- There is need to utilize this extracts to obtain compounds like farnesyl alcohol that are not based on existing synthetic antimicrobial agents to prevent/treat cancer and better antimicrobial against pathogenic species
- Further research to isolate specific compounds and elucidate the exact mode of action of compounds is required in order to attract their use in industrial practice.
- Further research is also needed to ascertain the safety of the long-term use of high levels of the *Vigna subterranea* nut.

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APPENDICES

Appendix A: MTT Test standard Procedure

Procedures:

1.0 MTT Test standard Procedure

- Pre-heat the MTT solutions at 37°C in a water bath before use.
- Remove the 96-well plate from the incubator and place it in the laminar flow bench.
- Wash the cells carefully with PBS and add 100 µl/well of fresh culture medium in order to avoid any interaction of the test compound with the chemical. Make sure the cell layer is not disturbed by the washing procedure.
- Add 10 µl of MTT to all wells, including the blanks
- Incubate the plate for 3 hours in a 37°C, 5% CO_2 incubator. Incubation time may be varied from 2 – 4 hours depending on cell type, maximum cell density and metabolic activity of cells.
- Add 100 µl of DMSO solubilization reagent to all wells, including the blanks and incubate at 37°C, 5% CO_2 overnight
- When necessary, mix very carefully. Avoid air bubble formation. Remove any bubbles prior to reading.
- Read the OD at 562 nm.

2.0 Preparation of antibiotic stock solutions

- Ceftriaxone antibiotics may be received as injection. Standard strains of stock cultures were used to evaluate the antibiotic stock solution.
- Stock solutions are prepared using the formula (1000/P) X V X C=W, where P=potency of the antibiotic base, V=volume in ml required, C=final concentration of solution and W=weight of the antimicrobial to be dissolved in V.

3.0 Preparation of dried filter paper discs

Whatman filter paper no. 1 is used to prepare discs approximately 6 mm in diameter, which are placed in a Petri dish and sterilized in a hot air oven.

The loop used for delivering the antibiotics/extract is made of 20 gauge wire and has a diameter of 2 mm. This delivers 0.005 ml of antibiotics/extract to each disc.

4.0 Application of Discs to Inoculated Agar Plates

The predetermined battery of antimicrobial discs is dispensed onto the surface of the inoculated agar plate.

Each disc must be pressed down to ensure complete contact with the agar surface.

Whether the discs are placed individually or with a dispensing apparatus, they must be distributed evenly so that they are no closer than 24 mm from center to center. Ordinarily, no more than 12 discs should be placed on one 150 mm plate or more than 5 discs on a 100 mm plate.

Because some of the drug diffuses almost instantaneously, a disc should not be relocated once it has come into contact with the agar surface. Instead, place a new disc in another location on the agar.

The plates are inverted and placed in an incubator set to 35°C within 15 minutes after the discs are applied.

5.0 Reading Plates and Interpreting Results

After 16 to 18 hours of incubation, each plate is examined. If the plate was satisfactorily streaked, and the inoculum was correct, the resulting zones of inhibition will be uniformly circular and there will be a confluent lawn of growth.

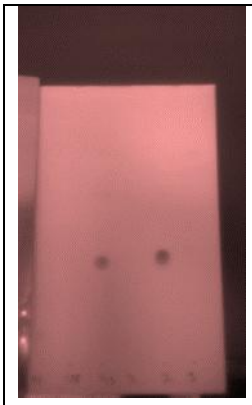
If individual colonies are apparent, the inoculum was too light and the test must be repeated. The diameters of the zones of complete inhibition (as judged by the unaided eye) are measured, including the diameter of the disc.

Zones are measured to the nearest whole millimeter, using sliding calipers or a ruler, which is held on the back of the inverted petri plate. The petri plate is held a few inches above a black, nonreflecting background and illuminated with reflected light. If blood was added to the agar base, the zones are measured from the upper surface of the agar illuminated with reflected light, with the cover removed.

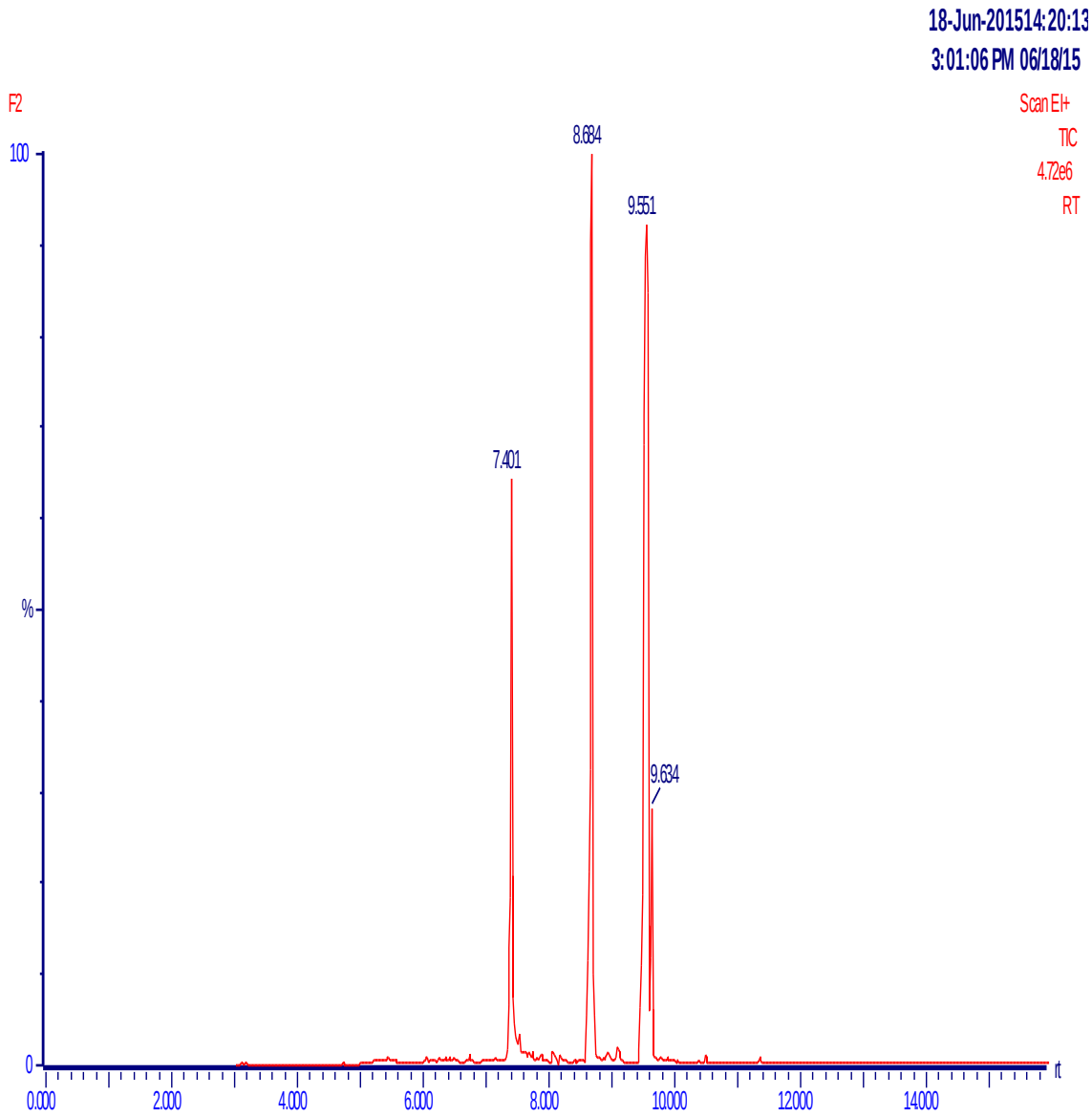
The zone margin should be taken as the area showing no obvious, visible growth that can be detected with the unaided eye. Faint growth of tiny colonies, which can be detected only with a magnifying lens at the edge of the zone of inhibited growth, is ignored. However, discrete colonies growing within a clear zone of inhibition should be sub-cultured, re-identified, and retested

The sizes of the zones of inhibition are interpreted by referring to Tables 2A through 2I (Zone Diameter Interpretative Standards and equivalent Minimum Inhibitory Concentration Breakpoints) of the NCCLS M100-S12: Performance Standards for Antimicrobial Susceptibility Testing: Twelfth Informational Supplement, and the organisms are reported as either susceptible, intermediate, or resistant to the agents that have been tested. Some agents may only be reported as susceptible, since only susceptible breakpoints are given.

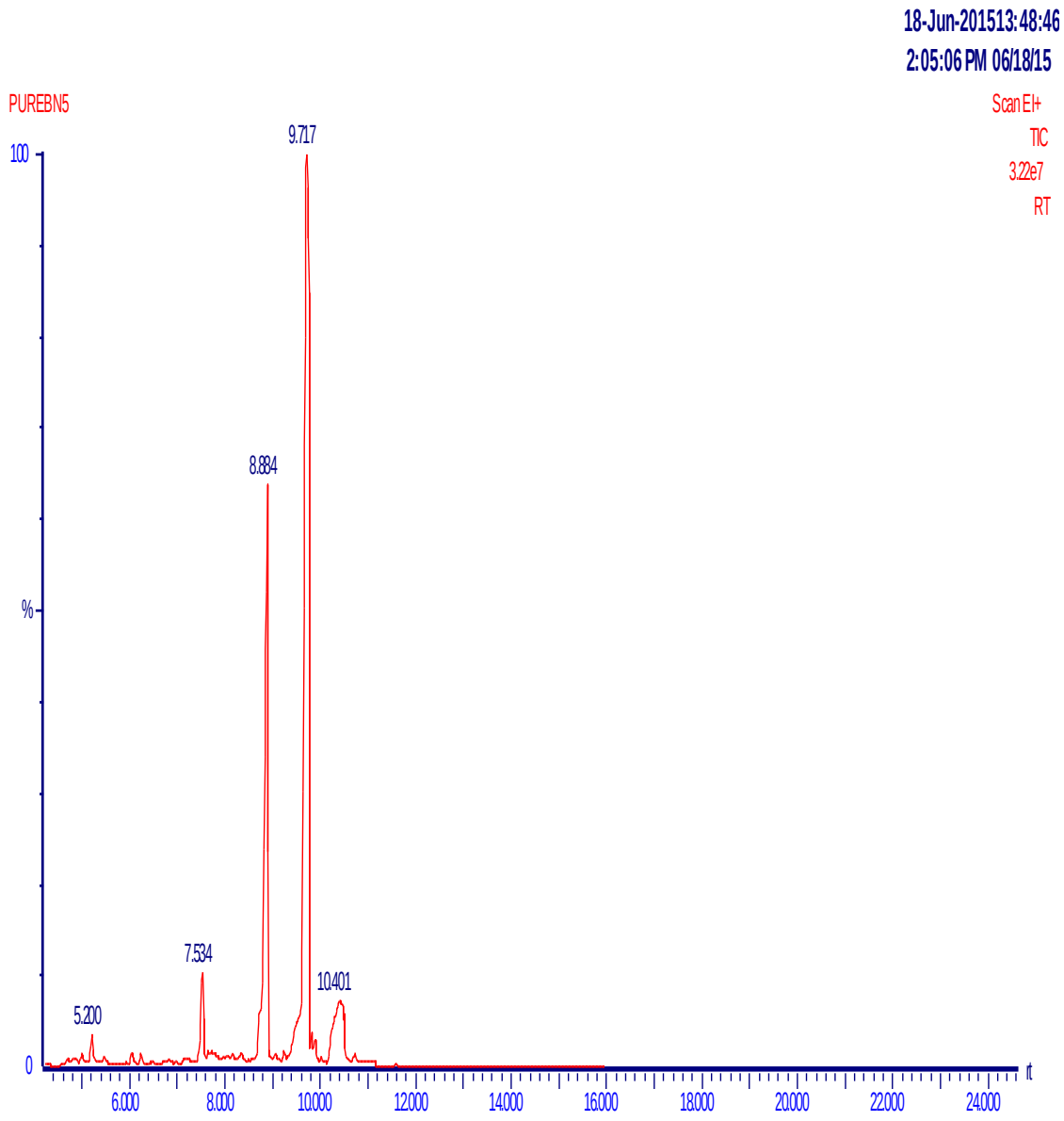
Appendix B: Thin layer chromatograph demonstration

	
F5 and 6 DCM: Me 98.2 Rf = Distance analyte travels Distance solvent front travels Rf = 1.3/2.8 =0.46	Fra 2 Hex: Me 9:1 Rf = Distance analyte travels Distance solvent front travels Rf= 1.4/2 =0.70

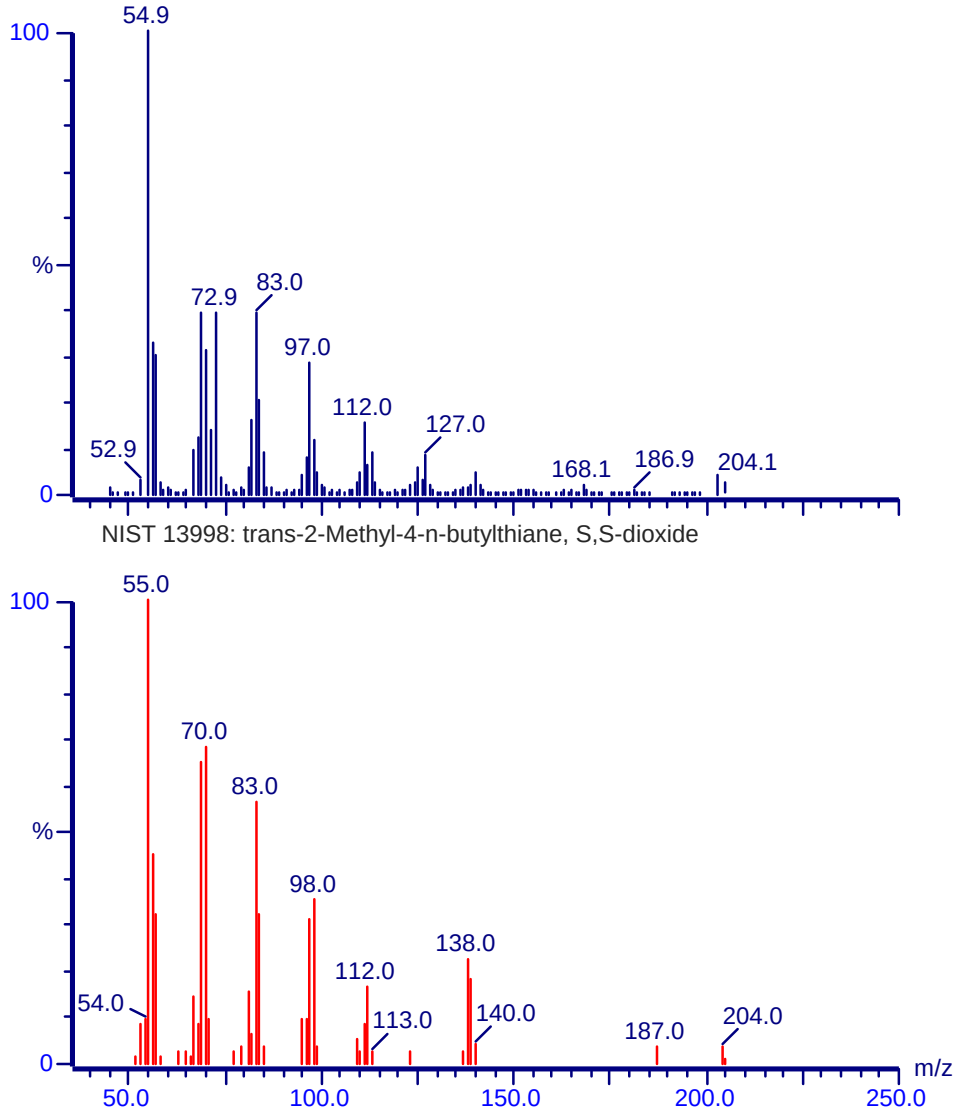
Appendix C 1: Chromatogram of Vigna subtarranea precleaned fraction F2 by GC-MS



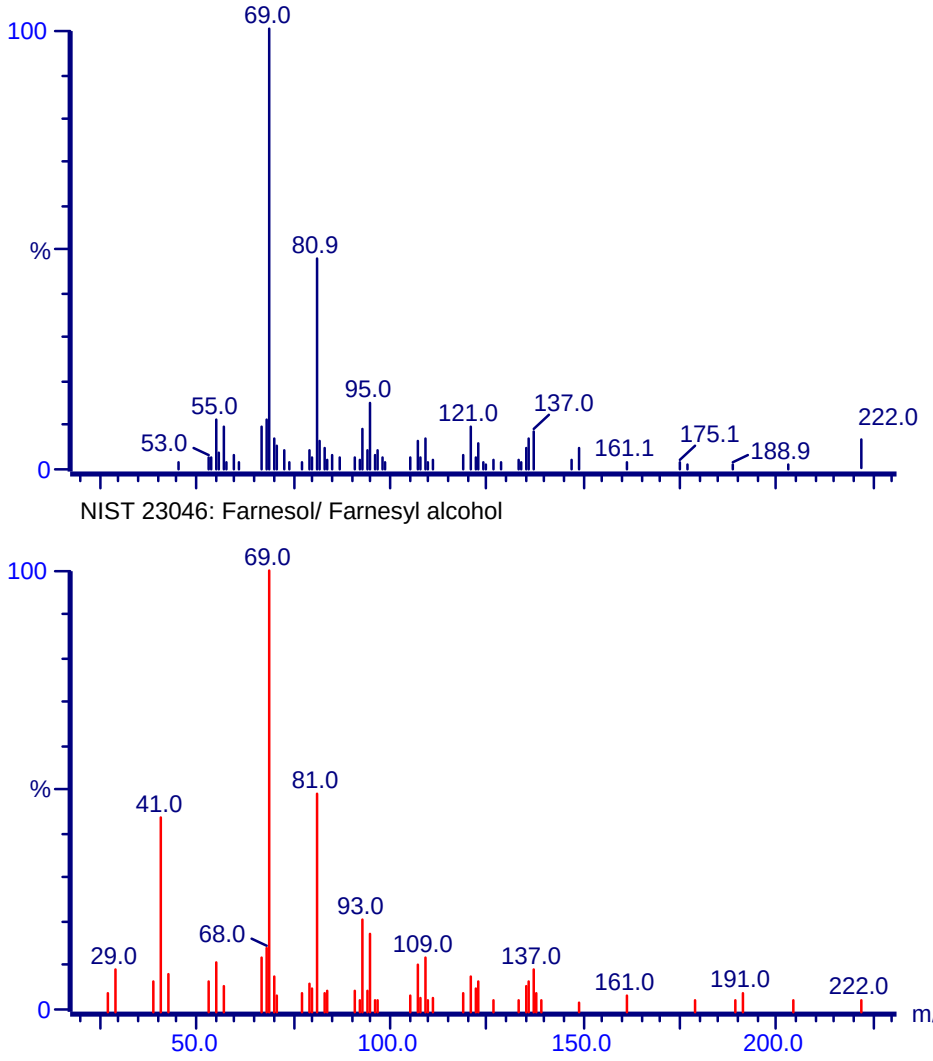
Appendix C 2: Chromatogram of Vigna subtarranea precleaned fraction F6 by GC-MS



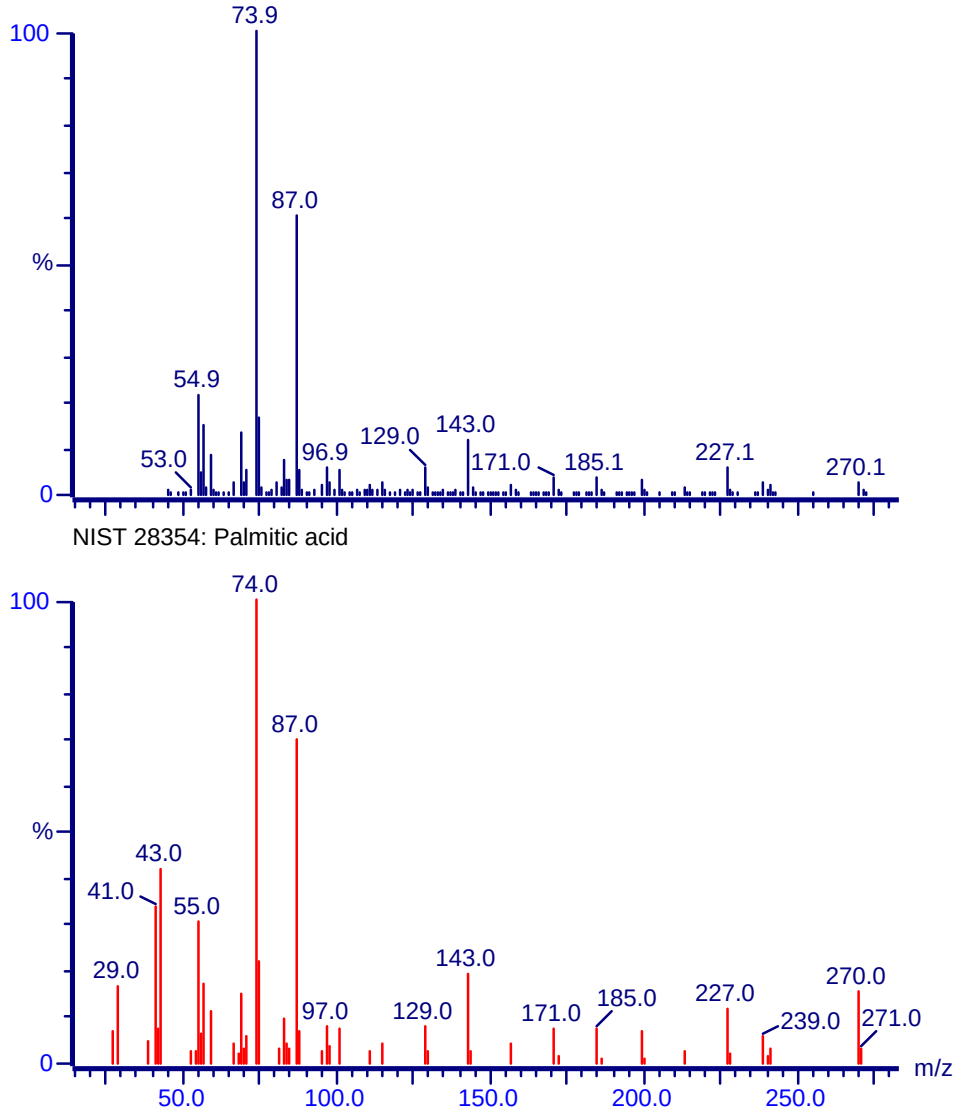
Appendix D 1: Part of the mass spectrum of trans-2-Methyl-4-n-butylthiane, S,S-dioxide. The elemental composition - C10H20SO2. The base peak was at m/z 54.9



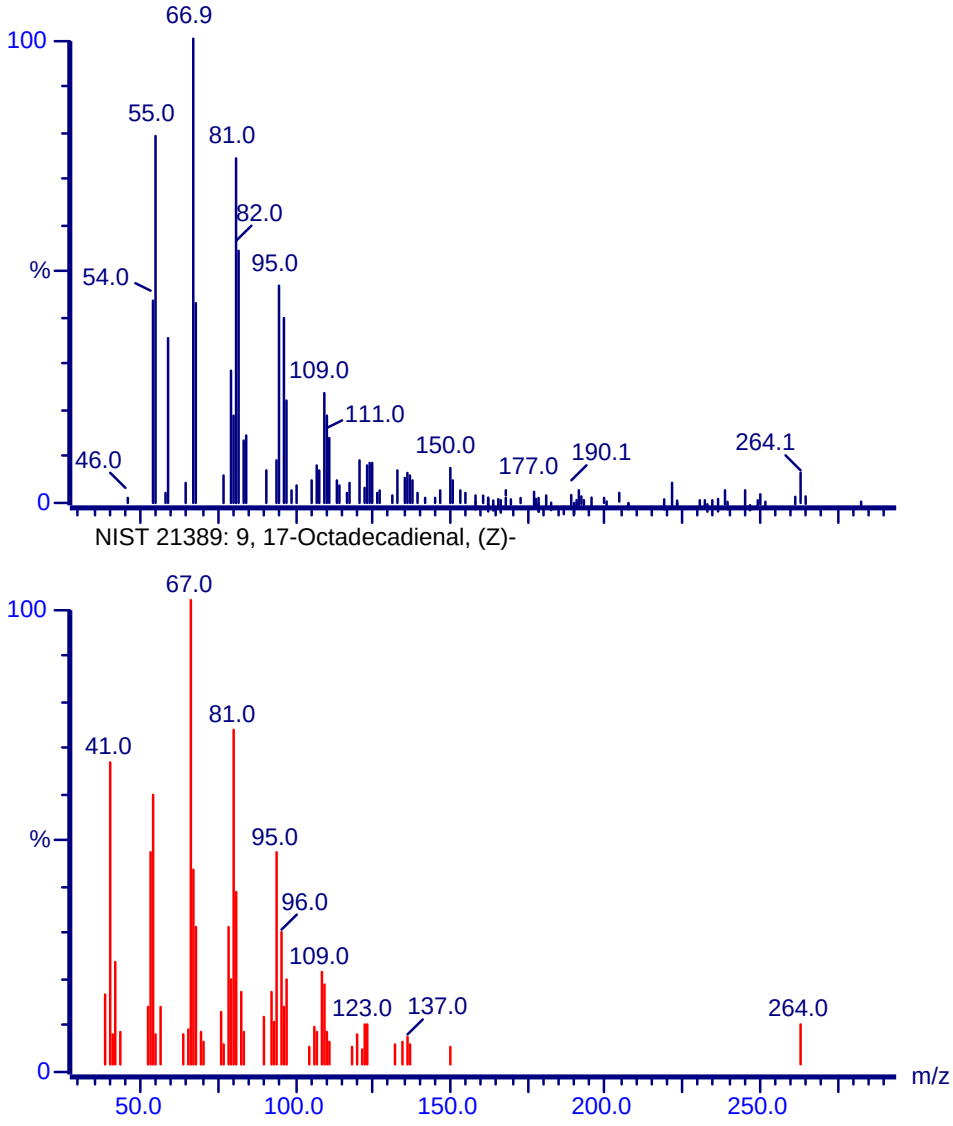
Appendix D 2: Part of the mass spectrum of farnesyl alcohol. The elemental composition -C15H26O. The base peak was at m/z 69.



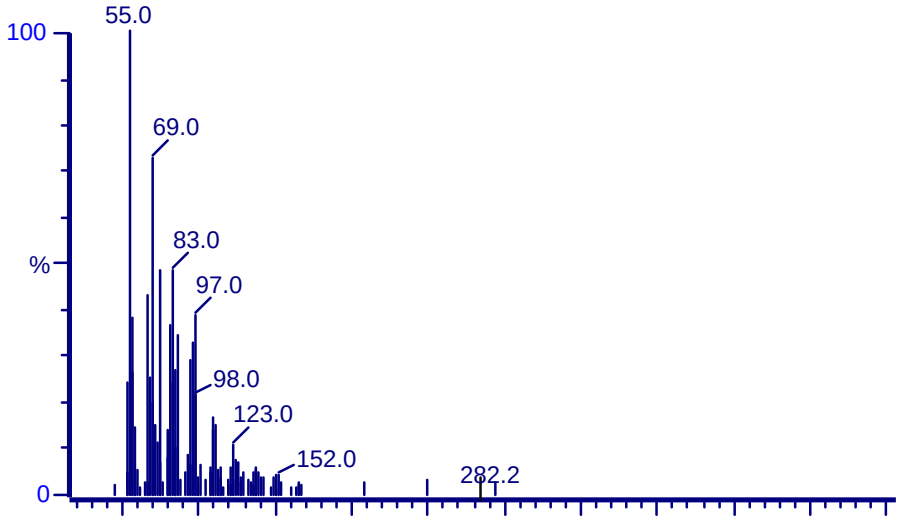
Appendix D3: Part of the mass spectrum of palmitic acid. The elemental composition -C16H32O2. The base peak was at m/z 73.9



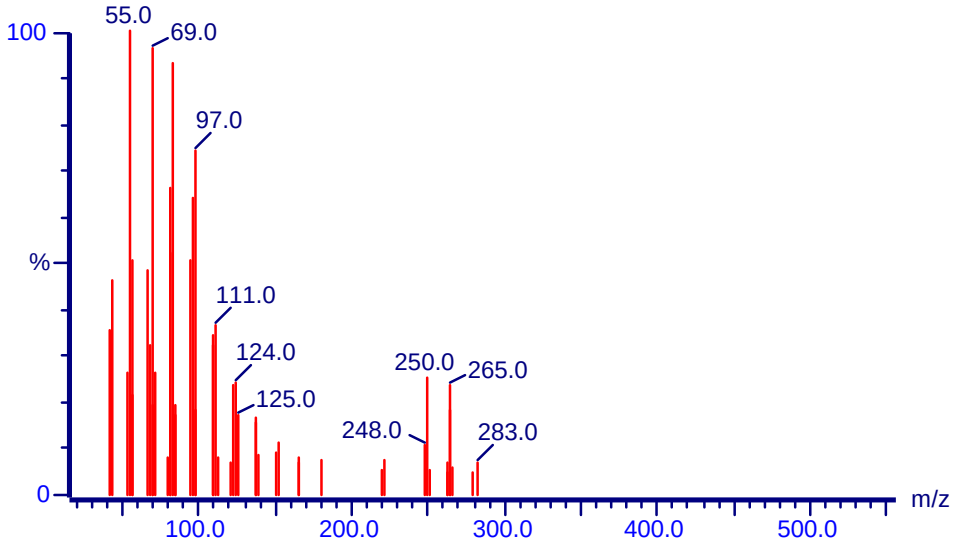
Appendix D4: Part of the mass spectrum of 9,17-Octadecadienal, (Z)-. The elemental composition was C18H32O. The base peak was at m/z 66.9



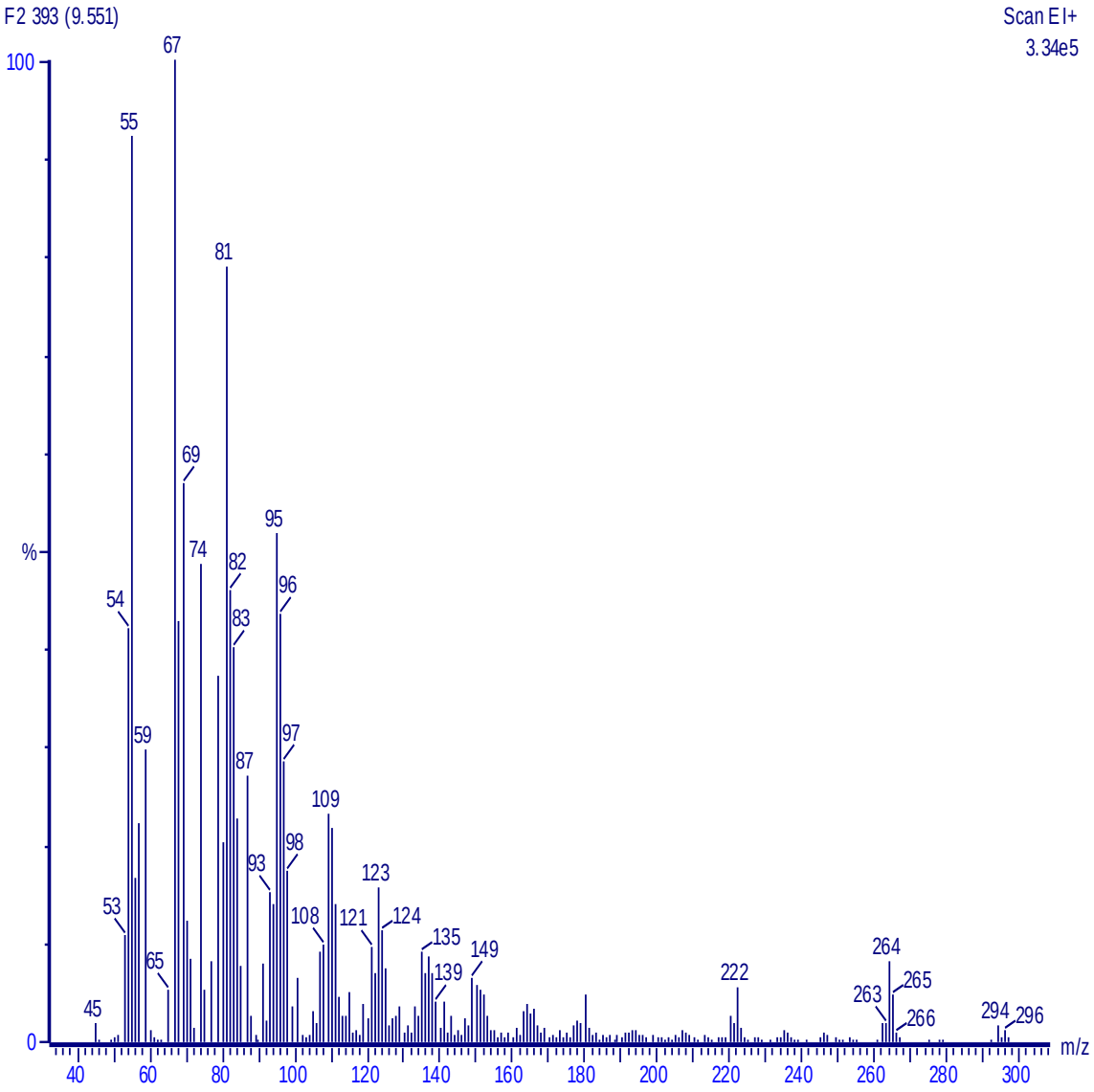
Appendix D 5: Part of the mass spectrum of Oleic acid. The elemental composition- C18H34O2. The base peak was at m/z 55.0



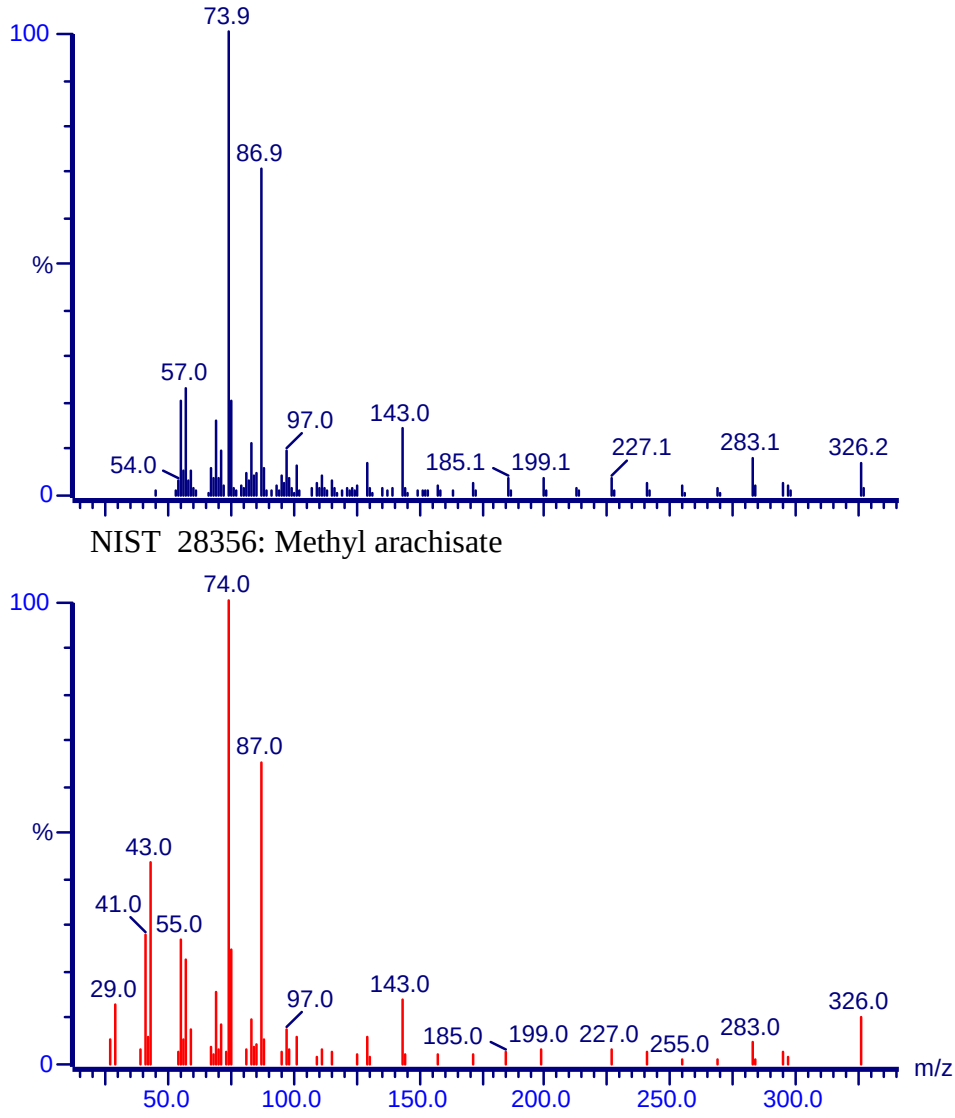
NIST 13925: Oleic acid or (Z)-9-octadecenyl ester or Cetiol



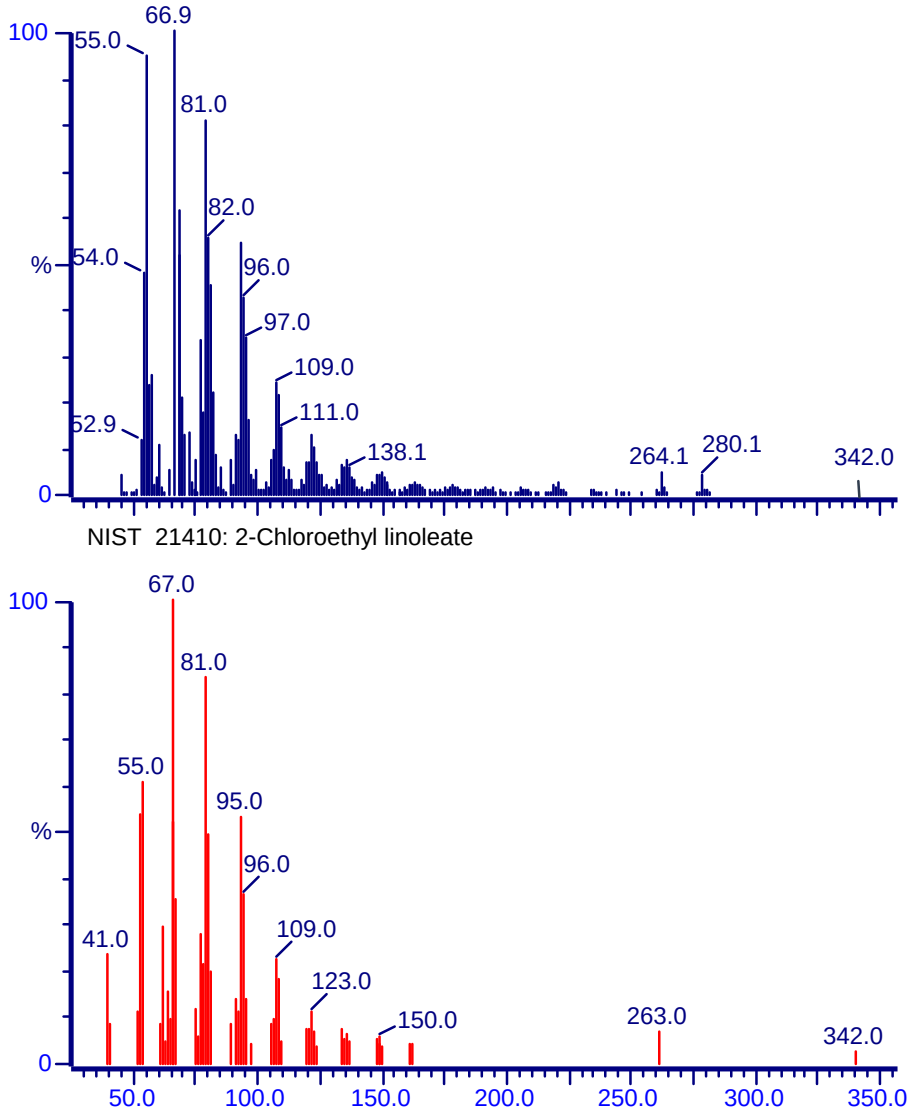
Appendix D 6: Part of the mass spectrum of 7-Octadecenoic acid methyl ester. The elemental composition of the ester- C19H36O2. The base peak was at m/z 67



Appendix D 7: Part of the mass spectrum of methyl arachisate ester. The elemental composition was C21H42O2. The base peak was at m/z 73.9



Appendix D 8 Part of the mass spectrum of 2-Chloroethyl linoleate. The elemental composition of the alcohol was C20H35O2Cl. The base peak was at m/z 66.9



Appendix D 9: Part of the mass spectrum of Methyl behenate or methyl docosanoate. The elemental composition of the ester was C23H46O2. The base peak was at m/z 73.9