# Evaluation of Anti microbial activity of Osyris lanceolata (East African Sandalwood)

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# DECLARATION

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

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# DEDICATION

This work is dedicated to my God for seeing me through it all, my parents for their constant support and my husband for encouraging me to its completion.

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## ABBREVIATIONS

ANOVA – Analysis of Variance

ATCC – American Type Culture Collection

CFU – Colony forming Unit

CHL - Chloroamphenical

Clin. - Clinical

CRDR – Center for Respiratory Research

GEN - Gentamycine

HPLC – High Performance Liquid Chromatography

ICIPE – International Center for Insect Physiology and Ecology

KAN - Kanamycin

KEFRI – Kenya Forestry Research Institutue

KEMRI – Kenya Medical Research Institute

MIC – Minimum Inhibitory Concentration

MTT - Methyltrityl

Etc – Et Cetera

# ABSTRACT

*Osyris lanceolata* (East African Sandalwood) is an evergreen shrub to small tree (1 – 6m) in the family Santalacea. The species has a relatively wide ecological distribution occurring in Eastern and Southern Africa. In Kenya, it grows in Coast, Eastern, Rift valley, Nyanza and Western provinces.

The aim of this work was to investigate anti microbial activity of the plant. The plant parts used were the roots, stem and stem bark. Polar and non-polar extracts were obtained from each plant part and these were used to screen for anti microbial activity and to obtain HPLC profiles.

Using the disc diffusion technique the extracts were screened against five bacteria and three fungi and the results showed that aqueous methanol extract and water extract had activity against Staphylococuss aureus. There was no significance difference in the activity of these extracts on the microorganism ($p>0.05$). The minimum inhibitory concentrations (MIC) value was a range between 294 – 301 µg/ml. The HPLC profiles displayed very large peak ares of polar constituents in aqueous methanol and water extracts and moderate peak areas of non-polar constituents. While the hexane and dichloromethane extracts displayed only moderate peak areas of non-polar constituents.

## CHAPTER 1

## INTRODUCTION

### 1.1 Background of plant.

#### 1.1.1 Geography

*Osyris lanceolata* is a dryland species with spatial distribution in Kenya and Tanzania. It occurs as isolated individuals, in close association with other woody species, and does not occur communally in large numbers. This could be attributed to its slow growth and host preference. It is found as a shrub on wooded rocky ridges or outcrops and on mountain slopes and as a tree in gorges, loofs and forest margins. It is common on rocky ridges in the Magaliesberg, North America (Palmer and Pitman, 1972) and in rocky sites where the original vegetation has been cleared (Beentje, 1994) as shown in Figure 1.1. It is also in margins of dry forests, evergreen bushland, grassland,thicket at 900 – 2550 m altitude (Pooley, 1993). In Kenya the species has been recorded in Oloitokitok, Amboseli, Makueni, Taveta, Chyulu hills, Narok, Mbeer

**Fig 1.1 The shrub growing between rocks**

### 1.1.2 Biology

*Osyris lanceolata* is a slender shrub or samll evergreen tree from 1 to 6 m tall as shown in Fig 1.2. It is a, multi-stemmed, spreading tree with a round to irregular canopy refered to in Fig 1.3. The dark branches and blue-green leaves contrast with one another. It is probably a partial root parasite, growing on the roots of other plants (Van Wyk and Van Wyk, 1997) and utilizing the root systems of these hosts, but does not produce its own chlorphyll (Thomas and Grant, 2002). As a result it is usually intimately associated with shrubs of other woody species. In Kenya it is known to have association with several species within the following genera: *Acacia*, *Albizia*, *Rapanea*, *Bridellia*, *Cordia* and *Teclea*.

**Figure 1.2 The shrub**

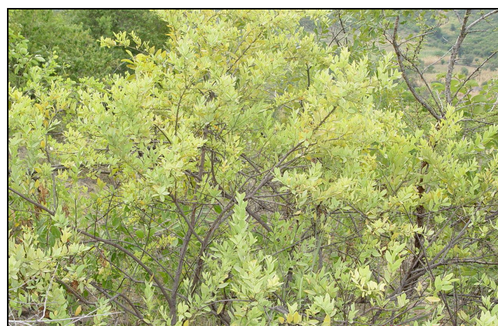


**Figure 1.3 Canopy of *Osyris lanceolata***

The leaves are alternately arranged (fig 1.4). They are small, simple, thick, leathery, rigid and tough and characteristically point upwards. They vary in size from 30-45 × 10-25 mm (Schmidt *et al.*, 2002) but their length may vary from 13-50 mm long (Pooley, 1993). They are lance-shaped or sometimes egg-shaped. The apex is broadly tapering to rounded with a fine, sharp tip. The base is broadly tapering. The leaf margin is entire and rolled. These hairless leaves are grey-green or blue green, smooth with a waxy bloom that can be rubbed off (Palmer and Pitman, 1972). The leaves often have an orange coloured margin (Thomas and Grant, 2002). The petiole attachment to the stem forms ridges running down the stem (Coates, 1977).

**Figure 1.4 Leaf arrangement**

*Osyris lanceolata* is usually multi-stemmed Figure 1.5 with the bark dark brown to blackish in color (Palmer and Pitman 1972) or grey and smooth (Van Wyke and Van wyk, 1997) Figure 1.6. The branches are erect, angular and rigid and the branchlets are greenish-blue and angular or square in section (Pooley, 1993). The twigs at the top of the canopy spread out from a single point (Thomas and Grant, 2002) and the twigs and leaves point upwards.

**Fig 1.5 Multi stemmed** **Fig 1.6 Barks**

Its flowers are unisexual and are in the form of short auxiliary panicles with small bracteoles. The tiny flowers are yellowish-greenish; they are borne in the axils of the leaves in short clusters of 2-3 flowers. The very small, greenish-yellow flowers are on long slender stalks (Schmidt *et al.*, 2002) this is shown in figure 1.7. All floral parts are in fours with stamens attached to the base of the fleshy perianth lobes. Male and female flowers are separate (Thomas and Grant, 2002), borne on different trees (Palmer and Pitman, 1972). Flowering time appears to be poorly documented with different authors

giving very different flowering periods: from March to August or even later (Coates, 1977), October to February (Pooley, 1993) and September to February (Schmidt *et al.*, 2002).

**Fig 1.7 The Flowers**

The fruit is a small one seeded drupe (Palmer and Pitman, 1972), about 15 × 10 mm in size. These fleshy, egg-shaped fruits are green at first, turning yellow and becoming bright red to purple-black when ripe and are crowned with a persistent calyx (Schmidt *et al.*, 2002) as shown in Figure 1.8. The fruits ripen between May and September (Pooley, 1993; Schmidt *et al.*, 2002).

**Fig 1.8 The fruit in the plant and the collected fruit**

### 1.1.3 Uses

Its fruit is edible but very bitter causing some dryness in the salivary glands. The roots give a strong red dye used to treat fibres for basketry. Among the Kamba community in Kitui, the wood is used to smoke milk containers to obtain an excellent aroma. The

wood is also a substitute for Asian sandalwood. At industrial level, the wood and bark oils are reportedly used to extract highly valued perfumes and other cosmetics (Mohamed and Musya, 2005). It is also used as an ingredient for quality lotions and rare soaps. The plant is highly valued by the local communities for its medicinal properties, providing income generation to the herbalists(Pamplona and Rogers, 2000). Locals are reported to use its bark powder to heal wounds. It is also reported to treat stomach aches, tonsils, diarrhoea, ulcers, snakebites and rashes (Mohamed and Musya, 2005). The species has been of little importance, until recently when it was captured in the limelight due to its overexploitation to meet the international demand for its perfumery and medicinal products in the treatment of hepatitis. The nature of it exploitation raises concern on its survival in the wild as it involves uprooting of the whole tree (destructuve harvesting).

## 1.2 Aspects of antimicrobial activity and its importance

An antimicrobial is a substance that kills or inhibits the growth of microbes such as bacteria, fungi, or viruses. Antimicrobial drugs either kill microbes or prevent the growth of microbes. A wide range of chemical and natural compounds are used as antimicrobials. Organic acids are widely used as antimicrobials in food products, e.g. lactic acid, citric acid and their salts. Traditional healers have long used plants to prevent or cure infectious disease. Many of these plants have been investigated scientifically for antimicrobial activity and a large number of plant products have been shown to inhibit the growth of pathogenic microorganisms (Eloff, 1998). A number of these agents appear to have structures and modes of action that are distinct from those of the antibiotics in current use, suggesting that cross-resistance with agents already in

use may be minimal (Eloff, 1998). So, it is important to study plants and plant products for activity against resistant bacteria.

The standard disk diffusion or Kirby Bauer test is used in clinical laboratories worldwide (Lemos *et al*., 1992). Since it was first described over 30 years ago, it has been refined to allow accurate and reproducible testing of most bacterial pathogens. It is relatively inexpensive, versatile, and easy to perform. In addition, it requires media, reagents, equipment and supplies that are readily accessible to most clinical laboratories. Instruments have recently become available that examine and measure zones and store zone measurements in electronic files. These have allowed high volume laboratories to perform disk diffusion tests more efficiently. Results generated from disk diffusion tests are qualitative (e.g. susceptible, intermediate, and resistant). Qualitative results are sufficient for many types of infections but are often not optimal for guidance in management of serious bacterial infections. In addition, the disk diffusion test cannot always reliably identify subtle decreases in susceptibility. For some bacteria, such as glycopeptide- intermediate *Staphylococcus aureus* and penicillin- intermediate or resistant *Streptococcus pneumoniae*, MIC methods are recommended to test them. Provided that clinical laboratory scientists perform the disk diffusion test according to standard recommendations, recognize its limitations, and communicate results effectively, it is a valuable test to consider for many bacterial pathogens (Hindler, 1999).

## 1.3 Problem statement

*Osyris lanceolata* is reported to be used as a medicinal plant to treat stomach upsets especially in young children in the local areas of Kitui and Kajiado. It is also used to treat snake bites and skin rashes as a paste is made and applied to the skin

areas(Mohamed and Musya, 2005). This study is interested in obtaining scientific evidence of inhibitory activity of the plant extracts on the microorganisms that cause the above mentioned ailments and other common microorganisms. This is because the evidence of local use of *Osyris lanceolata* extracts to treat disease and the active ingredients in the plant are not known. The study intends to answer the following question do *Osyris lanceolata* extracts have inhibitory activity against selected microorganisms?

## 1.4 Justification of the study

Since 2004, *Osyris lanceolata* has been one of the most highly valued and extremly over exploited medicinal plants in Kenya. The plant is much sought by businessmen from Tanzania, South Africa and their collaborators in Kenya. The species is poached even from security tight national parks in the southern drylands of Kenya. Apparently 150 tons of logs of the plant per month are being exported to India by a company in Mumbai(Mohamed and Musya, 2005). This shows that the plant has a high economic value and there is need to tap on this resource and protect it. The findings from the study will be used to sensitize the government to conserve the plant and prohibit its overexploitation. Detailed research involving chemical analysis is one of the sustainable management options for *Osyris lanceolata*. Results of the study may also assist in partnership with pharmacetical companies to provide an ingredient for the making of antibiotics. The results will also give us the evidence supporting the folklore knowledge of the use of *O. Lanceolata* to treat ailments locally. The plant also grows naturally in the arid and semi-arid areas which receive a mean annual rainfall of 500 – 700 mm per year. In these dryland areas the main land uses are subsistence dryland farming, agropastrolism, national park/reserves and isolated horticulture irrigation. This research is also expected to provide an alternative economic activity of farming of

*Osyris lanceolata* in large scale to provide raw material for pharmaceutical industries. At the community level this will increase their income and improve livelihood.

## 1.5 Objectives

### 1.5.1 General Objective

To evaluate the antimicrobial activity of *Osyris lanceolata*.

### 1.5.2 Specific Objectives

1. To obtain organic and aqueous extracts from the plant
2. To determine anti microbial activity of the plant extracts against selected microorganisms.
3. To determine the HPLC profiles of the extracts.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Use of Medicinal plants

Medicinal plants contain physiologically active principles that over the years have been exploited in traditional medicine for the treatment of various ailments(Sokmen *et al*., 1999; Kelmanson *et al*., 2000; Sirinivansal *et al*., 2001). Pharmaceutical industries have produced a number of new antibiotics in the last three decades, but resistance to these drugs by microorganisms has increased. In general bacteria have the genetic ability to transmit and acquire resistance to drugs, which are utilized as therpeutic agents. For years plants have been valuble sources of natural products for maintaining human health. More intensive studies have been carried out with plant extracts to determine their potential therapeutic properties in the last decade. According to World Health Organization and Santos *et al*., (1995) medicinal plants would be the best source to obtain a variety of drugs. About 80% of populations from developed countries use traditional herbal or plant based medicine. Therefore such plants should be investigated to better understand their properties, safety and efficacy (Elof, 1998).

The use of plant extracts with known anti microbial properties can be of great significance in therapeutic treatments. In the last few years, a number of studies have been conducted in different countries to prove such efficacy (Ikran and Inamul 1984; Almagboul *et al*., 1985; Kubo *et al*., 1993; Shapoval *et al*., 1994; Artizzu *et al*., 1995; Izzo *et al.,* 1995). Many plants have been used because of their anti microbial traits, which are due to compounds synthesized during the secondary metabolism of the plant. Examples of the compounds are phenol compounds, which are part of the essential oils (Jasen *et al*., 1987) as well as tannins (Saxen *et al*., 1994).

The anti microbial properties of plants have been investigated by a number of researchers worldwide. In Argentina, a research tested 122 known plant species used for therapeutic treatments (Anesin and Prez, 1993). It was documented that among the compunds extracted from these plants, twelve inhibited the growth of *Staphylococcus aureus*, ten inhibited *Escherichia coli* and four inhibiited *Aspergillus niger*. The anti microbial properties of compounds obtained from Parthenum argentatum Gray (Asteraceae) against *Candida albicans*, *Torulopsis*, *Hansemula*, *Klebsiella pneumoniae* and *Pseudomonous aeruginosa* was detected (Martinez *et al*., 1994 and Martinez *et al*., 1996). A more detailed study on anti microbial compounds was also done evaluating extracts from 120 plant species from 28 differeent families (Santos *et al*., 1990). It was documented that 81 extracts obtained from 58 plants were active against *S. aureus* and five extracts from four other plants inhibited the growth of *P. aeruginosa*. Another study (Lemos *et al*., 1992) detailed the anti bacterial and anti fungal activity of essential oils obtained from *Croton triangularis* (Asteraceae) leaves. The investigation of anti microbial activity as well as cell toxcicity of extracts from 30 plant species against five bacterial species and two fungi species was studied (Nascimento *et al*., 1990). It was concluded that ethanol extracts from 70% of the plants were toxic to cells and only one of the species of *Combretum duarteanum* showed anti microbial activity. The toxicity of extracts form *Arthemus sativa*, which is known to have anti microbial activity, has been studied (Carralho *et al*., 1988). The anti microbial activity of Mikania triangularis L. (Asteraceae) also known as "thin leaf guaco" was tested against five genera of bacteria and three genera of yeast and it showed activity against *Bacillus cereus*, *E. coli*, *P. aeruginosa*, *S. aureus* and *S. epidemidis* (Cruz *et al*., 1996).

## 2.2 Medicinal plants with specific anti microbial activity

*Escherichia coli*:

Enterohaemorrhagic Escherichia coli have increasingly emerged as pathogens that cause significant human diseases, including diarrhoea (Pai *et al*., 1998), haemorrhagic colitis (Riley, 1987), and occasionally complications such as haemolytic-uremic syndrome and thrombocytopenic purpura (Scotland *et al*., 1998; Griffin and Tauxe, 1991; Karmali, 1992).

Extracts from *Acacia catechu* L. (Fabaceae), *Psidium guajava* L. (Myrtaceae), *Punica granatum* L. (Punicaceae), *Quercus infectoria* Olivier (Fabaceae), *Uncaria gambir* Hunter (Rubiaceae), and *Walsura robusta* Roxb. (Walsuronoid) have been ahown to have activity against all strains of *Escherichia coli* O157:H7. Aqueous extract of *Holarrhena antidysenterica* and *Uncaria gambir* did not have anti bacterial effect while ethanolic extract produced zones of inhibition. In addition, ethanolic extract of *Holarrhens antidysenterica* also produced large zones of inhibition (11-13 mm)(Supayang *et al*., 2004).

*Staphhylococcus aureus*:

*Busera simaruba* L. (Burseraceae), *Haematoxylon brasiletto* Karst (Fabaceae), *Calophyllum brasiliense* Cambess (Guttiferae), and *Mammea americana* L. (Guttifereae) showed high activity against *Stapphylococcus aureus*. *Bursera simaruba* is a widely distributed tree in the tropical area in Mexico and is also well known for its applications as water decoctions or poultices made from the leaves against bacteria related diseases. *Haematoxylum brasiletto*, a tree distributed in dry tropical forests, had red heartwood. This morphological feature of red heartwood could probably be related to several medical applications, due to an association with blood or heart diseases.

*Haematoxylum brasiletto* is also known in certain localities as a febrifuge (Mullika *et al.*, 2005).

The extracts of *Senna alata* L. (Fabaceaea), *Eupatorium odoratum* L. (Asteraceaea), *Garcinia mangostana* L. (Guttiferae), *Barleria lupulina* botanical survey of India; (Asteraceaea), *Hibiscus sabdariffa* United States Interrnational Review Board; (Malraceae), *Garcinia mangostana* L. (Guttiferae) and *Eupatorium odoratum* L. (Asteraceae) showed anti bacterial activities against both *Propioibacterium acnes* and *Staphylococcus epidermidis* (Mullika *et al.*, 2005).

Mangostin is a xanthone derivative produced by guttiferaeous plants. Xanthone and its derivatives have activities against *Staphylococcus aureus* and methicillin-resistant *S. aureus* (Munekazu *et al.*, 1996). Seventeen plant extracts obtained from 16 different species belonging to the plant families namely: *Annonaceae*, *Combretaceae*, *Gnetaceae*, *Lauraceae*, *Leguminosae*, *Myristicaceae*, *Myrsinaceae*, *Myrtaceae*, *Piperraceae*, *Proteaceae*, *Rubiaceae*, *Rutaceae*, *Smilacaceae* and *Vochysiaceae* showed potent anti bacterial activity against *Staphylococcus aureus* (Ivana *et al*., 2006).

*Pseudomonas aeruginosa*:

Clinically significant infections with *P. aeruginosa* should not be treated with single antibiotic due to the fact that bacteria can rapidly develop resistance when such a single antibiotic is used. According to different reports, multiple drug resistances to *P. aeruginosa* are spreading hazards in the world and making the therapeutic management of these patients more problematic (McCallum *et al.*, 2001; Obritsch *et al.*, 2005; Sekiguchi *et al*., 2005; Naron-Veneiza *et al*., 2005). The effect of combinations of *Rhus coriaria* L. (Anacardiaceae) and *Thymus vulgaris* L. (Lameaceae) have anti bacteial enhancement (additive effect) against *P*. aeruginosa (Sekiguchi *et al*., 2005).

## 2.3 Medicinal plants of the drylands

*Thymus* species are wild and mostly found in the arid lands of Portugal. *Thymus lotocephalus*, which blossoms from April to June, is an endemic species of the Algarve region and can only be found in dry open areas and dry scrub, which are restricted to the Algarvian Barrocal (Faleiro *et al*., 2003).

*Acacia greggii* also known as catclaw is a member of the Fabaceae family; it is native to the southwestern United States and nothern Mexico. Catclaw occurs primarily in semi-desert grasslands and brushy rangelands largely confined to washes. Pods are used for treating conjuctivitis in the same manner as mesquite pods. The powdered pods and leaves make an excellent infused tea for diarhoea and dysentry, as well as a strongly astringent homeostatic and anti microbial wash. The straight powder will stop superficial bleeding and can also be dusted into moist, chafed body folds and dusted on infants for diaper rash (Dawson, 1944).

*Rumex hymenosepalus* is a common plant often found in sandy washes of the high western United States of America deserts. It helps modify and depress local inflammations caused by hives, contact dermatitis or chafing. They are also effective for relieving sunburns.

*Artemisia tridentate* is found in a large part of the high deserts of the western United States of America the entire plant is strongly anti microbial and anti parasitical. However, there is need for more studies on the therapeutic potential of other dryland plants. Plants from arid and semi-arid areas should be emphasized on as they also have much to offer in the fight against microorganisms.

### 2.4 Conservation of medicinal plants through research

The study of local knowledge about natural resources is becoming increasingly important in defining strategies and actions for conservation or restoration of residual forests. Reliance on medicinal plants creates the need to maintain and conserve biodiversity. Man's activities, for example, timber, fuel and construction poles threaten mecicinal plants. Also medicinal plants as components of various ecosystems are subjected to depletion and are threatened as a result of agricultural expansion, deforestation, over exploitation, destructive harvesting and habitat alteration. Sustainable management of medicinal plant species is important, not only because of their value as a potential source of new drugs but also due to reliance on medicinal plants for health care and increase of income to the household.

Despite the availability of modern medicines, most agro-cultural communities still use and retain an extensive pharmacopoeia of native plants (Prance, 1991). It is estimated that over 80% of rural people in Tanzania depend on traditional healers and herbs for their primary health care needs (Hamza, 1997; Dery *et al*., 1999). In the interior areas of western Himalaya plants become the only source of medicine and well-being. The importance of medicinal plants in traditional healthcare practices, providing clues to new areas of research and in biodiversity conservation is now well recognized. Out of the total 422,000 flowering plants reported from the world (Govaerts, 2001), more than 50,000 are used for medicinal purposes (Schippmann *et al*., 2002). Many renowed drugs of today would have gone into wider use decades ago if the folklore and traditions concerning certain plants had been taken seriously (Sapu, 2000).

# CHAPTER 3

# MATERIALS AND METHODS

## 3.1 Sample site

The plant samples were collected from Kitui and Ngong. Ngong is located in Kajiado district in Rift Valley province. The specific locality that the samples were collected was in Kibiko. The area was hilly, rocky and had a lot of dry bush land. The samples were picked from bush lands just after Kibiko secondary school in the farms bodering the escarpment along the forest edges as shown in Figure 3.1. Random collection was done as the plant is found scattered all over and there are very few samples available. Three areas were randomly identified and marked as zone 1,2 and 3 and the samples were collected. The local name for the shrub in this area is *Olesesiai* (Maasai). Kitui is located in Kitui district in Eastern province. The samples were collected from Kamandio-Malili (Miambani). The area was also hilly, rocky and dry bush land. The samples were picked from the forest reserve in Kamandio-Malili opposite the chief's camp. The local name for the shrub in this area is *Kithawa* (Kamba).

**Fig. 3.1 Kibiko area of Ngong**

## 3.2 Collection and preparation of samples

The mode of collection involved cutting twigs and stems with secateurs, panga and using a hoe for digging and cutting the roots. The samples were collected form three different plants in different places of the areas identified. All the samples were brought to the labaratory and cut into small pieces. The stem bark was separated from the stem using a knife and then kept in a brown paper on its own. These were then placed into separate brown paper in the following categories: Ngong roots, Ngong stem and Ngong stem bark, Kitui roots, Kitui stem and Kitui bark. Their fresh weight was recorded and the samples were oven dried at 60$^{o}$C for five days. After this the dried samples were weighed and their weights recorded then the samples were individually ground using a ball mill grinder.

## 3.3 Extraction

From each sample ground organic and aqueous extracts were obtained. This was done using the following procedure: An extraction solvent was prepared by mixing dichloromethane and ethanol at a ratio of 1:1 to a total volume of 2000 ml (Robert, 1993). Conical flasks of volume 500 ml were taken and labeled. To each of the flasks, 50 g of the individual plant samples was placed and 300 ml of the extraction solvent was added and this was left to soak for 15 h while shaking after every 3 h. Then filtration was done and and the filtrate stored at room temperature. The residues that were collected on the funnel was then placed back in clean conical flasks and 300 ml of methanol was added to the residue, shaken and left to soak for 5 h. Filtration was then done and the filtrate obtained was added to the first filtrate obtained as per the samples. This combined filtrate was then concentrated using a rotary evaporator. This was done

for about 4 h for each of the samples. The organic extracts obtained were collected in small tubes, labeled and weighed.

The residues were open air dried for 1 h and then placed in clean conical flasks and 300 ml of distilled water was added to each sample and left to soak for 15 h. After soaking filtration was carried out and the filtrate was kept in small conical flasks and these were placed in a freezer to solidify. On solidifying the filtrate was placed in a freeze-dryer for 4 days and an aqueous extract was obtained. This was stored, labelled and weighed. The residue was then discarded.

The organic and aqueous extracts obtained were then used in a trial bioactivity tests and the results promted a second extraction to be carried out. The second extraction was to result in production of individual organic extracts instead of a combined organic extract (Brian and Turner, 1975). This was done in hope that the activity of the individual organic extracts would give more insight into the anti microbial activity of the plant *Osyris lanceolata*. It was carried out as follows: 50 g of each of the dried ground samples were separately placed in labeled conical flasks and 150 ml of Hexane was added and left to soak for 15 h. After this each of the samples was filtered using Whatman ashless circles size 41 filter paper and the individual filtrates obtained were concentrated using Eyela rotary vacuum evaporator at 50$^{o}$C for 20 to 25 min. After concentration each extract obtined was weighed and stored.

The residues were left to dry for 1 h then placed in clean conical flastks and 150 ml of dichloromethane was added to each flask. This soaked the residue for 15 h after which each sample was filtered. The filtrate was concentrated at 40$^{o}$C in a time span of 9 to 16 min. The dichloromethane extracts obtained from each sample was stored in small tubes.

The residues were left to dry for 1 h and then placed in clean conical flasks. To each 150 ml of a mixture of methanol and water at a ratio of 1:1 was added to soak the residue for 15 h. This was then filtered and the filtrate concentrated at 50$^{o}$C for 30 min each. The aqueous methanol extract obtained was weighed, recorded and stored.

In the final step the residues were dried for 1 h then placed in a set of clean conical flasks and soaked in 150 ml of distilled water for 15 h. This was then filtered and the filtrate obtained were kept in the freezer to solidify. When each of the filtrates had frozen they were kept in the freeze-dryer FDU-830 to vaporize the water. This was done for 4 days and the water extract was obtained.

From this second extraction process 72 samples were obtained which were to be used for drug susceptibility testing. The extraction process was summarized as shown in Figure 3.2 below:

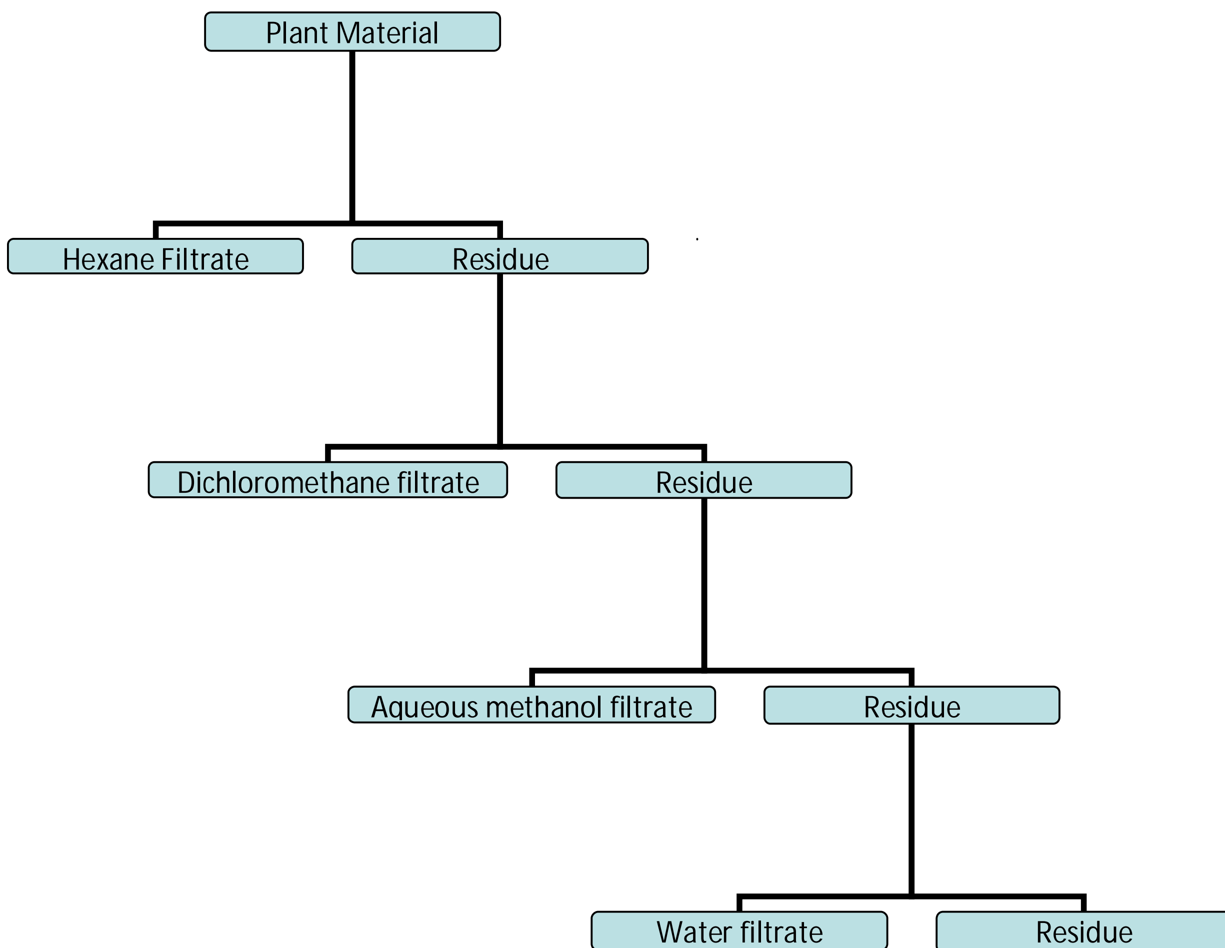



**Figure 3.2 A scheme of sequential extraction and the products obtained**

### 3.4 Anti microbial Testing

The microorganisms used were: ATCC 25923 *Staphylococcus aureus*, clinical isolate *Staphylococcus aureus*, ATCC 25922 *Escherichia coli*, clinical isolate *Salmonella typhi*, clinical isolate *Pseudomonas aeruginosa*, ATCC 90028 *Candida albicans*, clinical isolate *Microsporum gypseum* and clinical isolate *Cryptococcus neofumons* obtained from Center for Microbial Research, KEMRI.

Anti microbial screening of the plant extracts was undertaken in two phases according to the methods described by Clark *et al*. (1981):

1. Screening of the crude extracts (primary assay) to detect the presence or absence of activity. This was done against five bacterial strains and three fungal strains, and
2. Screening of crude extrracts (secondary assay) to determine their relative potency, expressed as minimum inhibitory concentration (MIC) value. This was done against the strains that gave positive response in the primary screening.

### 3.4.1 Preparation of nutrient agar and growing of bacteria cultures

Nutrient agar was prepared by dissolving 28 g of the agar in distilled water to make one liter of the solution followed by sterilization in an autoclave at 121°C for 20 minutes. Under aseptic conditions, in a laminar flow hood, portions of the sterilized nutrient agar medium 15 ml were dispensed into 90 mm pre-sterilized petri dishes to yield a uniform depth of 4 mm. The petri dishes were covered and allowed to cool at room temperature undisturbed until the culture medium hardened. They were then incubated at 37°C for 24 h in an inverted position to test their sterility. Using a sterile wire loop, bacteria cultures from stock cultures were scooped and spread on the nutrient agar surface with 3 fold dilutions and incubated at 37°C for 24 h aerobically. Colonies were picked and resuspended in normal saline (0.7% NaCl) to give a 0.5 MF solution (Gosh, 1994). Sterilized petri dishes were inoculated with 0.01 ml of the above culture media. Muller-hinton agar sterilized and cooled to 50°C was distributed by pipette (15 ml) into each inoculated petri dish and swirled to distribute the medium homogenously.

### 3.4.2 Preparation of Sabourand Dextrose Agar media and growing of fungal spores

Sabourand Dextrose Agar media was prepared by dissolving 65 g in distilled water to make a liter of the solution. This was then boiled for 1 min to completely dissolve the powder. It was then steam sterilized in an autoclave at 121°C for15 min. On cooling to

50$^{o}$C it was dispensed into sterile petri dishes under sterile conditions and left to solidify. This provided the medium for growing the fungal spores (Dhar and Bose, 1968). A spore suspension of 15 ml was placed into petri dishes.

### 3.4.3 Primary screening of the plant extracts

The extracts obtained from the sequential extraction process were injected into empty sterilized antibiotic discs of 6 mm diameter in amounts of 20μl. The discs were then oven dried at 50$^{o}$C for about 1 h to expel the solvent. These extracts were then firmly placrd on the inoculated petri dishes using sterile forceps under sterile conditions. They were then pressed down with slight pressure to ensure complete contact of the disc with the inoculated agar surface. The plates were incubated at 37$^{o}$C for 24h for the bacteria and 30$^{o}$C for 48 h for the fungi anaerobically in an inverted position. On each plate, an appropriate reference antibiobic assay disc was applied depending on test microorganism. The positive control for bacteria were Gentamicin and Kanamycin and for the fungi was Chloroamphenical at 20μl each. The negative controls were the extracting solvents hexane, dichloromethane, aqueous methanol and water at 20μl each (Chhabra and Uiso, 1991; McChesney *et al*., 1991). The zones of inhibition (if any) were measured. Each experiment was done in triplicate.

### 3.4.4 Secondary screening of the plant extract against the microoganisms that showed susceptibility

From the primary screening some microorganisms showed susceptibility to some of the plant extracts and this necessitated a secondary screening of the plant extracts. This was done to determine the minimal concentration of the plant extract that is able to inhibit activity of the microorganism. The secondary screening was carried out as

follows: the minimum inhibitory concentration analysis were conducted using broth dilution method according to the National Committee for clinical Laboratory Standard procedures for aerobic testing 2005. This was applied on extracts that proved their high efficacy against test microorganisms by disc diffusion method. The plant extract was dissolved in sterilized water and serially diluted to observe their activities at lower concentrations (Klepser *et al.*, 1996). Bacteria inoculum was added into the broth at the concentration of $10^6$ CFU/ml and then cultured at 37$^o$C. Every 4 h, the sample was taken for microbial count. The lowest concentration of each extract completely inhibiting the growth of test organisms in relation to the respective controls was considered as the MIC against that organism. This value is recorded in mg/ml.

### 3.5 HPLC profile determination of the plant extracts

The plant extracts showed inhibitory activity against some of the microorganisms and this caused some futher analysis on the chemical components that could constitute these extracts. Separation of the extracts using high Performance Liquid chromatography was applied (Eloff, 2000). The technique separated the chemical components of the plant extracts into organic and aqueous and the results were recorded as peak areas. The HPLC system was equipped with a model 600 binary pump contriller, a 717 auto sampler and a 996-photodiodide array detector (PAD). The chromatographic separation was performed using a thermo ODS hypersil column connected to a thermo ODS guard column. The auto sampler was set at room temperature.

The mobile phase consisting of 0.1% acetic acid in water (Solvent A) and 0.1% acetic acid in acetonitrile (Solvent B) was run with gradient elution at a flow ratee of 1ml/min. The linear gradient elution was set as follows solvent B increased from 0 to 5% in the first 5 mins, then increased to 20,90 and 100% in 5,10 and 10 min respectively, then

returned to 95% in 5 min and equilibrated for 15 min before the next injection. The injection volume was 20µl. The PAD was set for collection of spectral data from 210 to 350 nm. The chromatograms were obtained at 214nm. Quantification was done using a method based on the peak area of the analytes. The working solutions for standard curves as specific concentrations were prepared by diluting the stock solutions with methanol:water (50:50) containing 0.5% acetic acid (Murphy, 1993).

About 30 mg of the powdered sample was weighed in a 5 ml volmetric flask followed by the addition of 4.5 ml of extraction solvent (methanol:water, 80:20 v/v, containing 0.5% acetic acid). The flask was shaken and placed at room temperture for 30 min, and then sonicated for 15 min. After cooling to room temperature, the mixture was made to volume with the extraction solvent. 1ml of the final mixture was further diluted with the extraction solvent then 20µl of the supernatant was injected into the HPLC for analysis (Eloff, 2004). The experiment was conducted in triplicate.

### 3.6 Data analysis

The anti microbial activity of the various plant parts and extracts against microbes was analysed using analysis of variance following the method of Gomez and Gomez (1984). The software used include Gen stat, SPSS 11.0 and Excel. For the HPLC data was collected and analysed using Waters Millenium software.

# CHAPTER 4

# RESULTS

## 4.1 Extraction and yield obtained

The crude plant extracts obtained from the plant samples used were weighed. This weight was calculated as a percentage of the weight of plant sample used for the extraction. Average percentage yield showed that aqueous methanol was able to extract most crude extract out of the plant samples used followed by water (Table 4.1).

**Table 4.1: Average % yield of crude extracts of different plant parts**

| | | **Average % Yield** | | |
|---|---|---|---|---|
| **Site** | Extract | Roots | Stem | Stem bark |
| **Kitui** | Aq. Methanol | 3.3 | 3.0 | 3.9 |
| | Dichloromethane | 0.7 | 0.1 | 0.7 |
| | Hexane | 0.8 | 0.1 | 0.5 |
| | Water | 1.9 | 1.9 | 2.3 |
| **Ngong** | Aq. Methanol | 3.6 | 3.8 | 3.0 |
| | Dichloromethane | 0.5 | 0.3 | 0.3 |
| | Hexane | 0.6 | 0.3 | 1.3 |
| | Water | 2.9 | 3.5 | 2.8 |

Statistical analysis of the percentage yield data showed that in the Kitui samples there was significant difference ($p<0.01$) between plant parts. The stem bark had the highest yield followed by the roots and then the stem. Orghogonal comparisons showed that

there was no significant difference ($p=0.302$) in percentage crude yield between roots and stem whereas there was a difference ($p=0.007$) in percentage crude yield between roots and stem. It followed that there was a difference in percentage crude yield between stem and stem bark. There was also a significant difference ($p<0.01$) in mean crude yield percentage between the extracts. Aqueous methanol extract mean percentage yield was significantly different ($p<0.01$) from the others. Between the water and dichloromethane extracts there was significant difference ($p<0.01$), but there was no significant difference ($p>0.05$) between dichloromethane and hexane extracts mean percentage yield. On the interaction between extracts and plant parts there was no significant difference ($p=0.332$). this implied that the crude extracts obtained from each plant part by each extract was independent. This further explains that the mean percentage crude yield of each extract was not associated to plant part. This is further explained in the analysis of variance data on table 4.2.

**Table 4.2: ANOVA of source of variation for Ngong and Kitui**

| | **KITUI** | | | **NGONG** | | |
|---|---|---|---|---|---|---|
| **Source of variation** | df | m.s | Fpr | df | m.s | Fpr |
| **Plant part** | 2 | 0.870 | 0.001 | 2 | 0.055 | 0.794 |
| **Extract** | 3 | 6.534 | <.001 | 3 | 7.346 | <.001 |
| **Plant part. Extract** | 6 | 0.121 | 0.332 | 6 | 0.255 | 0.401 |
| **Residual** | 24 | 0.0998 | | 24 | 0.235 | |
| **Total** | 35 | | | 35 | | |

**Df represents the degrees of freedom; ms represents the mean square; Fpr represents the false positive rate.**

The above table also gives us data of samples from Ngong and we can see that there was significant difference ($p<0.01$) in mean percentage yield between extracts which concurs with data from Kitui as the extracts accounted for the highest percentage among the sources of variation. In comparing the extracts, there was a significant difference ($p<0.05$) between aqueous methanol extract and hexane and dichloromethane extract but no difference ($p>0.05$) in with water extract. Also there was no significant

difference ($p>0.05$) in mean percentage yield between dichloromethane and hexane. There was also no significant interaction effect ($p=0.401$) between extracts amd plant parts. Each of the plant extracts obtained was then subjected to screening to assess if it had any inhibitory activity against a number of microorganisms.

## 4.2 Bioassay

The results showed that aqueous methanol and water extracts had activity against two microorganisms ATCC Staphylococcus aureus and clinical isolate Staphylococcus aureus. This was seen by these extracts giving diameter zones of inhibition of more than 6mm long, this is shown in Figure 4.1 below. For the controls that were used the standard drugs also exhibited activity against the microorganisms but the extracting solvents showed no activity.

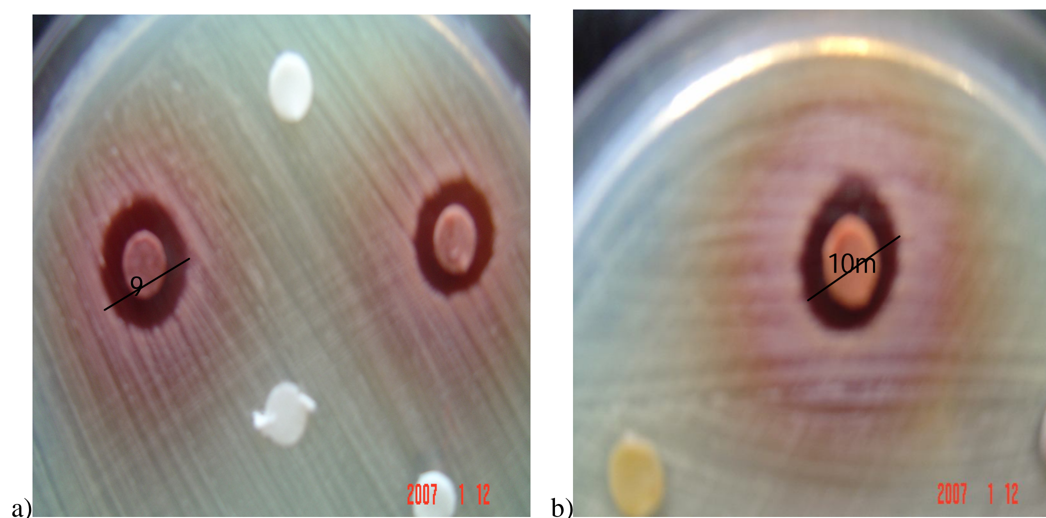



**Figure 4.1: Plant extract show inhibitory activity and gives a clear path around drug disc. The black line indicates the new dics diameter in this case 9 and 10 mm respectively. The first diagram a represents the crude plant extract and the second b represents a positive control. The white spot in a represents extract that lacks in hibitory activity**

The activity of the plant extracts is summarised in the following tables (Table 4.3 and 4.4). the results for Kitui and Ngong crude extract activity against selected microorganisms was similar except in Ngong where the Stem water extract exhibited activity against clinical isolate of *Salmonella typhi*. The standard drugs gentamycine and kanamycine had activity against the bacterial strains while the chloroamphenical had activity against the fungal strains. The discs that contained the extractants only, i.e (hexane, dichloromethane, aqueous methanol and water) did not show any activity against the microorganisms.

**Table 4.3 Antimicrobial activity of plant extracts from Kitui against selected microorganisms**

**ANTIMICROBIAL ACTIVITY IN EXTRACTS**

| | ROOTS | | | | STEM | | | | STEM BARK | | | | EXTRACTANTS | | | | DRUGS | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Microbe** | A | B | C | D | A | B | C | D | A | B | C | D | A | B | C | D | Gen | Kan | Chlo |
| **1** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + |
| **2** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + | + | - |
| **3** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + | + | - |
| **4** | - | - | + | + | - | - | + | + | - | - | + | + | - | - | - | - | + | + | - |
| **5** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + |
| **6** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + |
| **7** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + | + | - |
| **8** | - | - | + | + | - | - | + | + | - | - | + | + | - | - | - | - | + | + | - |

**(+) Inhibiton active inhibition zone diameter > 6mm; (-)No Inhibition inhibition zone diameter=6mm**

**Extracting solvent 20µl A: Hexane, B: Dichloromethane, C: Aqueous methanol, D: Water**

**Standard drugs Gen: Gentamycine, Kan: Kanamycine, Chlo: Chloroamphenical**

**Microbes 1: ATCC *Candida albicans*, 2: ATCC *Escherichia coli*, 3: ATCC *Pseudomonas aeroginosa*, 4: ATCC *Staphylococus aureus*, 5: clin *Cryptococcus neofumonus*, 6: clin. *Microsporum gypseum*, 7: clin. *Salmonella typhi*, 8: clin. *Staphylococus aureus***

**Table 4.4 Antimicrobial activity of plant extracts from Ngong against selected microorganisms**

**ANTIMICROBIAL ACTIVITY IN EXTRACTS**

| | ROOTS | | | | STEM | | | | STEM BARK | | | | EXTRACTANTS | | | | DRUGS | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Microbe** | A | B | C | D | A | B | C | D | A | B | C | D | A | B | C | D | Gen | Kan | Chlo |
| **1** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + |
| **2** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + | + | - |
| **3** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + | + | - |
| **4** | - | - | + | + | - | - | + | + | - | - | + | + | - | - | - | - | + | + | - |
| **5** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + |
| **6** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + |
| **7** | - | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - | + | + | - |
| **8** | - | - | + | + | - | - | + | + | - | - | + | + | - | - | - | - | + | + | - |

**(+) Inhibiton active inhibition zone diameter > 6mm; (-)No Inhibition inhibition zone diameter=6mm**

**Extracting solvent 20µl A: Hexane, B: Dichloromethane, C: Aqueous methanol, D: Water**

**Standard drugs Gen: Gentamycine, Kan: Kanamycine, Chlo: Chloroamphenical**

**Microbes 1: ATCC *Candida albicans*, 2: ATCC *Escherichia coli*, 3: ATCC *Pseudomonas aeroginosa*, 4: ATCC *Staphylococus aureus*, 5: clin *Cryptococcus neofumonus*, 6: clin. *Microsporum gypseum*, 7: clin. *Salmonella typhi*, 8: clin. *Staphylococus aureus***

Further analysis of the crude extracts to assess variation in disc diameter showed that the crude extracts that had an activity had minimal variation on inhibition of the microorganisms (Table 4.5). This was not significantly different ($p>0.05$). Those that did not show activity had a disc diameter of 6 mm thus no variation in inhibitory activity. The table 4.5 gives the variation on inhibitory activity on Staphylococcus aureus since this is the microorganism that showed susceptiblity to the drug. The other microbes show no effect to the crude plant extracts.

**Table 4.5: Variation in inhibitory activity**

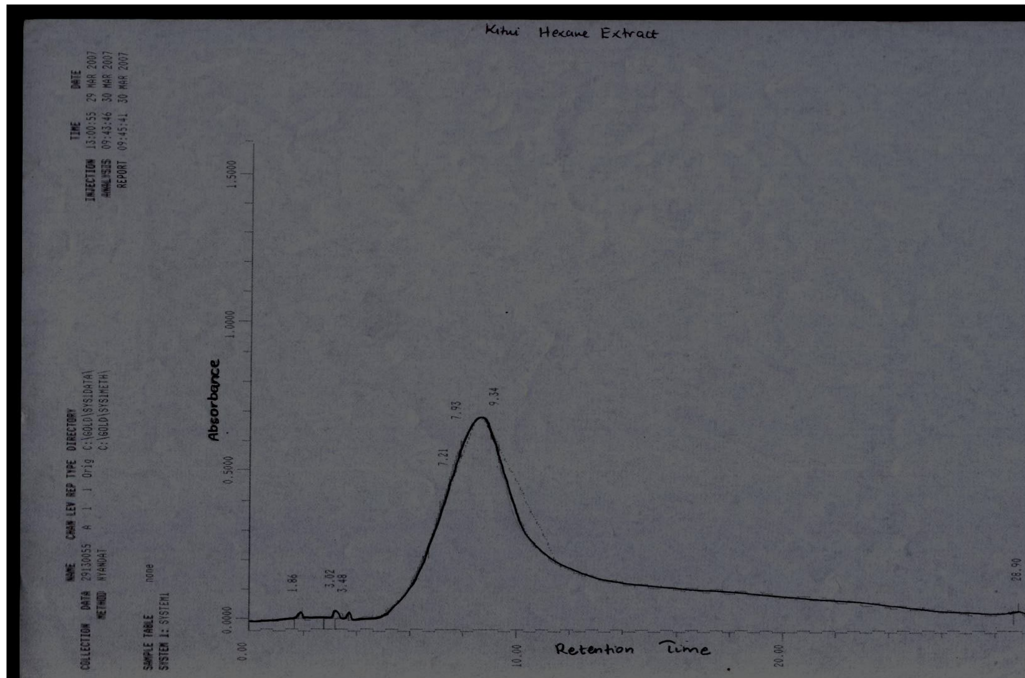
| INHIBITION ZONE DIAMETERS (mm) | | | | |
|---|---|---|---|---|
| **Site** | Plant part | Extract | ATCC *Staphylococuss aureus* | Clin. *Staphylococus aureus* |
| **Kitui** | Roots | Hexane | 6 | 6 |
| | | Dichlorometane | 6 | 6 |
| | | Aq. Methanol | 10.3 | 11 |
| | | Water | 10 | 10 |
| | Stem | Hexane | 6 | 6 |
| | | Dichlorometane | 6 | 6 |
| | | Aq. Methanol | 8 | 8.3 |
| | | Water | 7.3 | 7.3 |
| | Stem bark | Hexane | 6 | 6 |
| | | Dichlorometane | 6 | 6 |
| | | Aq. Methanol | 9.3 | 9.3 |
| | | Water | 8.7 | 9.3 |
| **Ngong** | Roots | Hexane | 6 | 6 |
| | | Dichlorometane | 6 | 6 |
| | | Aq. Methanol | 9.3 | 9.3 |
| | | Water | 9 | 9 |
| | Stem | Hexane | 6 | 6 |
| | | Dichlorometane | 6 | 6 |
| | | Aq. Methanol | 8.3 | 8.3 |
| | | Water | 8.3 | 8.3 |
| | Stem bark | Hexane | 6 | 6 |
| | | Dichlorometane | 6 | 6 |
| | | Aq. Methanol | 7.3 | 7.3 |
| | | Water | 7.3 | 7.3 |

## 4.3 Chromatographic analysis – HPLC

For this analysis all the extracts from Kitui and Ngong were analysed. The stem, bark and root extracts gave almost similar peak areas and thus for this analysis those of the stem were recorded. Under the current chromatographic conditions the organic and aqueous parts of the extracts were satisfactorily separated. The chromatograms show peak areas at varied retention times. The total run time for the chromatograms was 35 minutes and peak areas between 0 – 20 mins represent aqueous components of the extracts while those between 21 – 35 mins represent organic components of the crude

plant extracts. There was significant difference ($p<0.01$) in the results of the chromatograms due to the extracts. However, there was no significant difference ($p>0.05$) in the chromatographic results due to site or plant part. This results confirms those that were obtained from the extraction and the bioassay and we see that extract quantity and activity is not due to site of collection of extract or plant part it is obtained from but more on the crude extract itself.

In Figure 4.2 there is an appearance of three peak areas the first one is between 0 – 4 min, this represents some aqueous components in the hexane extract. The peak height in both the Kitui and Ngong sample is observed at less than 214 nm. A second peak of aqueous compounds that is appearing in this chromatogram at between 12 – 20 min. The last one which is the largest peak area is appearing between 24 – 30 min. This peak areas is observered at 214nm absorbance. The Ngong chromatogram seems to exhibit more clearly the chromatogram expected of a hexane extract than the kitui one due to the presence of two peaks between 0 – 20 min. These peaks may be due to contamination by impurities and the peak is a result of materials that are able to dissolve in the impurities.



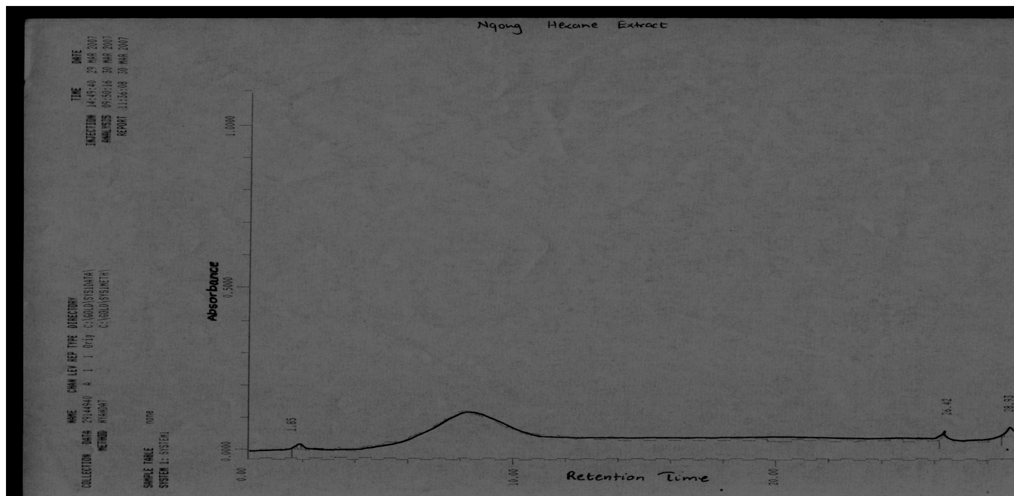




**Figure 4.2: Chromatograms of stem hexane extracts top; Kitui sample, bottom; Ngong sample**

Figure 4.3 shows the dichloromethane extracts between 0 – 4 min there is a small peak that is present in the Kitui sample but not the Ngong sample. Between 10 – 20 min there are two peak areas in the Kitui sample and one peak area in the Ngong sample. This could be a component that is specific to dichloromethane that is a compound that easily dissolves in dichloromethane and allows for its extraction from the plant

material. At 24 – 30 min is a large peak area that represents the organic compounds extracted from the plant material.

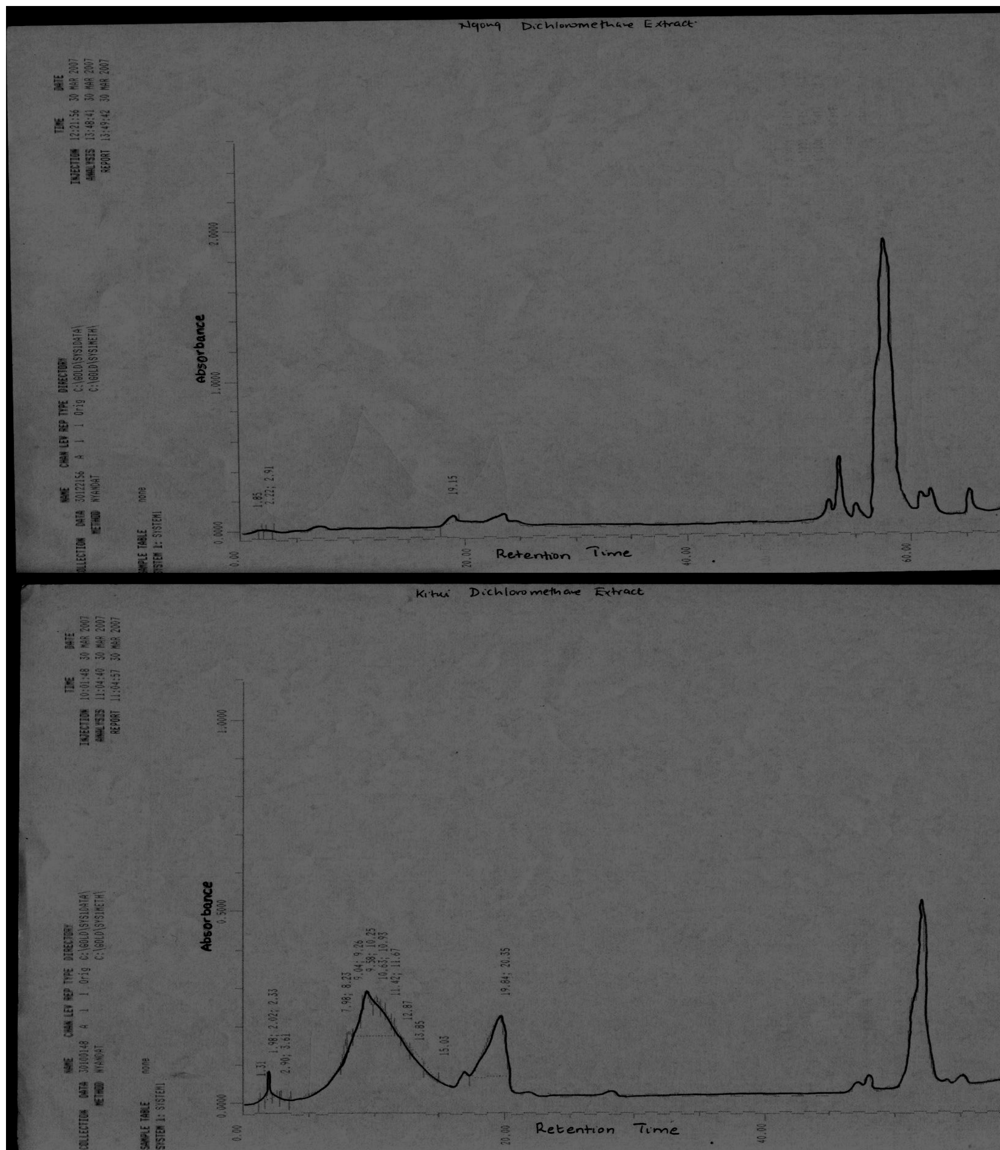



**Figure 4.3: Chromatograms of stem Dichloromethane extracts top;Ngong sample, bottom; Kitui sample**

Prominent increase in the proportion of medium polarity components is seen in Figure 4.4 as compared to the dichloromethane extracts. This is an expected observation. The aqueous components, which are observed in the peak area between 0 – 4 min have also increased in area and they are observed at almost 214 nm. The peak area between 24 –

30 min is small in both the Kitui and Ngong aqueous methanol extract and this shows less amounts of the organic components in the extracts.

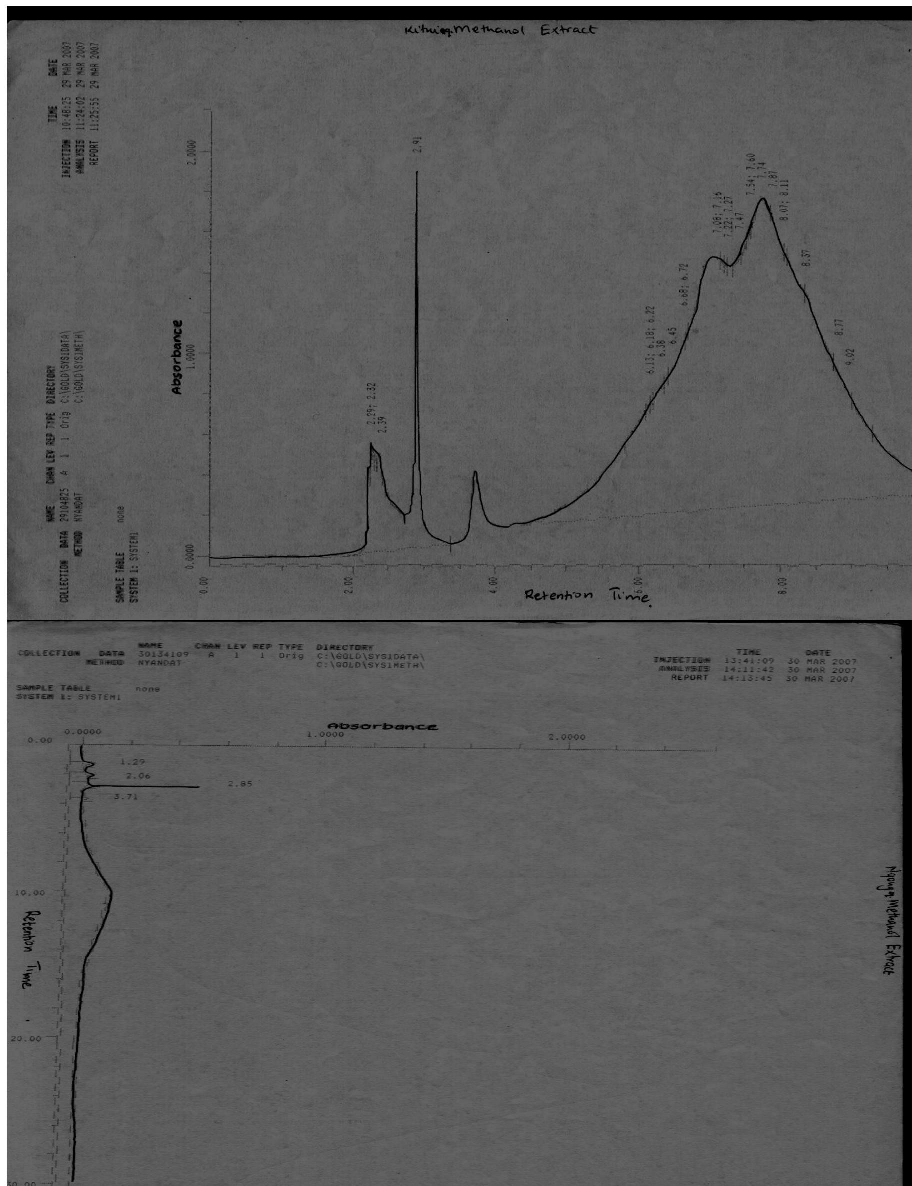
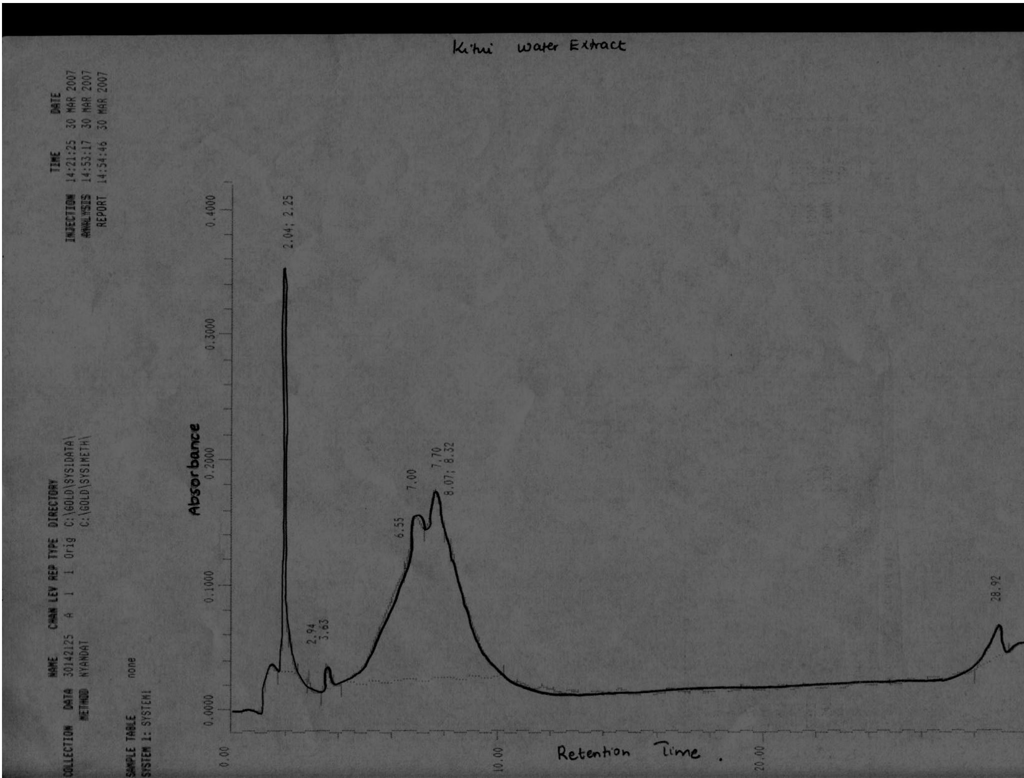



**Fig 4.4: Chromatogram of stem aq. methanol extracts top;Kitui sample, bottom;Ngong sample**

Figure 4.5 shows large peak areas for aqueous components in comparison to the medium and organic ones. The Ngong polar components are less than those of Kitui but more than the aqueous components of Ngong. These extracts show both the

aqueous and organic components and this could suggest that both polar are able to dissolve in water thus are extracted by water. In the Ngong extracts the large aqueous peak area could contain some impurities, which are soluble in water, as this large peak area is not expected.



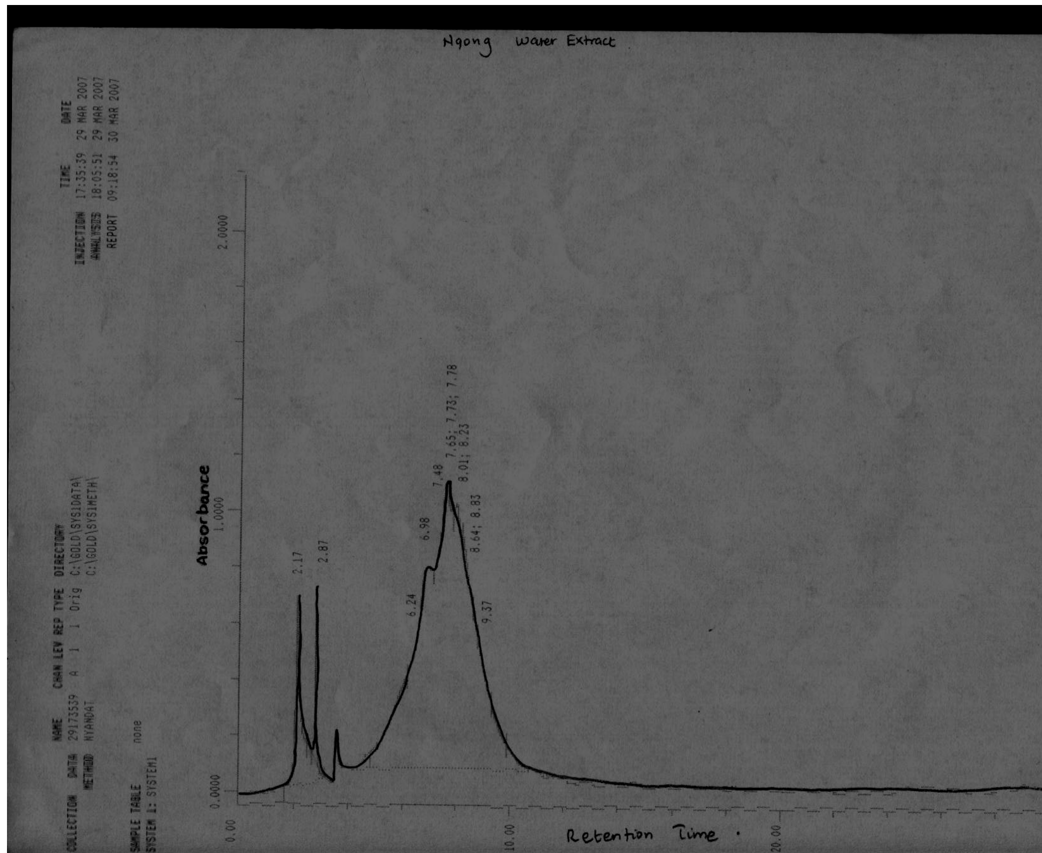



**Figure 4.5: chromatograms of stem water extracts top;Kitui sample, bottom; Ngong sample**

# CHAPTER 5

# DISCUSSION

## 5.1 Yield

*Osyris lanceolata* contains the active ingredient in all the three plant parts used in this experiment. It is seen that crude extracts of aqueous methanol and water are found in all three plant parts with minimal variation in their quantities. The hexane and dichloromethane extracts are also found in all the plant parts but these do not seem to contain the active ingredient. However the fact that all the crude extracts are found in all the three plant samples used is an indication of equal distribution of the secondary metabolites within the samples collected. The average percentages by which the crude extracts are obtained range from 0.1 to 3.9, these are an indicator of what the extracting solvents are able to absorb out of the plant samples and previous studies (Anesin and Prez 1993) show that this is about the expected quantity. Extracting solvents dissolve some specific components out of plant materials and when the solvent is evaporated the weight of the crude extract is not so much. However this small quantities have proven to be of much medicinal value (Elof, 1998).

The difference caused by geographical location on average yield is also observed to be minimal. Both localities were rocky and hilly and the samples were found on the forest margins. There was a slight difference in rainfall between the two areas. Some studies seem to suggest that geographical location has little impact on the quality and quantity of ingredients that plants produce which are used against microorganisms (Anesin and Prez, 1993). However this study cannot conclusively support such contibutions as Kitui and Ngong are almost similar in their geography and more areas need to be covered to be able to make such a conclusion. It is however worth noting that the two areas under

study do not seem to affect much the quantity of crude extract or the activity of the active crude extracts. This work supports traditional use of *Osyris lanceolata* in treatment of ailments as observed in Kitui and Ngong.

The role of extraction solvents cannot be belittled, the average percentage yield analysis, it is the extraction solvent that is seen to have the greatest impact on the amount of crude extract obatined. Plants contain physiologically active compounds and various extracting solvent and solvent mixtures are able to dissolve them out from plant samples (Somken *et al.*, 1999). Extracting solvents are specific on what they can dissolve. A mixture of extracting solvents may be able to disslove more substances and this could explain why aqueous methanol was able to obtain the highest percentage of crude extract. Studies show use of various solvents and solvent mixtures in extraction from plant materials (Shapoval *et al.*, 1994) and the varied amount of crude extracts obtined. There is no standard solvent system that is known to extract the highest amount of crude extract from any given plant material but it is valuable to know what amounts each solvent or solvent mixture is able to extract form a given weight of plant material. This is vital information in case of industrial application of such studies.

### 5.2 Bioassay

The study was designed to obtain preliminary information on the anti microbial activity of *Osyris lanceolata* on certain microorganisms. *Osyris lanceolata* is used in traditional medicine and the reports that it can treat skin disorders (Mohamed and Musya, 2005) especially those caused by *Staphylococcus aureus* were supported by the bioassay results of this study. The results showed that *Osyris lanceolata* water and aqueous methanol crude extracts had activity against both the standard strain and clinical isolates of *Staphylococcus aureus*. Stomach upsets caused by *Salmonella typhi*, which is a

pathogen that causes stomach upsets (Agunn *et al*., 2005), may not be treated by *Osyris lanceolata* crude extracts.

Only the aqueous methanol and water extracts showed activity against the microorganisms while the hexane and dichloromethane crude extracts did not show inhibitory activity. The broad anti microbial action of the aqueous extracts could be ascribed to the anionic components, which are naturally occurring in most plant materials (Darout *et al*., 2000). The other extracts showed no action as anti microbial agents. This may be due to little diffusion properties in the agar medium or effect of the active principal by the steps of extraction methods (El Astal *et al*., 2005). This is a good indicator for traditional healers as in most cases they use aqueous extracts (Eloff, 1998).

*Escherichia coli* showed no response to each of the extracts tested against it. Earlier studies show that *Escherichia coli* have moderate sensitivity to other plants namely *Helichrysum italicum* and *Phytilacca dodecanda* (Cowan, 1999). The observed resistance probably could be due to cell membrane permeability or due to other genetic factors (El Astal *et al*., 2005).

*Pseudomonas aeroginosa*, which is also resistant to different antibiotics, was also resistant to the plant extracts. This bacteria's control is very difficult by therapeutic means (Gislene *et al*., 2000).

*Staphylococcus aureus* was susceptible to the aqueous methanol and water crude extracts in this study. Other studies have also shown susceptiblity of the microbe to different plant extacts (Okemo *et al*., 2001; Madamombe and Afolayan, 2003). This could be due to the fact that the cell wall of gram-positive bacteria is less complex than that of gram-negative bacteria and lack the natural sieve effect against large molecules due to the small pores in their cell envelope (Gould and Booker, 2000).

*Candida albicans* has become resistant to the already limited toxic and expensive anti *Candida* agents available in the market. This factor necessitates the search for new anti fungal agents (Deborah *et al*., 2006). Yet the plant extract did not show activity against *Candida albicans*. It may be speculated here that the extracts would not be useful in the treatment of diarrhea caused by gastrointestinal *Candida* infection and skin lesions due to the fungal infection.

*Microsporum gypsum* did not show any response to any of the extracts of *Osyris lanceolata*, this could suggest to some extent lack of lapachol. This component when available in plant extracts is capable of inhibiting activity of *Micosporum gypsum* (Rasadah *et al*., 1998).

**5.3 Chromatographic analysis**

The chromatographic conditions allowed for the separation of the components of the extracts. For this study we were looking for clusters of either aqueous or organic componets and this was determined by the retention time. The extracts were crude and not pure and thus determinig the specific component of the extract shown by the peaks would not give true information on the extracts. The chromatograms were run for 35 min and typical retention times for aqueous components and organic components is between 0 – 20 min and 21 – 35 min respectively (Chang et al., 2008).

The hexane and dichloromethane extracts showed large peak ares of organic components and small peak ares for the aqueous components. This suggests presence of more organic components than the aqueous components. The aqueous methanol and water extracts showed almost similar amounts of aqueous and organic components. Relating this to the inhibitory activity, the aqueous methanol and water extracts were able to inhibit some of the microorganisms while the hexane and dichloromethane

extracts did not show any inhibitory activity. Seemingly presence of both aqueous and organic components in almost equal amounts has something to do with the inhibitory activity of the aqueous methanol and water crude extracts. Studies have been able to show that single components of extracts may not show inhibitory activity (Jembere and Hassanali, 2001) but that more than one component may exhibit synergistic effect thus causing inhibitory activity. In this case the non-polar components may not have inhibitory activity. However they may be able to activate the aqueous components causing them to exhibit inhibitory activity. Studies on synergistic effects of pure extracts have shown some interesting results and more studies need to be carried out on *Osyris lanceolata* extracts.

The bioassay results showed that aqueous methanol and water extracts contained an active ingredient against *Staphylococcus aureus*. From the chromatograms we could suggest that this active component is polar. This is because the organic component is found in all the extracts but the aqueous one is found in only the aqueous methanol and water extracts in large quantities. The polar component may on the other hand not contain the active ingredient, but may be largely responsible for the inhibitory activity against *Staphylococcus aureus*.

The chromatographic analysis was also able to give us a general view of the number of pure components that could be gotten from the crude extracts if separation and purification was done. This could be seen in the peak areas as smaller peaks were present within the lager peak areas. This provides a good basis for purification studies and HPLC results have normally been used to prepare and carry out purification experiments (Jembere and Hassanali, 2001). The chromatoographic analysis supports the bioassay results and give a basis for more experimental work on the plant *Osyris lanceolata*.

In general, the mechanism by which microorganisms survive the action of antimicrobial agents is poorly understood and remains debatable (Okemo *et al*., 2001). On the other hand, the chemical constituents of these extracts may have a causal role in protecting plants from microbial attack *in vivo*. Nevertheless, at least in part, if not all they should be valuable in the multi-chemical defense against bacterial attack. The compounds responsible for bioactivity are unknown at this point and isolation, purification and identification of bioactive compounds of *Osyris lanceolata* is crucial to a fuller understanding of the observed activity.

# CHAPTER 6

# CONCLUSION AND RECOMMENDATIONS

## 6.1 Conclusion

The study was designed to assess the anti microbial effect if any of *Osyris lanceolata* crude extracts on selected microorganisms. Using five bacteria and three fungi the study was carried out using the sequential extraction and disc diffusion techniques. The disc diffusion technique provides a quick and clear way of analysis of the actual ability of the plant extract to inhibit or not inhibit any microorganism. Further studies were done on the extracts by separation of the crude extracts using the high performance liquid chromatographic technique. The chromatograms were analyzed and the peak areas were categorised as either of polar or non-polar components based on the retention times that they exhibited and absorption was at 214nm with total run time of 35 min. The technique enabes a visual display of presence or absence of the components. This was used as an indicator of the likely nature of the active ingredient as either polar or non-polar(Chang *et al*., 2008).

The results clearly showed that the aqueous methanol and water crude extracts had inhibitory activity against *Staphylococcus aureus*. Hexane, dichloromethane, aqueous methanol and water extracts did not have inhibitory activity on any of the other microorganisms used in the study. The extracts that showed activity were from all three sample plant parts used. This gave an indication of equal distribution of the active ingredient among the sample parts used in the study. Further the variation of activity between Kitui and Ngong samples was minimal and this showed that in this case the geographical location did not affect the activity of the extracts. This is important as from our study plants grown in Ngong or Kitui will not vary in activity and will give an

assurance of high quality medicinal value. The results of the chromatographic runs showed us that all the plant extracts contained non-polar components but the active extracts contained the non-polar and polar components. This showed us that the polar components could be responsible for the inhibitory activity. The non-polar components were not responsible for activity but may have some role in enhancing the action of the polar components.

This study shows that *Osyris lanceolata* aqueous methanol and water crude extracts have inhibitory activity against *Staphylococcus aureus* both clinical and standard strains.

The study provides some basic information on the activity of *Osyris lanceolata* against microorganisms and also background information for further studies on the plant. No doubt, the plant has a great potential as a cash crop and a source of medicine in marginal arid and semi-arid areas of Kenya.

### 6.2 Recommendations

The present study has unraveled some information on activity of *Osyris lanceolata* on microorganisms but at the same time new questions have arisen. For instance, the actual active ingredient and its structure is not known and the mode of action of the plant extracts on the microorganism was beyond the scope of the current study. Thus, the results of this study have opened new areas of research namely:

1. Bioactive compounds of *Osyris lanceolata* should be isolated, purified and fully characterized.
2. Investigation on the mechanism of action of the isolated compound on the microorganism should be revealed whether it has any competitive advantage over the antimicrobial agents currently in use.

3. Pharmacokinetics studies on how fast the isolated compound is metabolized in the liver to harmless product should be carried out.
4. Futher studies on the inhibitory action of the organic extracts using surfactants that assist the extracts to diffuse in aqueous medium.

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