GENETIC VARIATION AND MEDICINAL ACTIVITY IN *OCIMUM GRATISSIMUM* L. OF KENYA

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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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To my three loving families based in Germany, Kenya and Malawi namely the Blin, Matasyoh, and Banda- Kamundi respectively. Your inspirations keep me going.

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
DNA	Deoxyribonucleic acid

ME	β-mercaptoethanol
DTT	Dithiothreitol
CTAB	Cetyl-trimethylammonium bromide
PVPP	Polyvinylpolypyrrolidone
SDS	Sodium dodecyle sulphate
NaCl	Sodium cloride
PVP	Polyvinylpyrrolidone
PCR	Polymerase chain reaction
RFLP	Fragment length polymorphism
DAF	Amplification fingerprinting
AP-PCR	Arbitrarily primed PCR
ISSRs	Simple sequence repeats
AFLP	Amplified fragment length polymorphism
RAPDs	Random amplified polymorphic DNA analysis

ABSTRACT

Hydro-distilled volatile oils from the leaves of *Ocimum gratissimum* L. (Lamiaceae) from 13 populations of different silvicultural zones were evaluated for antimicrobial activity against Gram positive (*Staphylococcus aureus, Bacillus spp*.) and Gram negative (*Escherichia coli, Pseudomonas aeruginosa, Samonella typhi, Klebsiella pneumoniae, Proteus mirabilis*) bacteria and a pathogenic fungus *Candida albicans*. All the essential oils were active to the tested microbials with different strength. The highest antimicrobial activity against Gram positive bacteria (*Staphylococcus aureus*) and Gram negative bacteria (*Pseudomonas aeruginosae* and *Proteus mirabilis*) was observed from the eastern Kenya (Meru) oil. Meru oil was overall the best and its effectiveness was consistent on nearly all the microbes tested. The oil from the plant growing in the coastal region of Kenya (Mombasa) showed the best effect only on Gram negative bacteria (*Escherichia coli* and *Proteus mirabilis*). Both oils (Meru and Mombasa) were dominated by monoterpenes accounting for 92.48 % and 81.37 % respectively. The monoterpene fraction was characterized by a high percentage of eugenol (68.8 %) for Meru oil and 74.10 % for Mombasa oil. The other major monoterpene was methyl eugenol (13.21 %). Camphor (0.95 %) was observed only in the Meru oil. (*Cis*)-Ocimene, (*trans*)-ocimene and β-pinene were present in both Meru and Mombasa oils. The sesquiterpenes present in fairly good amounts in both oils were germacrene D and

(*trans*)-caryophyllene. The minor sesquiterpenes were α-farnesene (0.85 %) and β-bisabolene (0.74 %) which were present in the Meru oil only.

After establishing the best storage conditions and genomic DNA extraction protocol for O. gratissimum L. which was the detergent SDS and the reducing agent dithiothreitol; genetic diversity studies involving twelve populations were perfomed using the amplified fragment length polymorphic (AFLP) markers. Six thousand, two hundred and thirty seven different AFLP bands were generated by the seven primers used. The total number of bands scored per primer ranged from 595 (ACT-CTG) to 1335 (ACT-CAA), with an average of 891 bands per primer. The size of the amplified fragments ranged from 50 to 472 base pairs (bp).

Estimates of Nei's unbiased genetic diversity showed some populations with similar gene diversity (Mill house II and Njoro with H = 0.13; Savona isle, Riat, Chesigei and Mariakani with H = 0.15; Kibarani and Roret with H = 0.10). Kibarani and Roret were the least diverse (H = 0.10) and Kiganjo as the most diverse (H = 0.19). The results showed some great variation in the levels of genetic diversity as also shown by Shannon's information index (I). There was some percentage polymorphic loci correspondence with the diversity estimates. In most cases, populations with high diversity estimate also showed high percentage polymorphic loci. The genetic variation was within populations as opposed to among populations. There was small genetic differentiation in the populations. The F_{ST} was zero.

CHAPTER 1

1.0 General introduction

1.1 Importance of Lamiaceae family

There are many plant species used as herbal medicine. Among them are the plants from the genus *Ocimum* in the family Lamiaceae (the mint family), in which many species are used as medicinal plants (Wren, 1968). The family has about 150 identified species in the tropics and warm temperate regions especially in Africa (Wren, 1968). The leaves are often hairy and posses epidermal glands which secrete volatile oils giving characteristic scents to many of the species
(*Ocimum basilicum,* http://www.ansci.cornell.edu/plants/medicinal/basil.html).

In the plant kingdom, the Lamiaceae family is the most important source of aroma compounds and flavourings
(*Ocimum basilicum,* http://www.ansci.cornell.edu/plants/medicinal/basil.html).
It is a relatively advanced group among the dicotyledons and is a family mainly of herbs and shrubs. Most *Ocimum* species can be grown and found throughout the world. However, they are not native to all areas of the world. For example, *Ocimum micranthum* originates from Central America
(*Ocimum basilicum,* http://www.ansci.cornell.edu/plants/medicinal/basil.html).

The Lamiaceae contain a rich array of secondary metabolites dominated by two classes of compounds; flavonoids and terpenoids, including monoterpenoids, sesquiterpenoids, diterpenoids and iridoid glycosides (The Bear's Byte, 2004). Many of its members and particularly of subfamily Nepetoideae produce essential oils, a complex group of secondary metabolites mainly composed of terpenoids and phenyl-propanoids (The Bear's Byte, 2004). The essential oils found in leaves, seeds, flowers and roots of *Ocimum* species are used as medicines. These oils have been shown *in vitro* to have antibacterial activity against *Staphylococcus aureus*, *Samonella enteritidis* and

Escherichia coli; antiseptic activity against *Proteus vulgaris, Bacillus subtilis* and *Salmonella paratyph*; and antifungal activity against *Candida albicans, Penicillium notatum* and *Microsporeum gyseum* (Nakamura *et al*., 1999; Matasyoh *et al*., 2007).

Oils from some *Ocimum* species have been shown to repel insects and have larvacidal activity against houseflies, blue bottle flies and mosquitoes. The effective concentration of the oil to kill 90 % of the larva ranges from 113-283 ppm (*Ocimum basilicum,* http://www.ansci.cornell.edu/plants/medicinal/basil.html).

From chemical analysis of the plant extracts, it is suggested that camphor, d-limonene, myrcene and thymol are some of the compounds in the oil that may provide the repellent properties. Eugenol and methylchavicol may be responsible for the larvacidal activity (*Ocimum basilicum,* http://www.ansci.cornell.edu/plants/medicinal/basil.html).

The genus *Ocimum* undergoes abundant cross-pollination resulting in large numbers of subspecies and varieties, which differ in essential oil composition and morphological characteristics (Murillo and Vina, 2003).

In *Ocimum*, unlike many other species, the composition, relative amounts of the constituents and the overall oil content may vary greatly from one species to another, one population to another, part of the plant, time of the day and season and even the method of extraction. The composition therefore may be due to genetic and environmental factors that influence genetic expression (Bernath, 1986).

1.2 Ethnobotanical studies

Studies on the utilisation of weed species as sources of traditional medicines in central Kenya identified four families of plants with high percentage of medicinal weed species namely Asteraceae 18.6 %, Solanaceae 9.3 %, Lamiaceae 9.3 % and Papilionaceae 6.6

% for conservation (Njoroge *et al*., 2004). *Ocimum gratissimum L.* of the family Lamiaceae is among the seven weed species identified as important medicinal herbs during a study by Njoroge *et al.*, (2004). The study has provided information on medicinal weed species for possible on-farm conservation hence the choice of *O. gratissimum* L in this study. However, for better and effective management purposes, there is a need for characterisation studies. That is to determine genetic variation within and between populations of this species. The information generated from such studies would be used to select useful genotypes that could be utilised by farmers. Therefore, this research work involved the characterisation of the medicinal activity and genetic variability of the populations of O. *gratissimum* L sampled from Kenya.

1.3 Problem statement

The increase in the cost of pharmaceutical drugs and the dire economic hardships facing most African countries has caused many of them to seriously consider the role of traditional herbal medicine in the provision of affordable health care. Research work on how these medicinal species can be domesticated by intercropping with food crops for sustainability is also important. This will ensure that the farmer has food as well as medicinal herbs from the same farm for use in the home. However, before such studies are effected, it is necessary to characterise the populations of the medicinal herb species. That is, characterisation of the medicinal plant, *O. gratissimum* L. will give important information about genetic variation within and between populations in Kenya. The information generated on genetic variability will be exploited to select useful genotypes that could be utilised as cultivars by the farmers. This would also provide information for efficient conservation and management of the species genetic diversity in Kenya.

1.4 Justification of the study

The economic importance of the *O. gratissimum* lies in its traditional use as a medicinal plant and through the commercial exploitation of its essential oil.

Incorporation of *O. gratissimum* L. in agriculture systems will help bring this medicinal plant close to the farmer or the majority of the population in addition to ensuring its sustainable utilisation.

However, before domestication, it is necessary to characterise the populations of the medicinal species to determine genetic variation within and between populations. The information generated from such studies would be exploited to select useful genotypes that could be utilised as cultivars by the farmers. This would also help to minimise batch to batch variation in extraction of medicinal fractions. It would also provide information for the efficient genetic conservation and management of the species.

1.5 Hypotheses

It is hypothesised that:

1. *Ocimum gratissimum* L. is genetically diverse.
2. There is variation of medicinal activity and medicinal ingredients in *O. gratissimum L*. within and between populations.

1.6 Objectives of the study

1.6.1 Overall objective

The overall objective was to determine the genetic diversity of the medicinal plant *O. gratissimum* L. of Kenya with the view of enhancing the utilisation and conservation of this valuable germplasm.

1.6.2 Specific objectives

1) To determine variation in medicinal activity within and between thirteen populations of *O. gratissimum* L. and identify those populations with enhanced medicinal activities

2) To determine genetic variation within and between thirteen Kenyan populations of the medicinal plant *O. gratissimum* L using molecular markers.

CHAPTER 2

2.0 Medicinal activity of *ocimum gratissimum* L. from kenya

2.1 *Ocimum gratissimum* L. species

Ocimum gratissimum L. belongs to the family Lamiaceae which is the most important source of aroma compounds and flavourings. Many species in this family, just like *O. gratissimum* L. are used as medicine (Wren, 1968).

Ocimum gratissimum L., native to Africa is a shrubby essential oil containing plant with medicinal, antimicrobial and antihelminthic properties (Charles and Simon, 1992). Essential oils are non-nitrogenous compounds, composed of monoterpenoid, sesquiterpenoids and phenyl propanoids (Hegnauer, 1967). They have the characteristic C_{10} and C_{15} structure arising from the linkage of biogenetic isoprene units. They evaporate on contact with air and hence are called volatile oils. They are stored in special organs called glandular trichomes in Lamiaceae (Bruni and Modesnesi, 1983).

The essential oils from the leaves, seeds, flowers and roots of the *Ocimum* spp. have been shown *in vitro* to have antibacterial activity against *Staphylococcus aureus*, *Salmonella enteritidis* and *Escherichia coli*, antiseptic activity against *Proteus vulgaris*, *Bacillus subtilis*, and *Salmonella paratyph* and antifungal activity against *Candida albicans, Penicillium notatum* and *Microsporeum gyseum* (Hegnauer, 1967; Matasyoh *et al*., 2007).

The anti bacterial activity of the essential oil of *O. gratissimum* L. was shown to be more potent than the reference antibiotics in some cases (Ndounga and Ouamba, 1997; Matasyoh *et al.,* 2008) and it was found to completely inhibit *Phytophthora palmivora* which causes black pod disease in cocoa in a similar way mediated by the commercial copper fungicide spray (Awuah, 1993).

However, the essential oils content in different species is influenced by genetic material, culture conditions and environment (Charles and Simon, 1990) and finally by crop and post crop processing (Paakkonen *et al*., 1990) hence the difference in activity.

2.2 Classification and general description of *Ocimum gratissimum* L.

O. gratissimum L. is a large family with over 250 genera and 6700 species (Thorne, 1992; Zomlefer, 1994; Mabberley, 1997). The family Lamiaceae is considered one of the most highly evolved of plant families, at least from the view point of floral structure (Hedge, 1992). It has eight subfamilies namely: Ajugoideae, Chloanthoideae, Lamioideae, Nepetoideae, Scutellarioideae, Teucrioideae, Viticoideae and Pogostemonoideae (Cantino *et al.*, 1992).

Nepetoideae is the largest subfamily in Lamiaceae with 133 genera and 3685 species (Thorne, 1992). This family and is characterised by hexacolpate, tri-nucleate pollen, lack of endosperm and iridoid glycosides, investing embryos, myxocarpy, a high volatile terpenoid content and highly unsaturated seed oils (Erdtman, 1945; Wunderlich 1967; Cantino and Sanders, 1986; Kaufmann and Wink, 1994; Wagstaff *et al*., 1995; Ryding, 1995). It contains most of the aromatic Lamiaceae and is the only one from the eight subfamilies in the new classification that has been divided into tribes i.e. Elsholtzieae, Lavanduleae, Mentheae and Ocimeae (Cantino *et al*., 1992).

O. gratissimum L. belongs to the tribe Ocimeae which contains approximately 35 genera (including genus *Ocimum*) and 1000 species. Members of the tribe are chiefly distributed in tropical and subtropical Asia, Africa and South America (Paton and Ryding, 1998). The generic name *Ocimum* is derived from the word Okimon, the Greek name for an aromatic herb and *gratissimum* means most pleasing. Thus, *Ocimum gratissimum* means the most pleasing aromatic herb. *O. gratissimum* L. has common English names such as African basil, East Indian basil, wild basil, tree basil and clove basil.

O. gratissimum L. native to Africa is a shrubby essential oil containing plant with medicinal, antimicrobial and antihelminthic properties (Charles and Simon, 1992). Mostly, it is a weed of roadsides and wasteland but also important in pastures. It is mainly propagated by seed. It prefers moist and fertile soils during growth, but will tolerate drought after flowering (Swarbrick, 1997).

In Kenya, *O. gratissimum* L. is known as *Kivumbashi* (Kiswahili), *Mukandu* (Kamba), and *Yoiyoia* (Marakwet). It is a much branched shrub of 1-4 meters high. It has a ribbed stem. Leaves are opposite with margins bluntly serrate and aromatic when crushed. They are light green above and pale green below (Figure 1.0).

Figure 1.0: *Ocimum gratissimum* L. leaf branches (Photo by Matasyoh).

Figure 2.0: Flowering *Ocimum gratissimum* L.stalk

(MountainValleyGrowers, http://www.mountainvalleygrowers.com/ocigratissimum.htm)

The inflorescence is terminal, with simple racemes and hermaphrodite flowers in whorls. The calyx is hairy with an oval upper lip and with four triangular teeth. The corolla is whitish or purplish with a four lobed upper lip. The flowers have four stamens each which are unequal and shorter than the style. The apex of the style is 2-fid (FAO, 1986). (Figure 2.0).

2.3 Distribution of *O. gratissimum* L.

Ocimum gratissimum is a widely distributed savannah species occurring on a wide range of soils at altitudes ranging from 100 to 2000 meters and in areas receiving between 500 to 1500 mm and more annual rainfall (FAO, 1986).

The species is widely distributed in tropical and southern Africa and has been recorded in Sierra Leone, Guinea, Ivory Coast, Nigeria, Cameroon, Zaire, Rwanda, Burundi, Sudan, Ethiopia, Somalia, Uganda, Kenya, Tanzania, Malawi, Zambia, Zimbabwe, Botswana, Angola, Namibia and South Africa (Figure 3.0) It is also found in N. Yemen and the Comoro Island. It has been introduced into the West Indies and South America.

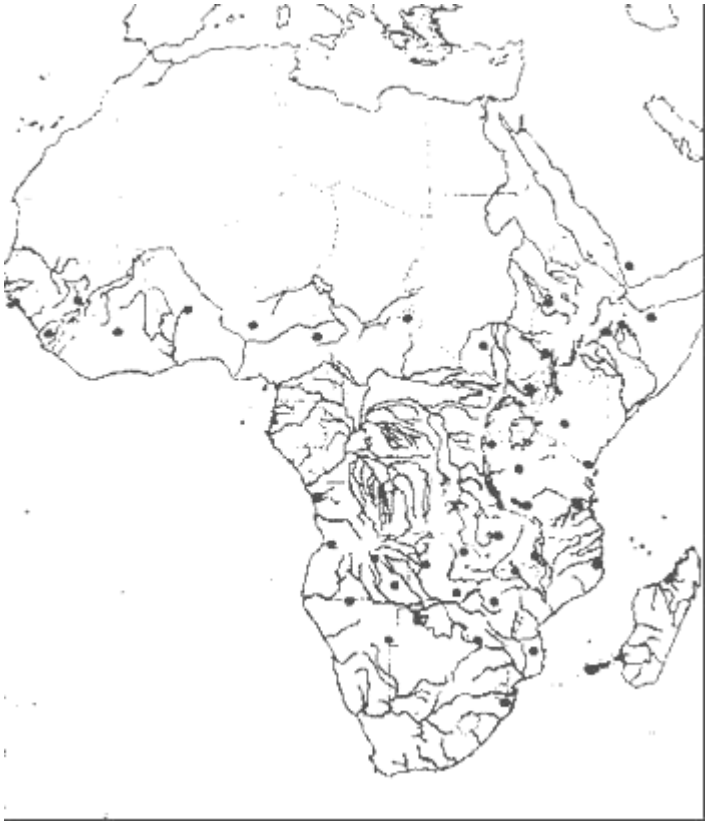


Figure 3.0: Distribution of *Ocimum gratissimum* L. (•) in Africa (FAO, 1986)

2.4 Uses of shrub species in the genus *Ocimum* (basil)

2.4.1 Medicine

Ocimum gratissimum L. is an important herbal medicine not only among Kenyan communities but also in the sub-Saharan Africa. The strongly scented leaves of this plant are rubbed between the palms and sniffed as a treatment for blocked nostrils (Kokwaro, 1993). The leaves are also used for treating abdominal pains, sore eyes, ear infections, coughs, barrenness, fever, convulsions, and as a tooth gargle, regulation of menstruation and as a cure for prolapse of the rectum (Watt and Breyer-Brandwijk 1962; Harjula 1980; FAO, 1986; Kokwaro, 1993). *O. gratissimum* L. has also been observed to have disinfectant and insecticidal properties (FAO, 1986). It is therefore used in making perfumes and in the preparation of some pharmaceuticals (FAO, 1986). *O. gratissimum* is rich in chemotypes such as eugenol and thymol. The young plants of *O. gratissimum* were found to have methyl eugenol as the major compound as opposed to (Z)-b-ocimene in mature ones (Lawrence, 1989; Sanda *et al.*, 1998; Yusuf *et al.*, 1998).

Several species and varieties of plants of the genus *Ocimum* have been reported to yield oil of diverse nature, commonly known as basilica oils. Lemos *et al*., (2005) reported some chemical compounds and active ingredients found in these plants such as eugenol, linalool, methyl cinnamate, camphor and thymol. Various species of *Ocimum* have been reported to have numerous medical uses (Mshana *et al*., 2000). For instance, Holy basil (tulsi: *Ocimum tenuiflorum; O. sanctum*) has been reported (Bhattathiry, http://www.alternative-healthguide.com/ayurveda/tulsi_basil.html) as having the following benefits:

1. Healing Power: The tulsi plant has many medicinal properties. The leaves are a nerve tonic and also sharpen memory. They promote the removal of the catarrhal matter and phlegm from the bronchial tube. The leaves strengthen the stomach and induce copious perspiration. The seed of the plant are mucilaginous.

2. Fever & Common Cold: The leaves of basil are specific for many fevers. During the rainy season, when malaria and dengue fever are widely prevalent, tender leaves, boiled with tea, are preventive against these diseases. In case of acute fevers, a decoction of the leaves boiled with powdered cardamom in half a liter of water and mixed with sugar and milk brings down the temperature. The juice of tulsi leaves can be used to bring down fever. Extract of tulsi leaves in fresh water should be given every 2 to 3 hours. In between, one can keep giving sips of cold water. In children, it is very effective in bringing down the temperature.

3. Coughs: Tulsi is an important constituent of many Ayurvedic cough syrups and expectorants. It helps to mobilise mucus in bronchitis and asthma. Chewing tulsi leaves relieves cold and flu.

4. Sore Throat: Water boiled with basil leaves can be taken as drink in case of sore throat. This water can also be used as a throat gargle.

5. Respiratory Disorder: The herb is useful in the treatment of respiratory system disorder. A decoction of the leaves, with honey and ginger is an effective remedy for bronchitis, asthma, influenza, cough and cold. A decoction of the leaves, cloves and common salt also gives immediate relief in case of influenza. They should be boiled in half a liter of water till only a quarter of the liter water is left and then drunk.

6. Kidney Stone: Basil has strengthening effect on the kidney. In case of renal stone the juice of basil leaves and honey, if taken regularly for 6 months will help expel them via the urinary tract.

7. Heart Disorder: Basil has a beneficial effect on cardiac disease victims and the weakness resulting from them. It reduces the level of blood cholesterol.

8. Children's Ailments: Common pediatric problems like cough cold, fever, diarrhea and vomiting respond favorably to the juice of basil leaves. If pustules of chicken pox delay their appearance, basil leaves taken with saffron will hasten them.

9. Stress: Basil leaves are regarded as an 'adaptogen' or anti-stress agent. Recent studies have shown that the leaves afford significant protection against stress. Even healthy persons can chew 12 leaves of basil, twice a day, to prevent stress. It purifies blood and helps prevent several common elements.

10. Mouth Infections: The leaves are quite effective for the ulcer and infections in the mouth. A few leaves chewed will cure these conditions.

11. Insect Bites: The herb is a prophylactic and also has preventive and curative effects on insect stings or bites. A teaspoonful of the juice of the leaves is taken and is repeated after a few hours. Fresh juice must also be applied to the affected parts. A paste of fresh roots is also effective in case of bites of insects and leeches.

12. Skin Disorders: Applied locally, basil juice is beneficial in the treatment of ringworm and other skin diseases. It has also been tried successfully by some naturopaths in the treatment of leucoderma.

13. Teeth Disorder: The herb is useful in teeth disorders. Its leaves, dried in the sun and powdered, can be used for brushing teeth. It can also be mixed with mustered oil to make a paste and used as toothpaste. This is very good for maintaining dental health, counteracting bad breath and for massaging the gums. It is also useful in pyorrhea and other teeth disorders.

14. Headaches: Basil makes a good medicine for headache. A decoction of the leaves can be given for this disorder. Pounded leaves mixed with sandalwood paste can also be applied on the forehead for getting relief from heat, headache, and for providing coolness in general.

15. Eye Disorders: Basil juice is an effective remedy for sore eyes and night-blindness, which is generally caused by deficiency of vitamin A. Two drops of black basil juice are put into the eyes daily at bedtime.

Apparently, all these benefits as reported by (Bhattathiry, http://www.alternative-healthguide.com/ayurveda/tulsi_basil.html) are also true for *Ocimum gratissimum* L. (Personal communication).

2.4.2 Food

Ocimum is also used as food. Mediterranean and Indochinese cuisines frequently use basil, the former frequently combining it with tomato. Basil is one of the main ingredients in pesto — a green Italian oil-and-herb sauce from the city of Genoa, its other two main ingredients being olive oil and pine nuts. The most commonly used Mediterranean basil cultivars are 'Genovese', 'Purple Ruffles', 'Mammoth', 'Cinnamon', 'Lemon', 'Globe', and 'African Blue'. Chinese also use fresh or dried basils in soups and other foods. In Taiwan, people add fresh basil leaves into thick soups. They also eat fried chicken with deep-fried basil leaves (Gernot Katzer, http://www.uni-graz.at/~katzer/eng/ocim_bas.html).

Basil is sometimes used with fresh fruit and in fruit jams and sauces, in particular with strawberries, but also raspberries or dark-colored plums. Arguably the flat-leaf basil used in Vietnamese cooking, which has a slightly different flavor, is more suitable for use with fruit (Gernot Katzer, http://www.uni-graz.at/~katzer/eng/ocim_bas.html).

2.4.3 Cultural uses of basil

There are many rituals and beliefs associated with basil. The French call basil "*herbe royale*". Jewish folklore suggests it adds strength while fasting. It is a symbol of love in present-day Italy, but represented hatred in ancient Greece. African legend claims that basil protects against scorpions while European lore sometimes claims that basil is a symbol of Satan (New Life Journal.com, 2006).

Holy Basil, also called 'Thulsi' or 'Tulsi', is highly revered in Hinduism and also has religious significance in the Greek Orthodox Church, where it is used to prepare holy water. It is said to have been found around Christ's tomb after His resurrection. The Macedonian Orthodox Church also uses basil (Macedonian) to prepare holy water and pots of basil are often placed below church altars.

In Europe, they place basil in the hands of the dead to ensure a safe journey. In India, they place it in the mouth of the dying to ensure they reach God. The ancient Egyptians and ancient Greeks believed that it would open the gates of heaven for a person passing on (New Life Journal.com, 2006).

In Nakuru, Kenya, every house of a Hindu has *Ocimum gratissimum* L. plant growing either in the compound in a place specially made for it or at the gate (Personal observation, 2005). It was said that this was common everywhere Hindus lived. They worship it because of its healing powers (Personal communication, 2006).

2.5 Antimicrobial Activity of *Ocimum gratissimum* L.

Ocimum gratissimum L activity against some disease causing microbes has been well documented (Matasyoh *et al.*, 2007; Nakamura *et al*., 1999). There are several disease causing microbes like bacteria, fungi and viruses. The following organisms, *Staphylococcus aureus, Salmonella typhi, Escherichia coli*, *Proteus mirabiliss, Bacillus spp, Klebsiella pnuemoniae,* and *Pseudomonas aeruginosae* cause most of the diseases suffered by humans. Hence this has warranted their selection as disease causing microbes to be used in this study.

The microbes involved in this study namely: *Staphylococcus aureus, Salmonella typhi, Escherichia coli*, *Proteus mirabiliss, Bacillus spp*, *Klebsiella pnuemoniae,* and *Pseudomonas aeruginosae* have been shown already to have resistance towards a number of antibiotics (Cornelis, 2008; Matasyoh *et al.,* 2008).

Antibiotic resistance is a growing problem. Some of this is due to overuse of antibiotics in humans. This is especially true in Africa including Kenya where unregulated use of antibiotics is prevalent and to a smaller extent some of it is probably due to the use of antibiotics as growth promoters (Johnson *et al.*, 2006). This in turn has led to the emergence of multi-drug resistant strains of microbes.

2.5.1 *Escherichia coli* (*E. coli*)

Escherichia coli (*E. coli*) is one of the main species of bacteria living in the lower intestines of mammals, known as gut flora. However, the bacteria are not confined to this environment, and specimens have also been located, for example, on the edge of hot springs. The *E. coli* strain O157:H7 is one of hundreds of strains of the bacterium that causes illness in humans (Karch *et al.*, 2005). *E. coli* can generally cause several intestinal and extra-intestinal infections such as urinary tract infections, meningitis, peritonitis, mastitis, septicemia and Gram-negative pneumonia.

2.5.2 *Klebsiella pneumoniae*

Klebsiella pneumoniae is a Gram-negative, non-motile, encapsulated, lactose fermenting, facultative anaerobic, rod shaped bacterium found in the normal flora of the mouth, skin, and intestines (Ryan & Ray, 2004). It is clinically the most important member of the *Klebsiella* genus of Enterobacteriaceae; *K. pneumoniae* can cause bacterial pneumonia. *Klebsiella* ranks second to *E. coli* for urinary tract infections in older persons. It is also an opportunistic pathogen for patients with chronic pulmonary disease, enteric pathogenicity, nasal mucosa atrophy, and rhinoscleroma. Feaces are the most significant source of patient infection, followed by contact with contaminated medical instruments.

2.5.3 *Salmonella enterica* Serovar Typhi

Salmonella enterica Serovar Typhi (historically elevated to species status as *S. typhi*) is the disease agent in typhoid fever. *Salmonella typhi* is a serovar of *Salmonella enterica* (formerly known as *Salmonella choleraesuis*) and the cause of the disease typhoid fever. The organism can be transmitted by the feacal oral route. It is excreted by humans in feaces and may be transmitted by contaminated water, food, or by person-to-person contact (with inadequate attention to personal hygiene).

2.5.4 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram-negative, aerobic, rod-shaped bacterium with unipolar motility (Ryan & Ray, 2004). An opportunistic human pathogen, *P. aeruginosa* is also an opportunistic pathogen of plants (Iglewski, 1996). Upon infection, sweet basil roots secrete rosmarinic acid, a multifunctional caffeic acid ester that exhibits in *vitro*

antibacterial activity against planktonic cells of both *P. aeruginosa* strains with a minimum inhibitory concentration of 3 μg mL^{-1}.

2.5.5 *Proteus mirabilis*

Proteus mirabilis is a Gram-negative, facultatively anaerobic bacterium. *P. mirabilis* is generally susceptible to most antibiotics apart from tetracycline however 10%–20% of *P. mirabilis* strains are also resistant to first generation cephalosporins and ampicillins. It is commonly found in the intestinal tract of humans. *P. mirabilis* is not pathogenic in guinea pigs or chickens. It has the distinction of being the only pathogenic organism with a virulence factor.

2.5.6 *Staphylococcus aureus*

Staphylococcus aureus, the most common cause of *staph infections*, is a spherical bacterium, frequently living on the skin or in the nose of a person, that can cause a range of illnesses from minor skin infections (such as pimples, boils, and cellulitis) and abscesses, to life-threatening diseases such as pneumonia, meningitis, endocarditis, Toxic shock syndrome (TSS), and septicemia. *S. aureus* is a Gram-positive coccus, which appears as grape-like clusters when viewed through a microscope and has largened, round, golden-yellow colonies, often with β-hemolysis, when grown on blood agar plates (Ryan and Ray, 2004). Today, *S. aureus* has become resistant to many commonly used antibiotics. *S. aureus* may occur as a commensal on human skin (particularly the scalp, armpits and groins); it also occurs in the nose (in about 25% of the population) and throat and less commonly, may be found in the colon and in urine. The finding of *Staphylococcus aureus* under these circumstances does not always indicate infection and therefore does not always require treatment (indeed, treatment may be ineffective and re-colonisation may occur). It can survive on domesticated animals such as dogs, cats and horses, and can cause bumblefoot in chickens.

S. aureus can infect other tissues when normal barriers have been breached (e.g. skin or mucosal lining). This leads to furuncles (boils) and carbuncles (a collection of furuncles). In infants *S. aureus* infection can cause a severe disease Staphylococcal scalded skin syndrome (SSSS) (Curran and Salihi, 1980).

S. aureus infections can be spread through contact with pus from an infected wound, skin-to-skin contact with an infected person, and contact with objects such as towels, sheets, clothing, or athletic equipment used by an infected person. *S. aureus* is one of the causal agents of mastitis in dairy cows. Its large capsule protects the organism from attack by the cow's immunological defenses.

2.5.7 *Bacillus* species

Bacillus is a genus of rod-shaped, Gram-positive bacteria and a member of the division Firmicutes. *Bacillus* species are either obligate or facultative aerobes, and test positive for the enzyme catalase (Turnbull, 1996). Ubiquitous in nature, *Bacillus* includes both free-living and pathogenic species. Under stressful environmental conditions, the cells produce oval endospores that can stay dormant for extended periods. These characteristics originally defined the genus, but not all such species are closely related, and many have been moved to other genera (Madigan and Martinko, 2005)

Two *Bacillus* species are considered medically significant: *B. anthracis*, which causes anthrax, and *B. cereus*, which causes a foodborne illness similar to that of *Staphylococcus.* A third species, *B. thuringiensis*, is an important insect pathogen, and is sometimes used to control insect pests. The type species is *B. subtilis*, an important model organism. It is also a notable food spoiler, as is *B. coagulans*.

It is known that the group of compounds in essential oil gives a problem to bacteria hence no development of resistance. The bacteria resistance deals with one component of a compound not as a group of compounds. For instance, *Staphylococcal* resistance to

penicillin is mediated by *penicillinase* (a form of β-lactase) production: an enzyme which breaks down the β-lactam ring of the penicillin molecule ((Jevons, 1961).

Considering that *Ocimum gratissimum* L has been widely used as a medicine in the world in addition to Kenya, it would be important to establish which chemotype of *O. gratissimum* L is basically used in Kenya and to what extent it is effective on different microbes commonly occurring in the society. This work involves isolation of crude material and essential oils from *O. gratissimum* L. leaves collected in Kenya in 13 populations (Figure 6.0) and testing their medicinal activity on broad-spectrum bacteria and fungi which cause most of the common diseases of man.

2.6 Bio-active compounds in plants

Plants contain an imposing variety of carbohydrates, nitrogen and sulphur containing compounds. Some of these, such as the fats and oils are deposited in specialised tissues and cells only at certain times in the life cycle and are important food reserves. Others, such as the waxes and the components of cutin and suberin, serve as protective coats over the plants' exterior. Still, others help perpetuate the species by facilitating pollination or by performing a defence role against other competitive organisms. Besides these compounds, certain plants produce many others, such as rubber without a defined function in the plant; as well as *tetrahydro cannabinol*, the active compound in marijuana (Salisbury and Ross, 1992). These latter compounds are not required for normal growth and development and therefore, they are referred to as secondary compounds or secondary products. Some of the secondary compounds found in plants that have curative properties are alkaloids and flavonoids (Salisbury and Ross, 1992).

2.6.1 Alkaloids

An alkaloid is strictly, a natural occurring amine produced by a plant (Carey, 2006). Many alkaloids have pharmacological effects on humans and other animals (Harborne, 1984). The alkaloids, of which some 5500 are known, comprise of the largest single class of secondary metabolites in plants for example, potatoes (Harborne, 1984). Many alkaloids can be purified from crude extracts by acid base extraction.

Alkaloids include those basic substances, which contain one or more nitrogen atoms, usually in combination as part of a cyclic system. They are usually derivatives of amino acids. They occur in a limited number of plants.

Alkaloids are often toxic to man. However, they are of special interest because of their dramatic physiological and psychological activities in humans and other animals (Salisbury and Ross, 1992). Most alkaloids are crystalline but a few (e.g. nicotine) are liquid at room temperatures and are usually colourless. A simple but by no means infallible test for alkaloids in fresh leaves or fruits is the bitter taste they impart to the tongue (Salisbury and Ross, 1992). The alkaloid quinine, for example, is one of the bitterest substances (Salisbury and Ross, 1992).

Some alkaloids have remarkable structural similarities with neurotransmitters in the central nervous system of humans, including dopamine, serotonin and acetylcholine (Lewis *et al*., 1977). The amazing effect of these alkaloids on humans has led to the development of powerful pain-killer medications, spiritual drugs, and serious addictions by people who are ignorant of the properties of these powerful chemicals (Lewis *et al*., 1977). For instance, the leaves, stem, root and fruits of *Datura* contain a battery of tropane alkaloids, the most potent of which are atropine, hyoscyamine and scopolamine (Lewis *et al*., 1977). These alkaloids affect the central nervous system, including nerve cells of the brain and spinal cord which control many direct body functions and behaviour of men and women (Lewis *et al*., 1977).

Others are mainly aromatic compounds, for example, the tropolone alkaloids of the autumn *Crocus* bulbs (Harborne, 1984). The alkaloids, for example morphine have a narcotic action, *reserpine* is a tranquilliser and *strychnine* is a nerve stimulant (Ikan, 1992).

The physiological roles of alkaloids in the plants that manufacture them are unknown. It has been suggested that they perform no important metabolic function, being merely by-products of other more important pathways (Salisbury and Ross, 1992). Nevertheless, several cases are known in which they are of ecological importance, providing some survival value to the plant (Robinson, 1979). For example, plants containing certain alkaloids are avoided by grazing animals and leaf-feeding insects. Others are used by danaid butterflies as substrates for synthesis of courtship pheromones (Keeler, 1975).

2.6.2 Flavonoids

Flavonoids are 15-carbon compounds generally distributed throughout the plant kingdom (Harborne, 1976; Hahlbrock, 1981) fulfilling many functions including producing yellow or red/blue pigmentation in flowers and protection from attack by microbes and insects (Harborne, 1965). More than 2000 flavonoids have been identified from plants. The widespread distribution of flavonoids, their variety and their relatively low toxicity compared to other active plant compounds (for instance alkaloids) mean that many animals, including humans, ingest significant quantities in their diet. Flavonoids have been found in high concentrations in butterflies and moths sequestered from dietary intake at the larval stage and then stored in adult tissues (Harborne, 2001).

Flavonoids are mostly known for their antioxidant activity (Meyers *et al.,* 2003). They show anti-allergic, anti-inflammatory, antimicrobial and anti-cancer activity (Yamamoto and Graynor, 2001). Consumers and food manufacturers have become interested in flavonoids for their medicinal properties, especially their potential role in the prevention

of cancers and cardiovascular disease (Meyers *et al.*, 2003). The citrus bioflavonoids include hesperidin, quercetin, rutin (a glycoside of quercetin) and tangeritin. In addition to possessing antioxidant activity and an ability to increase intracellular levels of vitamin C, rutin and hesperidin exert beneficial effects on capillary permeability and blood flow. They also exhibit some of the anti-allergy and anti-inflammatory benefits of quercetin. Quercetin can also inhibit reverse transcriptase, part of the replication process of retroviruses (Spedding *et al.*, 1989). Green tea flavonoids are potent antioxidant compounds, which reduce incidence of cancer and heart diseases. The major flavonoids in green tea are catechins (McKenna *et al.*, 2000). Grape skins also contain significant amounts of flavonoids as well as other polyphenols (Kenedy *et al.*, 2002).

Flavonoids absorb visible light and thus give these plants colour. There are three groups of flavonoids which are of particular interest in plant physiology. These are the anthocyanins, the flavonols and the flavones.

The anthocyanins are coloured pigments that commonly occur in red, purple and blue flowers and are also present in various other plant parts, such as certain fruits, stems, leaves and even roots. Most fruits and many flowers owe their colours to anthocyanins, although some, such as tomato fruits and several yellow flowers are coloured by carotenoids. Anthocyanins found in common foods protect against a variety of oxidants through a number of mechanisms. For example, red cabbage anthocyanins protect animals against oxidative stress from toxin paraquat (Igarashi *et al.*, 2000). Cyanidins found in most fruit sources of anthocyanins have been found to function as a potent antioxidant *in vivo* in recent Japanese animal studies (Tsuda, 2000). Anthocyanins have also an important function in flowers of attracting birds and bees to facilitate pollination (Harborne, 1976). They also play a role in disease resistance (Salisbury & Ross, 1992).

The flavonols and flavones are closely related to the anthocyanins, except that they differ in the central oxygen containing ring structure. Most of them are yellowish or

ivory-coloured pigments and contribute to the colour of flowers. These molecules are also widely distributed in leaves where they function as feeding deterrents (Salisbury and Ross, 1992).

Flavonols are generally found in woody angiosperms, for example, *Sambucus nigra.* Some of the common flavonols include kaemferol, myricetin and quercetin. Flavonol rich foods provide some protection against thrombosis (Cabrera *et al.,* 2002). Flavonols also reduces the risk for pancreatic cancer (Kono *et al.,* 1988). They also protect against coronary heart diseases (Nutrition Research Newsletter, 1997).

Flavones are generally found in herbaceous families, for example, Lamiaceae, Umbelliferae, and Compositae. Natural flavones include apigenin, luteolin and tangeritin. It is the yellow pigment found in plants. Apigenin is a flavone with potential chemopreventive actions (Dross *et al.,* 2003). Luteolin has been shown in animal studies to have anti-cancer, anti-inflammatory, anti-allergic effects and ant-asthma (Hiroshi *et al.,* 2002).

2.6.3 Essential oil / Terpenes

Essential oils can be defined as "volatile organic constituents of fragrant plant matter". They are extracted from plants by steam distillation, cold pressing or by using organic solvents. Such oils were originally called essential because they were thought to represent the very essence of odour and flavour. Essential oils are concentrated in various parts of the plant, for example, cinnamon oil in bark, garlic oil in bulbs, peppermint oil in leaves, fennel oil in seeds *etcetera.*

Essential oils are secondary metabolites. They have related chemical structures. Some are hydrocarbons, frequently terpenes. Others are oxygenated compounds such as alcohols, ketones, esters *etcetera.*

Terpenes in essential oils are usually monoterpenes consisting of two isoprene units or, less commonly, sequiterpenes consisting of three isoprene units.

Terpenes are a large and varied class of hydrocarbons, produced primarily by a wide variety of plants, particularly conifers, though also by some insects such as swallowtail butterflies, which emit terpenes from their osmeterium. They are the major components of resin, and of turpentine produced from resin. The name "terpene" is derived from the word "turpentine". When terpenes are modified chemically, for example, by oxidation or rearrangement of the carbon skeleton, the resulting compounds are generally referred to as terpenoids. Some authors will use the term terpene to include all terpenoids.

Terpenes and terpenoids are the primary constituents of the essential oils of many types of plants and flowers.

An essential oil is a concentrated, hydrophobic liquid containing volatile aromatic compounds from plants. It can be extracted by steam distillation. Other extraction processes to obtain aromatic plant compounds include expression, or solvent extraction. Essential oils are used in perfumery, aromatherapy, cosmetics, and incense, for flavouring food and drink, medicine and household cleaning products (Schnaubelt, 1999). They are valuable commodities to the fragrance and flavouring industries.

Essential oil is also known as volatile oil and ethereal oil. It may also be referred to as "oil of" the raw plant material from which it was extracted, such as *oil of clove*. The term essential is intended to indicate that the oil is the fragrant essence of the plant from which it is extracted and not in the more common sense of being indispensable (Sellar, 2001). It is not to be confused with essential fatty acids.

2.6.3.1 Function of plant essential oils/ terpenes

Medical use of vegetable oils has a long and distinguished history. Many oils that are used medicinally are essential oils, which are distilled rather than pressed or otherwise

extracted. Medical properties claimed by those who sell medicinal oils vary from skin treatments to remedies for cancer, and are often based on historical use of these oils for these purposes (Sellar, 2001).

The therapeutic action of essential oil is attributed to the naturally occurring chemicals within the plant. The essential oil of aromatherapy are those of proven value for the relief of stress related and certain other conditions amenable to this form of alternative and supportive holistic medicine (William, 1996). In aromachology, for example linalool, a monoterpene compound found in essential oil of many plant species is traditionally used as sedative. It has been demonstrated to be an anticonvulsant in several experiment models (Lahlou and Berrada, 2001).

Interest in such uses of essential oils has enjoyed a revival in recent decades with popularity of aromatherapy, in which oils are heated and volatilised.

Essential oils also have important functions like protection against predators (micro-organisms, fungi, insects and herbivores), attraction of pollinators, inhibition of germination and promotion of plant growth (Cowan, 1999).

The fragrance of a scented flower is due to the vapours of essential oils released from the specialised oil glands, the purpose of which is to attract pollinating insects (Cowan, 1999). Essential oils produced by plants which give fragrance when stroked, such as the mints, may in this way use the oil to deter hungry herbivorous animals and phytophagous insects whose noses and antennae will give warning of the proximity of inedible leaves (Heath, 2002). Some leaves, roots and barks have smelly molecules that are unappetizing or sickening and inhibit the growth of neighbouring plants, moulds and fungus (Heath, 2002). This is referred to as allelopathy.

Essential oil stored in the heartwood of oil bearing trees, such as cedar wood, rosewood and sandalwood, may preserve the integrity of the trunk against the ravages of

microorganisms and insects, in so doing maintaining the leaves at height sufficient to receive maximum sunlight (Pichersky and Gershenzon, 2002).

Essential oils also have commercial application not only in pharmaceutical and bio-medical fields, but are part of dyes, flavours, spices, teas, fragrances and insecticides (Verpoorte *et al.*, 2002). They have also been used to extend the shelf life of food suggesting the inhibition of bacteria, fungi and yeast.

2.6.3.2 Biosynthesis of essential oil/ terpenes

Terpenes are derived biosynthetically from units of isoprene, which has the molecular formula C_5H_8. The basic molecular formulas of terpenes are multiples of that; $(C_5H_8)_n$ where n is the number of linked isoprene units. This is called the *isoprene rule* or the C_5 *rule*. The isoprene units may be linked together "head to tail" to form linear chains or they may be arranged to form rings.

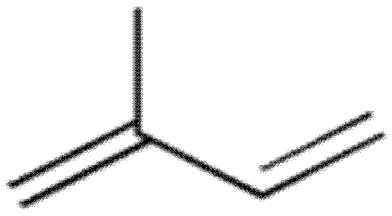
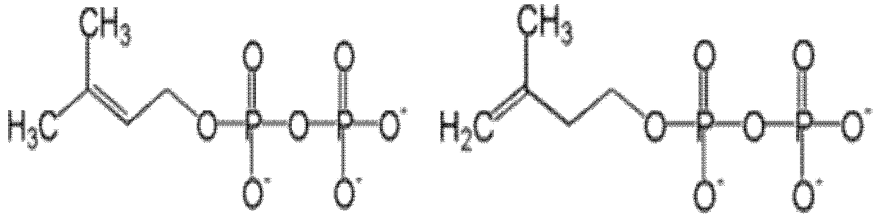


Figure 4.0: Isoprene

One can consider the isoprene unit as one of nature's preferred building blocks. Isoprene itself does not undergo the building process, but rather activated forms, isopentenyl pyrophosphate (IPP or also isopentenyl diphosphate) and dimethylallyl pyrophosphate (DMAPP or also dimethylallyl diphosphate), are the components in the biosynthetic pathway. IPP is formed from acetyl-CoA via the intermediacy of mevalonic acid in the β-hydroxy-β-methylGluteryl-CoA (HMG-CoA) reductase pathway. An alternative, totally unrelated biosynthesis pathway of IPP is known in some bacterial groups and the plastids of plants, the so-called MEP (2-Methyl-D-erythritol-4-phosphate)-pathway, which is initiated from C5-sugars. In both pathways, IPP is isomerized to DMAPP by the enzyme isopentenyl pyrophosphate isomerase (Simpson *et al.,* 1975).



Dimethylallyl pyrophosphate Isopentenyl pyrophosphate

Figure 5.0: Dimethylallyl pyrophosphate and Isopentenyl pyrophosphate

As chains of isoprene units are built up, the resulting terpenes are classified sequentially by size as hemiterpenes, monoterpenes, sesquiterpenes, diterpenes, sesterterpenes, triterpenes, and tetraterpenes.

Monoterpenes consist of *two isoprene* units and have the molecular formula $C_{10}H_{16}$. Monoterpene alcohol is also known as geraniol; the geranyl- prefix indicates two isoprene units.

Sesquiterpenes consist of *three isoprene* units and have the molecular formula $C_{15}H_{24}$. Sequiterpene alcohol is also known as farnesol; the farnesyl- prefix indicates three isoprene units (Chemical compound, 2008).

Synthetic variations and derivatives of natural terpenes and terpenoids also greatly expand the variety of aromas used in perfumery and flavors used in food additives (Schnaubelt, 1999).

2.6.3.3 Mode of action of essential oils/terpenes

Essential oil components reaching the cell in very low concentrations according to their physico-chemical properties and molecular shapes bind to special areas of cell membranes evoking specific effects by influencing the enzymes, carriers, ion channels and receptor proteins that are localised in these areas (Buchbauer, 1993). For instance, studies on skeletal and smooth muscle contraction have shown varied degree of specific action by depressing the contraction of the smooth muscle of the various segments of the intestines (Koul *et al*, 2004). For example, the essential oil of *Origanum compactum* Berth (Lamiaceae) showed insecticidal effect on *Drosophila melanogaster* adults. It also showed larvacidal effect on *Culex pipiens*, toxic effect on *Artemia salina* larvae. However, the potency of the pharmacological effect varied significantly (Lahlou and Berrada, 2001; Koul *et al.*, 2004).

Essential oils have also significant effects on microbes. Anti-microbial action of the monoterpenes is through diffusion into and damage of the cell membrane structures (Sikkema *et al.*, 1995). Monoterpenes are lipophilic and thus, will preferentially partition from an aqueous phase into membrane structures. This causes expansion of the membrane, increased fluidity disordering of the membrane structure and inhibition of

membrane embedded enzymes (Sikkemma *et al*., 1995). The report attributes this action to the interaction of the functional groups (phenol especially) with the microbial envelop while Helander *et al*., (1998) found that essential oils cause a deterioration of the cytoplasmic membrane. However, Bammi *et al.*, (1997) demonstrated an action of the essential oil of thyme on the cell life cycle. There were profound lesions in different micro-organisms (*Escherichia coli, Bacillus subtilis* and *Saccharomyces cereviseae;* Bammi *et al.*, 1997). Linalool inhibited spore germination and fungal growth. The inhibition of sporulation appeared to arise from respiratory suppression of arial mycelia (Sikkema *et al*., 1995).

Anti-microbial activity of volatile compounds results from the combined effect of direct vapour absorption by micro-organisms and indirect effect through the medium they absorbed the vapour (Moleyar and Narasimham, 1986). The vapour absorption by microorganisms is determined by their membrane permeability. Gram-negative micro-organisms are less susceptible to essential oil than Gram-positive bacteria because they possess an outer membrane surrounding the cell membrane (Ratledge and Wilkinson, 1988) which restricts diffusion of hydrophobic compounds through its lipopolysaccharide covering (Vaara, 1992).

Many essential oils are included in pharmacopoeias as having antimicrobial activity, including lemon oil, clove oil, thyme oil *etcetera* (Cowan, 1999). The antimicrobial under investigation in this project are plant essential oils.

2.6.3.4 Anti-microbial properties of essential oil

Tea tree oil is an example of an essential oil with known antimicrobial properties. It is obtained by steam distillation of the leaves of *Melaleuca alternifolia,* a tree which is a member of the family Myrtaceae which is native to Australia. It is known to be antibacterial, antifungal, and antiviral, anti- inflammatory and analgesic.

The major antimicrobial components in tea tree oil have been determined. These include terpinen-4-ol, a monoterpenoid alcohol, á- and ã-terpinenes and various other compounds. Terpinen-4-ol has been found to be more active in its pure form than in tea tree oil. It is more active against Gram positive bacteria than Gram negative (Cox *et al*., 2000). The main mode of action is disruption of the cell membrane which causes leakage of potassium ions (Cox *et al*., 2001). Other actions include inhibition of respiration in *E. coli, S. aureus* and *C. albicans*. There are numerous other examples of essential oils which have been shown to have antimicrobial activity.

For example, Origanum oil used as a food flavouring agent possess a broad spectrum antimicrobial activity due to in part to its high content in phenolic derivatives such as carvacrol and thymol (Manohar *et al*., 2001).

The components with phenolic structures are highly active against micro-organisms. Members of this class are known either to be bactericidal or bacteriostatic agents depending upon the concentrations (Pelczar *et al.*, 1993).

Previous studies have shown that the essential oils (EO) of four *Ocimum* species grown in Rwanda, i.e. *O. canum, O. gratissimum, O. trichodon* and *O. urticifolium*, display antimicrobial activity (Janssen *et al.* 1989). It has been reported that the volatile oil of this plant contains mostly phenols, particularly thymol (Oliver 1960; Sainsbury and Sofowora 1971) and that these are probably responsible for its reported antimicrobial action.

The essential oil (EO) of *Ocimum gratissimum* inhibited *Staphylococcus aureus* at a concentration of 0.75 mg/ml (Filho *et al.,* 1999). The minimal inhibitory concentrations (MICs) for *Shigella flexineri, Salmonella enteritidis, Escherichia coli, Klebsiella* sp., and *Proteus mirabilis* were at concentrations ranging from 3 to 12 mg/ml. The compound that showed antibacterial activity in the essential oil of *O. gratissimum* was identified as eugenol (Filho *et al.,* 1999).

Cryptococcal infection had an increased incidence in last years due to the explosion of acquired immune deficiency syndrome epidemic and by using new and effective immunosuppressive agents. All the extracts of *O. gratissimum* studied showed activity *in vitro* towards *C. neoformans* (Silva *et al.*, 2005). Based on the minimal inhibitory concentration values the most significant results were obtained with chloroformic fraction and eugenol. It was observed that chloroformic fraction inhibited 23 isolates (92%) of *C. neoformans* at a concentration of 62.5 µg / ml and eugenol inhibited 4 isolates (16%) at a concentration of 0.9 µg / ml (Silva *et al.*, 2005).

2.7 Other antimicrobial compounds

Any substance that kills or inhibits the growth of microbes such as bacteria (antibacterial activity), fungi (antifungal activity), viruses (antiviral activity) or a parasite (anti-parasitic activity) is called an antimicrobial. In addition to essential oils which have antimicrobial activities, others include: cations and elements, nitrofuranes, and the antibiotics.

2.7.1 Cations and elements

Many heavy metal cations such as Hg^{2+}, Cu^{2+} and Pb^{2+} have antimicrobial activities, but are also very toxic to other living organisms, thus making them unsuitable for treating infectious diseases (Levy, 1994).

Ionic silver ions are an excellent antimicrobial, with relatively low toxicity against non-target organisms. The antimicrobial properties of silver depend upon silver binding within the cell. Once inside the cell, silver ions begin to interrupt critical functions of the micro organism. A prime molecular target for the silver ion are enzymes. Enzymes become denatured because of conformational changes in the molecule that result from silver ion binding. Many of the enzymes that silver ions denature are necessary in the cellular generation of energy.

2.7.2 Nitrofuranes

The nitrofuranes have a common core furane substituted in position five of its structure by an essential function nitro for the antibiotic activity (Levy, 1994).The nitrofuranes acquire their antibacterial activity after the enzymatic reduction of their function nitro, catalysed by bacterial reductases which ensures their specificity of actions.

2.7.3 Antibiotics

Antibiotics are generally used to treat bacterial infections. The toxicity to humans and other animals from antibiotics are generally considered to be low. However, prolonged use of certain antibiotics can decrease the number of gut flora, which can have a negative impact on health.

Antibiotics are among the most commonly used drugs. They are also among the drugs commonly misused by physicians, for example, the usage of antibiotic agents in viral respiratory tract infection. This injudicious use of antibiotics has been the emergence of antibiotic-resistant pathogens, resulting in the emergence of serious threat to global public health. Most antibiotics are bacteriostatic in clinical use, although some may act as bactericides, particularly when administered in large quantities (Milton, 1990).

The former action inhibits the growth of pathogen and relies upon the immune system to immobilise any remaining bacteria. For example, chloramphenical, tetracycline *etcetera* act against bacteria by interfering with their ability to synthesise the proteins essential for their body components especially the cell wall (Milton, 1990). This leads to suppression of growth, interference with cell multiplication and ultimate death (Milton, 1990).

On the other hand, bacteriostatics like penicillins interfere with the ability of the bacterial cell to form a wall leading to the inevitable cellular disruption and death (Milton, 1990).

2.8 Biological assays

Biological assay or bioassay is a type of scientific experiment typically conducted to measure the effects of a substance on a living organism. Bioassays may be qualitative or quantitative. Qualitative bioassays are used for assessing the physical effects of a substance that may not be quantified, such as abnormal development or deformity. Quantitative bioassays involve estimation of the concentration or potency of a substance by measurement of the biological response that it produces (Joshua, 1992).

Bioassays are essential in the development of new drugs and monitoring pollutants in the environment. Many new remedies have been introduced on the basis of bioassay that is later replaced by chemical quantification when the active principle of the remedy has been characterised (Brander and Pugh, 1971). They are often the only practical means of standardizing antibiotic substances. The bioassay is measured by its effect on a biological system. These biological systems are usually microbes (unicellular or multi-cellular). Many medicinal agents, particularly those derived from natural products may consist of a mixture of biologically active substances (Joshua, 1992).

This work reports on the antibacterial and antifungal activities of essential oils of *Ocimum gratissimum* L. from Kenya using diffusion techniques on solid media.

2.9 Antimicrobial activity of the methanolic crude extract and essential oil extract from *O. gratissimum*

2.9.1 Materials and methodology

2.9.1.1 Preparation of methanolic crude extract from *O. gratissimum* L.

The fresh leaves of *O. gratissimum* L. from 13 different populations in Kenya (Figure 6.0) were collected. The leaves were dried in the shade (in a dark cupboard) to constant weight i.e. until the weight of the leaves could not change anymore. They were ground into powder using a grinding machine. Five grams of the ground sample from each population was soaked in 400 ml methanol for five days. After five days, the extract was filtered using a filter paper. The methanol was evaporated by leaving the extract stand for a week in a flask covered with foil paper which had perforations. The remaining crude extract was used for bio assays.

2.9.1.2 Extraction of essential oil from *O. gratissimum* L.

During the pre-liminary studies, fresh leaves of *Ocimum gratissimum* L. from Nakuru, Manyani area only. In the main study involving the antimicrobial activity of the essential oil extract of *O. gratissimum* from the 13 population of Kenya, fresh leaf samples were collected from 13 populations distributed throughout Kenya (Figure 6.0). Collection of the fresh leaf samples was done in Western Province, Rift Valley Province, Central

Province, Nyanza province and the Coast Province thereby sampling different silvicultural zones across the ecological amplitude of the species in Kenya. Collection sites were chosen mainly along the road where the herb is regularly found though a few collections were also made from cultivated fields. Sample collection details are found in appendix I. In cases where the herb could not be located easily, consultation with the local people around the villages in each district was done and sometimes the Kenya Agriculture Research Institute staff was consulted. Collection of the samples, which included leaves and seeds of the individual plant, was done during the flowering season in August, 2005. Collection was done only on plants that had not flowered.

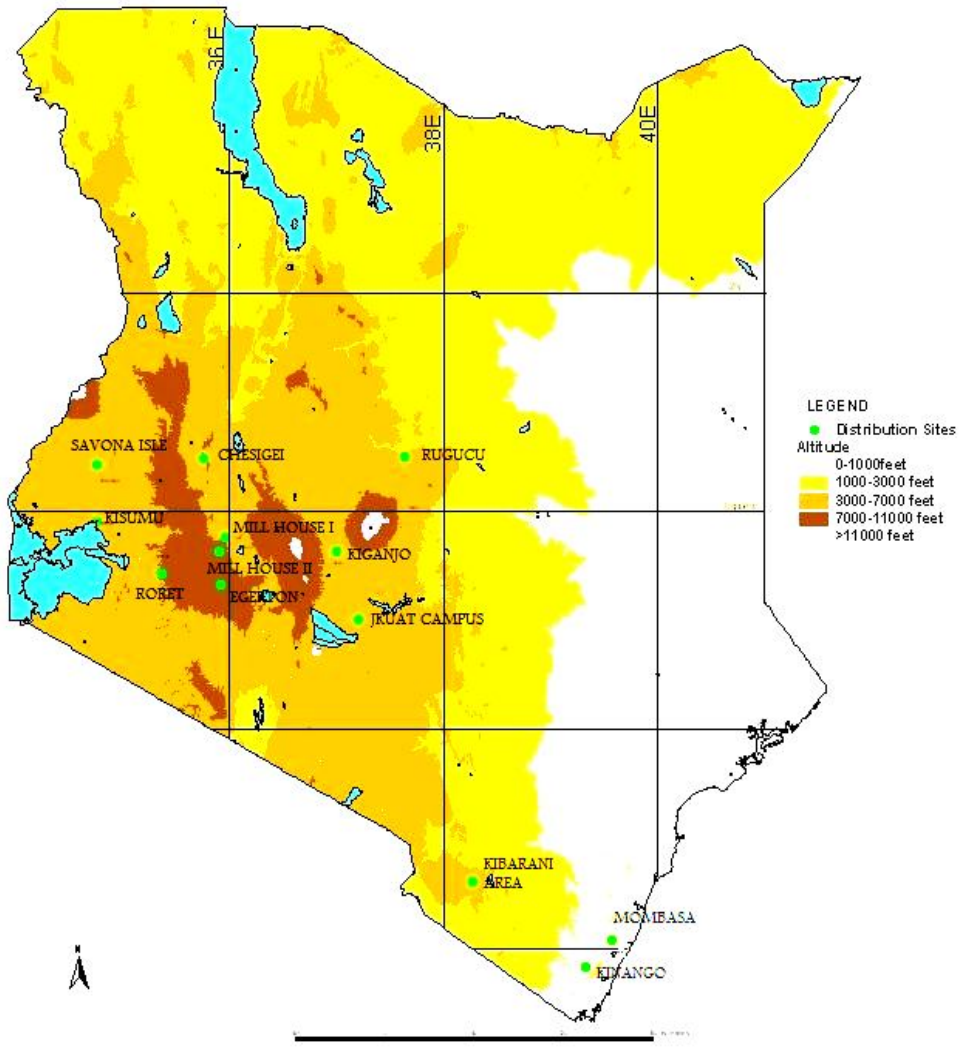


Figure 6.0: Sites where populations of *O. gratissimum L.* were collected in Kenya

For each population, leaf samples were collected from 25 mother herbs and a voucher specimen (805LG) was deposited at the Department of Botany, Egerton University, Kenya. After identification by a taxonomist, Dr. Kariuki S.T. from the Botany Department Egerton University, the leaves of the plants were plucked in preparation for

extraction. Both the fresh leaves from the pre-liminary study collection and the main study collection were subjected to hydro distillation in a Clevenger-type apparatus for a minimum of 4 hours.

Fresh leaves of the plants, *O. gratissimum* were plucked from the stems, then packed into a 2L flask up to three-quarter full then weighed. The weight of the fresh leaves was recorded before hydro distillation so that percentage weight of the oil per 100 g would be determined. About 500 ml of distilled water was added and then subjected to hydro-distillation, using modified Clevenger-type apparatus in accordance with the *British Pharmacopoeia* for a minimum of four hours. The resultant mixture of steam and essential oil was passed through a Lie big condenser, which is connected to a continuous flow of cold water as shown in Figure 7.0. Essential oils less dense than water separated as an upper layer, floating on the distillation water. The oil was then collected by decanting into sample bottles and dried using anhydrous sodium sulphate. The procedure was repeated until a reasonable amount of oil was obtained which was used for analysis and antimicrobial tests. The dried oil was weighed and the percentage yield calculated. The essential oil was obtained in a yield of w/w after drying over anhydrous Na_2SO_4. The oil was stored in a sealed glass vial (bijoux bottle) in a refrigerator at 4 $^{\circ}$C until required.

Figure 7.0: Hydro distillations of essential oils from *Ocimum gratissimum* in a Clevenger- type apparatus

2.9.1.3 Preparation of test plates for agar diffusion assay

The test plates were prepared using Sabouraud Dextrose Agar (SDA) for growth of fungi and Mueller Hinton Agar (MHA) for the growth of bacteria. The media was reconstituted using distilled water and then sterilized by autoclaving at 121 $^{\circ}$C for 15 minutes. It was then dispensed into Petri dishes aseptically (after cooling to 47 $^{\circ}$C) and left to solidify. The Petri dishes were then stored in the refrigerator at 4 $^{\circ}$C.

2.9.1.4 Preparation of the sensitivity discs

Blank sensitivity discs were prepared by dividing 100 sensitivity discs into each of the 13 bijoux bottles and sterilised in the oven by air-drying at 160 $^{\circ}$C for one hour. These discs were then soaked in one ml of the test samples from the 13 populations.

2.9.1.5 Determination of Antimicrobial activity of the methanolic crude extract and the essential oil extract

Antimicrobial test for the *O. gratissimum* L. was done using disc diffusion method (Baker *et al*., 1983). The micro organisms that were subjected to the bio-assays were strains of *Escherichia coli* ATCC25922, *Staphylococcus aureus* ATCC25923, *Pseudomonas aeruginosae* ATCC27853, *Klebsiella pneumoniae* (clinical isolate), *Proteus mirabilis* (clinical isolate), *Salmonella typhi*, *Candida albicans* and *Bacillus* spp. (samples from Kenya Medical Research Institute (KEMRI).

Antibaterial activity was determined on Mueller Hinton Agar (MHA), while the antifungal activity was determined on Sabouraud Dextrose Agar (SDA). The test organisms (inoculum) were inoculated in nutrient agar broth. The inoculum (bacteria or fungi) was standardized by adjusting the turbidity of an actively growing broth culture to match that of a barium sulphate standard i.e. standardized to a cell density of 1.5×10^8 (Mc Farland No. 0.5). Both the MHA and SDA plates were then inoculated with cotton-wool swabs moistened in the inoculated broth (bacteria or fungi suspension). 100 sterile

filter papers discs were soaked with 1 ml of either the methanolic extract or essential oil extract from *O. gratissimum* L. Each disc contained approximately 0.01 ml (10 µl) of the extract. The MHA Petri dishes were inoculated with the bacteria suspension aseptically and the SDA Petri dishes were inoculated with the fungi suspension aseptically. The individual plates sealed with laboratory para-film to avoid possible evaporation and contamination of the set up. Both the MHA and SDA Petri dishes were then incubated at 37 ° C for 24 hours. After 24 hours, the diameters of the inhibition zones were measured using calipers and expressed in millimeters. All procedures were done in triplicate. Methanol and sometimes sterile distilled water were used as controls.

Every bijoux bottle represented a population making a total of 13 populations of study.

2.9.1.6 Determination of the Minimum Inhibition Concentration (MIC) of the essential oil

The antimicrobial activity of the essential oil was tested according to the National Committee of Clinical Laboratory Standards (NCCLS, 1999) against the following micro organisms: *Escherichia coli* ATCC25922, *Staphylococcus aureus* ATCC25923, *Pseudomonas aeruginosae* ATCC27853, *Klebsiella pneumoniae* (clinical isolate), *Proteus mirabilis* (clinical isolate), *Salmonella typhi* (clinical isolate), *Candida albicans* (clinical isolate) and *Bacillus* spp. (clinical isolate). The test organisms (inoculum) were inoculated in nutrient agar broth. The inoculum (bacteria or fungi) was standardized by adjusting the turbidity of an actively growing broth culture to match that of a barium sulphate standard i.e. standardized to a cell density of 1.5×10^8 (Mc Farland No. 0.5). Serial dilutions of the essential oil (1, 1⁄2, ⅓, 1⁄4, 1⁄5…1⁄14) were done using 10 % TWEEN 80 in distilled sterile water which was also used as the control. The positive antibacterial and antifungal activities were established by the presence of measurable zones of inhibition after 24 hours of incubation at 37 ° C. Minimum inhibition concentration

(MIC) was defined as the lowest concentration that inhibited growth of the micro organism detected visually.

2.9.1.7 Gas chromatographic analysis of the essential oils

Gas chromatographic (GC) analyses of essential oils diluted in methyl tert.-butyl ether (MTBE) were performed on an Agilent GC-MSD apparatus equipped with an Rtx-5SIL MS ('Restek') (30 m x 0.25 mm i.d., 0.25 µm film thickness) fused-silica capillary column. Helium (at 0.8 ml/min) was used as a carrier gas. Samples were injected in the split mode at a ratio of 1:10 – 1:100. The injector was kept at 250°C and the transfer line at 280°C. The column was maintained at 50°C for 2 min and then programmed to 260°C at 5°C/min and held for 10 min at 260°C. The MS was operated in the El mode at 70 eV, in m/z range 42-350. The identification of the compounds was performed by comparing their retention indices and mass spectra with those found in literature (Adams, 1995) and supplemented by Wiley and Quadlib 1607 GC-MS libraries.

2.10 Data analysis

Analysis of variance was performed using Statistical Analysis System (1998). If significant P-values occurred, then comparison of means was done using Least Significant Differences Test (LSD Test).

2.11 Pre-liminary Results

2.11.1 Antimicrobial activity of methanolic crude extract from *O. gratissimum* L.

The methanolic crude extract from the 13 populations as follows: Njoro I, Njoro II, Mill House II, Kericho, Mill House I, Kabarnet, Meru, Nyeri, Kisumu, Kakamega, Thika, Taita taveta, and Mombasa (Figure 6.0) were found not to be active against all the seven bacterial strains (Table 1.0). The results showed that there was no inhibition zones on all the tested microbes namely *Escherichia coli* ATCC25922, *Staphylococcus aureus* ATCC25923, *Pseudomonas aeruginosae* ATCC27853, *Klebsiella pneumoniae* (clinical isolate), *Proteus mirabilis* (clinical isolate), *Salmonella typhi*, *Candida albicans* and *Bacillus* spp. Thus, the bacteria were resistant to the crude extract samples as denoted by zero in the table. However, the crude extract was active against the fungi, *Candida albicans* only (Table 1.0). The zones of inhibition ranged from 7 mm to 11 mm. Thus, the methanolic crude extract from some areas like Mombasa and Kisumu had enhanced activity as compared to some areas like Njoro I and Taita Taveta.

2.11.2 Antimicrobial activity of the essential oil from *O. gratissimum* L

The population of Nakuru, in Manyani area from which the essential oil was hydro-distilled showed activity on all the bacteria and fungi tested except for *Klebsiella pneumoniae* as indicated by its zone of inhibition which was zero (Table 2.0). The essential oil extract also showed varied activity on different microbes. It was very active on *Candida albicans*, the only fungi tested. The essential oil showed also enhanced activity on *Salmonella paratyph* which causes typhoid in humans. The results also showed that the standard antibiotic (chloramphenical) had high activity on

Staphylococcus aureus, *Escherichia coli*, *Bacillus* spp. and *Klebsiella pneumoniae.* However, it did not show activity on *Salmonella paratyph* and *Proteus vulgaris.*

Table 1.0: Mean antimicrobial activity of the crude extract from dried leaves of *Ocimum gratissimum* L from 13 different ecological zones (populations) of Kenya

MICRO ORGANISM	**INHIBITION ZONE (Mean)**													
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**	**L**	**M**	**N**
Gram positive														
Staphylococcus aureus ATCC25923	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bacillus spp. (Clinical isolate)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Gram negative														
Escherichia coli ATCC25922	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pseudomonas aeruginosae ATCC27853	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Klebsiella pneumoniae (Clinical isolate)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Proteus mirabilis (Clinical isolate)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Salmonella typhi (Clinical isolate)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Fungi														
Candida albicans (Clinical isolate)	7^{ab} *	10^{a}	9^{a}	9^{a}	8^{a}	10^{a}	8^{a}	10^{a}	11^{a}	10^{a}	8^{a}	7^{ab}	11^{a}	0

Key: A=Njoro I; B=Njoro II; C=Mill House II; D=Kericho; E=Mill House I; F=Kabarnet; G=Meru; H=Nyeri; I=Kisumu; J=Kakamega; K=Thika; L=Taita taveta; M=Mombasa; N=Distilled water. Clinical isolate from Kenya Medical Research Institute (KEMRI).

*Values in the same row (inhibition zone in mm) followed by the same letter do not differ significantly ($P<0.05$) by LSD test.

0= the microbe is not inhibited by the agent

Table 2.0: The mean antimicrobial activity of the essential oil from *O. gratissimum* L.

Population	Microbe	Inhibition Zone (mm)				
		Essential oil extract	Distilled water (control)	Methanol	Chloramphenical	Nystatin
Nakuru district-Manyani	*Proteus vulgaris*	11 ^{ab}*	0	0	0	0
	Staphylococcus aureus	10 ^{ab}	0	0	24 ^{ab}	0
	Escherichia coli	8 ^{b}	0	0	30 ^{a}	0
	Bacillus spp.	9 ^{b}	0	0	30 ^{a}	0
	Klebsiella pneumoniae	0	0	0	26 ^{ab}	0
	Salmonella paratyph	17 ^{a}	0	0	0	0
	Candida albicans	18 ^{a}	0	0	0	10 ^{a}

Notice: Chloramphenical is an antibiotic and Nystatin is an antifungal. Methanol is a control. 0= the microbe is not inhibited by the agent.

*Values in the same column (inhibition zone in mm) followed by the same letter do not differ significantly (P<0.05) by LSD test.

2.11.3 Antimicrobial activity of the essential oil extracts of *O. gratissimum* from the 13 populations of Kenya

The essential oil percentage yield of leaves from different population ranged from 0.16 - 1.40 % weight/weight (w/w) (Table 3.0). It was observed that the thicker and more velvet feel-like the leaves were, the less % oil yield. However, the amount of oil harvested had no effect on antimicrobial activity. Thus, for instance Meru oil was less in content as compared to Mill House II, however it did not mean the activity of the oil in Mill House II was high than that of Meru. The results showed Meru oil activity was consistent. Kabarnet oil was very low in quantity; however, its activity was much better than Njoro II which had the more or less similar amount (0.12).

Table 3.0: Population percentage yield of essential oil from *Ocimum gratissimum*

Population		Mean % yield weight/weight
1	Njoro I	0.78^{b}*
2	Njoro II	0.12^{d}
3	Mill House II	0.79^{b}
4	Kericho	0.21^{d}
5	Mill House I	0.83^{b}
6	Kabarnet	0.16^{d}
7	Meru	0.49^{c}
8	Kakamega	0.40^{cd}
9	Thika	0.52^{c}
10	Taita taveta	0.74^{b}
11	Mombasa	1.40^{a}
12	Kisumu	1.24^{a}
13	Nyeri	0.40^{cd}

*Values in the same column (inhibition zone in mm) followed by the same letter do not differ significantly ($P<0.05$) by LSD test

Table 4.0: Mean antimicrobial activity of the essential oil of *Ocimum gratissimum* L from 13 different ecological zones (populations) of Kenya

MICRO ORGANISM	**INHIBITION ZONE (Mean)**													
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**	**L**	**M**	**N**
Gram positive														
Staphylococcus aureus ATCC25923	20.0±0	20.0±0	13.5±0.7	20.0±0	15.0±0	21.0±1.4	26.6±5.7	22.0±4.0	25.3±1.2	19.3±1.2	25.3±4.6	15.0±1.4	15.0±1.4	24.5±0.7
Bacillus spp. (Clinical isolate)	21.0±1.4	20.5±0.7	16.0±1.4	22.0±0	16.5±0.7	25.0±1.4	22.3±1.5	17.3±2.5	21.7±1.5	16.7±1.2	21.7±1.5	18.3±2.5	19.0±1.7	30.0±0
Gram negative														
Escherichia coli ATCC25922	14.0±0	14.0±0	10.5±0.7	18.5±0.7	10.5±0.7	18.5±0.7	21.7±2.1	14.3±0.6	21.7±5.0	16.3±4.9	19.3±3.8	24.0±0	25.0±1.4	32.5±2.5
Pseudomonas aeruginosae ATCC27853	0	8.0±0	0	7.5±0.7	0	8.0±1.4	9.0±2.6	9.0±0	8.0±1.7	7.0±0	9.3±3.2	7.6±0.6	7.3±0.6	0
Klebsiella pneumoniae (Clinical isolate)	12.0±0	11.5±0.7	9.0±1.4	13.5±0.7	9.0±1.4	14.5±2.1	18.0±2.8	12.0±1.4	15.5±0.7	13.5±2.1	25.0±4.2	14.0±3.5	15.0±2.6	27.3±1.2
								11.3±0.6	12.7±2.1	10.3±1.2	13.0±1.7	13.3±1.2	15.3±2.5	0

Proteus mirabilis (Clinical isolate)	11.5±0.7	11.5±0.7	8.0±0	15.0±0	8.5±0.7	16.0±2.8	16.0±1.7							
Salmonella typhi (Clinical isolate)	13.5±0.7	13.5±0.7	10.5±0.7	18.0±2.8	10.0±0	18.5±2.1	20.5±0.7	13.5±0.7	17.5±0.7	10.5±0.7	18.5±0.7	20.0±1.7	18.6±2.3	0
Fungi														
*Candida albicans (*Clinical isolate)	*	*	*	*	*	*	*	*	*	*	*	*	*	0

Key: A=Njoro I; B=Njoro II; C=Mill House II; D=Kericho; E=Mill House I; F=Kabarnet; G=Meru; H=Nyeri; I=Kisumu; J=Kakamega; K=Thika; L=Taita taveta; M=Mombasa; N=Chloramphenical *=The organism was highly susceptible to the plant extract. Clinical isolate from Kenya Medical Research Institute (KEMRI). Sterile distilled water was a control.

Table 5.0: Variation in antimicrobial activity among locally collected populations of *O. gratissimum* L. in Kenya

	Inhibition Zone (mm)						
Population	*E. coli* (Gram –ve)	*K. pneumoniae* (Gram –ve)	*S.typhi* (Gram –ve)	*P. aeruginosae* (Gram –ve)	*P. mirabilis* (Gram –ve)	*S. aureus* (Gram +ve)	*Bacillus spp* (Gram +ve)
Chloramphenical	32.50^{a} *	28.00^{a}	6.00^{h}	6.00^{c}	6.00^{f}	24.50^{a}	30.00^{a}
Kabarnet	18.50^{cb}	14.50^{cb}	18.50^{bdc}	8.00^{bac}	16.00^{a}	21.00^{ba}	25.00^{b}
Kisumu	21.00^{cb}	15.50^{cb}	17.50^{ed}	8.50^{bac}	13.00^{bac}	25.00^{a}	22.50^{cb}
Thika	18.50^{cb}	25.00^{a}	18.50^{bdc}	10.50^{a}	13.50^{bac}	24.00^{a}	22.50^{cb}
Meru	22.00^{b}	18.00^{b}	20.50^{ba}	10.00^{ba}	16.50^{a}	25.00^{a}	22.50^{cb}
Kericho	18.50^{cb}	13.50^{cbd}	18.00^{dc}	7.50^{bac}	15.00^{ba}	20.00^{bac}	22.00^{cb}
Njoro I	14.00^{cd}	12.00^{cd}	13.50^{f}	6.00^{c}	11.50^{bcde}	20.00^{bac}	21.00^{cd}
Njoro II	14.00^{cd}	11.50^{cd}	13.50^{f}	8.00^{bac}	11.50^{bcde}	20.00^{bac}	20.50^{cd}
Mombasa	25.00^{b}	16.00^{cb}	20.00^{bac}	8.00^{bac}	16.50^{a}	15.00^{bc}	19.50^{cde}
Taita taveta	24.00^{b}	15.00^{cb}	21.00^{a}	8.00^{bac}	13.00^{bac}	15.00^{bc}	19.50^{cde}
Nyeri	14.00^{cd}	12.00^{cd}	13.50^{f}	7.50^{bac}	11.50^{bcde}	22.00^{ab}	18.50^{fde}
Kakamega	18.00^{cb}	13.5^{cbd}	10.50^{g}	6.50^{bc}	11.00^{cde}	19.00^{bac}	17.00^{fe}
Mill House I	10.00^{d}	9.00^{d}	10.00^{g}	6.00^{c}	8.50^{fed}	15.00^{bc}	16.50^{fe}
Mill House II	10.50^{d}	9.00^{d}	10.50^{g}	6.00^{c}	8.00^{fe}	13.50^{c}	16.00^{f}

*Values in the same column (inhibition zone in mm) followed by the same letter do not differ significantly ($P<0.05$) by LSD test.

Table 6.0: Minimum inhibition concentration (MIC) of the essential oil of *O. gratissimum* L. from Meru

Micro organism	Source	Inhibition Zone (mm)								
		75.00 ($\mu g \times 10^2$)	37.50 ($\mu g \times 10^2$)	25.00 ($\mu g \times 10^2$)	18.75 ($\mu g \times 10^2$)	15.00 ($\mu g \times 10^2$)	12.50 ($\mu g \times 10^2$)	10.70 ($\mu g \times 10^2$)	Chloramphenical (30µg)	MIC (mg/ml)
Gram negative										
E. coli	ATCC25922	21.7±2.1	15.0±0	11.0±1.4	10.0±1.4	9.5±2.1	9.0±1.4	7.5±0.7	28.0±0	107.1
S. typhi	[a]KEMRI	20.5±0.7	9.5±0.7	9.0±1.4	8.5±0.7	7.5±0.7	0	0	0	107.1
K. pneumoniae	[a]KEMRI	18.0±2.8	11.5±0.7	9.5±0.7	9.0±0	8.5±0.7	7.5±0.7	0	24.5±0.7	107.1
P. mirabilis	[a]KEMRI	16.0±1.7	10.0±0	8.5±0.7	8.0±0	7.5±0.7	0	0	0	107.1
P. aeruginosae	ATCC27853	9.0±2.6	0	0	0	0	0	0	0	750.0
Gram positive										
S. aureus	ATCC25923	26.6±5.7	13.0±1.4	12.5±0.7	11.5±0.7	11.5±2.1	8.5±2.1	8.0±1.4	23.5±2.1	93.75
Bacillus spp.	[a]KEMRI	22.3±1.5	11.0±1.4	8.0±0	7.5±0.7	7.0±0	0	0	24.5±0.7	187.50
Fungus		75.00 ($\mu g \times 10^2$)	5.35 ($\mu g \times 10^2$)	5.00 ($\mu g \times 10^2$)					Nistatin 100µl	
C. albicans	[a]KEMRI	S	8.5±0.7	7.0±0					10 mm	

Key: 0= the microbe is not inhibited by the agent; S= susceptible. [a]KEMRI=clinical isolate from Kenya Medical Research Institute

The essential oil from 13 populations of Kenya was evaluated for antimicrobial activity against pathogenic strains of Gram positive (*Staphylococcus aureus* and *Bacillus spp.*) and Gram negative (*Escherichia coli, Pseudomonas aeruginosae, Salmonella typhi, Klebsiella pneumoniae* and *Proteus mirabilis*) bacteria. The essential oil was found to be active against all the bacteria strains. It also showed a marked antifungal activity against *Candida albicans*. *Candida albicans* was highly susceptible to the plant extract. Although the concentration of the oil were generally in the range of 100 times or more than the standard antibiotic (Chloramphenical), they showed marked antibacterial and antifungal activities as evidenced by their zones of inhibition (Table 4.0). There were also cases where the essential oils were more active than standard antibiotic Chloramphenical (Tables 5.0 and 6.0). For instance, the essential oil was active against Gram negative bacteria, *Pseudomonas aeruginosae, Proteus mirabilis* and *Salmonella typhi* but Chloramphenical was resistant (Table 6.0 and Figure 8.0). Similarly the essential oil was able to clear all the fungi, *Candida albicans* (Figure 9.0) in the plate. Nystatin had an inhibition zone of 10 mm on *Candida albicans*.

The results also showed that the activity of the oil varied significantly from one population to another on different microbes (Table 5.0). For instance the essential oil from Meru, Mombasa and Taita Taveta showed high activity against Gram negative microbes (*Escherichia coli* and *Salmonella typhi)* and moderate activity on *Pseudomonas aeruginosae, Klebsiella pneumoniae* and *Proteus mirabilis* (Table 4.0 and 5.0). The essential oil from Meru had high activity on almost all the microbes as compared to the other populations which would show high activity on one microbe but show least activity on the other microbes (Table 5.0). The essential oil from Njoro I, Njoro II, Mill House I and Mill House II (all these are areas in one geographical region) showed least activity on almost all the microbes (Table 5.0).

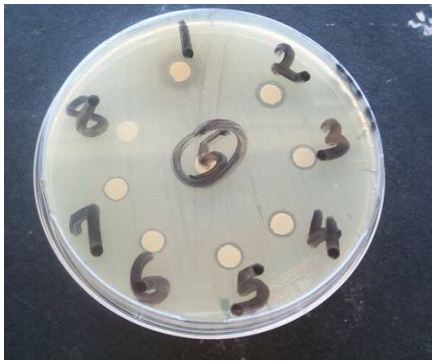


Figure 8.0: Inhibition zones of the essential oil on *Proteus mirabilis* namely 1, 2, 3, 4, 5, 6, 7 comprising of different essential oil concentrations; 8 is distilled water and 10 % TWEEN 80 as a control and Chloramphenical centre (paper disc).

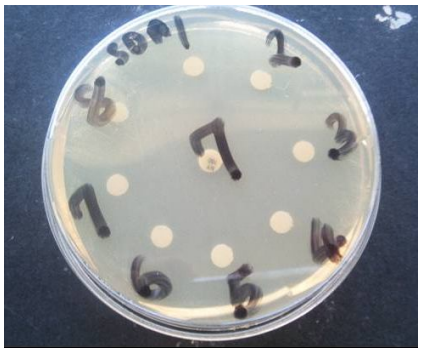


Figure 9.0: A plate of *Candida albicans* susceptible to the essential oil of *Ocimum gratissimum* L (1-8).

These four populations are all from Nakuru district. Mill House I and Mill House II are separated by a distance of 200 meters; while Njoro I and Njoro II are apart by 100 meters. These populations showed low inhibition zones in general in all the microbes (Table 5.0).

Among the Gram negative bacteria, the essential oil was active against all of them namely: *E. coli, P. mirabilis, K. pneumoniae, P. aeruginosae and S. typhi*. However, the standard antibiotic, Chloramphenical (30 µg), showed no inhibition zones on *P. mirabilis, P. aeruginosae* and *S. typhi*. Thus, these microbes were resistant to reference antibiotic Chloramphenical. The essential oil had relatively the same

activity response to Gram positive bacteria *S. aureus* as in Chloramphenical. However, Chloramphenical showed high activity towards *Bacillus* spp. which varied significantly from the activity of the essential oil from all the populations (Table 5.0).

Although, the concentration of the oil were generally in the range of 100 times or more than the standard antibiotic (Chloramphenical), it is important to note that the percentage of the active ingredient in the oil was not 100 percent as in chloramphenical. Therefore, in general, the essential oils were more effective than the standard antibiotic. The essential oils from all the populations showed activity on both Gram negative and Gram positive bacteria in addition to the fungi (*Candida albicans*) as evidenced by their zones of inhibition (Table 4.0).

The Minimum inhibition concentration (MIC) was done on Meru population only (Table 6.0 and Figures 10.0, 11.0, 12.0, 13.0, 14.0, 15.0). Meru essential oil showed very significant activity on all microbes which was comparable or better than that of reference antibiotic Chloramphenical (Table 5.0). The MIC for the Gram negative bacteria was 107.1 mg/ml except for *Pseudomonas aeruginosae* which had MIC of 750 mg /ml (Table 6.0). In the case of *Pseudomonas aeruginosae,* the essential oil was active only when it was neat (Figure 13.0), that is, when it was not diluted as shown on sensitivity disc number one only (Figure 13.0). All the other sensitive discs 2-8 did not show inhibition zone.

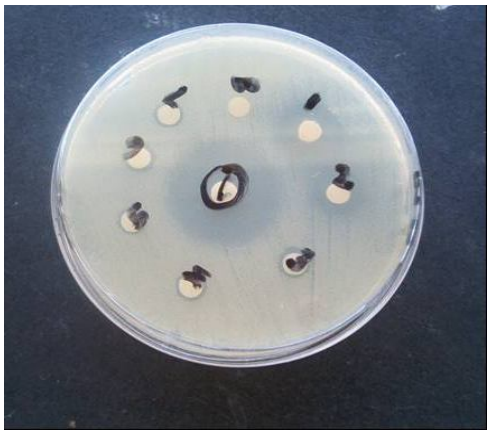


Figure 10.0: Minimum inhibition zone of the essential oil from Meru on *Escherichia coli* (1-7) comprising of different essential oil concentrations with 8 as distilled water and 10 % TWEEN 80 (control). At the centre as chloramphenical (paper disc).

Figure 11.0: Minimum inhibition zone of the essential oil from Meru on *Klebsiella pneumoniae* (1-7) comprising of different essential oil concentrations with 8 as distilled water and 10 % TWEEN 80 (control) and at the centre as chloramphenical.

Figure 12.0: Minimum inhibition zone of the essential oil from Meru on *Proteus mirabilis* (1-7) comprising of different essential oil concentrations with 8 as distilled water and 10 % TWEEN 80 (control) and at the centre as chloramphenical.

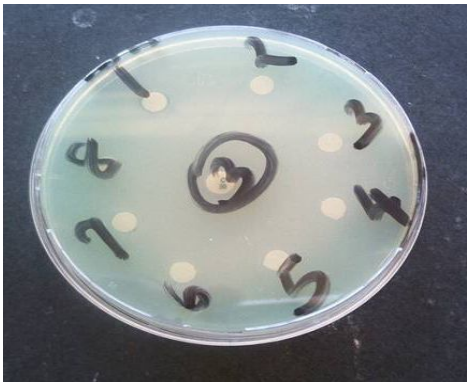


Figure 13.0: Minimum inhibition zone of the essential oil from Meru on *Pseudomonas aeruginosae* (1-7) comprising of different essential oil concentrations with 8 as distilled water and 10 % TWEEN 80 (control) and at the centre as chloramphenical. The microbes were all susceptible to the oil.

The MIC for the Gram positive bacteria was 93.75 mg/ml for *Staphylococcus aureus* ATCC 25923 and 187 mg/ml for *Bacillus* spp. (Table 6.0, Figures 14.0 and 15.0). The MIC for the fungus *Candida albicans* was 53.57 mg/ml (Table 6.0, Figure 9.0).

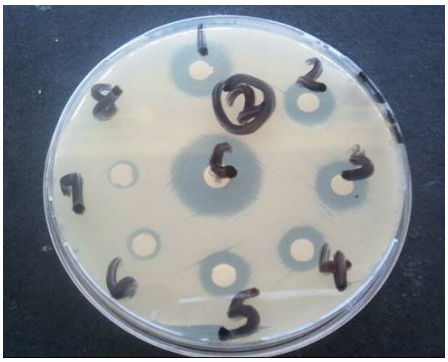


Figure 14.0: Minimum inhibition zone of the essential oil from Meru on *Staphylococcus aureus* (1-7) comprising of different essential oil

concentrations with 8 as distilled water and 10 % TWEEN 80 (control) and at the centre as chloramphenical.

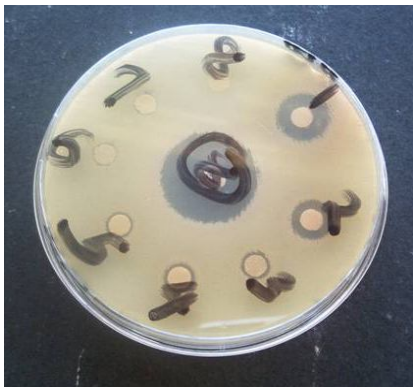


Figure 15.0: Minimum inhibition zone of the essential oil from Meru on *Bacillus* spp. (1-7) comprising of different essential oil concentrations with 8 as distilled water and 10 % TWEEN 80 (control) and at the centre as chloramphenical.

Table 7.0: Chemical composition of *Ocimum gratissimum* L. leaves oil

Compound	KI	% Concentration		Method of identification
Monoterpenes		*MERU*	*MOMBASA*	
β- Pinene	978	1.10	1.27	RI, GC-MS
cis-Ocimene	1037	7.47	6.00	RI, GC-MS
trans- Ocimene	1050	0.94	0.00	RI, GC-MS
Camphor	1143	0.95	0.00	RI, GC-MS
Eugenol	1356	68.81	74.10	RI, GC-MS
Methyl eugenol	1401	13.21	0.00	RI, GC-MS
	Total	92.48	81.37	
Sesquiterpenes				
trans-Caryophyllene	1430	1.69	3.70	RI, GC-MS
Germacrene-D	1487	4.25	8.74	RI, GC-MS
α- Farnese	1504	0.85	0.00	RI, GC-MS

β- Bisabolene	1508	0.73	0.00	RI, GC-MS
	Total	100.00	93.81	

KI – Kovat index

Table 7.0 shows the constituents identified by GC-MS analysis, their retention indices and area percentages of the essential oil from *Ocimum gratissimum* L. ≥100 % of the volatile oils which were identified in the sample from Eastern Kenya (Meru) and the coastal region of Kenya (Mombasa) districts. The oil was dominated by eugenol, which accounted for 68.81 % (Meru) and 74.10 % (Mombasa) and methyl eugenol in Meru district only (13.21%). Minor components included *cis*- Ocimene, Germacrene-D, *trans*- Caryophyllene and β- Pinene (Table 7.0).

2.12 Discussion

During the pre-liminary results, Table 1.0 showed that the dried methanolic crude extracts from *O. gratissimum* L. had no activity towards the bacterial microbes tested. However, they had some activity on the fungus *Candida albicans*, with a higher activity than that of standard antifungal nystatin.

On the other hand, the essential oil from *O. gratissimum* L. showed activity on broad-spectrum bacteria and fungi (Table 2.0). In some instances, the essential oil was more active than the standard antibiotic. There are some microbes which were not inhibited by chloramphenical like *Salmonella paratyph* but the essential oil was able to inhibit the growth of the microbe. It is well established that some plants contain compounds able to inhibit the microbial growth (Naqui *et al.*, 1994; Matasyoh *et al.*, 2007). These plant compounds can have different structures and different action when compared with antimicrobials conventionally used to control the microbial growth and survival (Nascimento *et al*., 2000). The results also showed that in most instances the inhibition zones by chloramphenical were bigger than those of the essential oil. The essential oil contains more compounds within it and it could be that the proportion of the compound that inhibits a particular microbe is small as compared to chloramphenical which is a pure substance.

Essential oils are volatile substances contained in several plant organs. They play several essential functions for the plant survival including defense against invader micro organisms (Cowan, 1999). In addition, many plant derived essential oils are known to exhibit antimicrobial activity against a wide range of bacteria and fungi (Janssen *et al.*, 1986, Matasyoh *et al.*, 2007).

During the study on antimicrobial activity of the essential oil extract of *O. gratissimum* from the thirteen populations of Kenya, the difference in activity of the oils from the different populations could be attributed to geographical locations (Table 5.0). The chemical composition of the essential oil of *O. gratissimum* can change accordingly with geographical distribution and day time of collection (Lemos *et al.*, 2005).

The analysis of the essential oils from the Eastern region (Meru) and coastal region (Mombasa) of Kenya by GC-MS revealed a major compound (68.8 %) and (74.1 %) respectively with a Kovat's index of 1356 (Table 7.0). The compound was identified as eugenol as also reported previously (Nakamura *et al*., 1999; Lemos *et al*., 2005). The compound (eugenol) has been demonstrated to have antibacterial (Nakamura *et al,*. 1999; Adebolu and Oladimeji, 2005) insecticidal (Chavan and Nikam, 1982), antihelminthic (Pessoa *et al.,* 2002), nematicidal (Chatterje *et al.,* 1982) and antifungal (Lemos *et al.*, 2005) activities.

Other reports have shown chemical composition percentages similar or higher than for this study (Lemos *et al.*, 2005) with eugenol (57.82 %) followed by α-bisabolene (17.19 %) and thymol (9.8 %); (Iwalokun *et al*., 2001a and 2001b) with essential oil obtained from the seeds of *O. gratissimum* containing thymol and eugenol in amounts ranging from 32 % to 65 %; Nakamura *et al.,* (1999) reported eugenol (67 %) as a major component ; (Keita *et al.,* 2000) reported thymol (46 %) p-cymeme (12 %) and γ-terpene + trans-sabiene hydrate (17 %) for *O. gratissimum* in the Republic of Guinea.

The genus *Ocimum* has been reported to yield oil of diverse nature commonly known as basilica oils. Lemos *et al.,* (2005) reported some chemical compounds and active ingredients found in these plants such as eugenol, linalool, methyl cinnamate, camphor, and thymol. Guenther, (1948) observed that *Ocimum gratissimum* oils could be divided into two groups: thymol and eugenol-rich chemotypes. Since that time, review by Lawrence (1997) indicated other chemotypes characterised by high contents linalool/methyl chavicol (Lawrence, 1992; De medici *et al*., 1992; Fun *et al.,* 1990) and methyleugenol/eugenol (Vostrowsksy, 1990). Other chemotypes are characteirised by high contents of geraniol (Charles and Simon, 1992), methyl cinnamate (Fun *et al.,* 1990), ethyl cinnamate (Ali and Shamsuzzaman, 1968) and citral (Hegnauer, 1967). According to the eugenol, methyl eugenol content, the extracted oil from this study could be classified as the eugenol/methyl eugenol chemotype which has not been reported.

Eugenol is a monoterpene, and so are methyl eugenol, *cis*-ocimene, ß-pinene, camphor and *trans*-ocimene. Methyl eugenol also has antifungal and antibacterial activities (Wright, 2002), in addition to its central nervous system depressant with anaesthetic, hypothermic, myore-relaxant and anticonvulsant properties (EMEA, 2004). *Cis*-ocimene, which was found in appreciable amounts in this study, has been found to have antibacterial activity and *beta*-pinene has antifungal activites (Wright, 2002). Research into the antimicrobial actions of monoterpenes suggests that they diffuse into and damage the cell membrane structures (Sikkema *et al.,* 1995). The sesquiterpene found in appreciable amounts like germacrene D has been reported to activate a major type of antennal receptor neuron of the tobacco budworm moth *Heliothis virescens* (Rostelien *et al.,* 2000). It is known that plants release hundreds of volatile oils that are important in interactions with insects or other organisms, for instance pollination.

Lima *et al.,* (1993) tested 13 essential oils obtained from plants against dermatophytes. *O. gratissimum* was found to be the most active in inhibiting 80 % of the dermatophyte strains tested and producing zones greater than 10 mm diameter.

Similarly, Okafor and Nwosu (1995) reported the antifungal of extracts of ten medicinal plants collected from Southeastern Nigeria against seven pathogenic fungi. According to the report, *O. gratissimum* L. inhibited the growth of *Trichophyton rubrum* and *T. mentagrophytes*. Antidiarrhoel activities of the leaf extracts of *O. gratissimum* have also been reported (Ilori *et al.*, 1986). Iwalokun *et al.*, (2003) studied the effects of *O. gratissimum* L. essential oil at sub-inhibitory concentration on virulent and multi drug resistant *Shigella* Strains from Lagos. In addition, Mbata and Saikia, (2005) showed that *O. gratissimum* oils have properties that can inhibit the growth of *Psychrophils* and heat resistant organisms and suggested there was need for the use of this plant and its derivatives for the primary purpose of flavouring foods and antimicrobial activities.

The minimum inhibition concentration (MIC) (Table 6.0) which was determined from the essential oil from Eastern Kenya (Meru) only, showed very good activity and the results were comparable or sometimes better than the standard antibiotic (chloramphenical). The essential oil showed variable activities against tested bacteria. The highest antimicrobial activity was observed on Gram positive bacteria as opposed to Gram negative bacteria although the oil was effective on all microbes tested. The oil inhibited *S. aureus* at an MIC of 93.7 mg/ml. In contrast to the relatively low MIC of the oil for Gram positive bacteria, Gram negative bacteria belonging to the genera *Escherichia, Salmonella, Klebsiella, Proteus* and *Pseudomonas* were inhibited by the oil with MICs ranging from 107 to 750 mg/ml. It has been already shown that the antimicrobial activity of the volatile compounds results from the combined effect of direct vapour absorption on micro-organisms and indirect effect through the medium that absorbed the vapour (Moleyar and Narasimtram, 1986; Bassole *et al.*, 2005). The vapour absorption on microorganisms is determined by their membrane permeability. Gram negative micro-organsims are less susceptible to the essential oils than Gram positive ones because they posses outer membrane surrounding the cell membrane (Ratledge and Wilkinson, 1988) which restricts diffusion of hydrophobic compounds through its lipopolysaccharide covering (Vaara, 1992).

Meru essential oil gave the best consistent results in their effectiveness as compared to the other 12 remaining populations (Table 5.0). Even though the MIC for the Meru oil is greater than that of the standard reference antibiotic, it should be realised that the essential oil comprises of many other compounds and not only eugenol (Table 7.0). This implies that pure eugenol from *O. gratissimum* could show higher inhibition than the crude essential oils. This was also observed by Lemos *et al.* (2005).

2.13 Conclusion

According to the results on activity of both the crude extracts and essential oils of *O. gratissimum* L., on microbes, the essential oils generally had activity on both the bacteria and the fungi tested.

This study has shown the composition of the essential oil of *Ocimum gratissimum* growing in Meru, Eastern Kenya and Mombasa, the coastal region of Kenya and the antimicrobial activity of the essential oil in 13 populations of Kenya. This indicates that this plant can be used as a herbal medicine in the management of ailments caused by these microbes like intestnal infections, pulmonary diseases, typhpoid fever, skin diseases. The pharmacological evidence and chemical composition results from this study serve as a link between scientific findings and traditional empirical knowledge. The results also confirm existing traditional empirical knowledge that the studied plants, which are used as herbal medicine locally, contain essential oils which can be used to control various pathogenic microbes which are a threat to human health in the Sub – Saharan region.

The study has also shown that geographical regions play an important role in the antimicrobial activity and chemical composition of the oil. Meru generally gave consistent and best results. It showed a remarkable activity on Gram negative bacteria (*E. coli, K. pneumoniae, S. typhi, P. mirabilis*) and Gram positive bacteria (*S. aureus* and *Bacillus spp.*) in addition to a fungal pathogen, *Candida albicans*. This indicates possible genetic differentiation between the populations, hence the

need for genetic studies. Alternatevely, the efficacy of Meru population could be environmental. Overall, the essential oil of *O. gratissimum* from the 13 populations showed activity, although there was variation in their efficacy.

CHAPTER 3

3.0 Genetic variation in *ocimum gratissimum* L from kenya

3.1 Introduction

Ethno-botanical studies are often significant in revealing locally important plant species especially for the discovery of crude drugs (Cox and Balick 1996; Flaster, 1996). They are also a suitable source of information regarding useful plants that can be targeted for domestication. Domestication of important medicinal plants in

Eastern Africa has been seen as a way of increasing income and availability of the curative products to healers and other resource users (Dery and Otsyina, 2000).

In Kenya, 2.9 million people live within 5 km of forest areas thereby exerting high pressure on forests such that out of the original closed canopy indigenous forest cover of 6.8 million hectares, only 1.24 hectares is left (Wass, 1995). As most rural people in Kenya turn to use of traditional medicinal plants that include important weeds, it is important to consider their genetic conservation. Four plant families with high percentage of medicinal weed species namely: Asteraceae 18.6 %, Solanaceae 9.3 %, Lamiaceae 9.3 % and Papilionaceae 6.6 % have been identified for conservation in Central Kenya (Njoroge *et al.*, 2004). The medicinal weed species in this region are dominated by herbs (65.33 % or 49 species), shrubs (32 % or 24 species) and trees (2.667 % or 2 species) (Njoroge *et al.*, 2004). *Ocimum gratissimum L.* is among the seven weed species identified as important medicinal herbs. A study by Njoroge *et al*., (2004) has provided information on medicinal weed species for possible on-farm conservation. However, an understanding of the extent and distribution of genetic variation within and among indigenous medicinal plant populations is essential for determining appropriate genetic management strategies, both for their utilization and conservation. This would also allow appropriate sampling strategies to be devised that would efficiently capture genetic diversity for field trial evaluation, *ex* and *circum situ* conservation. Furthermore, it would allow the distribution of inherently diverse planting material to farmers for domestication.

Ocimum gratissimum under study in this case belongs to the Lamiaceae family, which has close to 252 genera and 6700 species (Mabberley, 1997) most of which are used as medicine (Wren, 1968). The leaves are often hairy and posses epidermal glands which secrete volatile oils giving characteristic scents to many of the species. The essential oils found in leaves, seeds, flowers and roots of *Ocimum* species are used as medicine. Under *in vitro* conditions, the oils have shown to have antibacterial activity against Gram positive bacteria such as *Staphylococcus aureus* (ATCC 25923), and *Bacillus* species, and Gram negative bacteria including *Escherichia coli* (ATCC 25922), *Salmonella typhi, Pseudomonas aeruginosae, Proteus mirabilis, Klebsiella pneumoniae* (ATCC 27853), *Salmonella enteriditis, Shigella flexineri,* as well as activity against such pathogenic fungus as *Candida albicans* (Nakamura *et al.*, 1999; Matasyoh *et al.*, 2007).

Due to its potential as a traditional medicine, incorporation of *O. gratissimum* L. into agro forestry systems would not only make the species accessible to the majority of the rural population that uses it but also contribute to its genetic conservation. Like most countries in Sub-Saharan Africa, access to health services in Kenya is beyond the reach of most of the rural population. However, before widespread domestication of the species is implemented, it would be important to determine its genetic diversity in Kenya so that useful genotypes that could be used as cultivars by farmers can be selected thereby also facilitating the efficient conservation, management and utilization of the species genetic diversity. The techniques available for use in determination of genetic diversity in plant species can be divided into three classes

based on the choice of traits or markers for assay; morphological, biochemical and more recently the deoxyribonucleic acid (DNA) technology.

3.2 Factors affecting DNA extraction

The application of DNA technology in agricultural research has progressed rapidly over the last twenty years, especially in the area of cultivar identification and characterisation (Nybom, 1990) as well as determination of population diversity in many plant species ((Muluvi *et al*., 1999; Cardoso *et al*., 2000; Ipek and Simon, 2001; Nan *et al*., 2003; Chen and Yang, 2004; Lei *et al*., 2006). The application of this powerful tool in some plant species has however been constrained by lack of efficient nucleic acids isolation techniques. The extraction of the nucleic acids is difficult in a variety of plant species because of the presence of secondary metabolites that interfere with DNA isolation and subsquent molecular biology procedures such as DNA restriction, *in-vitro* amplification and cloning (Sghaier and Mohammed, 2005). A large number of secondary metabolites such as tannins, alkaloids, phenolics and terpenes which are responsible for the valuable pharmacokinetic properties of medicinal plants interfere with the nucleic acids isolation process and tend to copurify with DNA as well as interact irreversibly with proteins and nucleic acids (Katterman and Shattuck, 1983). Problems encountered in the isolation and purification of high molecular weight DNA from certain medicinal and aromatic plant species include degradation of DNA due to endonucleases, co-isolation of highly viscous polysaccharide and inhibitor compounds like polyphenols and other secondary metabolites which directly or indirectly interfere with

subsequent enzymatic reactions (Weishing *et al*., 1995). Good extraction procedures for the isolation of DNA should idealy yields adequate and intact undegraded DNA of reasonable purity. Various protocols for DNA extraction have successfully been applied to many plant species (Dellaporta *et al*., 1983; Rogers and Benedich 1985; Doyle and Doyle, 1990; Edwards *et al*., 1991; Ziegenhagen and Scholz, 1993).

The major difference in these protocols mainly concerns the ingredients (and also the pH) and therfore composition of the extraction buffers used. For example EDTA is generally included in DNA isolation buffers and storage solutions, since this compound chelates bivalent cations and thereby inhibits metal-dependent Dnases that are likely to randomly degrade DNA. Reducing agents such as β-mercaptoethanol (ME) and dithiothreitol (DTT) are also usually included in the DNA isolation buffers to inhibit oxidation process, which either directly or indirectly causes damage to DNA. DTT (Cleland's reagent) is an unusually strong reducing agent, owing to its high conformational propensity to form a six-member ring with an internal disulfide bond. DTT is used as a reducing agent for thiolated DNA. The terminal surfur atoms of thiolated DNA have a tendency to form dimmers in solution, especially in the presence of oxygen. Because of the vast differences between plant species in their secondary metabolism, each plant species may require its relevant protocol depending on the demand of the level of DNA purity. For example, Doyle and Doyle (1990) have used the cetyl-trimethylammonium bromide (CTAB) to isolate DNA with the reducing agent β-mercaptoethanol in addition to proteinase K which removes protein. Others like Reichardt and Rogers (1993) have used CTAB at high concentration. CTAB is a cationic detergent, which solubilises membranes and forms

a complex with DNA (Sghaier *et al*., 2005). CTAB also deters DNase activity and also removes polysaccharides. Edwards *et al*., (1991) have used Sodium dodecyl Sulfate (SDS) and phenol instead of CTAB as a detergent for the same function in DNA isolation. SDS extraction protocol is just but a modified version of CTAB with various alterations to increase the efficiency of removing proteins from the extracted DNA. In the protocol, there is the use of dithiothreitol (DTT), which reduces proteins at millimolar levels. SDS is a detergent which is (or a close relative, of sodium lauryl sulphate) often found in everyday shampoos, where it solubilises grease and oils. In the DNA preparation, it breaks up the lipids in the membranes to free the DNA from the cell (Mahoney, 2005). Polyvinylpolypyrrolidone (PVPP) has also been used successfully to remove polyphenols along with a high molar concentration of sodium cloride (NaCl) to inhibit co-precipitation of polysaccharides and DNA. The conventional DNA isolation procedures are sometimes further modified to provide DNA suitable for several kinds of analyses (Wang and Taylor, 1993; Ziegenhagen and Scholz, 1998). Because of the varied biochemical composition of plant tissues and species, it may be virtually impossible to supply a single isolation protocol which is optimally suited for each plant species. Thus, different plant taxa often may not permit optimal DNA yields from one isolation protocol (Weishing *et al*., 1995). Even closely related species may require quite different isolation procedures (Weishing *et al*., 1995). In addition to a reliable DNA extraction method, the storage of plant tissues for DNA extraction is also important. Most of the protocols are recommend for isolation of DNA from fresh tissues, but sometimes the samples collected from remote and rare locations may consist of plant parts in dry or semi-dry conditions (Khanuja *et al*., 1999). This necessitates the development of protocols for

the isolation of DNA from different plant organs, including dry tissues. Therefore, under these conditions, a good extraction procedure and good plant tissue storage conditions are important in order to extract good quality and quantity of DNA.

O. gratissimum L. contains an array of secondary metabolites dominated by two classes of compounds; flavonoids and terpenoids including monoterpenoids, sesquiterpenoids, diterpenoids and iridoid glycosides (The Bear's Byte, 2004). These compounds make DNA extraction difficult in addition to the problems of storing the plant tissue samples before DNA extraction.

In this study therefore, different *Ocimum gratissimum* L. tissue storage conditions and four different DNA extraction protocols were compared for effectiveness and efficiency in yielding optimal DNA concentrations and quality prior to genetic variation studies using different molecular markers.

3.3 Measurement of genetic variation/diversity

Genetic diversity refers to any variation in the nucleotides, genes, chromosomes, or whole genomes of organisms.

Most plant species are diploid, having two sets of chromosomes, and therefore two alleles for each gene though some can be haploid, triploid, or tetraploid genomes. Within any single plant species, there may be variation between the two (or more)

alleles for each gene (Harrison *et al*., 2004). This variation is introduced either through mutation of one of the alleles, or as a result of sexual reproduction. During sexual reproduction, offsprings inherit alleles from both parents and these alleles might be slightly different, especially if there has been migration or hybridization of organisms, so that the parents may come from different populations and gene pools. Also, when the offspring's chromosomes are copied after fertilisation, genes can be exchanged during sexual recombination (Harrison *et al*., 2004).

The presence of unique genetic characteristics distinguishes members of a given population from those of any other population. Large populations will usually have a greater diversity of alleles compared to small populations. This diversity of alleles indicates a greater potential for the evolution of new combinations of genes and, subsequently, a greater capacity for evolutionary adaptation to different environmental conditions (Harrison *et al*., 2004). In small populations, the individuals are likely to be genetically, anatomically, and physiologically more homogeneous than in larger populations and less able to adapt to different environmental conditions. Genetic diversity is, therefore, a key component for conservation efforts associated with population management (Andayani *et al*., 2001).

The genetic improvement of any organism depends on the existence, nature and the extent of the genetic variability available for manipulation. Genetic diversity can be assessed using different available marker techniques. Genetic markers can be classified into three classes; morphological, biochemical and DNA based markers.

3.3.1 Morphological markers

Morphological markers displaying Mendelian inheritance have been for a long time used to characterise genetic variation (Wilde *et al.*, 1992). However, most morphological descriptors can only be assessed at maturity (Wilde *et al.*, 1992) which makes their application time consuming. In addition, these descriptors are greatly influenced by environmental factors and therefore may not reflect true genetic similarities or differences between germplasm (Dawson *et al.*, 1993). Furthermore, there may be limited polymorphism of morphological traits in cultivated germplasm (Matus and Hayes, 2002). Thus, morphological traits can only estimate genetic parameters on measurable traits but not on individual genes. Therefore, these markers can neither provide information on what particular genes and how many of them are involved in genetic diversity nor how much of phenotypic variation can be explained by genetic variation in these genes. Despite these limitations, morphological markers are still an important measure for genetic variation.

To overcome these problems, various techniques involving the uses of molecular markers for the detection of genetic variation have been devised (Avise, 1994). These approaches include biochemical analysis and DNA based analyses. The latter include techniques such as RFLPs analysis (Botstein *et al.*, 1980) and those involving the polymerase chain reaction (PCR), such as random amplified polymorphic DNA (RAPDs; Williams *et al.*, 1990); simple sequence repeats (SSRs;

Tautz, 1989); inter simple sequence repeats (ISSRs; Zietkiewicz *et al.*, 1994) and amplified fragments length polymorphism (AFLPs; Vos *et al.*, 1995).

3.3.2 Biochemical techniques

Biochemical markers examine the products of the genes and can either be scored at the protein level (e.g. isozymes) or at the organic chemical level (e.g. secondary plant metabolites such as terpenes).

3.3.2.1 Isozymes

The term isozyme was derived by two early researchers (Markert and Moller, 1959) to describe different molecular forms of enzymes with the same substrate specificity. These are based on multiple forms of an enzyme which differ in electrophoretic mobility. Electrophoresis is a method of separating the different forms of enzymes present in plant tissue samples. The procedure uses an inert gel matrix (either starch or polyacrylamide) as the medium to separate and immobilize the proteins. Sections of the gel are then placed into different substrate solutions, and the resulting zones of enzyme activity cause a distinct color zone in the gel. The type of patterns produced by the different forms of the same enzyme (isozymes) is permanently recorded in the gel. Because different enzyme forms (isozymes) are inherited co-dominantly at individual loci, it is possible to determine the genotype of an individual from its electrophoretic profile (Weeden and Wendel, 1990; Avise, 1994).

Isozymes have been employed more than any other marker technique to characterise patterns of genetic diversity and differentiation in plant species. It is because of the relative technical simplicity of the approach and its inexpensive nature as compared to other molecular techniques (Avise, 1994; Tanksley and Orton, 1983) that have made it popular. Isozymes have extensively been applied to assess patterns of genetic variation in a range of medicinal plant species including *Garcinia* spp., *Labisia pumila*, *Parkia speciosa* and *Aquilaria malaccensis*, (IPGRI Newsletter, 1997), *Podophyllum hexandrum* Royle, an endangered medicinal herb of North-western Himalaya, (Bhadula *et al*., 1996) and *Allium sativum* L. (Ipek and Simon, 2001). However, for most plant species including those that are inbred, isozymes are often less polymorphic than other types of molecular markers listed below.

3.3.3 Molecular Markers

3.3.3.1 Extraction of Genomic DNA

The isolation of intact, high molecular-mass genomic DNA is essential for many molecular biology, applications including long PCR, endonuclease restriction digestion, Southern blot analysis, and genomic library construction. Many protocols are available for the extraction of DNA from plant materials (Saghai-Maroof *et al*., 1984; Doyle and Doyle 1987; Webb and Knapp, 1990). Experience has shown that plant protocols need to be tailored to each plant species (and sometimes even each tissue) due to the presence of secondary metabolites that vary in quantity and

composition. These phytochemicals may not only hinder the *su moto* application of the other methods, but they may also interfere with subsequent amplification and even restriction-digestion of the isolated DNA. Sometimes, the contaminants are not detectable but are sufficiently high to interfere in the analysis of polymorphism.

Medicinal plants which are also aromatic such as *Ocimum gratissimum* L. are particularly rich in a myriad of natural products and they require additional efforts to find an appropriate DNA isolation procedure for reliable and consistent results. For instance, *Artemisia annua* L. which is an annual herb native to Asia is a medicinal and aromatic plant rich in the myriad of natural products (Sangwan *et al.,* 1998). The protocol for isolation of DNA from the metabolite-rich species *A. annua* comprised of steps like the addition of polyvinylpyrrolidone (PVP) in order to remove polyphenols and use of high NaCl concentration in DNA extraction buffer in order to remove polysaccharides (Lodhi *et al.,* 1995). In addition to that, a column chromatography step was added to further purify the DNA from any residue contamination (Sangwan *et al.,* 1998). However, though the DNA obtained was of high yield it was not restrictable. An additional purification step through a quick small DE-column was necessary to completely eliminate impurities from the DNA (Sangwan *et al.,* 1998). Similarly, for latex-containing Asteraceae (*Cichorioideae*) species, standard protocols and commercially available kits do not produce efficient yields of high-quality amplifiable DNA (Michiels *et al.,* 2003). A cetyltrimethyl ammonium bromide protocol has been optimised for isolation of genomic DNA from latex-containing plants. The modified protocol has key steps which include the use of etiolated leaf tissue for extraction and an overnight 25 degrees and Chloroform:

isopropanol precipitation step (Michiels *et al.,* 2003). The purified DNA has excellent spectral qualities and is efficiently digested by restriction endonucleases; and is suitable for long-fragment PCR amplification (Michiels *et al*., 2003). Abdin *et al.,* (2007) also reported a protocol for isolation of genomic DNA from dry and fresh roots of medicinal plants suitable for RAPD and restriction digestion. The method involved a modified CTAB procedure using 3 % CTAB, 4 % β-mercaptoethanol, 2M NaCl and 5 % PVP. The extraction was carried out at 70 °C. It was observed that a slight increase in the concentration of the above constituents of the isolation buffer and temperature helped in the removal of secondary metabolites and polysaccharides from the DNA preparation (Abdin *et al.,* 2007). The isolated DNA was higher in quantity and purity when compared with DNA extracted by the methods of Dellaporta *et al.,* (1983); Doyle and Doyle (1990).

3.3.3.2 Types of DNA based molecular markers

There are various types of DNA-based molecular techniques (Joshi *et al*., 1999; Powell *et al.,* 1996; Botstein *et al*., 1980) that are available for characterising germplasm with each method differing in principle, application, type and amount of polymorphism detected, cost and time required. The markers detect polymorphism by assaying subsets of the total amount of DNA sequence variation in a genome (Powell *et al.*, 1996). DNA-based marker systems have several advantages over other marker types. For instance, they are not affected by the environment and they can be detected in all tissues at all stages of development (Sorriano *et al.,* 2005). They are

the most reliable tools for measurement of genetic variability because in addition, they are also useful for individual DNA genotyping (Bling, 2000).

DNA based marker systems can be classified into three major groups; hybridization-based methods, polymerase chain reaction (PCR)-based methods, and sequencing-based methods. These marker systems differ in their information content (ability to distinguish between genotypes) and the number of loci that each can provide for silmutaneous analyser per experiment (multiplex ratio). The utility of a given marker system is however a balance between the level of polymorphism (information content) and the extent to which the assay can detect multiple polymorphism. Powell *et al.*, (1996) derived a convinient estimate (marker index) of marker utility that is a product of the information content and multiplex ratio.

3.3.3.2.1 Hybridization-based methods

Hybridization based DNA methods include restriction fragment length polymorphism (RFLP) (Botstein *et al.*, 1980) and variable number tandem repeats (Nakamura *et al.*, 1987). Labeled probes such as random genomic clones, cDNA clones, probes for microsatellite (Litt and Luty, 1989) and minisatellite (Jeffrey *et al.*, 1985) sequences are hybridized to filters containing DNA which has been digested with restriction enzymes. Presence or absence of bands upon hybridization detects polymorphisms.

3.3.3.2.1.1 Restriction fragment length polymorphisms (RFLPs)

This is one of the older alternative DNA-based procedures for the detection of polymorphism. It has the potential to produce numerous genetic markers (Tanksley *et al*., 1989; Paterson *et al*., 1991).

In RFLP analysis, genomic DNA is isolated, cut using restriction endonucleases, size-fractionated on gels, transferred to a filter by blotting (Southern, 1975) and probed with clones from the genomic region of interest (Aquadro *et al*., 1992). Though RFLPs have been used extensively for fingerprinting some crop plants (Powell *et al*., 1991), their exploitation has been limited in perennial tree species mainly owing to the level of resourcing, technical expertise and the quantity and quality of DNA (Wachira *et al*., 1995). For some plant species, low levels of polymorphism (Kochert *et al*., 1991), has limited the use of this marker. Another potential disadvantage of the RFLP technique is that it only indicates the presence or absence (dimorphic nature) of cleavage sites and therefore does not give a great deal of genotypic information.

3.3.3.2.2 Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR)–based assays are generally much less expensive and reveal greater amounts of polymorphism (Paterson, 1996; Staub and Serquen, 1996). These assays involve *in vitro* amplification of particular DNA sequences or

loci, with the help of specific or arbitrary oligonucleotide primers and thermostable DNA polymerase enzyme (Vosberg, 1989).

Thus, the PCR is a test tube system for DNA replication that allows a "target" DNA sequence to be selectively amplified, or enriched, several million-fold in just a few hours. Within a dividing cell, DNA replication involves a series of enzyme-mediated reactions, whose end result is a faithful copy of the entire genome. Within a test tube, polymerase chain reaction uses just one indispensable enzyme- the DNA-polymerase- to amplify a specific fraction of the genome.

PCR-based techniques where random primers are used include random amplified polymorphic DNA (RAPD) (Williams *et al*., 1990; Welsh and McClelland, 1990), arbitrarily primed PCR (AP-PCR) (Welsh and McClelland, 1991) and DNA amplification fingerprinting (DAF) (Caetano-Anolles *et al*., 1991; Caetano-Anolles *et al*., 1994).

Other more specific PCR based marker systems include the inter-simple sequence repeats (ISSRs) and amplified fragment length polymorphism (AFLP).

3.3.3.2.2.1 Random amplified polymorphic DNA analysis (RAPDs)

The RAPD assay involves the amplification of DNA fragments from a template of genomic DNA using short oligodeoxynucleotide primers of arbitrary nucleotide

sequence and polymerase chain reaction procedure (Welsh and McClelland, 1990; Devos and Gale, 1992; Kahl, 2001).

RAPDs were the first arbitrary primed PCR markers to be developed (Williams *et al.*, 1990) and since then, there have been a number of modifications made, predominantly in primer length and detection methodology.

The RAPD technique is based on the amplification of unknown DNA sequences using short oligonucleotide primers of arbitrary sequence. There is a high probability that genomic DNA contains several primer binding sites close to one another in an inverted orientation. The RAPD technique essentially scans for these inverted repeats and amplifies intervening DNA segments of variable length (Hardys *et al*., 1992).

DNA polymerase is used to amplify DNA segments between closely spaced sequences (<2 Kb) and complementary to the short random oligomers (typically 10-mers). The amplification products are then resolved on agarose gels and polymorphisms normally occur as the presence or absence of a specific fragment. RAPD polymorphism results from changes in the primer-binding site in the DNA sequence.

The RAPD technique does not require the knowledge of the genome sequence nor does it involve radioactivity (Chalmers *et al*., 1992; Dawson *et al*., 1993; De Bustos, 1998). It is relatively simple, fast and permits analysis of large number of individuals at a reasonable expense (Duarte *et al*., 1999; Maciel *et al*., 2001). Like all PCR based

methods, it requires small amounts of DNA (<10 ng), which can be extracted from dried leaf material, facilitating field collection of samples, particularly when this is difficult.

Despite the above advantages, RAPDs analysis has limited repeatability and may result in the amplification of repetitive DNA sequences (Devos and Gale, 1992; Penner *et al.*, 1993). Reproducibility problems may however, be overcome if care is taken to ensure consistent reaction conditions during amplification. In addition, since RAPDs are dominant markers, they can not directly reveal heterozygous character states and therefore require special statistical techniques for analysis. There is also a lack of cross-transferability and it must be acknowledged that in some cases, amplified fragments that are of the same length may not necessarily be of the same sequence (it is not homologous). This is because gel electrophoresis separates DNA fragments quantitatively (according to size), but not qualitatively (according to base sequence) (Ford-Lloyd & Painting, 1996). Some of these latter problems can be overcome by cloning and partially sequencing the fragments to turn these into sequence characterised amplified regions (SCARs) (Paran & Michelmore, 1993), which are usually more robust than RAPDs. Alternatively, these limitations can be reduced by scoring an appropriate number of RAPD fragments for statistical analyses (usually >30) (Lynch and Milligan, 1994).

RAPD analysis has been employed widely to assess genetic variation within a range of medicinal plant species (Al-Zahim *et al.*, 1997; Padmesh *et al.*, 1999; IPGRI Newsletter, 1997). RAPDs have been used to characterise and describe three species

of the medicinal plant *(Scutellaria)* (Hosokawa *et al.*, 2000); samples of *Cannabis sativa* from different sources have been distinguished using RAPDs (Jagadish *et al.*, 1996); while the genetic diversity in North American ginseng (*Panax quinquefolius* L.) has been estimated using the same marker (Bai *et al.*, 1997).

3.3.3.2.2.2 Inter Simple Sequence Repeat (ISSR) analysis

Inter simple sequence repeats (ISSRs) (Zietkiewicz *et al.*, 1994) polymorphism is a specific primer-based polymorphism detection system, where a terminally anchored primer specific to a particular simple sequence repeat (SSR) is used to amplify the DNA between two opposed SSRs of the same type. Polymorphism occurs whenever one genome is missing in one of the SSRs or has a deletion or insertion that modifies the distance between the repeats.

The ISSR technique uses simple sequence repeat primers, for example, $[AC]_n$ to amplify regions between their target sequences (Zietkiewicz *et al.*, 1994; Kahl, 2001). The primers may be anchored at their 3' or 5' ends by addition of one or two extra nucleotides, for example, 5' $[AC]_n$3'

The technique exploits the abundant and random distribution of simple sequence repeats (SSRs) in plant genomes by amplifying DNA sequences between closely linked SSRs.

ISSRs are semi-arbitrary markers amplified by PCR in the presence of one primer complementary to a target micro-satellite. Micro-satellites are usually more or less proportionally dispersed in the genome. Regions with a greater abundance of these sequences called "SSR hot spots" (Zietkiewicz *et al.*, 1994; Bornet *et al*., 2002) have been found to serve as ISSR markers. The 3'-anchored primers may be used to amplify regions between two SSRs with compatible priming sites (Yang *et al*., 1996). Work by Zietkiewicz *et al.*, (1994) shows that more complex banding patterns can be achieved using 5'-anchored primers that incorporate the SSR regions in their amplification products, and by combining 3'- and 5'- primers.

ISSR are useful for detecting genetic polymorphism, and have been used to fingerprint closely related individuals (Zietkiewicz *et al.*, 1994) and for genetic diversity studies (Tsumura *et al.*, 1996). The PCR amplifies the sequence between two SSRs, yielding a multilocus marker system useful for fingerprinting, diversity analysis and genome mapping (Zietkiewicz *et al.*, 1994). Though the ISSR technique does not require prior knowledge of the SSR target sequences and radiolabelling, it shows high levels of polymorphism and is also highly reproducible (Kojima *et al*., 1998; Bornet & Brachard, 2001). ISSR markers have been successfully used for determination of genetic variation in *Rhodiola crenulata* eastern (Lei *et al*., 2006); *Rhodiola alsia* (*Crassulaceae*) (Chen and Yang, 2004) and *Primula obconica* (*Primulaceae*) (Nan *et al*., 2003).

In the ISSR assay, each band corresponds to a DNA sequence delimited by two inverted micro-satellites. Like RAPDs, ISSR markers are quick and easy to handle.

The simplicity of ISSR markers predetermines them for gene tagging. Though the ISSR technique lacks co-dominance and exhibits the consequent resolution of effectively bi-allelic loci (band presence vs absence) it is still thought to be a convenient supplement to RAPD markers (Tsumura *et al*., 1996).

3.3.3.2.2.3 Amplified fragment length polymorphism (AFLP)

Amplified fragment length polymorphism (AFLP) (Zabeau, 1993) is a technique that is based on the detection of genomic restriction fragments by PCR amplification. Adaptors are ligated to the ends of restriction fragments followed by amplification with adaptor-homologous primers. AFLP has the capacity to detect thousands of independent loci and can be used for DNAs of any origin or complexity (Kumar, 1999).

The amplified fragment length polymorphism (AFLP) technique combines components of RFLP analysis with PCR technology (Vos *et al*., 1995). Prior to PCR amplication, two restriction endonucleases, a rare cutter and a frequent cutter, digest the genomic DNA. Adaptors of known sequence are then ligated to the DNA fragment to generate template DNA for PCR. Primers complementary to the adaptors are used to amplify the restriction fragments. Extra nucleotides added at the 3` end of the PCR primers allows the selective amplification of only those restriction fragments starting with nucleotides homologous to those of the primers. Therefore,

AFLP fingerprints can be tailored to produce patterns of desired complexity. In principle both the restriction enzymes and the nature and number of selective nucleotides in the primers can be varied. Consequently, an unlimited number of markers can be generated. The PCR-amplified fragments can then be separated by gel electrophoresis and banding patterns visualised. Sometimes, fluorescent-labeled primers are used in the selective amplification stage to allow for fragment detection in the sequences (Vos *et al*., 1995).

The reproducibility of the AFLP profiles is assured by using primer sequences of at least 16 nucleotides at stringent amplification conditions. The reproducibility of the AFLP technique is further demonstrated by the consistency in relative band intensities. However, any quantitative differences in band intensities between the lanes are easily used to recognise the homozygous presence of AFLP marker allele (Van Eck *et al*., 1995).

The advantages of the AFLP technique include the production of large number of polymorphisms, its ability to reveal multiple polymorphisms and high marker index (Powell *et al*., 1996; Wachira *et al*., 2001). Its ability to differentiate individuals in a population makes the technique useful for paternity analysis, gene flow studies and cultivar research. Other advantageous features of AFLPs are that it requires very small amount of DNA; no sequence information is required (Vos *et al*., 1995); the technique is fast (it has the capacity to analyse many markers in a short time; Hartl and Seefelder, 1998); results are reproducible and standard kits are available (Robinson and Harris, 1999). In addition, the single greatest advantage of the AFLP

technology is its sensitivity to polymorphism detection at the total-genome level. The major limitation of the technique is in band homology (Karp *et al*., 1997).

AFLP markers have been successfully applied to assess genetic diversity within a range of medicinal plant species; for example in *Allium sativum* L. (Ipek, and Simon, 2001), *Moringa* species (Muluvi *et al*., 1999), *Euterpe edullis* Mart. (Cardoso *et al.*, 2000) *et cetera*. AFLP has been used in other fields of study like in molecular evolution and diversity in *Bacillus anthracis* (Keim *et al*., 1997); genetic diversity of *Hibiscus tiliaceus* (*Malvaceae;* Tang *et al*., 2003) and in globe artichoke (*Cynara cardunculus* L. var. scolymus L.; Portis *et al*., 2004).

3.3.4 Other PCR based markers

A number of Sequence Tagged Site (STS) markers, useful as anchoring loci between crosses, have been developed. The most important of these is the microsatellite or simple sequence repeat (SSR) marker (Paterson, 1996). For example, sequence-tagged microsatellites sites (STMS) markers have been developed for the medicinal plant Madagascar periwinkle (*Catharanthus roseus* (L.) for characterisation (Bhatia *et al*., 2005). In fact, the STMS markers of *C. roseus* also amplified corresponding loci in a related species (*C. trichophyllus*) suggesting conservation of the loci across the genus. These markers have proven useful for genetic diversity analysis and linkage map construction in *C. roseus* (Bhatia *et al.*, 2005). Additionally, many other STS markers (Staub *et al.*, 1996) have been developed for use, including CAPs (cleaved amplified polymorphic sequences), SCARs (sequence characterised

amplified regions), ASAPs (allele-specific associated primers), and ESTs (expressed sequence tags), a subset of STSs derived from cDNA (Paterson, 1996).

3.3.5 Sequencing-based markers

DNA sequencing can also be used as a definite means for identifying species. Variation due to transversion, insertion or deletion can be assessed directly and information on a defined locus can be obtained. Genetic variation occurs extensively at the single nucleotide level (SNP) and direct sequencing can therefore efficiently identify such SNPs depending on the relative closeness of the organisms being compared. Other sequencing-based strategies include analysis of the variable internal transcribed spacer (ITS) sequences of ribosomal DNA (rDNA). For instance, molecular marker rDNA (ribosomal DNA) ITS (internal transcribed spacer) region was used in order to establish a simple method for the molecular identification of the medicinal plants of *Dendrobium chrysanthum* and *D. fimbriatum* (Kaiya *et al.*, 2005). Also, Khan *et al.*, (2006) have reported universal primers designed from the 16S rDNA sequences of phytoplasmas and which were applied in an efficient and effective nested PCR-based system. The primers were also useful for detection of associated phytoplasma. In addition to this, more than 300 nucleotides of 25S rDNA 5' terminal region have been determined, and the specific nucleotides can be used for the identification of the medicinal plant duzhong (*Eucommia ulmoides*; Chen *et al.*, 1998).

3.3.6 Application of molecular markers in herbal drug technology

DNA-based molecular markers have proved their utility in fields like taxonomy, physiology, embryology, genetics among others.

3.3.6.1 Genetic variation and genotyping

It has been well documented that geographical conditions affect the active constituents of medicinal plants and hence their activity profiles (Oleszek *et al.*, 2002). Many researchers have studied geographical variation at the genetic level using molecular markers. Estimates of genetic diversity are particularly important inputs in the design of crop improvement programmes, for management in designing crop improvement programs and for management of germplasm resources as well as in the development of conservation strategies. RAPD-based molecular markers have been found to be useful in differentiating various accessions of *Taxus wallichiana* (Shasany *et al*., 1999), neem (Farooqui *et al*., 1998), *Juniperus communis* L. (Adams *et al*., 2002), *Codonopsis pilosula* (Fu *et al*., 1999), *Allium schoenoprasum* L. (Friesen and Blattner, 2000), *Andrographis paniculata* (Padmesh *et al.*, 1999) collected from different geographical regions. Similarly, different accessions of *Cannabis sativa* (Kojoma *et al.*, 2002) have been classified using ISSR markers and those of *Arabidopsis thaliana* L. Heynh. (Barth *et al*., 2002) have been differentiated using cleaved amplified polymorphic sequence and ISSR markers. Inter- and intra-species variation has also been evaluated using DNA-based molecular markers.

Interspecies variation has been studied using RFLP and RAPD in different genera such as *Echinacea* (Kapteyn *et al.*, 2002), *Curcuma* (Chen *et al.*, 1999) and *Arabidopsis* (Lind-Hallden *et al.,* 2002). RAPD and RFLP have also been applied for the characterisation of *Epimedium* (Nakai *et al.*, 1996) at the genetic level. RAPD has served as a tool for the detection of variability in Jojoba (*Simmondsia chinensis* L. Schneider; Amarger *et al.*, 1995), *Vitis vinifera* L. (Tessier *et al.,* 1999) and tea (*Camellia sinesis*; Wachira *et al.*, 1995).

The successful application of molecular marker tools in the plant genetic diversity studies is often dependent upon optimised strategies for the isolation of fairly good quality undegraded genomic DNA.

3.3.6.2 Separation and detection of DNA fragments

The DNA profiling techniques discussed in the proceeding sections of the thesis may also differ in the techniques of separation and detection of fingerprints profiles. Three different media are used to separate and visualise DNA fragments. They are agarose, denaturing or non-denaturing polyacrylamide gel electrophoresis (PAGE) and capillary systems (Diers *et al.*, 2003). The separated fragments are detected by silver staining, ethidium bromide or fluorescently labeled primers detected by a laser (Diers *et al.,* 2003).

Agarose gel electrophoresis separates DNA fragments according to size (Myers *et al.*, 1985). Agarose gels have the disadvantage of inaccurate sizing and lack of high-

resolution power (Buhariwalla and Crouch, 2004). Most agarose gels are made between 0.7 % and 2 %. A 0.7 % gel will show good separation (resolution) of large DNA fragments (5-10 kb) and a 2 % gel will show good resolution for small fragments (Scherf and Hernandez-Rivas, 1997).

Polyacrylamide gel electrophoresis is the method of choice for separating nucleic acids less than 500 base pairs in length (Gels, 2007). Polyacrylamide forms gels with pores of much more controlled and uniform size than does agarose. Consequently, polyacrylamide gels can be used to separate molecules that differ in size by as little as 2 % of their molecular weight (Gels, 2007).

The ABI PRISM® 377 DNA Sequencer (Applied Biosystems) automatically analyses DNA molecules labeled with multiple fluorescent dyes. After samples are loaded onto the system's vertical gel, they undergo electrophoresis, laser detection, and computer analysis. Electrophoretic separation can be viewed on-screen in real-time, and final data can be output in a variety of formats. The 377 system accepts gel plates in four different lengths for flexibility in sample analysis (ABI 377 & ABI 3700 DNA Sequencers, 1998). It has a higher resolution power than the agarose gel and can detect up to a two base pair difference between fragments and also takes much less quantities of product as compared to the agarose gels. For instance, for the agarose gels, up to 10-15 µl of PCR product is required for visualisation while for the ABI Prism 377 DNA Sequencer (Applied Biosystems), a total of 1.5 µl of PCR product is enough to accurately size fragments.

The ABI 3730 DNA Sequencer (Applied Biosystems) is a fully automated capillary based system, high-throughput, capillary electrophoresis systems used for analysing fluorescently labeled DNA fragments that allows for the unattended analysis of samples in 96 or 384 well PCR plates (Appied Biosystems 3730/3730xl DNA Analyzer-Sequencing Chemistry Guide). It has the reproducibility of accurate sizing of microsatellite alleles to within ±0.3 base pairs which is an advantage over the ABI 377 (Buhariwalla and Crouch, 2004). The ±0.3 base pair size range is an important criterion for large scale germplasm genotyping projects (Wenz *et al.*, 1998). In addition, the system has the advantages of automated filling of capillaries. Therefore, the matrix does not have to be manually loaded on it. Thus, automated sample loading and rapid electrophoresis (Buhariwalla and Crouch, 2004). These systems also expose the worker to less toxic chemicals during handling and disposal (Buhariwalla and Crouch, 2004).

There is some work which has been reported on characterisation of other *Ocimum* species (Dwivedi *et al.*, 2006) but not *Ocimum gratissimum* L. This work therefore, reports on the genetic variation (characterisation) of *Ocimum gratissimum* L. from Kenya using RAPD, ISSR and AFLP markers.

3.4 Optimal leaf storage conditions and genomic DNA isolation in *Ocimum gratissimum* L.

3.4.0 Materials and Methodology

3.4.1 Determination of optimum Storage conditions for *O. gratissimum* L. leaves

A natural population of *O. gratissimum* was identified at Mill House, Njoro, Nakuru district 0 °19'S; 36 °E for sampling. Samples of this species were collected from three mother palnts and exposed to four different leaf storage conditions as follows; 1) storage in a freezer at –76 °C; 2) oven drying at 50 °C for 48 hrs and then storage at room temperature in a dark room; 3) air drying and then storage in a dark room at room temperature; 4) rapid drying using silica gel followed by storage in a dark room at room temperature. Before storage treatments, the leaf tissues were separated into two batches: one batch was cleaned with sterile distilled water (SDW) while the other was not cleaned. From each batch, leaf tissues were sampled for the storage treatments i.e.. All the samples were left under these conditions for a month and total genomic nucleic acids isolated.

3.4.2 DNA extraction from *O. gratissimum* L. leaf

Genomic DNA was isolated from the *Ocimum* specimens using four protocols; 1) the modified hexadecyltrimethylammonium bromide (2 x CTAB) mini preparation method described by Doyle and Doyle (1990), with 1 % 2-mercaptoethanol as a reductant; 2) the 2 x CTAB-extraction buffer which contained 6.5 mM dithiothreitol (DTT) as the reducing agent; 3) the modified Sodium dodecyl sulphate (SDS) mini

preparation method of Edwards *et al*., (1991) with 1 % 2-mercaptoethanol and 4) the (SDS) mini preparation method of Edwards *et al*., (1991) with 6.5 mM dithiothreitol as reductant. All DNA isolation was carried out in three replicates per treatment. The methods are as detailed below:

a) The hexadecyltrimethylammonium bromide (2 x CTAB) mini preparation method with 1 % 2-mercaptoethanol or 6.5 mM dithiothreitol

Fresh leaves (500 mg) were weighed into an eppendorf tube and then rapidly lyophilised in liquid nitrogen for two minutes. The leaves were ground in 600 µl of preheated (65 °C) extraction buffer (100 mM Tris-HCl pH 8.0; 20 mM EDTA pH 8.0, 1.4 M NaCl, 2 % CTAB and 1 % 2-mercaptoethanol). The mixture was homogenised with an eppendorf homogeniser and 10-20 mg of polyvinylpolypyrrolidone (PVPP) added to it and the contents incubated for 45 minutes at 65 ° C and vortexed every 15 minutes. 500 µl of chloroform: isoamyl alcohol (24:1) was added and mixed by constant swirling for 10 min and centrifugation at 14,000 rpm for five minutes. The supernatant was removed to a clean tube. The previous step was repeated and the supernatant was transferred again to a clean micro tube. The nucleic acids were precipitated by addition of 600 µl ice cold isopropanol and pelleted by centrifugation at 14,000 rpm for five minutes. The DNA pellet was washed twice with 1000 µl cold 70 % ethanol. The pellet was then

air dried and later re-suspended in 100 µl sterile distilled water and incubated in water bath overnight at 55 °C.

b) The hexadecyltrimethylammonium bromide (2 x CTAB) mini preparation method with 6.5 mM dithiothreitol

The same protocol described in (a) above was followed to isolate DNA from leaves of *Ocimum* using the 2 x CTAB minipreparation methods with 6.5 mM dithiothreitol. In this latter protocol, the 1 % 2-mercaptoethanol was replaced with 6.5 mM dithiothreitol as reductant.

c) The modified Sodium dodecyl sulphate (SDS) mini preparation method with 1 % 2-mercaptoethanol

Fresh leaves (500 mg) were weighed into an eppendorf tube and then rapidly frozen in liquid nitrogen for two minutes. The weighed leaves were ground in 800 µl extraction buffer (200mM Tris Hcl pH 7.5; 25 mM EDTA pH 8.0; 250mM NaCl, 10 % SDS (sodium dodecyl sulphate), and 1 % 2-mercaptoethanol). The mixture was homogenised with an eppendorf homogeniser and 10-20 mg of polyvinylpolypyrrolidone (PVPP) added to it. Another 400 µl of the extraction buffer was added and the homogenate vortexed followed by centrifugation at 14,000 rpm for two minutes in order to pellet the plant debris. The supernatant was removed to a clean tube. An equal volume of chilled phenol: chloroform: isoamyl alcohol (25:24:1) was added to the supernatant. The samples were mixed well to emulsify

and then centrifuged at 10 000 rpm for 10 min. The aqueous layer was transferred to a clean tube. The nucleic acids were precipitated by addition of 600 µl ice cold isopropanol and centrifuging at 14,000 rpm for five minutes. The resultant DNA pellet was washed twice with 1000 µl cold 70 % ethanol. The pellet was then air dried and later re-suspended in 100 µl sterile distilled water and incubated in a water bath overnight at 55 °C.

d) The modified Sodium dodecyl sulphate (SDS) mini preparation method with 6.5 mM dithiothreitol

The same protocol described in (c) above was followed to isolate DNA from leaves of *Ocimum* using the SDS minipreparation method with 6.5 mM DTT. In this protocol, the 1 % 2-mercaptoethanol was replaced with 6.5 mm DTT as reductant.

3.4.3 Purification of DNA

The DNA samples isolated using the above methods were purified as detailed. To each tube, 500 µl chloroform: Iso-amyl alcohol (CIA 24:1) was added and the contents mixed by shaking for 15 minutes, followed by centrifugation at 12000 rpm

for 15 minutes. The aqueous phase was transferred to a new tube and then 200 µl 1M NaCl-TE added to the old tube and shaken for 15 minutes. The old tube was centrifuged for 15 minutes at 12000 rpm. The aqueous phase was transferred to the new tube and mixed, followed by centrifugation at 12000 rpm for 15 minutes in order to settle any remaining debris. The supernatant was then transferred to a new tube. Ice cold isopropanol (700µl) was added to the sample and mixed gently, and centrifuged at 10,000 rpm for 5 minutes and the supernatant discarded. Cold 75% Ethanol (1000 µl) was added to the pellet to wash it. This was repeated thrice, and contents centrifuged at 5000rpm for five minutes. The Ethanol was discarded and the pellet air dried. The pellet was re-suspended in 200 µl sterile distilled water (SDW) and incubated overnight at 55 °C.

3.4.4 RNase treatment

The DNA was treated with DNase free Ribonuclease A (10 mg/ml). RNase (10 µl of 10 mg/ml; Sambrook *et al.,* 1989) was added to 100 µl of resuspended DNA pellet and then incubated at 37 ºC over night. Equal volume of ice-cold absolute ethanol was added to each sample and then centrifuged at 10,000 rpm for 10 minutes to re-precipitate the DNA. The supernatant was poured off and the DNA pellets air-dried and re-suspended in 100µl double sterile distilled water (dSDW).

3.4.5 Evaluation of quality and quantity of DNA

The DNA was quantified and examined for intactness by assessing ethidium bromide fluorescence using the gel electrophoresis method (Qiagen, 1997). The yield of DNA per gram of leaf tissue extracted was also quantified spectrophotometrically using a Bio-photometer at 260 nm. The purity of DNA was determined by calculating the ratio of absorbance at 260 nm to that of 280 nm (Sambrook and Russell, 2001).

3.4.5.1 Ethidium bromide fluorescence

Five microlitres of each of DNA was mixed with 5 µl of 1X gel loading buffer III (0.01 % bromophenol blue (w/v), EDTA Ph 8.0 (0.5 M) and 50 % glycerol (v/v)) and run in 1 % agarose gels containing ethidium bromide (0.5 µg/ml) buffered in 1X TBE (1M Tris – HCl [Ph 7.5], 1M Boric acid and 0.5 M EDTA (pH 8.0)] in a horizontal electrophoresis apparatus. Standards of 10 ng, 25 ng, 50 ng, 75 ng and 100 ng of uncut and unmethylated lambda (λ) DNA were also loaded and the gels run at 80 V for 1.30 hr. Gels were viewed under a UV Transilluminator Bio Doc-It™ System and photographed using its UVP printer (Mitsubishi). DNA concentration of the isolated *Ocimum* samples was estimated by comparing DNA band size and ethidium bromide staining intensity of the test samples against the λ standards.

3.4.5.2 Spectrophotometric determination

A Bio photometer (Eppendorf) was used to determine DNA concentration of *Ocimum gratissimum* samples. A 1:50 dilution of DNA in dSDW was made and the absorbance (optical density) measured at 260 nm. DNA concentration was calculated as follows:

DNA conc. of sample (µl/ml) = A_{260} x dilution factor x 50, where

A_{260} is the absorbance of DNA sample at 260 nm. DNA purity was estimated from the optical density (OD) ratios for the DNA samples at 260 nm and 280 nm when compared to the OD_{260} / OD_{280} for pure DNA preparations, which is equal to 1.8.

3.4.6 Results

3.4.6.1 Four different DNA extraction protocols

The results obtained showed that there was no intact quantifiable DNA obtained for most samples in all the four storage treatments using the 2 x CTAB with mercaptoethanol as assayed using the mini gel electrophoresis method. Any DNA obtained was degraded and was therfore low in molecular weight as can be seen from the smears. There were no differences between DNA samples from leaf tissue treated under different storage regimes (Figure 16.0 a, i). Rinsing of the leaves in water caused loss of DNA in most samples due to the soft velvet nature of the leaf which led to rotting of the leaf in the process of storage, (Figure 16.0 a, ii). Samples that yielded some DNA only had degraded low molecular weight DNA and this was

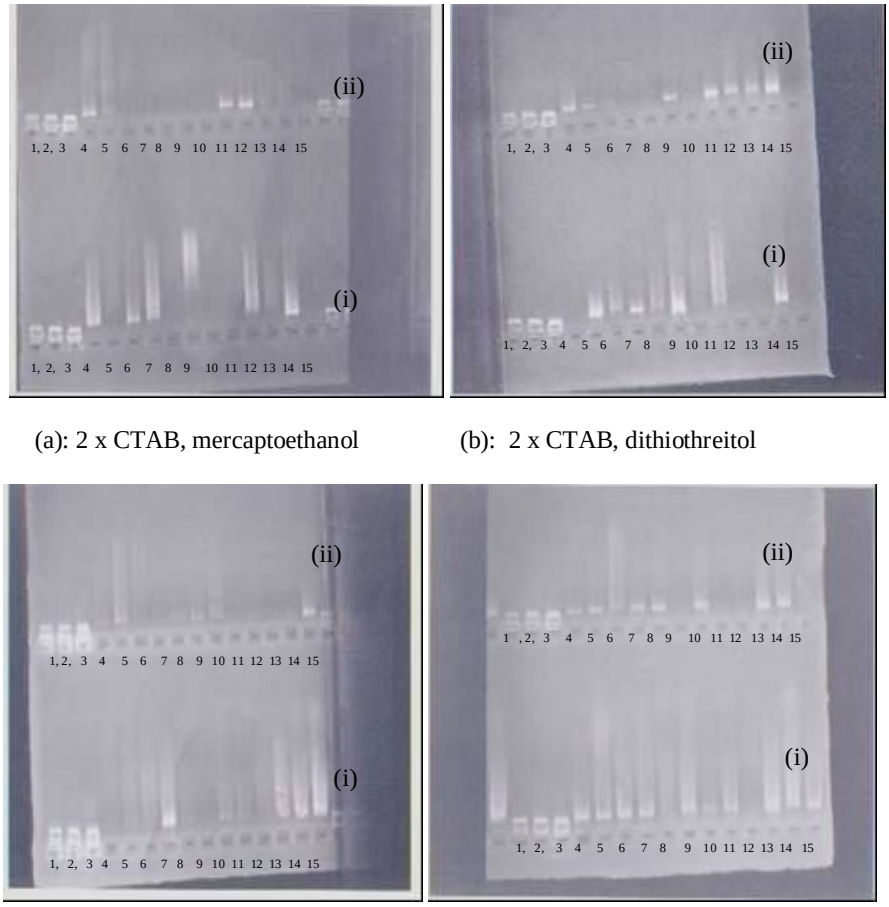
observed in samples which were air-dried and later kept in the dark cupboard, lanes 11and 12 (Figure 16.0 a, ii).

When 2 x CTAB was used with dithiothreitol (DDT), efficiency of DNA isolation was inconsistent (Figure 16.0 b, i and ii). However, with DDT more samples yielded some DNA when compared to the 2 x CTAB with mercaptoethanol treatment. These results indicated some improvement in getting DNA when the reducing agent dithiothreitol (DDT) was used. For the non water rinsed leaf, the rapid leaf drying methods (silica gel, oven drying) consistently gave quantifiable DNA. Leaf cleaning with sterile distilled water prior to storage and isolation of the total genomic DNA also resulted in low molecular weight DNA (Figure 16.0 b, ii). Rinsed air dried and frozen leaf tissue consistently gave quantifiable DNA.

The use of SDS with 2-mercaptoethanol to isolate genomic DNA from *Ocimum gratissimum* gave very poor results. Although some DNA was successfully isolated from the non rinsed leaf, this was only low in molecular weight and was degraded (Figure 16.0 c, i). This DNA was isolated only from the leaf tissue as well as from the rapidly dried tissue. For the leaf tissue that was rinsed with water prior to DNA isolation (Figure 16.0 c, ii), there was no DNA in all the leaf treaments.

The use of SDS buffer with DTT gave good DNA yield from all the samples from the non water rinsed leaf tissue and the DNA was largely intact and undegraded and was therefore high in molecular weight (Figure 16.0 d, i). However, the rinsed leaf samples did not yield as much DNA (Figure 16.0 d, ii) as those samples which were not rinsed (Figure 16.0 d, i).

Good quality DNA would give a quantification of ratio of 1.8 – 2.0, using the spectrophotometric determination. However, none of the above methods gave such high values, the range obtained being 1.4 -1.6 (Appendix II).



(a): 2 x CTAB, mercaptoethanol (b): 2 x CTAB, dithiothreitol

(c): SDS, mercaptoethanol (d): SDS, dithiothreitol

Fig 16.0: DNA isolation protocol a) 2 x CTAB with mercaptoethanol; b) 2 x CTAB with dithiothreitol; c) SDS with mercaptoethanol; (d) SDS with dithiothreitol.

1) In the diagram, (i) showed non rinsed leaf samples: lanes 1, 2, 3 were lambda (λ) DNA in concentrations of 50, 100 and 200 ng/ respectively;

lanes 4, 5, 6 were silica gel dried leaf; lanes 7, 8, 9 were oven dried leaf; 10, 11, 12 were air dried leaf; and 13, 14, 15 were frozen leaf samples.

2) In the diagram, (ii) showed rinsed samples: lanes 1, 2, 3 were λ DNA in concentrations of 50, 100 and 200 ng, respectively; lanes 4, 5, 6 were silica gel dried leaf; lanes 7, 8, 9 were oven dried leaf; 10, 11, 12 were air dried leaf; and 13, 14, 15 were frozen leaf samples.

3.4.7 Discussion

DNA extraction from leaves of *Ocimum gratissimum* was complicated probably by the abundance of secondary metabolites. This is also true with many other medicinal plants for instance *Terminalia arjuna* (Sarwat *et al*., 2006) where isolation of purified DNA proved to be a major bottleneck. The same has also been experienced with other species like *Theobroma cacao* (Haymes *et al.*, 2004), *Vitis vinifera* (Hanania *et al*., 2004), *Tagetes minuta* (Hills and van Staden, 2002), *Eucalyptus* spp., *Pinus* spp., *Araucaria cunninghamii* (Shepherd *et al*., 2002), *Davidia involuctata* (Li *et al*., 2002), *Anthurium andreanum* (Buldewo and Jaufeerally-Fakim, 2002), *Drosera rotundifolia, Artemisia dracunculus* (Pirttila *et al*., 2001) *etcetera*.

Extraction of DNA from *O. gratissimum* using 2 x CTAB and the reducing agent mercaptoethanol yielded some degraded DNA in both the rinsed leaf samples dried in silica gel and frozen samples. The results of those leaf samples treated using 2 x CTAB and the reducing agent dithiothreitol had a much higher DNA yield both for the rinsed frozen samples and non rinsed silica dried samples. There was some

scanty low molecular weight DNA in oven dried leaf non rinsed samples and air dried rinsed samples. There are several possible reasons to such a result. It could be that the plant *O. gratissimum* has a lot of proteins in addition to the presence of polyphenols. The presence of the buffer CTAB helps in the release of DNA from its nuclear membrane. However, in the presence of the reducing agent mercaptoethanol (ME), the DNA extracted was of low molecular weight as compared to that obtained using the reducing agent dithiothreitol (DTT). The reducing agent DTT reduces proteins at the millilolar levels (Ruegg and Rudinger, 1977; Ask a Scientist Molecular Biology Archive, 2005) which would otherwise interfere with DNA extraction, hence the improved DNA with DTT. It could also be that the DNA which was extracted with DTT was thiolated DNA. With DTT, the thiolated DNA is protected from forming dimers with other thiolated DNA in the presence of oxygen. DTT is a strong reducing agent, and it removes almost all the oxygen atoms preventing the formation of dimers and hence prevents degradation of DNA (Cleland, 1964). Though mercaptoethanol may have sulphur, it is likely that the atom is not available as a terminal atom and hence can not be available to reduce the oxygen atoms which become readily available in the buffer and which oxidise the thiolated DNA which then forms dimers with other thiolated DNA ultimately causing degradation.

When the genomic DNA was extracted from *Ocimum gratissimum* using SDS and the reducing agent DTT, good quality DNA was realised. In addition to the strong reducing agent DTT, SDS which is a negatively charged ionic detergent is able to break down the lipid made membrane of the nucleus thus releasing the DNA. SDS is

also able to coat proteins with the negative charges thereby denaturing them (Harewood and Wolff, 1973). It is known that when SDS is added to proteins (with heat); it disrupts interactions between side groups and disrupts all of the disulfide bonds that stabilise the higher order structure of proteins. SDS then coats the proteins thus preventing any of the higher order structures from re-forming (Harewood and Wolff, 1973). Therefore, with the removal of proteins by SDS and DTT, the removal of oxygen by DTT which would otherwise have degraded the thiolated DNA, the removal of polyphenols by PVPP and phenol, the chelating of bivalent cations by EDTA thereby inhibiting metal dependent DNases that degrade DNA randomly, intact high molecular weight DNA was successfully extracted from *Ocimum gratissimum*. According to Jobes *et al.*, (1995), in the presence of PVPP, phenolics adhere to DNA in solution forming a coloured extract around the DNA that becomes cleaner after the addition of the detergent SDS. The addition of high molar concentration of NaCl increases the solubility of polysaccharides in ethanol, effectively decreasing co-precipitation of polysaccharides and DNA (Fang *et al.*, 1992; Aljanabi *et al.*, 1999). The addition of DTT also helps the removal of polyphenolics and other contaminants (Kumpatla *et al*., 2004).

In this study, it was shown that SDS with DTT worked better than any other combination of the tested reagents. Thus, the only extraction protocol that proved successful was the SDS based isolation protocol (Edwards *et al*., 1991). This protocol was used with dithiothreitol as a reducing agent as was also reported by Waldschmidt *et al*., (1997). The DNA obtained was restrictable and useful for AFLP

analysis. This study showed that some detergents work better in conjunction with specific reducing agents during DNA extraction for some plants.

This study also established that the success of genomic DNA extraction was dependent on the leaf sample treatment prior to extraction. Thus, the method of tissue storage is also very important in order to obtain good quality DNA (Thomson, 2002; Bhattacharjee *et al.,* 2004). It was observed that the samples which were silica gel dried as well as those that were freeze dried gave high molecular weight DNA as opposed to those samples which were air dried and oven dried. The silica gel dried as well as freeze dried leaves maintained the integrity of the leaf cells and therefore no compounds or enzymes which could degrade DNA were released as a consequence of plasmolysis of vacoules. It may therefore be concluded that these leaf pre-treatments prevented chemical reaction among the cell contents of the leaves prior to DNA extraction.

The leaf samples which were air dried (slowly drying) and oven dried (rapid drying at high temperature) could have resulted in plasmolysis and breakdown of the tonoplast thereby releasing terpenes which are the major secondary metabolites of Ocimum. The released terpenes could have then been oxidised into terpenoids which consequently damaged organelle membranes, for instance, lysosomes which contain numerous membrane bound enzymes including nucleases like DNase. Sikkema *et al.*, (1995) demonstrated that terpenes damage cell membrane structures because they are lipophilic (lipid loving). When cells are disrupted, major cytoplasmic compounds can come into contact with nuclei and other organelles resulting in degradation of nuclei

contents (Loomis, 1974). For example in their oxidised forms, polyphenols covalently bind to DNA giving it a brown color and making it useless for most research applications (Katterman and Shattuck, 1983; Guillemaut and Maréchal-Drouard, 1992). One method commonly used to avoid problems with polyphenols involves freezing the tissue during or prior to homogenization (Katterman and Shattuck, 1983; Leutwiler *et al.*, 1984). The presence of these compounds renders studies difficult due to the long and tedious DNA extraction procedures required though with even these procedures, rarely is good DNA obtained. It is therefore, plausible that in this experiment even before extraction, the terpenes released from the vacuoles of air and oven dried tissue rapidly got oxidised (in the absence of the strong reducing agent DTT) to terpenoids which then attacked the membrane bound vesicles that contained the enzymes, thereby causing the degradation of DNA which was not protected after release. That explains why the DNA extracted from air and oven dried leaf tissue either had little or only low molecular weight DNA for both the 2 x CTAB and SDS buffers using either mercaptoethanol or DTT as reducing agents. It is known that, success in the extraction of DNA depends also on the methods of sampling in the field and preservation of the samples in the laboratory (Drabkova *et al.*, 2002; Feres *et al.*, 2005). *Ocimum gratissimum* species exhibited oxidation (leaf fermentation) when left under humid conditions for some time after collection, which meant that it was necessary to dry or freeze them as fast as was possible to better preserve the DNA.

It is also important to realise that among these samples which gave the best DNA, the rinsed samples seemed affected. Thus, the DNA extracted from the non rinsed

samples was much better than the DNA from the rinsed samples. Apart from the rotting of the leaf samples as a contributing factor, it is possible that the presence of water on the leaf samples caused some biochemical reactions. Rinsing may have caused the hydrolysis of complex carbohydrates as well as oxidation of compounds of the phenylpropanoid pathway which are predominant in this spcies. Therefore the oxidation of the phenolic compounds could have led to the breakdown of some membrane bound vesicles leading to partial oxidation of some DNA prior to extraction.

3.4.8 Conclusion

This study revealed that, the methods of storage after leaf sample collection and the composition of DNA extraction buffers have a major influence on the efficiency and effectiveness of DNA isolation from *Ocimum gratissimum* and consequently the success of intended molecular studies.

Collecting conditions and preservation of samples are important for the quality of DNA (Ribeiro and Lovato, 2007) as also observed in this study. Good quality DNA from *O. gratissimum* L. was obtained from the non-water rinsed and silica gel dried leaf or frozen leaf samples using a buffer containing the detergent SDS with the reducing agent dithiothreitol. Though cleaning of leaf samples before DNA extraction is recommended for good DNA yield, (Thomson, 2002; Bhattacharjee *et al.*, 2004), this study revealed that, care must be taken when dealing with soft velvety leaves such as those of *Ocimum gratissimum* because the water may lead to rotting of the leaves and oxidation of secondary metabolites leading to poor yields of nucleic

acids as observed in this study. Probably, it would be recommended to swab the leaves with cotton wool wet with alcohol prior to preserving in order to dust off the leaf.

3.5 Genetic variation studies in *Ocimum gratissimum* from Kenyan populations

3.5.0 Materials and Methodology

3.5.1 Leaf sample Collection

In order to study the genetic variation of *O. gratissimum* L., fresh leaf samples were collected from 13 populations as seen in (Tables 8.0 & 9.0) distributed throughout Kenya (Figure 6.0).

Collection sites were chosen mainly along the road where the herb is regularly found though a few collections were also made from cultivated fields. Sample collection details are found in appendix I.

For the selected herbs, the colour of the leaves, flowers and bark was observed and recorded using the Royal Horticultural Society Colour Chart (Appendix I). This was done in order to find out whether there was any phenotypic variation that could be correlated to genetic variation in succeeding studies. The collected leaf samples were cleaned with distilled water, wiped dry with cotton wool and then stored in two parts. One part was stored in a freezer at –76 ° C and the other part in silica gel. This method of preservation allowed several months of storage without affecting the yield and quality of the extracted DNA (Milligan, 1994).

3.5.2 Genomic DNA extraction using SDS method

Genomic DNA was isolated from the leaf samples using the Sodium dodecyl sulfate protocol of Edwards *et al*., (1991) with slight modifications as described in section 3.2.1.2.d. and purified as described in section 3.4.3.

Table 8.0: Source of *O. gratissimum L.* in Kenya

Population	**Co-ordinates of the mapped sites**		**Natural Vegetation**	**Altitude metres above sea level**	**Average annual Rainfall (mm)**
	Eastings	**Northings**			
Kakamega (Savona Isle)	34.75	0.42	Rainforest	1500-1850	1100-2700
Kisumu (Riat)	34.75	-0.1	Mountain grassland and bush land	1200-1500	800-1400
Nakuru			Mountain	1850-2150	800-1400

(Mill house I and II) (Njoro I and II)	35.92 -0.25 35.93 -0.37	grassland and bush land		
Meru (Rugucu)	37.4 0.43	Bush land and thicket	2150-2450	1000-1600
Nyeri (Kiganjo)	37.0 -0.38	Mountain forest	1850-2150	800-1400
Thika (JKUAT)	37.2 -1.0	Mountain grassland and bushland	1200-1500	600-1100
Taita Taveta (Kibarani)	38.37 -3.4	Bushland and thicket	900-1200	450-900
Mombasa (Kinango)	39.33 -4.18	Mangrove swamp	0-900	800-1400
Kabarnet (Chesigei)	35.74 0.48	Bushland and thicket	1850-2150	800-1400
Kericho (Roret)	35.33 -0.58	Mountain forest	1850-2150	1100-2700

Table 9.0: Ecological regions where *O. gratissimum L.* leaf samples collected from 13 populations

Population Town	Population location name	Population type	Average weight of 100 seeds (g)	N
Kericho	Roret near Bureit	In cultivated field	0.047	25
Kakamega	Savona Isle resort	Natural near river Isikhu	0.039	25
Kisumu	Riat (5km from Kisumu town towards	Natural near a road	0.037	25

	Kakamega			
Nakuru	Mill House I	In a cultivated field	0.051	25
Kabarnet	Chesigei	Natural near a road	0.036	25
Meru	Rugucu	Natural near a road	0.046	25
Nyeri	Kiganjo	In a cultivated field	0.061	25
Taita taveta	Kibarani area	Natural near Mukachi river	0.035	25
Mombasa	Mariakani towards east south coast Kinango	In a cultivated field near a stream	0.035	17
Nakuru 1	Njoro I	Natural near a road	0.051	25
Nakuru 2	NjoroII	Natural near a stream	0.051	25
Nakuru 3	Mill house II	Natural near a railway line	0.051	25
Thika	JUAT campus	Near a field	0.029	25

In all populations, seeds were collected also. Voucher number 805LG. N denotes the sample size of each population.* Predominant vegetation of collection site in Appendix I.

3.5.3 RNase digestion

The samples were treated with RNase as described in section 3.4.4.

3.5.4 Evaluation of quality and quantity of DNA

The DNA concentration and purity was determined as described in sections 3.4.5.1 and 3.4.5.2 respectively.

3.5.5 The RAPD-Polymerase Chain Reaction (PCR)

Appropriate dilutions of the genomic DNA were made in sterile double distilled water to a final DNA concentration of approximately 2.5 ng / µl for the RAPD-PCR reactions. These dilutions were stored at -20 ºC and were only thawed twice before discarding, in order to maintain the quality of DNA.

3.5.5.1 Optimisation of RAPD (PCR)

RAPD-PCRs were optimised for the following variables; *Taq* DNA polymerase concentration, Magnesium ion concentration, annealing temperature and template DNA concentration. All the optimisation reaction used the same four and sometimes five DNA samples derived from the following germplasm; KAK 6, KIS 34, NAK 59, MOM 192 and KER 206 in that order. Four different oligonucleotide primers were used namely OPW-04, OPW-07, AB4-13 and OPV-17 (Sequence of primers in Table 11.0). All primers were synthesised in the International Livestock Research Institute (ILRI). In the PCR optimisation reactions, all factors were kept constant except for the variable being optimised.

(a) Optimisation of Taq DNA concentration

Five DNA samples were used as named above (section 3.6.1.6.1). Taq DNA polymerase (Perkin Elmer, USA) of varying concentrations was used for the

optimisation process. The concentrations used were: 0.5 units (U), 1.0 U, 2.0 U, 3.0 U, 4.0 U and 5 U per PCR of 10 µl final volume. Each concentration of *Taq* DNA tested, used all the four primers.

(b) Optimisation of Magnesium Chloride concentration

Four DNA samples as described above were used. Magnesium chloride of varying concentrations were tested for PCR optimisation: 1.5 mM, 2.0 mM, 2.5 mM, 3.5 mM, 4.0 mM, and 5.0 mM .

(c) Optimisation of DNA concentrations

The concentration of template DNA in the PCR was also optimised in order to establish the optimal working concentration for RAPD-PCR. The concentrations tested were 2.5 ng, 5.0 ng, 10 ng and 20 ng per 10 µl PCR final volume. Five DNA samples were used in the optimisation assays.

(d) Optimisation of annealing temperature

The optimal primer annealing temperature was determined by using the optimised *Taq* DNA polymerase concentration, Magnesium chloride concentration and template DNA concentration as was defined following the experiments described while varying the annealing temperaturein different PCR tubes above:

Master Mix for a 10 µl PCR volume

DNA volume (2.5 ng)	0.5 µl (from a 1:50 dilution)
Taq polymerase (0.5U)	0.1 µl
dNTPs (200 µM)	0.5 µl
$MgCl_2$ (3.5mM)	0.7 µl
10 X Reaction Buffer	1.0 µl
Primer	1.0 µl (20 ng/µl)
SDW	6.2 µl

The annealing temperatures that were tested were: 36 °C, 38 °C, 40 °C, 42 °C and 44 °C. Details of optimisation reactions are summarised in Table 10.0.

Table 10.0: Optimisation conditions for RAPD markers

Primer Concentration (20 ng / µl)	Primer volume (pmol)	DNA samples (ng)	$MgCl_2$ (mM)	dNTPs (mM)	Taq (U)	Annealing temperature (° C)
OPW-04 OPW-07 AB4-13 OPV-17	0.2	2.5	1.0	0.2	0.5 1.0 2.0 3.0 4.0	42 ° C

					5.0	
OPW-04 OPW-07 AB4-13 OPV-17	0.2	2.5	1.5 2.0 2.5 3.5 4.0 5.0	0.2	0.5	42 ° C
OPW-04 OPW-07 AB4-13 OPV-17	0.2	2.5 5.0 10.0 20.0	3.5	0.2	0.5	42 ° C
OPW-04 OPW-07 AB4-13 OPV-17	0.2	2.5	3.5	0.2	0.5	36 ° C 38 ° C 40 ° C 42 ° C 44 ° C

3.5.5.2 RAPD reaction

PCR reactions involving 10-mer arbitrary oligonucleotide primers for RAPD analysis contained approximately 2.5 ng of genomic DNA, 200 µM each of dATP, dCTP, dGTP and dTTP (Borehringer Mannheim, Germany), 100 µM primer, 10x PCR Buffer II (Perkin Elmer, USA-10Mm Tris-HCl Ph 9.0, 50 mM KCl, 0.1 % Triton X-100, 1.3 % BSA), 3.5 mM $MgCl_2$ (Perkin Elmer,USA) and 1.0 U of Taq DNA

polymerase (Perkin Elmer, USA) in a final volume of 10 µl. Amplification was carried out in a Micro Amp Optical 96-Well Reaction Plate (Eppendorf) using an MJ Research Thermal Cycler (Inc. PTC- 200) programmed as follows: initial denaturation step of 5 min at 93 º C, followed by 40 cycles of 1 min at 93 º C, 1.5 min at 42 º C, 1 minute at 72 º C with a further final extension step of 10 min at 72 º C. The initial long pre-amplification step at 93 º C is required to activate the enzyme, Taq DNA polymerase. A negative control of sterile distilled water was incorporated in every PCR run. The reproducibility of the RAPDs was tested by repeating a subset of similar samples across PCR runs. Only those bands which showed consistent amplification were considered in this study.

24 RAPD primers (Table 11.0) were screened for polymorphism in the *Ocimum gratissimum* germplasm and the ones that produced good results were selected for further analysis. Each of the 24 RAPD primers were tested against a selected sample of 20 DNA samples and those that gave clear banding patterns were chosen for the study.

Table 11.0: RAPD primers screened for polymorphism in *Ocimum gratissimum* L.

PRIMER	**5' SEQUENCE 3'**
AB4-04	AAGTCCGCTC
AB4-04	GTCAGAGTCC
AB4-19	GGTGCACGTT
OPB-07	GGTGCACGTT

OPC-02	GTGAGGCGTC
OPD-05	TGAGCGGACA
OPD-10	GGTCTACACC
OPD-18	GAGAGCCAAC
OPD-20	ACCCGGTCAC
OPE-01	CCCAAGGTCC
OPF-01	ACGGATCCTG
OPG-06	GTGCCTAACC
OPV-19	GTCAGTGCGG
OPV-02	AGTCACTCCC
OPV-03	CTCCCTGCAA
OPV-08	GGCGAAGGTT
OPV-15	GAGGGCCAGT
OPV-18	GAGGTCCACA
OPW-02	ACCCCGCCAA
OPW-03	GTCCGGAGTG
OPW-04	CAGAAGCGGA
OPV-06	ACGCCCAGGT
OPV-14	AGATCCCGCC
OPV-17	ACCGGCTTGT

All primers were synthesised in the International Livestock Research Institute (ILRI), Kenya.

3.5.5.3 RAPD-PCR Product resolution

RAPD-PCR products were resolved by electrophoresis on 1.5 % agarose gels in 1 X TBE buffer by running at 150 V for 3:30 hrs. Five microlitres of 1X loading buffer

was added to the PCR products and 15 µl of this mixture run on the gel. Products were visualized under UV light after staining for 30 min in a 1 µg/mL ethidium bromide, and then photographed using a UV light machine Bio Doc-It™ System UVP printer (Mitsubishi). Products were sized against 100 bp ladder.

3.5.6 Inter simple sequence repeats (ISSR-PCR)

Dilution of the genomic DNA were prepared in sterile double distilled water to a final DNA concentration of approximately 2.5 ng / µl just like for the RAPD-PCR reactions. These dilutions were stored at -20 ºC and were only thawed twice before discarding, in order to maintain the quality of DNA.

3.5.6.1 ISSR optimisation

The optimisation of the ISSR-PCR variables followed the same procedure as that in section 3.6.1.6.1.

3.5.6.2 ISSR reaction

The PCR amplification reactions for ISSRs included 100 nM 17-19mer arbitrary 3' anchored ISSR primer, 200 nM dNTPs (Roche, Applied Biosystems), 3.5 mM

Magnesium chloride, 10X PCR Buffer II (Perkin Elmer, USA-10Mm Tris-HCl pH 9.0, 50 mM KCl, 0.1 % Triton X-100, 1.3 % BSA), 0.5 units of Taq DNA polymerase (Perkin Elmer, USA), 2.5 ng of genomic DNA in a final volume of 20 µl. The thermocycler (an MJ Research Thermal Cycler, Inc. PTC- 200) was programmed as follows: Initial denaturation step of 5 minutes at 94 ºC, followed by 40 cycles of 30s at 94 ºC, 45s at 57 ºC, 2 min at 72 ºC and a final 10 minutes extension at 72 ºC. The initial long pre-amplification step at 94 ºC was required to activate the *Taq* DNA polymerase.

The PCR products were re-amplified using the same reaction primers as in the first PCR to increase the resolution of the PCR products. Reproducibility of the ISSR analytical procedure was investigated with repeated analysis of several samples. Only those bands which showed consistent amplification were considered in this study.

16 anchored ISSR primers were screened and the ones that produced good results were selected for further analysis. Each of the 16 ISSR primers were tested against the selected DNA samples and those that gave clear banding pattern were chosen for the study. The sequences of ISSR primers selected and used in this study are shown in the Table 12.0 below.

Table 12.0: ISSR primers screened for polymorphism in *Ocimum gratissimum* L.

PRIMER NAME	5’ SEQUENCE 3’
808	$(AG)_8C$
810	$(GA)_8T$
816	$(CA)_8T$
813	$(CT)_8T$
842	$(GA)_8TG$
851	$(GT)_8CG$
850	$(GT)_8TC$
849	$(GT)_8CA$

All primers were synthesised in the International Livestock Research Institute (ILRI), Kenya

3.5.6.3 ISSR-PCR product resolution

ISSR-PCR products were resolved by electrophoresis on 2.5 % agarose gels in 1X TBE buffer, by running at 150 V for 3:30 hrs. Five microlitres of the loading dye was added to the PCR products and 15 µl of this mixture loaded and run on the gel. PCR products were visualized under UV light after staining for 30 min in a 1 µg/mL ethidium bromide, washed briefly with tap water and then photographed using a UV photographing machine Bio Doc-It™ System UVP printer (Mitsubishi). The products were sized against 100 bp DNA ladder.

3.5.7 Amplified fragment length polymorphism analysis (AFLP- PCR)

The AFLP method was carried out following the standard procedure as described by Vos *et al*., (1995) adapted in the AFLP ® Plant Mapping protocol of the Applied Biosystems (ABI), USA.

3.5.7.1 Template preparation and adaptor ligation

Before carrying out the AFLP analysis, the suitability of the restriction enzymes chosen to cut the *Ocimum gratissimum* genomic DNA was first tested. This was carried out by digesting the genomic DNA with *Mse*I (frequent-4-base cutter-TAA) and *Eco*RI (rare-6-base cutter-AATTC) restriction enzymes separately and then in combination. 20 µl of the genomic DNA was incubated for 2 hours at 37 ºC with 2 µl of *Eco*RI/*Mse*I [1.25 units/µl each in 10mM Tris-HCl (pH 7.4), 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mg/ml bovine serum albumen (BSA), 50 % glycerol (v/v), 0.1 % Triton® X-100], and 5 µl of 5X reaction buffer [50 mM Tris-HCl (pH 7.5), 50 mM Mg-acetate, 250 mM K-acetate]. Five microlitre of each of the digested products sample in 5µl of 1X blue dye was run on 1.5 % agarose gel in 1 x TBE to check for complete digestion with DNA size markers (100 bp ladder).

A restriction-ligation enzyme master mix was prepared by combining 1 µl of T_4 DNA ligase [1 unit/µl in 10 mM Tris-HCl (ph 7.5), 1 mM DTT, 50 mM KCl, 50 %

(v/v) glycerol] with 24 µl adapter/ligation solution [*Eco*RI/*Mse*I adapters, 0.4 mM ATP, 10 mM Tris-HCl (pH 7.5), 10 Mm Mg-acetate, 50 mM K-acetate]. The adapter pairs were first annealed to make them double stranded by heating the tubes in a water bath at 95 ºC for five minutes. The tubes were then cooled to room temperature over a 10-minute period. This reaction mixture was then incubated at room temperature overnight. The restriction-ligation products were then diluted by adding 18.9 µl of 1X TE to 10µl of the reaction mixture in a 1.5 ml micro-centrifuge tube to give the appropriate concentration for subsequent PCR. The diluted products were stored at 4 ºC for use within one month or – 20 ºC for longer period.

3.5.7.2 Pre-selective amplification

PCR amplification of the adapter-ligation restriction fragments was performed for subsequent selective amplification. The PCR reactions were performed in a 20 µl volume consisting of 4.0 µl diluted restriction-ligation DNA products, 1.0 µl AFLP pre-selective primer pairs (EcoR1+ A and Mse1+ C) and 15 µl core mix. Pre-amplification was carried out at initial hold time of two minutes at 72 ºC followed by 20 cycles of 20 seconds at 94 ºC, 30 seconds, at 56 ºC, and 72 ºC and a further hold time of 30 minutes at 60 ºC.

3.5.7.3 Verification of successful amplification

To check the success of the pre-selective amplification reaction, 10 µl of pre-selective amplification products was mixed with 2 µl of 10X loading dye and run on

1.5 % agarose gel in 1X TBE buffer at 4 V/cm for 3.30 hrs. The gel was stained with 1 µg/ml ethidium bromide and viewed on a UV transilluminator. The presence of a smear of products from 100-1500bp meant that the pre- amplification was successful. For each sample, 10 µl of the pre-selective amplification product was then diluted with 19.0 µl 1X TE buffer, and stored at 4 ºC.

3.5.7.4 Selective amplification reaction

Selective amplification was conducted using various combinations of two AFLP primers specific for *Eco*RI +3 and *Mse*I +3 primer adapters on a test panel of representative samples. These primers have three additional 3' nucleotides. The *EcoRI* primers are florescent-labelled (Table 13.0). Initially, 16 primer combinations were used for screening in a representative sample of four accessions (Table 13.0). Seven of the best primers (*) showing high polymorphism were then used in full analysis of the test germplasm. The selective PCR was performed in 20 µl volumes consisting of 3 µl of the diluted pre-selective amplification products, 1 µl florescent-labeled *Eco*RI primer, and 1 µl of *Mse*I primer and 15 µl of AFLP core mix. The selective PCR amplification was programmed for an initial two minutes at 94 ºC followed by one cycle of 94 ºC for 20 seconds, 66 ºC for 30 seconds and 72ºC for two minutes. This cycle was repeated 12 times with a lowering of the annealing temperature of 1 ºC per cycle. This was followed by 20 cycles of 94 ºC for 20 seconds, 56 ºC for 30 seconds and 72°C for two minutes and a further hold time of 30 minutes at 60 ºC.

Table 13.0: AFLP selective amplification primers screened in *Ocimum gratissimum* L

Mse I Primers					
EcoRI Primers		-CAA	-CAC	-CTG	-CAG
	-ACT	√*	√	√*	√
	-ACA	√*	√	√*	√
	-ACC	√*	√*	√	√
	-AGC	√*	√	√	√

√ implies primer combination during screening

* implies primer combination that detected high polymorphism for further analysis

All primers were purchased from Applied Biosystems -ABI, (Germany).

3.5.7.5 Selective amplification product resolution

AFLP analysis was advanced by development of automated capillary array systems sequencers. A capillary array system from Applied Biosystems-ABI (Forster City, CA, USA) was used. LICOR Biosciences (Lincoln, NE, USA) and Amersham Biosciences (Picataway, NJ, USA) are most common for sequencing and fragment analysis (Rinehart, 2004).The ABI capillary systems located at the International

Livestock Research Institute (ILRI), in Nairobi, Kenya, was used to resolve the selective amplification products in this study.

Samples were prepared for analysis on ABI 3130*xl* genetic analyzer from the selective amplification products. The ABI 3130*xl* genetic analyzer is a fluorescent-based 48 capillary detection systems that use polymers as the separation matrix. Apart from the reproducibility of accurate sizing of base pairs which is an important criteria for large scale germplasm genotyping projects (Wenz *et al*., 1998); the system has also the advantage of automated filling of capillaries so the matrix does not have to be manually loaded on it, automated sample loading and rapid electrophoresis (Buhariwalla and Crouch, 2004). These systems also expose the worker to less toxic chemicals during handling and disposal (Buhariwalla and Crouch, 2004).

Each run consisted of 96 samples. Samples were loaded through the use of an autoloader, which transfered a small aliquot of purified sample from a 96 well plate. A loading buffer mix was prepared by adding 12 µl of Gene Scan 500 LIZ internal size standard (ABI) to 1 ml (1000 µl) deionised formamide HiDi. Nine microlitres of the size standard mix was added to 1 µl of the selective amplification products in a MicroAmp PCR Plate. From the *ABI PRISM* 3730 / ABI 3130*xl* genetic analyzer, the sample data was directed to the GeneMapper Software version 3.0 which analysed and displayed the sizing results as electrograms and tabular data.

All the primers produced peaks that could easily be interpreted. The Southern algorithm automatically calculated the fragment sizes using the GeneMapper Software version 3.0. The software was used to score the alleles. Category bins were created in GeneMapper to be able to group peaks based on the sizes of the allele. The category was defined by size in base pairs and an automatic standard deviation which was a maximum shift that a fragment could show across the individual capillaries and still be scored as the same allele. Athreshold of the peak height was set at 50 - 500 relative fluorescent units (rfu) such that any peak that was below this was treated as an artifact and was not scored by the software (Palsson *et al.*, 1999). Category bins and peak height threshold ensured accurate allele scoring.

3.5.8 Data analysis

Various statistical methods were used to analyse population genetic diversity as revealed by the arbitrary primers. The analysis was based on both individual and population frequency data. Arlequin software version 2.000, POPGENE Ver. 1.32 and GenA1Ex 6 were used in the study.

3.5.9 Genetic structure study

Each polymorphic band was considered as a locus and identified by the presence or absence of the band. The RAPD and ISSR products were scored by visual inspection of gel images as a matrix of product presence (1) and product absence (0) in a

spreadsheet (Microsoft Excel 97). From the automated sequencers, the AFLP products were scored using the Gene mapper package version 3.0 as a matrix of product presence (1) and product absence (0) in a spreadsheet (Microsoft Excel 97. Data in spreadsheets was then appropriately configured as input files for analysis using different population diversity software packages. Analysis was undertaken based on the individual and the population frequency data.

3.5.9.1 Nei's unbiased diversity

Population allele frequency data and diversity values estimated assuming Hardy-Weinberg equilibrium were calculated and analysed with POPGENE Ver. 1.32 (Yeh *et al.*, 1999) using Nei's unbiased statistic (Nei, 1987):

$$H = n\,(1- \sum [p_i^2])/\,(n-1)$$

Where n = number of individuals analysed and p*i* is the frequency of the *i*th allele. Diversity values were averaged across loci.

3.5.9.2 Nei's genetic distance and cluster analysis

Genetic distance (D) between population frequency data set was generated with POPGENE 1.32 (Yeh *et al.*, 1999) from the equations of Nei (1978);

$$D = -1n\,[J_{XY} / {}_{(}J_X J_{Y)}{}^{½}]$$

Where, J_x, J_y and J_{xy} are the arithmetic means of the individual loci identities $\sum x_i^2$, $\sum y_i^2$ and $\sum x_i y_i$ over all loci, respectively.

Cluster analysis based on genetic distances was undertaken according to equations of Nei (1978) using unweighted pair-group method with arithmetic averaging (UPGMA; Sneath and Sokal, 1973) to generate a dendrogram showing relationships among populations. The degree of polymorphism was also quantified using Shannon's index of phenotypic diversity (King and Schaal, 1989).

The ARLEQUIN software version 2.000 (Schneider *et al.,* 1997) and GenA1Ex 6 were used to partition genetic variation into within and between populations components according to an analysis of molecular variance (AMOVA; Excoffier *et al.*, 1992) based on Euclidean distance. Significance values were assigned to variance components based on the random permutation (5000 times) of individuals assuming no genetic structure.

3.5.10 Results

3.5.10.1 Quality of DNA

The genomic DNA of fresh leaves of *O. gratissimum* L. after gel electrophoresis (Figure 17.0) which used the SDS extraction method by Edwards *et al.*, (1991) in section 3.2.1.2 (d). This was part of the genomic DNA from 140 samples of *O. gratissimum* L. which were used for the study (Figure 17.0).

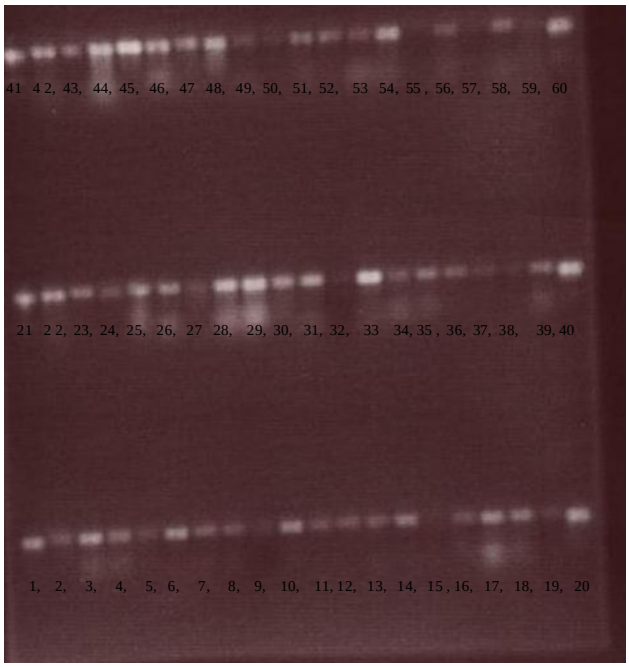


Figure 17.0: Lanes 1-60 showing DNA of *O. gratissimum* run on an agarose gel to check its quality before RNase treatment.

Figure 18.0 shows the genomic DNA samples after RNase treatment, together with the serial dilutions of the uncut Lambda (λ) DNA.

The spectrophotometer readings at 260 nm and 280 nm for the 140 samples of *O. gratissimum* ranged between 1.4 to 1.6 which was slightly below the expected 1.8. Data is in Appendix II.

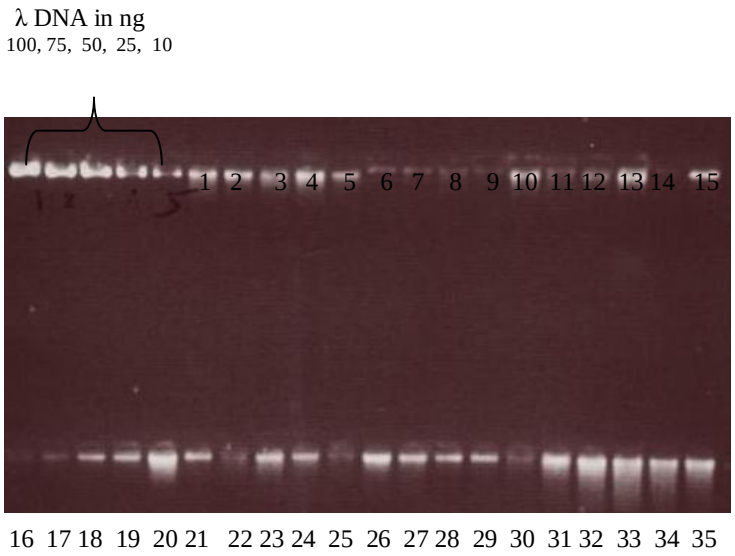


Figure 18.0: Genomic DNA quantification using gel electrophoresis after RNase treatment. Serial dilutions of uncut Lambda DNA (λ DNA) are represented in the first five lanes at 100 ng 75 ng, 50 ng, 25 ng and 10 ng total DNA. The other lanes 1-35 represent RNA free DNA samples from *O. gratissimum* L.

3.5.10.2 Optimisation conditions of primers for RAPD and ISSR markers

The results on optimisation of *Taq* DNA polymerase concentration showed that positive amplicons were most consistently produced at a DNA polymerase concentration of 0.5 units (Figure 19.0)

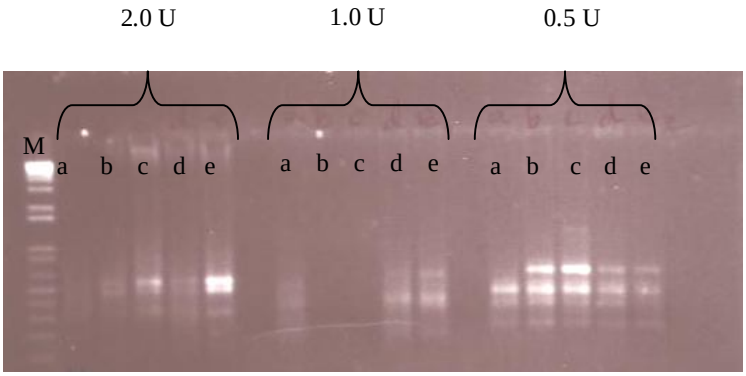


Figure 19.0: Gel showing amplicons from different *Taq* polymerase concentrations namely: 2.0 U, 1.0 U and 0.5 U at an annealing temperature of 42 °C. The same genotypes (a-e) of *O. gratissimum* are used in the three experiments. M is 100 bp DNA ladder. Primer OPW 04 was used.

The 0.5 U concentration of *Taq* DNA polymerase was adopted during the optimisation of $MgCl_2$ concentration. At 3.5 mM concentration of $MgCl_2$, more samples were amplified as compared to the other concentrations (Figure 20.0).

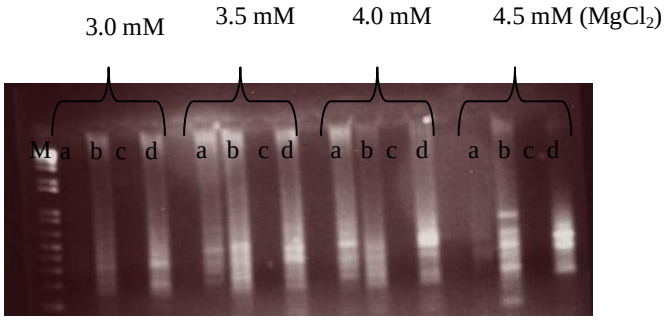


Figure 20.0: Gel showing amplicons from different $MgCl_2$ concentrations namely: 3.0 mM, 3.5 mM, 4.0 mM and 4.5 mM at annealing temperature 42 °C. The same genotypes (a-d) of *O. gratissimum* are used in the four experiments. M is 100 bp DNA ladder. Primer OPW 04 was used.

The template DNA concentration which was found to give repeatable and scorable RAPD-PCR amplicons was 2.5 ng (Figure 21.0).

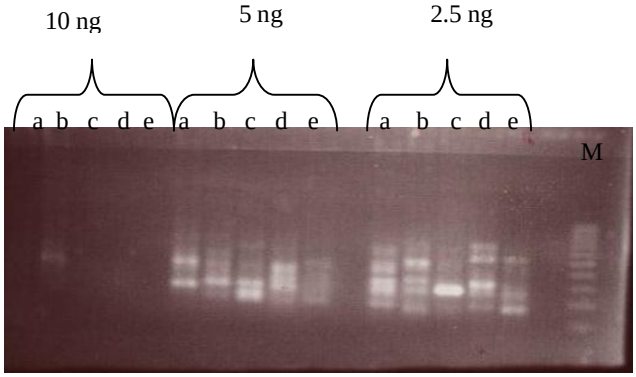


Figure 21.0: Gel showing amplicons of DNA concentrations namely: 10 ng , 5 ng and 2.5 ng at annealing temperature 42 °C. The same genotypes (a-e) of *O. gratissimum* are used in the three experiments. M is 100 bp DNA ladder. Primer OPW 04 was used.

Using the standardised variables, RAPD-PCR analysis was attempted for the entire test germplasm set. RAPDs analysis of the full set of the samples however showed a lot of irreproducibility. Every repeat of the similar primer gave a different picture of fragment (amplicons) presentations. Due to these unrepeatable results, ISSR markers

were adopted for analysis. Sixteen ISSR primers were screened; however the results did not give profiles that were scorable at all in all the primers (Figure 22.0). Even when the usage of the PCR products was adopted (Figure 23.0), the bands could not be scored either. Therefore, ISSR however also failed to give good results as shown in Figures 22.0 and 23.0.

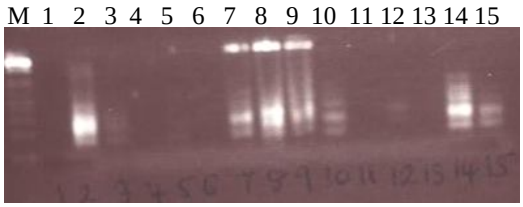


Figure 22.0: ISSR Analysis of 15 samples (lanes 1-15) from Kakamega using Primer 810 with sequence $(GA)_8T$ and M is 100 bp DNA ladder.

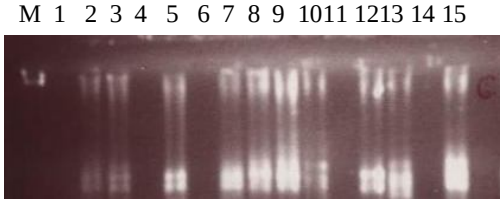


Figure 23.0: ISSR Analysis of 15 PCR product material samples (lanes1-15) from Kakamega using Primer 810 with sequence $(GA)_8T$, and M are 100 bp DNA ladder.

The AFLP marker was then adopted for screening the *Ocimum gratissimum* germplasm.

3.5.10.3 Genetic relationships in *Ocimum gratissimum L.* populations as revealed by AFLPs

The template DNA from *Ocimum gratissimum* germplasm was successfully restricted by the two restriction enzymes (*EcoR*I and *Mse*I) (Figure 24.0).

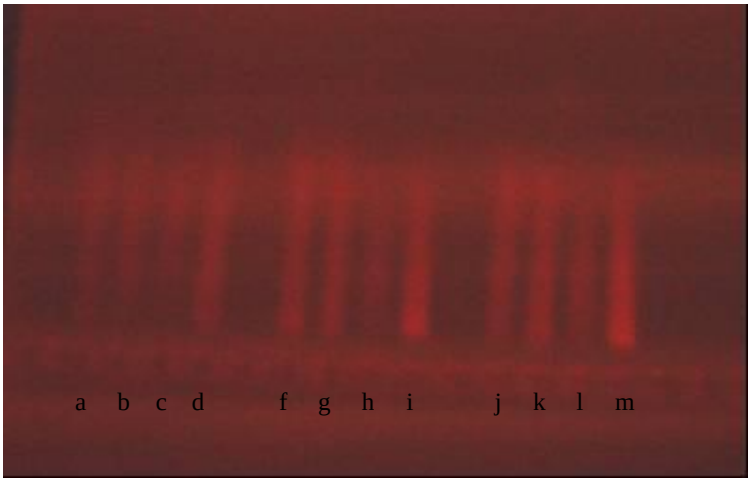


Figure 24.0: Lanes a-m are part of isolated genomic DNA of *Ocimum gratissimum* after restriction digestion by two restriction enzymes (*EcoR*I and

*Mse*I). Smears show digested DNA. Resolution on agarose gel electrophoresis.

When the restricted template DNA was PCR amplified using non-selective adapter complementary AFLP primers, positive amplicons were produced (Figure 25.0). A smear was observed for all the test DNA samples. The smear indicated that there were many amplicons present which needed to be selected again in order to separate them further for a clear resolution.

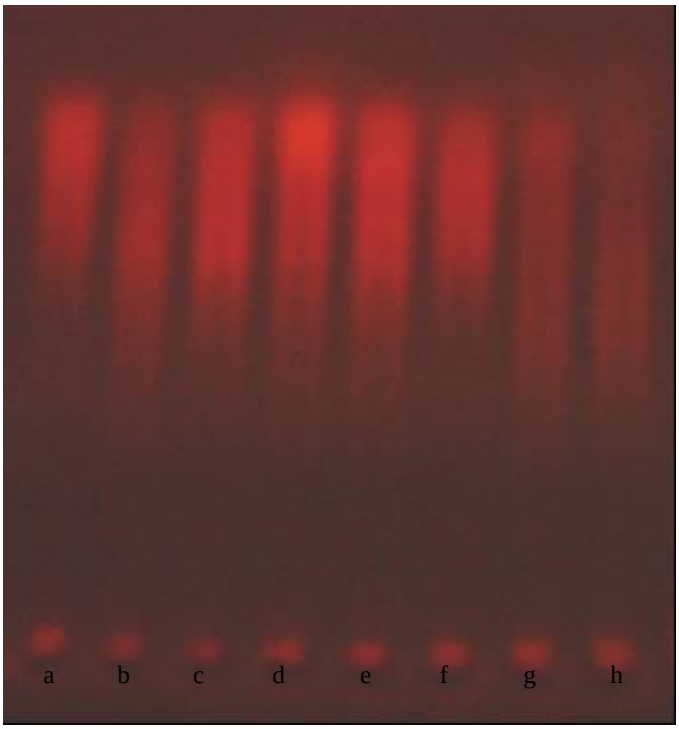


Figure 25.0: Part of the preselective amplification products (10 µl/ lane) of some of the *O. gratissimum* samples (Lanes a-h). Resolution on agarose gel electrophoresis. Smears show amplification.

A second AFLP-PCR assay was set using selective AFLP primers (Table 13.0) and the pre-amplified DNA was used as template for amplification. Resolution of the resulting PCR products revealed numerous fragments (Figure 26.0).

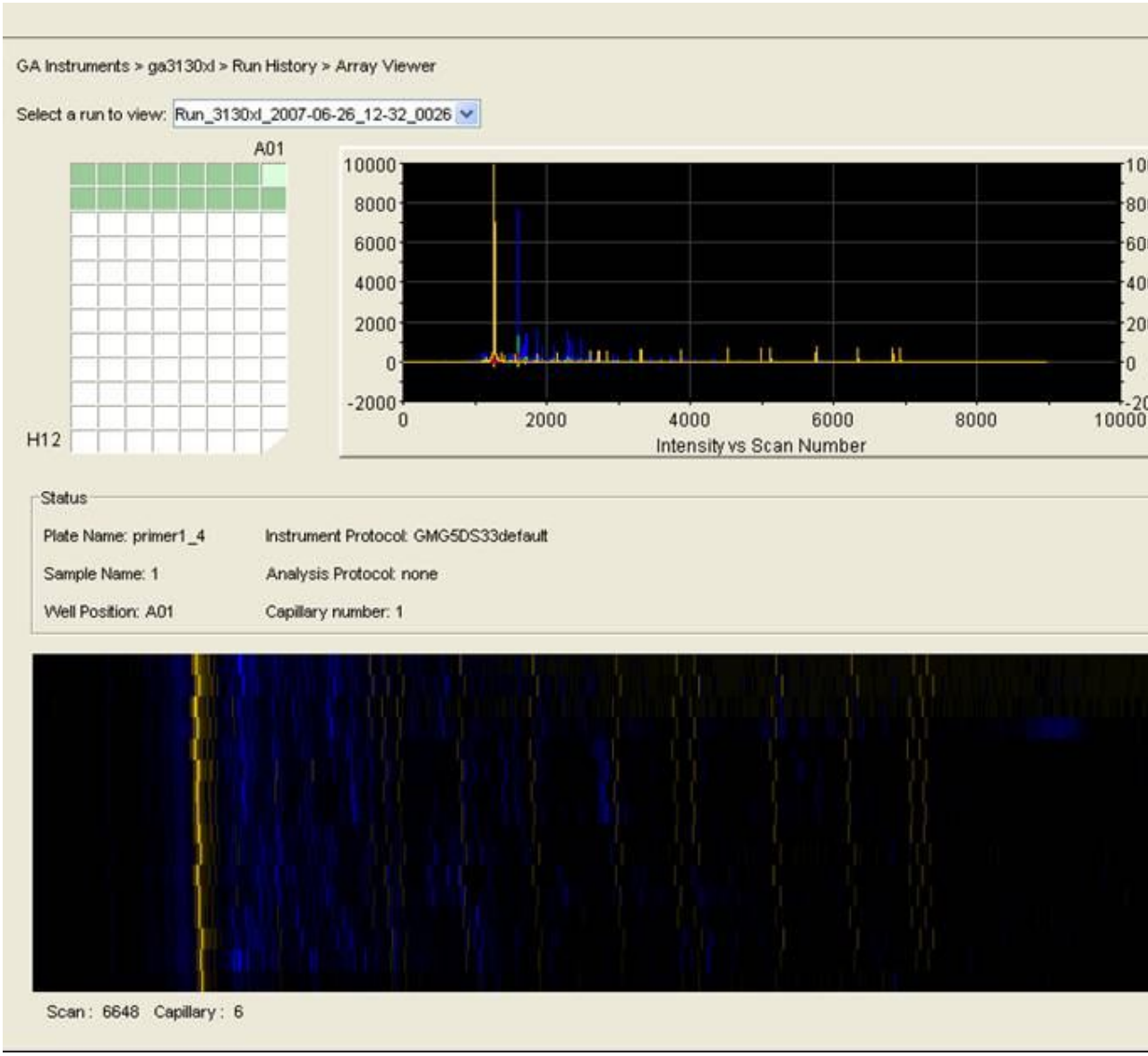


Figure 26.0: Raw results from the ABI 3130*xl* genetic analyzer. The top panel shows the Genescan500 LIZ size standard YELLOW peaks and the products as blue peaks (*Eco*RI-ACT [FAM] and *Mse*I-CAA, CAC, CTG and CAG), from the smallest size- to the largest on the extreme right. The size range is 35, 50, 75, 100, 139, 150, 160, 200, 250, 300, 340, 350, 400, 450, 490, and 500. In the lower panel, the same size standards as yellow bars and the products as blue bars. Top most lane is capillary one

.Six thousand, two hundred and thirty seven different AFLP polymorphic bands were generated by the seven primers analysed (Table 14.0) from 140 samples. The total number of bands scored per primer ranged from 595 (ACT-CTG) to 1335 (ACT-CAA), with an average of 891 bands per primer. The size of the amplified fragments ranged from 50 to 472 base pairs (bp). The number of scorable markers was highest for primer 5'ACT-CAA 3' and lowest for primer sequence 5' ACT-CTG 3'.The highest fragment range was in primer sequence ACC- CAC. Table 14.0 summarises this data.

Primer	**5' Sequence 3'**	**Number of polymorphic loci**	**Fragment range**
1	ACT-CAA	1,335	50-289
2	ACT-CTG	595	50-253
3	ACA-CAA	1,123	50-129
4	ACA-CTG	862	50-208
5	AGC-CAA	717	50-306
6	ACC-CAA	963	50-123
7	ACC-CAC	642	50-473
Total		6237	50-473
Mean/primer		891	

Table 14.0: Codes and sequences of primers analysed, total number of bands analysed and fragment size

Estimates of Nei's unbiased genetic diversity (Table 15.0) show some populations with similar gene diversity (Nakuru-Mill house II and Nakuru-Njoro with H = 0.13; Kakamega, Kisumu, Kabarnet and Mombasa with H = 0.15; Taita Taveta and Kericho with H = 0.10). Taita Taveta and Kisumu were the least diverse (H = 0.10) and Nyeri was the most diverse (H = 0.19). The results show some great variation in the levels of genetic diversity as also shown by Shannon's information index (I) (Table 15.0). There is some percentage polymorphic loci correspondence with the diversity estimates. In most cases, populations with high diversity estimate also show high percentage polymorphic loci (Table 15.0).

Table 15.0: Mean diversity estimates (H) and Shannon's information index (I) for twelve populations of *Ocimum gratissimum* L. species sampled in Kenya analysed using AFLP markers based on Nei (1987) statistics.

District	**Population Name**	**Sample Size**	**na***	**ne***	**h***	**I***	**% loci**
Kakamega	Savona Isle	7	1.46	1.25	0.15	0.22	45.6
Kisumu	Riat	10	1.60	1.25	0.15	0.24	59.9
Nakuru	Mill house I	14	1.52	1.24	0.14	0.22	52.1
Kabarnet	Chesigei	14	1.64	1.25	0.15	0.15	63.6
Nyeri	Kiganjo	9	1.65	1.31	0.19	0.30	65.0
Meru	Rugucu	14	1.67	1.30	0.18	0.28	67.3
Thika	JKUAT	14	1.63	1.25	0.16	0.25	62.7
Taita Taveta	Kibarani	14	1.53	1.147	0.10	0.17	53.0

Mombasa	Mariakani	9	1.55	1.24	0.15	0.23	54.8
Kericho	Roret	13	1.41	1.17	0.10	0.16	41.0
Nakuru	Mill house II	14	1.51	1.22	0.13	0.21	51.2
Nakuru	Njoro	7	1.44	1.22	0.13	0.21	44.2

* na = Observed number of alleles

* ne = Effective number of alleles [Kimura & Crow (1964)]

* h = Nei's (1973) gene diversity

* I = Shannon's Information index [Lewontin (1972)]

% loci = Percentage polymorphic loci

Summaries of analyses of molecular variance by Arlequin software version 2.000 (Table 16.0) and GenA1Ex 6 (Table 17.0) based on twelve populations indicated that most variation was found among individuals within populations (100 %, Arlequin software version 2.000; 71 %, P< 0.001, GenA1Ex 6). According to Arlequin software version 2.000, there was no variation among populations and similarly although there was 29 % variation among populations according to GenA1Ex 6, it was not significant.

Table 16.0: Analysis of molecular variance (AMOVA; Arlequin software version 2.000, Shneider *et. al.*, 2000) based on AFLP markers for 140 individuals sampled from 12 populations of *Ocimum gratissimum* L from Kenya. AMOVA, Degrees of freedom (df), mean squared deviation (MSDs) and the % variance are shown

```
------------------------------------------------------------------------
--
 Source of                  Sum of      Variance         Percentage
 variation        d.f.           squares       components          of
variation
------------------------------------------------------------------------
--
 Among
 groups                 3               1.500            0.00000  Va
0.00

 Among
 populations
 within
 groups                 8               4.000            0.00000  Vb
0.00

 Within
 populations       127               63.500            0.50000  Vc
100.00
------------------------------------------------------------------------
--
 Total          138         69.000        0.50000
------------------------------------------------------------------------
--
 Fixation Indices
      FSC :      0.00000
      FST :      0.00000
      FCT :     0.00000
------------------------------------------------------------------------
--
```

Table 17.0: Summary of AMOVA by GenA1Ex 6

Summary AMOVA Table								
Source	**df**	**SS**	**MS**	**Est. Var.**	**%**	**Stat**	**Value**	**Prob**
Among Pops	11	1262.982	114.817	8.187	29%			
Within Pops	127	2601.795	20.487	20.487	71%	**PhiPT**	0.286	0.001
Total	138	3864.777	135.303	28.674				

Out of the 16 selective AFLP primers which were screened, 7 primers were selected for further study (Table 13.0). The 7 primers selected showed a high frequency of polymorphism and were therefore used to screen the entire *Ocimum* germplasm. The

dendrogram (Figure 27.0) based on Nei's genetic distance (D) (Table 18.0) show two major clusters and there are four sub-clusters which do not necessarily correspond to geographical distances between the populations. Mombasa population (coast region) clusters together with Nakuru and the Kericho population (rift valley region) is the most disparate. Clustering is not related in any way to geographical distribution, for example, with coastal populations (Taita taveta) clustering with populations from central Kenya (Nyeri, Meru and Thika). In this cluster, the central region populations clustered together forming one distinct group (Nyeri, Meru and Thika) and one population from Taita Taveta (coast region) was the most disparate. Then, there is the cluster in which the Kisumu population from western region is standing distinctly on its own. The population from Kakamega, (western region) clustering together with Nakuru and Kabarnet populations from the rift valley region

Table 18.0: Nei's Unbiased Measures of Genetic distance (Nei, 1978) from 7 AFLP markers for 12 populations of *Ocimum gratissimum* L. sampled from Kenya. Population Identity: 1-Kakamega, 2-Kisumu, 3-Nakuru, 4-Kabarnet, 5-Nyeri, 6-Meru, 7-Thika, 8-Taita Taveta, 9-Mombasa, 10-Kericho, 11-Mill House and 12-Njoro.

POP ID	1	2	3	4	5	6	7	8	9	10	11	12
1	****											

2	0.0085	****									
3	0.0053	0.0170	****								
4	0.0146	0.0298	0.0057	****							
5	0.0274	0.0463	0.0232	0.0101	****						
6	0.0451	0.0697	0.0422	0.0287	0.0020	****					
7	0.0502	0.0707	0.0417	0.0268	0.0035	0.0022	****				
8	0.0430	0.0718	0.0363	0.0212	0.0166	0.0197	0.0123	****			
9	0.0904	0.1371	0.0910	0.0798	0.0604	0.0463	0.0470	0.0575	****		
10	0.1464	0.1859	0.1473	0.1332	0.1014	0.0843	0.0909	0.1066	0.0425	****	
11	0.1194	0.1611	0.1204	0.1011	0.0759	0.0622	0.0678	0.0857	0.0214	0.0283	****
12	0.1446	0.1828	0.1502	0.1302	0.0973	0.0806	0.0914	0.1181	0.0390	0.0377	0.0167
****											

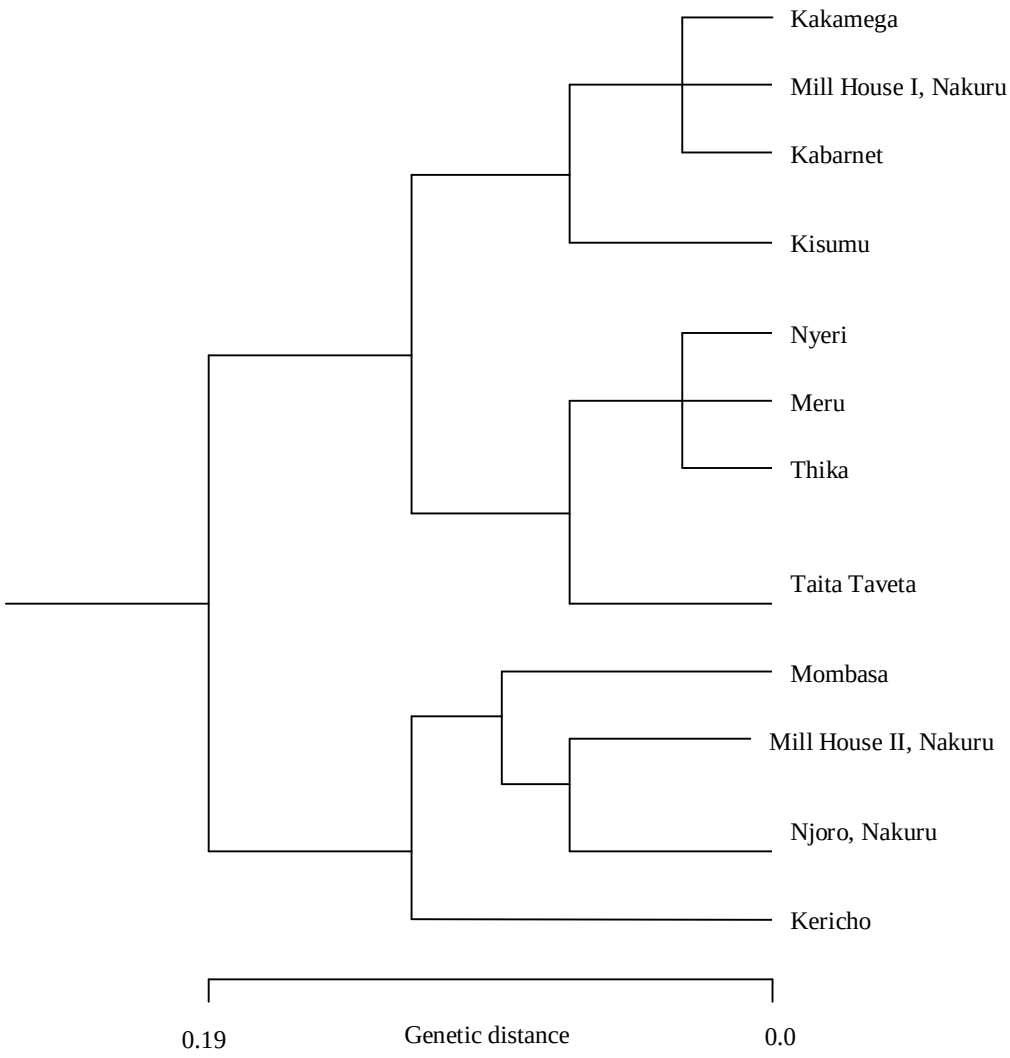


Figure 27.0: Dendrogram based on Nei's (1978) Genetic distance: Method = UPGMA, Modified from NEIGHBOR procedure of PHYLIP Version 3.5 from 6237 AFLP markers for 12 populations of *O. gratissimum* L. sampled from Kenya.

3.5.11 Discussion

3.5.11.1 Quality and yield of genomic DNA extracted using the SDS method

Nucleic acids are routinely quantified spectrophotometrically by measuring the absorbance at 260 nm. Inside a spectrophotometer, a sample is exposed to ultraviolet light at 260 nm and a photo-detector measures the light that passes through the sample. The more light absorbed by the sample, the higher the nucleic acid concentration in the sample. The aromatic rings absorb extremely maximally and rather characteristically with a peak between 255 and 260 nm. Tyrosin and Tryptophan confer absorption at 280 nm as proteins. Thus the A_{260}:A_{280nm} ration of nucleic acid extract should exceed 1.8 if it is to be considered free from proteins. The 260/280 UV Absorbance ratio averaged between 1.4 and 1.6 slightly below the required 1.8 indicating presence of some secondary metabolites including proteins after all the DNA cleaning process.

In addition to proteins, many DNA isolation procedures also yield large amounts of RNA, especially 18S and 25S rRNA (Rogers and Bendich, 1985; Doyle and Doyle, 1987; Mejjad *et al.*, 1994). Large amounts of RNA in the sample can chelate Mg^{2+} and reduce the yield of the PCR. DNA isolates from *Ocimum* also had large quantities of RNA which were however easily removed by treatment with RNase A. Although, the DNA yields of *O. gratissimum* based on the A_{260}:A_{280nm} ratio of nucleic acid extract did not exceed 1.8, implying it still had impurities, the DNA

worked well with the AFLP marker. It is most likely that the numerous dilutions involved in the AFLP procedure, for instance, during template DNA preparations, restriction step, ligation step and pre-amplification step ended up diluting DNA polymerase inhibiting metabolites cleaning up the DNA and making it suitable for the analysis.

3.5.11.2 RAPD-PCR and ISSR-PCR analysis

The amplification profiles for specific oligonucleotide primers in RAPD-PCR assays are greatly dependent on specific conditions of reactions; banding patterns thus vary extensively because of inconsistencies in a number of reaction parameters. However, fully optimised, RAPD-PCRs can be reliable, cost-efficient molecular methodology in phylogenetic studies. RAPD has been used to analyse genetic variation in several species (Dawson *et al*., 1993; Yeh *et al*., 1995) and RAPD-based linkage maps have been published (Tulsieram *et al*., 1992).

In order to optimise the RAPD and ISSR technique for *O. gratissimum*, several concentrations of $MgCl_2$, template DNA and *Taq* DNA polymerase were tested. At each test, the conditions of dNTPs, primers and PCR reaction buffer, using the same amplification profile in the thermocycler remained constant. Different plant species require different optimisation conditions for reproducibility of banding patterns generated using the standardised RAPDs or ISSRs. For instance, in *O. gratissimum* RAPD-PCRs, the best $MgCl_2$ concentration was found to be 3.5 mM while in the genetic studies of *Cuban triatominae,* the best fingerprinting pattern was obtained

using 2.5 mM $MgCl_2$ (Fraga *et al.*, 2005). Lower (1.5 mM) and higher (3, 3.5 and 4 mM) $MgCl_2$ concentrations had significant effects upon the RAPD profiles produced revealing fewer and non-specific bands (Fraga *et al.*, 2005). The concentration of $MgCl_2$ is known to affect PCR (Ellsworth *et al.*, 1993).

This is true with all the other PCR variables. Whereas in this study, the optimal *Taq* DNA polymerase concentration was 0.5 U, it was 2.0 U for *Cuban triatominae* (Fraga *et al.*, 2005). Fraga *et al.*, 2005 established that the optimal template DNA concentration for successful RAPD-PCR amplification in *Cuban triatominae* was 25 ng. In this study, 5 ng templates DNA were optimal. Dilution of the *Ocimum* template DNA has the effect of also diluting any contaminating metabolites such as terpenes or terpenoids that would inhibit the activity of the *Taq* DNA polymerase.

The annealing temperature and number of thermal cycles were other parameters assayed in this study. It has been reported that higher temperatures prevent amplification by 10-mer primers while low temperatures may result in many non-specific RAPD-PCR products (Williams *et al.*, 1990). However, in this case 42 ºC ensured a maximal number of primer binding events.

After all the optimisation was done, the selected oligonucleotide primers failed to repeat results. As such, the primers did not have the potential to be used in RAPD-PCR analysis for *Ocimum* differentiation. Similarly, no repeatable and scorable ISSR amplicons were obtained for all the test germplasm to enable a comprehensive genetic diversity study.

3.5.11.3 Genetic relations in *Ocimum gratissimum* L. populations as revealed by AFLP-PCRs

The data in Table 14.0 indicated the presence of different degrees of polymorphism within each primer. These results show that AFLP markers can be generated at great speed, as illustrated by the high number of polymorphic loci in all markers generated through a few primers. Because of the nearly unlimited number of markers that can be generated with AFLP-PCR, using a series of different primer combinations, at least some AFLP markers will be located in variable regions (Vos *et al.,* 1995) and thus reveal even minor genetic differences within any given group of organisms.

The AFLP assay was able to provide all the information necessary for genetic variation studies and the results showed high polymorphic loci percentage in most of the population (Table 15.0). The results on estimates of Nei's unbiased gene diversity show some populations with similar gene diversity, others with very low and others with very high gene diversity (Table 15.0). The Shannon's Information index (I) also gave a similar order of population diversity. This order of population diversity could be related to the origin of the seeds for this species. The results showed some populations with similar gene diversity (Table 15.0). Nyeri (Kiganjo) was the most diverse followed by Meru (Rugucu) population. In Table 15.0, Nyeri, Meru and Thika (central region) which have generally high genetic diversity as well as high Shannon's information index are located in the same region. It is possible that the seeds for *O. gratissimum* were introduced to the other areas from the central

region. The low diversity seen in Kericho could be because of introduced seed based on only a few cultivars or seed from a restricted source as has also been shown in other studies (Wachira *et al.*, 2001). Low population densities have been linked to reduction in population heterozygosity and gene diversity (Dusan, 1992) which is attributed to restricted maternal mother plant and genetic drift. It is also known that mating between related individuals that is inbreeding, further accelerates the reduction in heterozygosity as it reduces the effective population size (Hamrick and Godt, 1989). Kericho (Roret) also showed the least percentage polymorphic loci. AFLP polymorphism is due to point mutations in the restriction sites or in the selective nucleotide and small insertions / deletions within the restriction fragment (Vos *et al.*, 1995). It could mean that the Kericho population is not mutating as fast as the other populations. It is also known that the number of generations in a year matters a lot. The high the number the increase in appearance of mutations and therefore increase in divergence between individuals within populations (Roux *et al.*, 2007). So if this *O. gratissimum* happens to be growing in a farm which is cultivated regularly like Kericho where the samples were collected, the number of generations is reduced as compared to an idle land overgrown with *O. gratissimum* and other weeds. The low genetic diversity observed in Kericho and Kakamega as indicated by many the many genetic parameters in Table 15.0 could be due to the high amount of rainfall in those two areas. The phylogenetic closeness between Mill House II, Nakuru, Njoro and Mombasa as shown on the dendogram could among other things be due to similarity in the average annual rainfall since all have rainfall ranging between 800 and 1400 mm. All this has important implications to genetic resource management and any conservation of the species. Therefore, it becomes important

when collecting germplasm to consider many individuals across the ecological amplitude of a population in order to avoid loss of genetic resources.

Nested AMOVA indicated significant genetic variation ($P<0.001$) within populations (Tables 16.0 and 17.0). Partitioning of the genetic diversity revealed that overall, most of the variation resided within populations (100 %, Arlequin; 71 %, GenA1Ex6). This finding indicates that this species is an out crossing plant. Out crossing plants retain considerable variability and that most variation is exhibited within populations (Hamrick *et al*., 1979) as was also found in *Cannabis sativa* (Mandolino *et al*., 2001); *Camellia sinensis* (Wachira *et al*., 2001); *Ilex paraquareensis* (Gauer and Cavalli-Molina, 2000). The low divergency among the populations could be explained by the occurence of gene flow among populations, but this seems not to be the current situation because the populations analysed are about 300 Km or more distant from each other except the Nakuru populations which are < 30 Km apart.

Genetic differentiation among populations is principally a function of gene flow among populations via pollen and seed dispersal. F_{ST} (which is the degree of gene differentiation among population in terms of allele frequency) was zero (Table 16.0) which means the genetic differentiation was small. As earlier discussed that the origin of seed material seems to be playing an important role in the results being observed, the study seems to reveal that after the initial introductions of the seed from central region to the other areas through human activities most likely, there has been minimal exchange of germplasm resources by seed or plant material and

therefore populations from different areas are trying to evolve independently as islands of genetic diversity. However, not enough time has passed to allow mutation and differentiation among the populations.

On the other hand, this could be due to life history characteristics of the species which according to Hamrick *et al.*, (1979) and Hamrick and Godt (1989) can affect the genetic structure of plant populations and have effects on genetic variation. By observation, *Ocimum gratissimum* is long lived, perennial and the results have shown that it is an out crossing species. A positive association between these characters and genetic variation has been established (Hamrick, 1979). These characters allow a high within population diversity.

By observation, *Ocimum gratissimum* is an insect-pollinated plant. There is no information about its seed dispersal. Alternatively, an explanation to this high inter population similarity levels could be due to geographical isolation. The geographical isolation of these populations may have begun in the early 19^{th} century with the colonisation by settlers, who deforested the native areas and became more intense when there was a reduction of natural forests to increase plantation areas intended for large-scale crops. It is unlikely that forests with *O. gratissimum* were continuous, but the populations were probably geographically closer allowing gene flow between them. So the plants analysed could have been living when the populations' were not yet isolated, or could be plants of only a few generations where no much mutation has occurred after isolation, thus giving insufficient time to allow differentiation among the populations. It is also possible that such groupings or clustering is as a

result of long distance dispersal events and direct overland migrations facilitated by climate fluctuations (White, 1983).

The dendogram (Figure 27.0) showed that (Nyeri, Meru and Thika) cluster together. This is expected because all these populations come from the same region, the central region of Kenya. However, there are some clusters like the four distinct groups comprising of the two Nakuru populations (Mill House II and Njoro) which cluster together, and then Kericho as a distinct group and then Mombasa. Despite the geographical proximity of Mill House I to Mill House II and Njoro (< 30 Km), Mill House II and Njoro are seen to be genetically closer to Mombasa (> 600 Km). This could be due to germplasm exchange as earlier discussed. Genetic distance which measures the difference between two genes, proportional to the time of separation from a common ancestor was able to give a similar picture as one observed in the dendrogram. For instance the Thika, Meru and Nyeri cluster together and this is reflected in their genetic distance which is small. For instance, Thika and Meru is 0.0022; Meru and Nyeri is 0.0020. However, Meru and Kericho is 0.0843 which means there are differences.

3.5.12 Implications to genetic management of the *Ocimum gratissimum* L. in conservation

Analysis on molecular variation (AMOVA) showed that most of the genetic variation is retained within populations rather than among population.

Population genetic diversity is also high and therefore for conservation purposes sampling intensively within a few populations will capture the genetic variation. Thus, sampling should focus on a few populations with many individuals across the ecological amplitude of a population. The phytochemical studies have shown that Meru (Rugucu) material is superior in terms of antimicrobial activities (Table 5.0). Although, phytochemicals are greatly influenced by environment and therefore may not form the basis of strategies for the genetic conservation of *Ocimum*, it would still be of value to consider how well conservation can maintain this important attribute of the Meru population. If there would be any consideration of commercial production of drugs, the Meru population could be ideal because of its medicinal effectiveness, hence the need for special attention to this population. Therefore, planting programmes should ensure the genetic distinction is retained.

3.5.13 Conclusion

This study has shown that *Ocimum gratissimum* contains a high content of secondary metabolites which made it difficult to extract high quality and content of DNA. It has also been shown that although the DNA extracted using SDS method with the reducing agent DTT could not give reproducible results in RAPD analysis or scorable amplicons in ISSR analysis, it gave very good results for AFLP analysis.

These results suggest that AFLP marker is very good also especially for those species which have a high amount of secondary metabolites like *Ocimum gratissimum*.

Partitioning of the genetic diversity revealed that overall, most of the variation resided within populations. This suggests that sampling intensively within a few populations especially those that have a high genetic diversity (based on their level of heterozygosity) for conservation purposes would capture the genetic variation.

CHAPTER 4

4.0 General discussion and conclusion

4.1 Genetic variation and the medicinal activity of *Ocimum gratissimum* L in Kenya

The methanolic crude extract from the dry leaves of *O. gratissimum* was found to be effective on test fungi (*Candida albicans*) only. It had no effect on test bacteria (*Escherichia coli* ATCC25922, *Staphylococcus aureus* ATCC25923, *Pseudomonas aeruginosae* ATCC27853, *Klebsiella pneumoniae* (clinical isolate), *Proteus mirabilis* (clinical isolate), *Salmonella typhi* and *Bacillus* spp.). *O. gratissimum* contains essential oils which were found to be very effective against all the test bacteria. They are volatile oils giving it a characteristic aroma. Because of its efficiency in inhibiting the growth of microbes, it could be used to treat several diseases of man caused by the test bacteria. However, research must be done in order to establish the dosage to effectively destroy the microbes in man. It is likely that the crude extract was not as active as the essential oil because the most active ingredients against

microbes reside in the essential oil. Therefore, extraction of the essential oil or usage of the fresh leaf material as medicine is the best way to utilise the active igrediients.

The phytochemical analyses on the active ingredients in the herbal therapy showed that some populations were more active against antimicrobial test organisms than others. Such variation would generally be expected from a species with a wide geographic distribution. For any trait of interest, observed differences among individuals may be due to differences in the genes coding for this trait or may be the result of variation in environmental condition. In many cases it is a combination of the two. It was also shown oils from Meru (Rugucu) which was the most active against microbes tested contained eugenol and methyl eugenol as opposed to Mombasa oils which had eugenol only as a major compound. Although the Nyeri population was generally the most diverse, its phytochemical activity on microbes was lower when compared to the other populations of the central province. Interestingly, Mombasa and Taita Taveta are geographically in the same region and their effectiveness towards the test microbes was similar, however, they show very different population diversity within their populations (0.15 and 0.10 respectively).

DNA extraction in *O. gratissimum* is very challenging. However, the extraction of DNA using SDS and the reducing agent DTT gave recommendable DNA. SDS seems to be better than CTAB buffer in this case. SDS works by dissolving the lipid containing nuclear membrane of *O. gratissimum* cells releasing the DNA. The reducing agent DTT also worked better than mercaptoethanol in this case. DTT is able to protect DNA from oxidation after release from the cells. It is able to protect

the DNA from the many enzymes and secondary metabolites available in the cells enclosed in their own organelles which become available after maceration of the cells during DNA extraction. Mercaptoethanol does not seem to be a strong reducing agent in this case such that in its presence the DNA is oxidized fast and is either unavailable or is of low molecular weight.

The storage conditions of *O. gratissimum* prior to DNA extraction was a very crucial treatment. Freezing of the leaf material maintains most of the cell's environment and so therefore there is no oxidation which occurs before maceration of the leaf during DNA extraction. Similarly, silica gel drying worked well too as a storage treatment prior to DNA extraction. This could be because of the way silica gel absorbs the moisture (water) from the leaf without affecting the make up of the cells. Oven drying and air drying seem to be harsh storage treatments for this species. The treatments are harsh in the sense that they encourage a number of chemical processes which affect the DNA in general. Air drying seems to affect the DNA probably because it takes long and in the process a lot of oxidation in the leaf occurs and the DNA becomes oxidised. The oven drying is extremely fast and therefore could be that the metabolic processes of the leaf are affected, rendering some reactions which degrade the DNA. It could be that during these processes, the secondary metabolites and even some enzymes in addition to whole cell environment becomes unfavourable for DNA to maintain its integrity. It is possible that oxidation takes place to most secondary metabolites and hydrolisation takes place to the DNA structure and hence no DNA extracted or DNA of low molecular weight obtaind.

Due to the integrity of the DNA which was extracted, it is clear that more work needs to be done in order to establish the best DNA extraction method for this species which would yield high quality and quantity DNA. In addition, the RAPD and ISSR markers need more optimisation. However, it is clear that AFLP marker does work well in circumstances where DNA yields are low and probably containing some impurities. It appeared the dilutions in the AFLP procedure play an important role in also reducing the concentration of impurities in the extracted DNA.

There was a high level of genetic diversity in the populations. High levels of genetic variation are associated with a wide geographical distribution, out breeding, high numbers of chromosomes and polyploidy. *O. gratissimum* is widely distributed and has shown to be an out crossing. The genetic variation was within a population as opposed to among populations. Therefore, conservation of the genetic resources would focus on a few populations with many sampled individuals in the population. *O. gratissimum* has a low level of genetic differentiation. F_{ST} the degree of gene differentiation among populations in terms of allele frequency was zero meaning that genetic differentiation (G_{st}) was small.

Therefore, considering all the factors observed in this study, selection for domestication and even conservation of the species would be effective at the population level. Thus, single population selection or even a few plants will not represent the whole genetic variability in *Ocimum gratissimum.* There appears to be a need to maintain sufficiently large populations in natural habitats to conserve genetic diversity in *O. gratissimum* and avoid genetic erosion. For *ex situ* conservation of

this plant species, focus should be on seed collection from as many individuals as possible within a large population to ensure that genotypes adapted to a range of soil types, soil moisture regimes and temperatures are represented in any new populations.

The greatest genetic diversity is located within populations of central Kenya origin i.e. Meru, Nyeri and Thika. Therefore, any selection for ex-situ conservation or any improvement program must target these populations.

4.2 Essential oil activity, properties and genetic diversity

The results in Table 5.0 showed that there is a lot of variation in antimicrobial activity among locally collected populations. There are populations like Meru whose essential oils had antimicrobial activity that was consistent and high across all the microbes that were tested.

There were also populations like Taita Taveta and Mombasa whose oils had high activity only on particular microbes (*E. coli* and *S. typhi*), in this case only Gram negative bacteria. Kabarnet, Kericho and Njoro I for instance, showed high activity on Gram positive bacteria only (*S. aureus* and *Bacillus spp.*). In the case of Kisumu, the essential oils showed high activity on only one Gram negative bacteria (*E. coli*) and all the Gram positive tested (*S. aureus* and *Bacillus spp.*). This could be attributed to basically the compositions of the oils due to either geographical locations or climatic conditions like the amount of rainfall annually or both. The

results of the composition of oils from Meru and Mombasahave demonstrated this (Table 7.0). In these results, it was shown that Meru oil contained some compounds which were not found in the Mombasa oils like trans-ocimene, Camphor, Methyl eugenol, α- Farnese and β- Bisabolene. These variations in the composition of oils could have contributed to the variation to medicinal activity.

During the genetic diversity studies, the results also showed that there was variation in genetic diversity within populations emphasizing the variation in medicinal activity from different populations. For instance, Meru whose diversity was the greates (Table 15.0) showed also high medicinal activity and even its activity was consistent. Mombasa also showed some greater diversity as compared to Kericho for instance, and this is also reflected in its medicinal activity. Therefore, there was some correlation seen in this study between medicinal activity and genetic diversity, hence the need to consider both these areas before any sampling for conservation is effected. This would help to capture the important qualities for conservation.

4.3 Recommendations

Almost all populations showed some activity towards the different microbes tested. It would be recommended that the oils which showed high activity as compared to others undergo *in vivo* tests so that dosages for the particular microbes are established.

Also, isolation of individual compounds in essential oil could also be done and their antimicrobial activity evaluated.

Essential oils could have greatest potential use as a food preservative since they have been shown to inhibit bacteria, fungi and yeast, therefore research on this could be carried out.

It would also be important to study the shelf life of essential oils, in order to assess their rate of deterioration if it is to be used as medicine.

It would be recommended that further work is done on DNA extraction of this species so that a protocol is established which could have high quality DNA for RAPD and even ISSR analysis. The AFLP marker is expensive as opposed to RAPDs which is cheap. Although RAPD are characterised by irreproducibility of results if the assay is well standardised, it gives good results and it still remains a cheap method especially in developing countries where cost is an issue. It would be recommended that more optimisation work is done on the ISSR marker before applied so that optimal conditions are found for a particular species.

Conservation of *Ocimum gratissimum* L. would involve intensive selection of material within a population especially those showing high genetic diversity.

It would also be recommended to have experiments to check if cultivars from Meru were brought to Kericho; the medicinal activity would remain the same.

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APPENDIX I

1. COLLECTION OF SAMPLES IN KAKAMEGA (KAK)

Collected samples near Savona Isle Resort near river Isikhu in Kakamega on the 18th February, 2005

The area is characterised by green vegetation. It is near a river called Isikhu.
The site is next to Savona Isle Resort. There is a lot of *Lantana camara* growing together with *Ocimum gratissium* L. and a number of guava trees.

General seed colour was a mixture of brown group 200 B ,C and greyed-orange group 166 A.
The colour of the bark is brown group 200 B.
The colour of the flowers was brown group 200 C when dry and is yellow-green group 148 B when fresh.
The leaf colour was yellow-green 148 A top and greyed-green group 197 B below.

2. COLLECTION OF SAMPLES IN KISUMU

Collected samples at RIAT, 5 km from Kisumu town on the 19th February, 2005

It is a very stony area with *Lantana camara* growing all around. The place was very dry . A valley near by which was dry at the moment but has water running during the rainy season.

General seed colour was a mixture of greyed orange group 166 A and brown group B.

The colour of the bark is brown group 200 C.

The colour of the flowers is grey-brown N199 when dry and yellow green group 148 B when fresh.

The leaf colour is yellow green 148 A top.

The leaf colour is greyed green group 195 A below.

3. COLLECTION OF SAMPLES IN KABARNET

Collected samples at Chesigei in Kabarnet district on 21-February,2005.

Ocimum gratissimum is called Kapmatebar or Tumotoplagog in Tugen. The area is hilly and with a lot of vegetation and characterised by the presence of *Lantana camara*.

General seed colour was a mixture of brown group 200 B and greyed-orange 166 A group.

The colour of the bark is brown group 200 D.

The colour of the flowers is greyed-brown group N199 D.

The leaf colour was yellow-green group 148 A top.

The leaf colour was greyed-green group 194 A below

4. COLLECTION OF SAMPLES IN NAKURU

Collected samples in Nakuru district, Mill house area on 22-February, 2005.

It is a dry field with patches of *Lantana camara* and *Plectranthus barbatus.* The field is flat.

General seed colour was a mixture of greyed-orange group 177A and brown group 200B.

Colour of the bark is brown group 200 B.

Colour of the flowers is brown group 200 D.

Leaf colour is yellow-green group 148A top and greyed-green group 195 A below.

5. COLLECTION OF SAMPLES IN NYERI

Collection of samples in Kiganjo area in Nyeri district on 24-February, 2005.

The area is dry. The plants looked water stressed .The leaves were not as Velvet feeling as the others . They are thin in thickness and light smooth. The flowers of most plants had dried off and dessicated. The collection was done in a field along the road. The population was big. The plants under shade were very tall >3.5<4 cm but

those well exposed to the sunshine were short. The plant found common growing with *Ocimum* was *Lantana camara* and *Datula sp.*

General seed colour was a mixture of brown group 200 B ,C and greyed orange group 166 A.

The colour of the bark was greyed orange group 166 A or brown group 200 D.

The colour of the flowers was yellow green group 148 A.

The leaf colour was yellow green group 148 A top .

The leaf colour was greyed green group 194 A below.

6. COLLECTION OF SAMPLES IN MERU

Collection of *Ocimum gratissimum* L. samples in Rugucu area in Meru district on 24-February,2005

Meru has a big population of *Ocimum gratissimum* L.and the vegetation around these bushes common is *Tithonia, Lantana camara* and *Plectranthus barbatus* (Maikoya). The leaves are velvet-like and soft.The population of *Ocimum* varies from a height of >0.5>2.5<3m. The area was hot and dry at the time of collection. However, there was a lot of green vegetation.The *Ocimum gratissimum* had flowered but most of it had not shade its seeds. The

flowers had not dried and they have violet brown colour.

General seed colour was a mixture of greyed-orange group 166 A, B , C.

The colour of the bark was brown group 200 D.

The colour of the flowers was grey-brown N199 A, C group.

The leaf colour was green group 137 C top.

The leaf colour was greyed-green group bottom 195 A.

7. COLLECTION OF SAMPLES IN MOMBASA

Collection of samples (*Ocimum gratissimum* L.) from Mombasa On 10-March, 2005 in Mariakani towards Kinango (East South Coast) in a field next to a stream.

This area was very dry at the time of collection. The stream was also very dry. The nearby area had signs of a bush fire. The population was so small. The leaves were very thin and smooth.

General seed colour a mixture of brown group 200 B ,C and greyed-orange group 166 A.

The colour of the bark was brown group 200 C.

The colour of the flowers is grey-brown group N199C.

The leaf colour was yellow-green group 148 A top and greyed-green group 197 B.

8. COLLECTION OF SAMPLES IN TAITA TAVETA

Collection of *Ocimum gratissimum* L. samples from Taita Taveta district on 9-March near river Mukachi at Wundanyi from Mwatate function Kibarani area.

The area is along the road on a valley with Mukachi river passing on the valley with scattered plants of *Lantana Camara* and *Tithonia sp.* It is on a hilly area .The place is fairly dry. The river is about 200 m high to the road. The leaves are soft and velvet like.

General seed colour a mixture of brown group 200 B and greyed-orange group 160 A.

The colour of the bark was brown group 200 C.

The colour of the flowers was yellow green group 152 B when fresh and brown group 200 C when dry.

The leaf colour was yellow green group 148 A top .

The leaf colour was greyed green group 194 A below.

9. COLLECTION OF SAMPLES IN THIKA

Collection of *Ocimum gratissimum* L. samples from Thika district, Juja on 8-March, 2005 at JKUAT compass near a field.

The area was dry. The collection was from juveniles. The plant had been cut down or sometimes browsed by cows. The leaves were soft and velvet like. No much vegetation around this area.

General seed colour was brown group 200 B,C.

The colour of the bark was brown group 200 C.

The colour of the flowers was brown group 200D.

The colour of the leaf was yellow-green group 148 A on top and greyed-green group 195 A below.

10. COLLECTION OF SAMPLES IN KERICHO

Collection of *Ocimum gratissimum* L. in Kericho at on 15th March, 2005.

The collection was done in a cultivated land which had been harvested. It was during the dry season. The Ocimum was growing in patches i.e. scatterred in the farm. The samples had no seeds.

Lower surface of the leaf – Yellow green group No 147C

Upper surface of the leaf – Yellow green groupNo 146B

Stem bark - Greyed orange group No 177C

Flower - Greyed purple group No 183B

APPENDIX II

DNA CONCENTRATION AFTER RNASE TREATMENT

Sample name	**OD_{260} / OD_{280}**	**Concentration in ng/µl**
1. KAK 2	1.41	45.6
2. KAK 8	1.52	40.2
3. KAK 14	1.50	42.5
4. KAK 16	1.40	39.6
5. KAK 18	1.56	67.0
6. KAK 22	1.53	49.5
7. KAK 25	1.49	54.2
8. KIS 28	1.40	38.0
9. KIS 32	1.57	61.8
10. KIS 33	1.52	41.5
11. KIS 34	1.41	38.6
12. KIS 36	1.62	72.8
13. KIS 40	1.51	55.6
14. KIS 41	1.41	39.4
15. KIS 42	1.48	46.1
16. KIS 46	1.41	35.1
17. KIS 49	1.51	50.5
18. NAK 77	1.39	32.8
19. NAK 79	1.48	47.4

20. NAK 81	1.41	34.3
21. NAK 83	1.43	35.2
22. NAK 85	1.53	42.3
23. NAK 87	1.54	42.2
24. NAK 89	1.52	40.1
25. NAK 90	1.54	56.6
26. NAK 91	1.41	37.6
27. NAK 92	1.47	37.0
28. NAK 93	1.49	40.5
29. NAK 95	1.43	35.2
30. NAK 97	1.42	33.6
31. NAK 98	1.56	58.8
32. KAB 51	1.54	42.1
33. KAB 52	1.49	40.8
34. KAB 54	1.42	33.8
35. KAB 55	1.47	38.4
36. KAB 57	1.49	40.4
37. KAB 59	1.55	45.3
38. KAB 60	1.54	42.1
39. KAB 63	1.57	59.4
40. KAB 64	1.46	38.4
41. KAB 65	1.47	41.3
42. KAB 66	1.61	67.3
43. KAB 68	1.40	28.8

44. KAB 69	1.41	34.6
45. KAB 71	1.56	52.6
46. NYERI 103	1.54	51.5
47. NYERI 107	1.59	62.2
48. NYERI 111	1.56	54.3
49. NYERI 113	1.54	50.8
50. NYERI 115	1.57	53.4
51. NYERI 116	1.61	64.6
52. NYERI 117	1.55	53.8
53. NYERI 125	1.54	50.9
54. NYERI 127	1.48	49.5
55. MERU 129	1.47	42.0
56. MERU 130	1.47	42.2
57. MERU 134	1.55	51.2
58. MERU 135	1.55	51.1
59. MERU 138	1.46	40.8
60. MERU 139	1.45	
61. MERU 140	1.48	45.2
62. MERU 141	1.41	34.9
63. MERU 142	1.41	34.8
64. MERU 143	1.46	41.4
65. MERU 144	1.43	37.8
66. MERU 145	1.56	56.2
67. MERU 146	1.46	41.0

68. MERU 148	1.44	40.9
69. THIKA 202	1.42	34.1
70. THIKA 203	1.46	41.0
71. THIKA 204	1.39	29.6
72. THIKA 205	1.36	22.5
73. THIKA 207	1.39	31.5
74. THIKA 208	1.44	40.2
75. THIKA 212	1.41	33.0
76. THIKA 213	1.46	41.0
77. THIKA 218	1.43	37.5
78. THIKA 219	1.44	38.6
79. THIKA 220	1.37	27.6
80. THIKA 221	1.44	40.7
81. THIKA 224	1.56	46.7
82. THIKA 225	1.41	38.8
83. TAITA 176	1.40	37.4
84. TAITA 177	1.41	33.0
85. TAITA 178	1.39	29.7
86. TAITA 181	1.56	51.4
87. TAITA 183	1.46	42.0
88. TAITA 184	1.41	34.2
89. TAITA 188	1.48	47.1

90. TAITA 189	1.38	26.6
91. TAITA 190	1.37	23.2
92. TAITA 195	1.37	23.3
93. TAITA 197	1.41	34.9
94. TAITA 198	1.40	32.7
95. TAITA 199	1.40	31.4
96. TAITA 200	1.42	33.8
97. MOM 152	1.40	30.6
98. MOM 154	1.40	32.7
99. MOM 155	1.38	29.6
100. MOM 156	1.39	29.9
101. MOM 157	1.44	40.7
102. MOM 158	1.38	27.5
103. MOM 173	1.38	29.4
104. MOM 163	1.36	25.3
105. MOM 175	1.47	46.0
106. KER 226	1.46	44.1
107. KER 227	1.49	47.9
108. KER 228	1.55	53.8
109. KER 229	1.53	51.9
110. KER 231	1.50	50.1
111. KER 233	1.58	58.8
112. KER 235	1.55	54.9
113. KER 237	1.47	45.9

114. KER 238	1.48	46.4
115. KER 241	1.49	48.3
116. KER 245	1.64	120.1
117. KER 246	1.63	109.8
118. KER 247	1.60	69.0
119. KER 248	1.46	44.0
120. MH II 251	1.44	41.0
121. MH II 252	1.47	45.7
122. MH II 253	1.43	38.2
123. MH II 254	1.61	68.8
124. MH II 255	1.62	66.3
125. MH II 256	1.49	49.8
126. MH II 257	1.49	49.8
127. MH II 258	1.52	50.6
128. MH II 259	1.44	39.5
129. MH II 261	1.44	40.7
130. MH II 263	1.42	35.3
131. MH II 267	1.46	44.1
132. MH II 269	1.42	37.9
133. MH II 270	1.42	36.1
134. NJR II 291	1.53	51.6
135. NJR II 293	1.47	48.1
136. NJR II 294	1.53	51.3

137. NJR II 295	1.55	52.8
138. NJR II 300	1.58	55.3
139. NJR II 303	1.59	57.8
140. NJR II 306	1.62	64.4